# Surfactant Modification of Lipases for Lipid Interesterification and Hydrolysis Reactions

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**ABSTRACT:** Commercial grade lipases from *Rhizopus japonicus, R. delemar,* and *Rhizomucor miehei* were modified by surfactant (sorbitan monostearate), and their protein recovery and interesterification, and hydrolysis activities were investigated. By repeating the lipase modification processes three times, total protein recoveries of 17–35% could be obtained. The original lipases had no interesterification activities at all; however, all modified lipases in the first process had significant interesterification activities. In the hydrolysis reactions, all modified lipases obtained from the first process showed about three times higher specific activities than the original lipases. The modified lipases obtained from the second and third processes had lower specific interesterification and hydrolysis activities than the lipases from the first one. These results suggest that the surfactant modification process is effective not only for interesterification but also lipase purification.

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**KEY WORDS:** Enzymatic hydrolysis, enzymatic interesterification, modified lipase, protein recovery, *Rhizomucor miehei, Rhizopus delemar, Rhizopus japonicus,* sorbitan monostearate, surfactant.

Functional oils and fats, MCT (medium-chain fatty acid triglycerides), polyunsaturated fatty acids (EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid), and low-calorie triglycerides are receiving attention for their nutritional benefits. MCT consist mainly of octanoic and decanoic acids, which are more easily hydrolyzed by pancreatic lipases than long-chain triglycerides, and are commonly used for medical applications (1). EPA and DHA have been reported to have beneficial therapeutic and nutritional effects (2,3). Low-calorie triglycerides, such as Caprenin® and Salatrim®, have been studied for use in snack foods and confectionery (4). Enzymatic processes using lipase are a useful technique for the production of nutritionally valuable oils and fats because of their specificity and high activity at low temperatures. They are also advantageous compared to conventional chemical processing, which requires a high temperature (5,6). As a means to improve the activity and stability of lipases, the use of an immobilization carrier and the attachment of polyethylene glycol or fatty acid to lipase have been investigated (7–9). In the last few years, researchers have also studied surfactant-modified lipase, which is a complex of lipase and surfactant (10–13). This surfactant-modified lipase was obtained by the aggregation of lipase and surfactant in water. It was assumed that the hydrophilic radicals of the surfactant attached to the enzyme surface and that the hydrophobic radicals arranged themselves on the outer side of the enzyme. By this modification, the enzyme becomes soluble or well dispersed in the organic solvent and can have high activity in the organic solvent. In our previous publications, the surfactant-modified lipase showed a much higher interesterification activity than the original lipase and surfactant mixture (14).

During the lipase modification process, the percentage of the recovered protein in the modified lipase was only 10%, and the recovered hydrolysis activity was less than 50% (14). To obtain the modified lipase efficiently, higher recoveries of protein and activity are required. In this paper, the lipase modification process was repeated with the same lipase in order to recover more protein. The activities of hydrolysis and interesterification, as well as the protein contents of the modified lipases and residuals, were analyzed in each process. In addition, the characteristics of surfactant modification of lipase are discussed.

## EXPERIMENTAL PROCEDURES

*Materials.* Original lipase: Lipase Saiken 100, *Rhizopus japonicus*, 10.0% protein concentration, was purchased from Nagase Biochemicals Co., Ltd. (Kyoto, Japan). Lipase D Amano 20, *Rhizopus delemar*, protein concentration of 27.7%, was supplied from Amano Pharmaceutical Co., Ltd. (Nagoya, Japan). Lipozyme 10000 L, *Rhizomucor miehei*, 5.0% protein concentration was supplied from Novo Nordisk A/S (Bagsvaerd, Denmark). Surfactant: Emazol S-10 (F), sorbitan monostearate, was supplied from Kao Co., Ltd. (Tokyo, Japan). Protein concentration was determined by the Hartree method (15).

Chemicals: Tripalmitin of more than 99% purity was purchased from Sigma Chemical Co. (St. Louis, MO). 1,2-Dipalmitoyl-3-stearoyl glycerol (PPS) and 1,3-distearoyl-2-palmitoyl glycerol (SPS) of more than 98% purity were a gift from Unilever Research Colworth Laboratory (Bedford,

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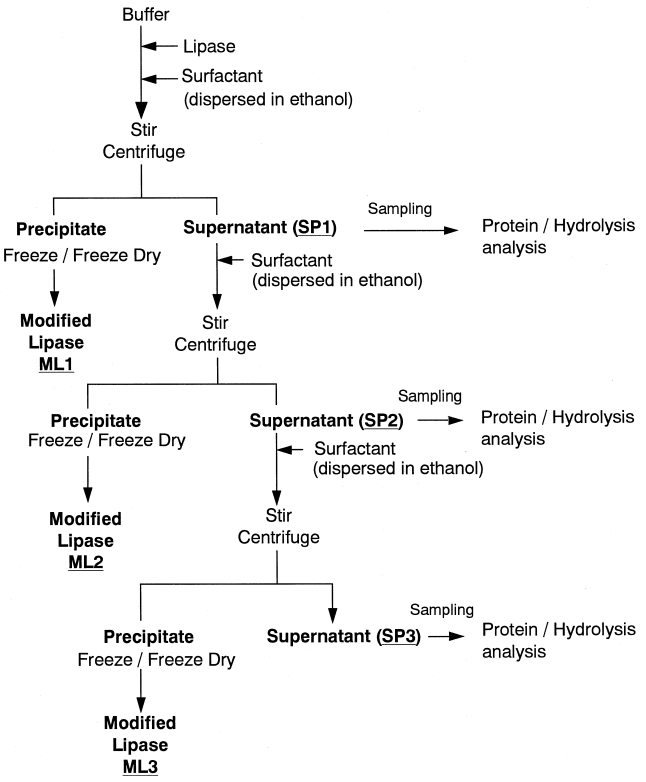


**SCHEME 1**

United Kingdom). All other analytical-grade chemicals were purchased from Wako Pure Chemical Ind. (Tokyo, Japan).

*Lipase modification process.* The batch modification process was repeated three times as shown in Scheme 1. First, each original lipase, containing 300 mg protein, was dissolved in 1 L of Mcllvaine buffer solution (16). Table 1 shows the lipase amount and the buffer pH used for the modification process. The pH used in the experiment was adjusted to optimize the modified lipase activity for each lipase (12). Surfactant (0.75 g Emazol S-10(F), sorbitan monostearate) dispersed in 20 mL ethanol at 40°C was added to the lipase solution. The mixture was stirred at 600 rpm and 5°C for 24 h. A precipitate was obtained by centrifugation at 9,000 × *g* and 5°C for 20 min, frozen, and freeze-dried. The modified lipase (ML1) was obtained as a powder.

Using the supernatant (SP1), the second lipase modification process was carried out. Surfactant (0.75 g) dispersed in 20 mL ethanol at 40°C was added to SP1. The mixture was stirred at 600 rpm and 5°C for 24 h. The precipitate was obtained by centrifugation at 9,000 × *g* and 5°C for 20 min, frozen, and freeze-dried. The modified lipase (ML2) was obtained as a powder. The supernatant (SP2) was used for further lipase recovery process. The modified lipase, ML3, was obtained from the supernatant, SP2, by the same procedure as for ML2.

**TABLE 1**
**Amount of Lipase and Buffer Used for Lipase Modification Process**

| Lipase[a] | Amount of lipase (g/L) | Protein amount (mg/L) | Buffer pH |
|---|---|---|---|
| Lipase Saiken 100 | 3.00 | 300 | 5.0 |
| Lipase D Amano 20 | 1.10 | 305 | 6.0 |
| Lipozyme 10000 L | 6.00 | 300 | 6.0 |

[a]Lipase Saiken 100 *(Rhizopus japonicus)* (Nagase Biochemicals Co., Ltd., Kyoto, Japan); Lipase D *(Rhizopus delamar)* (Amano Pharmaceutical Co., Ltd., Nagoya, Japan); Lipozyme 10000 L *(Rhizomucor miehei)* (Novo Nordisk A/S, Bagsvaerd, Denmark).

*Interesterification experiment.* The substrates of tripalmitin (500 mg) and stearic acid (500 mg) were dissolved in 50 mL *n*-hexane, in which the water concentration was reduced to below 10 mg/L by molecular sieve. The modified lipase was added to the reaction mixture, and the interesterification reaction was carried out with stirring at 500 rpm and 40°C for 24 h. As a control, the interesterification reaction was also carried out using original lipase with the addition of the surfactant to the reaction mixture. The initial water concentrations of the reaction mixture were below 30 mg/L. Samples were taken from the reaction system at appropriate time intervals and were filtered (LCR13-LH, 0.5 μm pore size, Nihon Millipore Ltd., Tokyo, Japan) for gas chromatography (GC) injection. GC analysis was performed on a GC 14AH gas chromatograph (Shimadzu Co., Kyoto, Japan) equipped with a capillary column (ULBON HR-TGC1, 0.25 mm internal diameter, 25 m column length, 0.1 μm film thickness, Shinwa Chemical Industries, Ltd., Tokyo, Japan) and flame-ionization detection (FID). The operating procedure was the same as described in a previous paper (12). The concentrations of diglycerides and monoglycerides in the reaction mixture were determined by thin film chromatography-FID (TLC-FID) system using an Iatroscan MK-5 detector and Iatrocorder TC-11 (Iatron Laboratories Inc., Tokyo, Japan). The hydrogen and air flow rates were 0.16 and 2.0 L/min, respectively. Chromarod SIII TLC rods coated with boric acid were prepared according to the method described by Itoh and Sugai (17). Samples (1 μL) were applied to five TLC rods, and the average values of five data were used. The rods were placed in a closed tank containing 50 mL benzene, 20 mL chloroform, 0.7 mL acetic acid, and 0.5 mL acetone until the solvent front had traveled 10 cm (approximately 30 min).

Interesterification activity was described as the specific reaction rate constant, $k^*$ [$m^6$/(mol·gPr·s), where gPr is grams of protein], which was based on two-substrates, two-products reversible reaction systems, assuming second-order reaction kinetics (14). The value $k^*$ reflects the conversion rate of the tripalmitin to PPS and SPS per gram of protein of the lipases.

*Hydrolysis experiment.* Hydrolysis activities of original, modified, and supernatant lipases were analyzed by tributyrin assay, using a pH-stat titrator, VIT 90 Video Titrator (Radio Meter, Analytical A/S, Copenhagen, Denmark) (18). Tributyrin and emulsification reagent (mixture of sodium chloride, potassium dihydrogen phosphate, glycerol, and gum arabic) were mixed vigorously, then used as the substrate. Lipases were dissolved in water by stirring for an hour, then added to the substrate. Hydrolysis activity was described by LU (lipase unit). One lipase unit is the amount of lipase that hydrolyze 1 μmol butyric acid/min from emulsified tributyrin.

*Water concentration analysis.* Water concentration of the interesterification system was analyzed by Karl Fischer water determination, 684 KF Coulometer (Metrohm, Herisau, Switzerland). The sample for water analysis from the interesterification reaction system was injected into the coulometer before the filtration.

*Protein content analysis.* The protein contents of the original and modified lipases and the supernatants were analyzed according to the Hartree method (15). A spectrophotometer (UV-2101 PC; Shimadzu Co., Kyoto, Japan) was used for absorbance measurement.

All experiments were done twice. Each result was almost similar, however, the data shown in this paper are the average of the results.

## RESULTS AND DISCUSSION

*Protein recovery and interesterification activity.* The protein recovery and interesterification activity in modified lipase by the lipase modification process are shown in Table 2. In the case of the lipase from *Rhizopus japonicus*, protein recovery in the ML1 was 11.8% by the first lipase modification process. By means of the second and third lipase modification processes, protein recoveries of the ML2 and ML3 were 3.34 and 2.64%, respectively, which were much lower than that of the ML1. As a whole, 48.5 mg protein was recovered in 3.43 g modified lipase, the protein concentration was 1.38%, and the protein recovery reached 17.1%. In the control test without lipase addition, no precipitate was observed. The interaction between lipase and surfactant gave a significant amount of precipitate, and most of the surfactant was transferred to the precipitate.

The original lipase of *Rhizopus japonicus* had no interesterification activity at all. Nor was activity observed upon addition of surfactant to the reaction mixture. However, after the first lipase modification, the ML1 showed high interesterification activity. Figure 1 shows the time course of the interesterification of tripalmitin and stearic acid in *n*-hexane using the ML1. The concentrations of tripalmitin and stearic acid decreased; and palmitic acid, PPS, and SPS were produced with time. Tristearin (SSS) was not produced at all. The reaction system seemed to reach steady state after 2-h reaction. The interesterification activity of the ML1 was $80.0 \times 10^{-9}$ $m^6/(mol \cdot gPr \cdot s)$. The interesterification activity of ML2 decreased to $24.0 \times 10^{-9}$ $m^6/(mol \cdot gPr \cdot s)$. The concentration of diglycerides produced was about 4 wt% of the triglycerides after 24-h reaction. Monoglycerides were not produced at all. In the ML3, the interesterification reaction did not occur

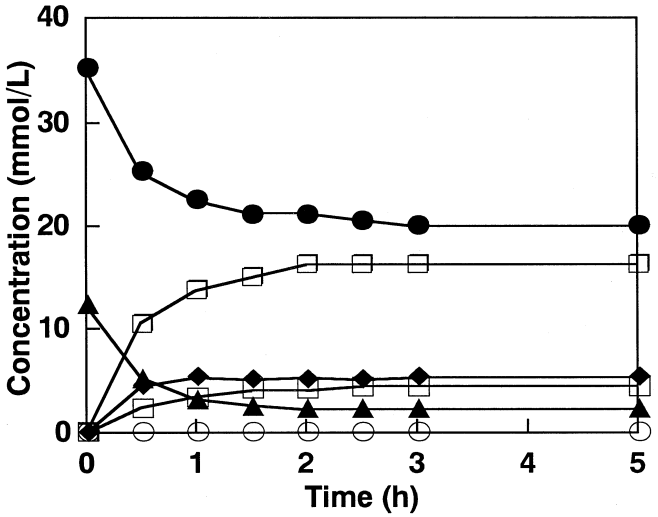


**FIG. 1.** Interesterification reaction time history using the modified lipase 1 (ML1) obtained from *Rhizopus japonicus* lipase and sorbitan monostearate. Substrates were 500 mg of tripalmitin and 500 mg of stearic acid. The reaction was carried out with 500 rpm stirring at 40°C in *n*-hexane. ❒, Palmitic acid; ●, stearic acid; ▲, tripalmitin; ◆, 1,2-dipalmitoyl-3-stearoyl glycerol; □, 1,3-distearoyl-2-palmitoyl glycerol; ○, tristearin.

**TABLE 2**
**Protein Recovery and Interesterification Activity in Modified Lipase[a]**

| Modification process | ML amount (g) | Protein content (%) | Protein recovery in each process (%) | Interesterification activity $k^* \times 10^9$ [$m^6/(mol \cdot gPr \cdot s)$] |
|---|---|---|---|---|
| *Rhizopus japonicus* | | | | |
| Lipase[b] | — | 10.0 | — | 0 |
| 1st | 1.12 | 2.80 | 11.8 | 80 |
| 2nd | 1.10 | 0.77 | 3.34 | 24 |
| 3rd | 1.21 | 0.53 | 2.64 | 0 |
| *Rhizopus delemar* | | | | |
| Lipase[b] | — | 27.7 | — | 0 |
| 1st | 1.28 | 2.38 | 10.5 | 13 |
| 2nd | 1.17 | 3.78 | 16.8 | 2.9 |
| 3rd | 1.39 | 1.99 | 12.9 | 0.8 |
| *Rhizomucor miehei* | | | | |
| Lipase[b] | — | 5.0 | — | 0 |
| 1st | 1.49 | 1.49 | 7.88 | 6.4 |
| 2nd | 1.38 | 1.38 | 8.10 | 0.6 |
| 3rd | 1.32 | 1.32 | 14.1 | 0.1 |

[a]ML amount, total dry weight of recovered modified lipase (ML); gPr, grams of protein. See Scheme 1 for details.
[b]Lipase before modification.

within a period of 24 h. The interesterification activities of the supernatant, the SP1, SP2, and SP3 (Scheme 1) of *Rhizopus japonicus,* were also investigated. The SP1, SP2, and SP3 were frozen, freeze-dried, and used for interesterification reaction with sorbitan monostearate in *n*-hexane; no reaction products were observed at all during 24-h reaction (data not shown). The surfactant, which is combined with lipase, was not to be one of the substrates for interesterification and hydrolysis. As a blank test for the interesterification reaction, only the modified lipase was stirred in *n*-hexane at 500 rpm and 40°C for 24 h. After GC analysis of the final product, neither related surfactant nor chemicals derived from the sorbitan monostearate were detected. In the blank test for the hydrolysis reaction, no surfactant or chemicals derived from the sorbitan monostearate were detected.

In the case of lipase from *Rhizopus delemar*, protein recovery of the ML1 was 10.5%. By the second and third processes, protein recoveries of the ML2 and ML3 were 16.8 and 12.9% respectively; a similar amount of protein was recovered in each modification process. As a whole, 102 mg protein was recovered from 3.84 g modified lipase; the protein concentration was 2.66%, and the protein recovery reached 35.4%.

The original lipase from *R. delemar* had no interesterification activity at all. Nor was activity observed with addition of the surfactant to the reaction mixture. However, after the first lipase modification, the ML1 showed interesterification activity of $13.0 \times 10^{-9}$ $m^6/(mol \cdot gPr \cdot s)$. The concentration of diglycerides was about 4 wt% of the triglycerides, and monoglycerides were not produced after 24-h reaction. The interesterification activities of ML2 and ML3 were $2.90 \times 10^{-9}$ and $0.80 \times 10^{-9}$ $m^6/(mol \cdot gPr \cdot s)$, respectively.

When lipase from *Rhizomucor miehei* was used for the lipase modification process, protein recovery of the ML1 was 7.88%. Following the second and third processes, protein recoveries of the ML2 and ML3 were 8.10 and 14.1%, respectively. As a whole, 77.5 mg protein was recovered in 4.19 g modified lipase, its protein concentration was 1.85%, and the protein recovery reached 27.3%.

In the case of *R. miehei,* the original lipase had no interesterification activity at all. Nor was activity observed with addition of the surfactant to the reaction mixture. However, after the first lipase modification, the ML1 showed interesterification activity, and it was $6.40 \times 10^{-9}$ $m^6/(mol \cdot gPr \cdot s)$. The concentration of diglycerides was about 12 wt% of the triglycerides, and monoglycerides were not produced after 24 h of reaction. The interesterification activities of the ML2 and ML3 were $0.60 \times 10^{-9}$ and $0.10 \times 10^{-9}$ $m^6/(mol \cdot gPr \cdot s)$, respectively. The concentration of diglycerides was three times higher than those in the modified lipases obtained from *Rhizopus japonicus* and *R. delemar*.

The protein recovery of *R. japonicus* was not significantly increased by the repeated modification processes. However, in the case of *R. delemar* and *Rhizomucor miehei*, the lipase recoveries increased significantly with lipase modification processes. *Rhizopus japonicus* seems to have different characteristics of association with surfactants from the other two lipases. The optimal weight ratio of *R. japonicus* and sorbitan monostearate during the modification process to obtain the highly active modified lipase has been investigated (12). At a ratio of lipase protein to surfactant between 0.2 and 2.5, protein recoveries were kept constant at 10–12%. From these results, we speculated that a limited part of protein in *R. japonicus* was associated with the surfactant. The modified lipase in this study was obtained by the interaction of lipase and surfactant in aqueous media, which seems important in order for modified lipase to give the interesterification activity. We speculate that significant lipase modification by the surfactant did not occur in *n*-hexane, so there was no interesterification activity. SP1, SP2, and SP3 did not have interesterification activities, which is probably due to the low concentration of surfactant–lipase complex.

**TABLE 3**
**Hydrolysis Recovery in Modified Lipase by the Lipase Modification Process**

| Modification process[a] | Activity[b] in SP (LU) | Specific activity in SP (LU/mg Pr) | Residual activity in SP (%) | Recovered activity[b] in ML (LU) | Specific activity in ML (LU/mg Pr) | Recovered activity (%) |
|---|---|---|---|---|---|---|
| *R. japonicus* | | | | | | |
| Initial | 11,200 | 38.1 | — | — | — | — |
| 1st | 1,530 | 5.87 | 13.7 | 3,570 | 106 | 31.9 |
| 2nd | 431 | 1.75 | 3.90 | 549 | 64.4 | 37.8 |
| 3rd | 0.00 | 0.00 | 0.00 | 159 | 25.0 | 38.2 |
| *R. delemar* | | | | | | |
| Initial | 9,100 | 30.5 | — | — | — | — |
| 1st | 5,090 | 19.0 | 55.9 | 2,340 | 76.8 | 25.7 |
| 2nd | 3,950 | 18.1 | 43.4 | 641 | 14.5 | 32.7 |
| 3rd | 3,250 | 17.4 | 35.7 | 465 | 16.8 | 37.8 |
| *R. miehei* | | | | | | |
| Initial | 52,700 | 179 | — | — | — | — |
| 1st | 18,400 | 67.6 | 34.8 | 11,200 | 500 | 21.2 |
| 2nd | 11,600 | 47.6 | 22.1 | 2,080 | 96.6 | 25.1 |
| 3rd | 10,200 | 49.4 | 19.3 | 1,060 | 31.4 | 27.1 |

[a]As illustrated in Scheme 1.
[b]SP, supernatant; LU, lipase units (see the Experimental Procedures section); for other abbreviations see Table 2.

*Hydrolysis activity recovery.* The hydrolysis activity recoveries in modified lipases are presented in Table 3. Initial activities show the total hydrolysis activities of original lipases in the pH-adjusted buffer solution. In the case of *R. japonicus*, activity of 3,570 LU was recovered in the ML1 from the initial activity of 11,200 LU by the first process, i.e., the activity recovery was 31.9%. In the second and third processes, activities of 549 and 159 LU were recovered in the ML2 and ML3, respectively, and total recovered activity reached 38.2%. The specific hydrolysis activity of original *R. japonicus* was 38.1 LU/mg protein. The specific activity of the ML1 increased to 106 LU/mg, which was about three times of the initial specific activity. The specific activities of ML2 and ML3 increased 10–15 times compared to those of SP1 and SP2. From these results, it can be concluded that the proteins having higher hydrolysis activity (mainly lipase) were recovered selectively in the modified lipases from the original lipase, and the other proteins with less activity remained in the supernatants. These results suggest that the surfactant modification process may be a useful lipase purification process for *R. japonicus*.

In the case of *R. delemar*, an activity of 2340 LU was recovered in the ML1 from the initial activity of 9100 LU by the first modification process, i.e., the activity recovery was 25.7%. In the second and third modification processes, 641 and 465 LU were recovered in the ML2 and ML3, respectively, and total activity recovered reached 37.8%. By the first modification, the specific lipase activity of the ML1 was increased to 76.8 LU/mg, which was 2.5 times that of the original activity. However, in the second and third processes, the specific activities of the ML2 and ML3 were similar to specific activities of the SP1 and SP2.

For *Rhizomucor miehei* lipase, the recovered hydrolysis activity in the ML1 was 11,200 LU from the initial activity of 52,700 LU by the first modification process, i.e., the activity recovery was 21.2%. By the second and third modification processes, 2,080 and 1,060 LU were recovered in the ML2 and ML3, respectively, and total activity recovered was 27.1%. By the first modification process, the specific activity of the ML1 increased 2.8 times from the original activity.

For all *Rhizopus japonicus*, *R. delemar,* and *Rhizomucor miehei* lipases, the specific hydrolysis activities of the ML1 increased 2.5–2.8 times higher than the original lipases. More than 60% of the total recovered activity was found to be obtained in the ML1 by the first lipase modification process in each lipase. In the second and third processes of *R. delemar* and *R. miehei*, specific activities of the modified lipases were almost the same as the respective specific activities of supernatant lipases, which implies that there is little selectivity for proteins. However, in the case of the second and third processes of *R. japonicus*, the specific activities of modified lipases were 10 to 14 times higher than the respective specific activities of the supernatant lipases, which suggests that surfactant interacts with lipase in higher selectivity.

In checking the activity balance of *R. japonicus*, the total recovery of hydrolysis activity was 38.2% after the third modification process; however the residual activity in the SP3 showed no activity at all. During modification, 61.8% of the initial activity was lost. The total activities in all modified lipases and the residual activity in the SP3 were also lower than the initial total activity in *R. delemar* and *R. miehei*, which might be explained by two reasons: (i) The modified lipase is thought to be a complex of lipase and surfactant that is not easily soluble in water. In the hydrolysis experiments, the activity of the nonsoluble modified lipase might be estimated to be lower than that of soluble lipase. (ii) Some deactivation or activity inhibition may occur due to the surfactant modification effect.

As explained above, both hydrolysis and interesterification activities of ML1 were much higher than in ML2 and ML3 for all three lipases. All the specific hydrolysis activities in the ML1 were about three times higher than the original specific activities, suggesting that the modification process is effective not only for interesterification but also for lipase purification. Further investigation of lipase purification by surfactant modification is required.

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