Oil–Water Interfacial Activation of Lipase for Interesterification of Triglyceride and Fatty Acid[1]

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ABSTRACT: Lipase usually has little interesterification activity in organic solvents, probably owing to the absence of an oil-water interface. Lipases were processed in a two-phase hydrocarbon–water system that had an oil–water interface. Crude lipase (from *Rhizopus japonicus*) in a buffer and a small volume of aliphatic hydrocarbon as an oil phase were mixed and then lyophilized to remove the aqueous and oil phases. The interfacially processed lipase has a remarkable interesterification activity in *n*-hexane compared to crude native lipases. We postulate that this activation is caused by the oil–water interface, i.e., the interface between hydrocarbon and water makes the lipase lid open and enables the lipase to work effectively in *n*-hexane. Several different hydrocarbons were investigated as an oil phase, and *n*-tetradecane was found to be the best for interesterification. Activated lipase was successfully inactivated in a water suspension without an oil–water interface, and the inactivated lipase could be reactivated. We demonstrated that the oil (hydrocarbon)–water interface induced reversible activation to lipase for interesterificaiton.

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KEY WORDS: Interesterification, interfacial activation, lipases, oil-water interface, organic solvent.

The modification of the structure and the composition of oils and fats by enzymatic interesterification is currently of great industrial interest (1,2). In this reaction, an exchange of acyl residues between a triglyceride and a fatty acid (acidolysis), an alcohol (alcoholysis), or an ester (transesterification) produces a new triglyceride mixture. Conventionally, these reactions are promoted using sodium metal or sodium alkoxide to catalyze acyl migration among triglyceride molecules and to produce a randomly distributed fatty acyl residue among triglyceride molecules. Studies have demonstrated the potential of using lipase for various interesterification reactions in organic media (2,3).

The first attractive feature of lipase is that it is safe for use in food and medical applications. The second is the potential specificity of reactive position and the specificity toward different fatty acids. Lipase-catalyzed interesterification owing to enzyme specificity provides a precise control on the incorporation of the desired fatty acid at a specific glyceride chain position. The reaction also takes place under mild reaction conditions and is therefore preferable to the harsh and nonspecific chemical catalysis or to random physical blending. However, lipase has several problems for industrial application, one of which is the very low activity of lipase in organic solvents. Lipase can catalyze the hydrolysis reaction in aqueous mixtures but the substrates are generally insoluble in water. For industrial applications, syntheses such as esterification and interesterification reactions are best carried out either in organic media or in nonsolvent systems in which the water content can be controlled (4). Several techniques have been developed to improve the performance of lipase in organic solvents. Immobilized lipase (5), biphasic system (3), reversed micellar system (6,7), modification of lipase surface using polyethylene glycol (8), surfactant-coated lipase (9–11), and fatty acid-modified lipase (12) are techniques that have been used to improve the activity. However, the mechanism of lipase activation by these treatments has not yet been elucidated.

Recently the X-ray crystallographic structures of several lipases have been identified (13–15). These studies showed that lipases have an α-helical fragment (termed the "lid") that covers the active site. It is known that lipid hydrolysis by lipase is activated by an oil (triacetin or ethyl butyrate)–water interface (16). Following the X-ray crystallographic analysis, it has been suggested that opening of the lid might occur during oil–water interfacial activation, hence allowing substrates access to the active site (17). Usually, lipase shows a very low activity in organic solvents, the reason for which is also thought to be the closed lid. In organic solvents, most lids of the lipase molecules will be closed because of the absence of oil-water interface. That is why it is necessary to use an excess amount of lipase for catalysis of various reactions in or-

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ganic media. If the lids can be kept open in advance, lipase is expected to have a high activity in organic solvents.

Based on this hypothesis, we prepared lipase that had been activated by an oil–water interface to enable catalysis of interesterification reactions in organic media. Various conditions of this oil–water interfacial activation were investigated to optimize the enzymatic activity in organic solvents.

MATERIALS AND METHODS

Materials. All chemicals were purchased from Wako Pure Chemicals Ltd. (Tokyo, Japan), except for the following. Crude lipase Saiken 100 (from *Rhizopus japonicus*) was purchased from Nagase Biochemicals (Kyoto, Japan). The enzyme contains 6.0 wt% protein as determined by the Lowry method (18). Crude lipase Asahi (from *Chromobacterium viscosum*) was purchased from Asahi Chemical Industry Ltd. (Tokyo, Japan). The enzyme contains 2.4% protein. Tripalmitin (PPP) and stearic acid were purchased from Sigma (St. Louis, MO). 1,2-Dipalmitoyl 3-stearoyl glycerol (PPS) and 1,3-distearoyl 2-palmitoyl glycerol (SPS) were gifts from Unilever (Colworth Lab, United Kingdom). Before use, the *n*-hexane was dried on molecular sieves (200 g molecular sieves 4 Å/3 L *n*-hexane) to give a water content of 25 mg/L. Water content was measured with a Karl Fischer titrator 684 KF (Coulometer, Metrohm, Herisau, Switzerland).

Oil–water interfacial activation of lipase. Crude lipase Saiken 100 (20 mg) was dissolved in 5 mL of McIlvaine buffer (pH 7) and 0.25 mL of aliphatic hydrocarbon was then added. The two-phase mixture (a dispersed solution) had an oil–water interface and was stirred for 1 h at 40°C with a magnetic stirrer. Then it was lyophilized to remove both the water and aliphatic hydrocarbon. The oil–water interfacially activated lipase was obtained as a dry residue with salt from the mixture. No aliphatic hydrocarbon was observed by gas chromatographic (GC) analysis when the oil–water interfacially activated lipase was suspended in *n*-hexane. As an oil phase, the following hydrocarbons were used for activation: *n*-hexane, *n*-octane, *n*-decane, *n*-dodecane, *n*-tetradecane, *n*-hexadecane, and *n*-octadecane. These hydrocarbons were selected since they are liquid at ambient temperature and have a high volatility during lyophilization.

Interesterification reaction. Interesterification reactions of PPP and stearic acid catalyzed by either nonactivated or activated lipase were performed in 25 mL of *n*-hexane at 40°C. The concentration of PPP was 11.2 mmol/L and that of stearic acid was 35.2 mmol/L. Samples (0.25 mL) were periodically withdrawn from the reaction system and filtered with a 0.5 μm filter (LCR 13 LH; Millipore Co., Bedford, MA). The interesterification reaction products, PPS and SPS, and the substrate (PPP) were analyzed using a GC-14AH gas chromatograph (Shimadzu Co., Kyoto, Japan) equipped with a capillary column (URBON HR-TGC1; Shinwa Chemical Industries, Ltd., Tokyo, Japan) and flame-ionization detector as described previously (11). The interesterification activity [mol/(h·g-protein)] was evaluated as the average of the initial PPS production rates, which were calculated from PPS concentration at 1, 2, and 3 h according to previous work (19).

Hydrolysis activity. Lipase-catalyzed hydrolysis reactions were carried out at 30°C and pH 7.0 in 20 mL of a glycerol/water (5.4:4.6, vol/vol) solution containing gum arabic (6.0 g/L), NaCl (0.30 mol/L), KH_2PO_4 (3.0 mmol/L), and 0.5 mL tributyrin as substrate. Release of free butyric acid was monitored by continuous titration with NaOH using a pH-stat (632 Digital-pH-Meter, 614 Impulsomat and 665 Dosimat; Metrohm Ltd.).

RESULTS AND DISCUSSION

Activation and inactivation of lipase. Figure 1 shows the typical time course for the interesterification of tripalmitin and stearic acid by the oil–water interfacially activated lipase from *R. japonicus* in *n*-hexane with low water content (25 mg/L), in which *n*-tetradecane was used for oil–water interfacial activation. Significant amounts of PPS and SPS were produced. The activity of the activated lipase was determined to be 48 mmol/(h·g-protein). Control lipase was also prepared using the same procedure as the activated lipase but without an oil phase, i.e., just lyophilized from buffer solution. The activity of the control lipase was 2.5 mmol/(h·g-protein), and crude native lipase had no activity. Thus, the oil (*n*-tetradecane)–water interfacial activation was found to be effective in stimulating the enzymatic interesterification reaction. Previously we reported fatty acid modification for lipase activation (12). The total retrieved activity after the oil–water interfacial activation method described here was 70% higher than that of the fatty acid modification. The difference is likely due to the fact that the oil–water interfacial activation method allowed recovery of all the crude lipase used initially. In contrast, in the fatty acid modification method it was necessary

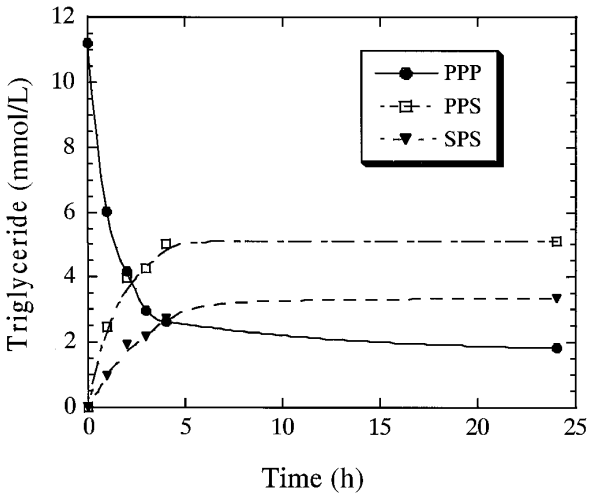


FIG. 1. Interesterfication reaction by oil–water interfacially activated lipase (20 mg) between tripalmitin (PPP) and stearic acid to produce 1,2-dipalmitoyl 3-stearoyl glycerol (PPS) and 1,3-stearoyl-2-palmitoyl glycerol (SPS) in *n*-hexane.

to collect the precipitate of fatty acid-lipase complex, and part of the crude lipase used initially was lost at this step.

As described in the introduction, it has been reported that a lipase (from porcine pancreas) was activated by an oil (triacetin or ethyl butyrate)–water interface so as to catalyze lipid hydrolysis reaction in an oil–water two-phase system. X-ray crystallographic studies of the lipase structure suggest that the interfacial activation of lipase is caused by lid opening at the oil–water interface, which allows substrates to approach the active site (17). In our study it was speculated that the lids of native and control lipases were kept closed because of the absence of an oil–water interface, making the active site inaccessible to the substrates in organic solvents (Scheme 1). Therefore, those nonactivated lipases had little interesterification activity in *n*-hexane. In the case of the activated lipase, the lids would be opened by the oil–water interface and be kept open through lyophilization. The lids would be also kept open in *n*-hexane and not be closed easily because the stability of the protein structure is influenced mainly by water and polar solvents (20). Hence the active site could be accessible to the substrates in *n*-hexane, so the activated lipase had a high interesterification activity.

If the foregoing hypothesis on lipase activation is correct, it would be possible to close and open the lid of the lipase for inactivating the activated lipase and re-activating the inactivated lipase. The oil (*n*-tetradecane)–water interfacially activated lipase was added to water and mixed at 4°C for 24 h (inactivation process). Then the mixture was lyophilized again. The dry residue was composed of lipase and salt, the same as the activated lipase. The interesterification activity of this dry residue was only 3% of that of the activated lipase under the same reaction conditions. This revealed that the activated lipase was easily inactivated by water in the absence of an oil–water interface. Derewenda *et al.* (21) reported that the lid of lipase had a high mobility in an aqueous solution and that specific molecular interactions with hydrophobic molecules affected and/or stabilized the lid. There was a subtle equilibrium between the two conformations (open and closed) of lipase. Their results and theory account for our results.

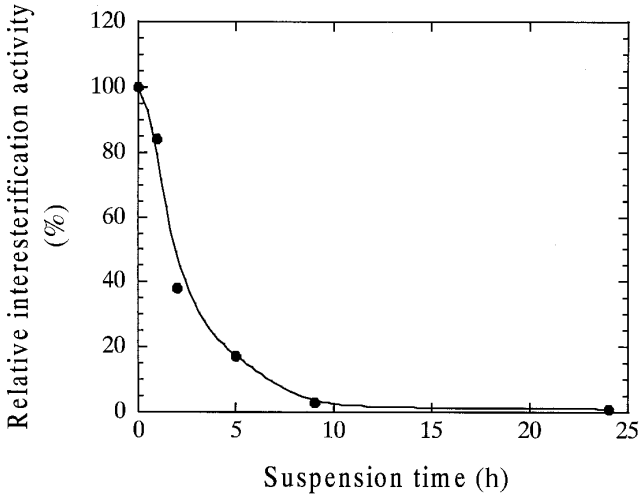
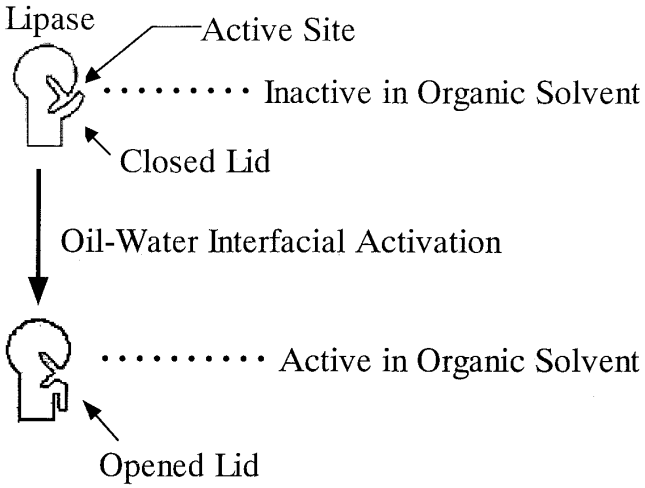


FIG. 2. Effect of the suspension time of interfacially activated lipase in water on its subsequent interesterification activity.

The effect of the inactivation process time on the inactivation was investigated and is shown in Figure 2. The interesterification activity decreased gradually as a function of the inactivation process time. This suggests that the lid of the activated lipase is closed gradually in water because of the absence of an oil–water interface. Hence the lipase was inactivated and could not catalyze the interesterification reaction in *n*-hexane. This inactivated lipase was processed again using the oil (*n*-tetradecane)–water interfacial activation method. This interfacially re-activated lipase had an activity 10 times higher than that of inactivated lipase, although total activity was reduced to 28% of the first activated lipase. This re-activation would also involve lid opening. Table 1 shows relative interesterification and relative hydrolytic activities for crude native, activated, inactivated (suspended in water for 24 h), and re-activated lipases. The lipase lost 36% hydrolytic activity after the first activation and an additional



SCHEME 1

TABLE 1
Relative Interesterification and Hydrolysis Activities for Crude, Activated, Inactivated, and Re-activated Lipase Saiken 100

	Relative interesterification activity (%)[a]	Relative hydrolysis activity (%)[b]
Crude native lipase (Saiken 100)	0	100
Native lyophilized	5	75
Activated lipase	100	64
Inactivated lipase	3	52
Re-activated lipase	28	49

[a]Each interesterification activity is expressed as a percentage based on the interesterification activity of activated lipase.
[b]Each hydrolysis activity is expressed as a percentage based on the hydrolytic activity of crude native lipase. Lipase Saiken 100 supplied by Nagase Biochemicals (Kyoto, Japan).

10% hydrolytic activity after the inactivation process. These results show that it is inevitable for lipase to be partly denatured through the process of oil–water interfacial activation and lyophilization. This is probably because the contact with the hydrocarbon and lyophilization will cause not only interfacial activation but also denaturation. Therefore, interfacial re-activation gave the lipase only a partly recovered interesterification activity. This result revealed that the lipase activation due to the oil–water interface was partly reversible and supported our hypothesis on lids opening and closing for interesterification activity. This result suggests that the closing of the lipase lid is caused by water, and the opening of the lipase lid is caused by an oil–water interface in oil–water two-phase system. Both closing and opening cannot take place in organic solvents, which agrees with the report that interfacial activation of lipase was caused by an oil (methyl butyrate)–water interface, and not by the oil–alcohol interface in a triolein-ethanol two-phase system (21). That is why the activation and inactivation of lipase here was carried out in the presence of an aqueous phase, and the activated lipase had a high activity in organic solvent. It was also reported that the conformational changes of the enzyme occurred readily in water, and enzyme had great thermal stability in organic media (20).

Effect of buffer pH during the oil-water interfacial activation process. The oil–water interfacial activation was carried out at various pH values, to investigate the effect of the buffer pH on interesterification activity. Figure 3A shows the relationship between interesterification activity and buffer pH. The pH value of the buffer used for the activation process strongly affected the interesterification activity. The highest activity was obtained at pH 7, and the activity decreased drastically at lower pH values. Figure 3B shows the relationship between the triolein hydrolysis activity of crude native lipase and the pH of the reaction medium. This activity also had a peak at pH 7. Figures 3A and 3B are very similar. Both show that pH 7 gave the highest activity, and a lower pH (around 5) gave a very low activity. It has been reported that when an enzyme is dispersed in organic media as a heterogeneous catalyst, the enzymatic activity greatly depends on the pH of the buffer solution at lyophilization (22). This is thought to be because the dissociation of amino acid residues around the lipase's active site is strongly associated with conformational change. The optimal pH, which is pH 7 in this case, caused the best dissociation, which probably leads to efficient lid opening and allows the active site to catalyze easily. Therefore the activated lipase can "remember" the pH environment of the aqueous phase to which the lipase was finally exposed.

Effect of various hydrocarbons as an oil phase on interesterification activity. The effect of using a variety of aliphatic hydrocarbons as an oil phase for the activation of interesterification was investigated (Fig. 4). All hydrocarbons used—*n*-hexane, *n*-octane, *n*-decane, *n*-dodecane, *n*-tetradecane, *n*-hexadecane, and *n*-octadecane—were liquid at 40°C and therefore could generate an oil–water interface. Activity was significantly dependent on the hydrocarbon used for activation. The activity increased remarkably according to the carbon

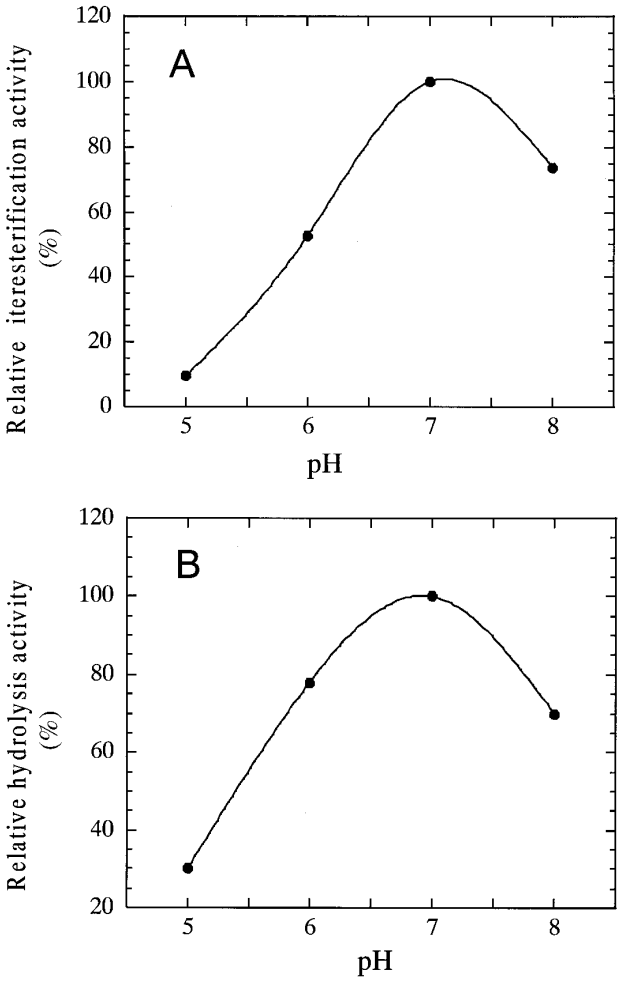


FIG. 3. Effect of buffer pH during the oil–water interfacial activation process on interesterification activity of the activated lipase (A) and the relationship between the hydrolytic activity of crude native lipase and pH of reaction medium (B).

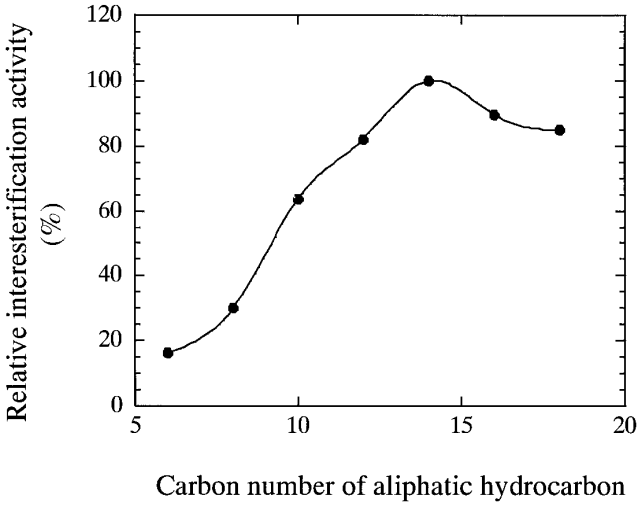


FIG. 4. Effect of various kinds of aliphatic hydrocarbons as an oil phase for activation of interesterification activity. Each interesterification activity was expressed as a percentage based on the interesterification activity of *n*-tetradecane.

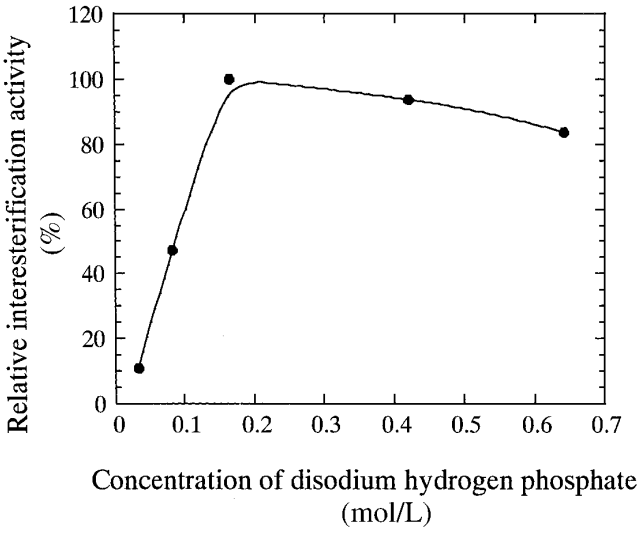


FIG. 5. Effect of buffer concentration during the oil–water interfacial activation process on interesterification activity. The pH was kept 7.0 in all cases.

number of the aliphatic hydrocarbon, but over *n*-tetradecane [48 mmol/(h·g-protein)] the activity shifted to decrease gradually, as shown in Figure 4. This suggests that *R. japonicus* lipase has a certain affinity for the *n*-tetradecane–water interface.

Effect of buffer concentration on interesterification activity. The effect of buffer concentration during the oil–water interfacial activation process on interesterification activity was studied (Fig. 5). Buffer concentration (based on disodium hydrogen phosphate) was investigated from 0.03 to 0.65 M, while the buffer pH was constant at 7.0. The activities were almost constant at concentrations higher than 0.16 M. However, below 0.16 M buffer, activity decreased, and the activity at 0.03 M buffer was only 5.0 mmol/(h·g-protein), which was 10–15% of the highest activity. This suggests that lipase requires a minimum buffer concentration (0.16 M) for interfacial activation.

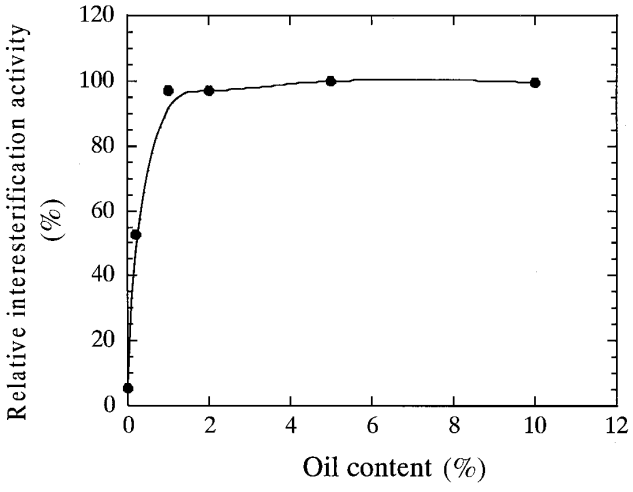


FIG. 6. Effect of oil content during the oil–water interfacial activation process on interesterification activity.

TABLE 2
Interesterification Activities [mmol/(hÆg-protein)] of Crude Lipase Saiken 100 and Asahi in *n*-Hexane

Lipases	Native powder	Native lyophilyzed	Activated by $C_{14}H_{30}$
Saiken 100 *(Rhizopus japonicus)*	0	2.5	48
Asahi *(Chromobacterium viscosum)*	0	2.8	25

[a]Lipase Asahi supplied by Asahi Chemical Industry Ltd. (Tokyo, Japan). For other supplier see Table 1.

*Effect of oil (*n*-tetradecane) content during the oil–water interfacial activation process on interesterification activity.* The effect of the oil (*n*-tetradecane) content during the oil–water interfacial activation process was investigated from 0.2 to 10 vol/vol % for the interesterification reaction (Fig. 6). The activities were constant for contents higher than 1%. At 0.2% oil, the activity was reduced to half. This suggests that the concentration of lipase in this experiment required a certain interfacial area to be activated, with a 1% oil phase providing sufficient interfacial area for successful lipase activation.

Different lipases. Crude lipase Asahi (from *C. viscosum*) was also investigated for oil (*n*-tetradecane)–water interfacial activation. Although the structure of the lipase from *R. japonicus* has not yet been elucidated, the crystal structure of *C. viscosum* lipase has been published (15), and it was found that this lipase has a lid. The results are shown in Table 2. The crude native lipase Asahi had no interesterification activity in *n*-hexane, but after oil–water interfacial activation it displayed 25 mmol/(h·g-protein) interesterification activity. This result also strongly supports our hypothesis of lid opening for interesterification activity. Therefore, this oil–water interfacial activation technique may be generally applicable to lipases.

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