Changes in Hydrolysis Specificities of Lipase from *Rhizomucor miehei* to Polyunsaturated Fatty Acyl Ethyl Esters in Different Aggregation States

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ABSTRACT: Hydrolysis specificities of lipase from *Rhizomucor miehei* were compared for various fatty acyl ethyl esters. Activity yields of immobilized lipases, measured with 1 mM substrate, were more than 100%. Differences in hydrolysis rate and affinity for the substrates between lipase preparations were also typically higher during hydrolysis of substrates at 100 mM than at 1 mM, indicating better mass transfer effects for 1-mM substrates. The native lipase showed higher affinity for polyunsaturated fatty acid substrates at 1 mM than at 100 mM. Hydrolysis rates for 1-mM substrates were observed with immobilized lipases, fixed on anion exchange resin with glutaraldehyde and on cation exchange carrier with carbodiimide, and suggested some modification of the basic amino acid related to the lid of *R. miehei* lipase. Activation with these bifunctional reagents was not observed for 100-mM substrates, indicating that interfacial activation always occurred because of aggregation of 100-mM substrates. These results show that lipase from *R. miehei* recognizes molecular aggregation of lipids, and that various changes occur in the hydrolysis specificities for fatty acids. *JAOCS 74,* 1395–1399 (1997)

KEY WORDS: Aggregation, fatty acid, immobilized lipase, interfacial activation, lipase, polyunsaturated fatty acid, specificity.

The aggregate structure of polyunsaturated lipids under aqueous conditions is of great interest. Investigation of the oxidative stability of 1 mM polyunsaturated fatty acids (PUFA) under aqueous conditions showed that the highest stability was achieved by docosahexaenoic acid (22:6n-3, DHA), followed by eicosapentaenoic (20:5n-3), arachidonic (20:4n-6), α-linolenic (18:3n-3), γ-linolenic (18:3n-6) and linoleic (18:2n-6) acids, indicating that stability increases with increasing degree of unsaturation (1). However, most data indicate that the oxidative stability of these PUFA is inversely proportional to the number of bis-allylic hydrogens in the molecule. Therefore, DHA is susceptible to lipid peroxidation in the air. The structural patterns of 1 mM DHA under aqueous conditions may stabilize the molecule, but little is known about the structure of PUFA in aqueous solution.

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Lipase attacks the ester bonds that bind hydrophobic and hydrophilic residues in amphipathic lipids. The amphipathic nature of lipids is the origin of many substrate aggregates, such as monolayers, micelles, liposomes, and membranes. Aggregate formation depends on the lipid concentration, temperature, the nature of the solute, and lipid structure (2). Porcine pancreatic lipase activity is greatly increased at the water/lipid interface, suggesting that interfacial activation of lipase occurs (3). The catalytic triad Ser-144, His-203, Asp-257 of *Rhizomucor miehei* is buried completely beneath a short helical segment (residues 82–96), or lid (4). X-ray crystallographic analysis of the lipase–inhibitor complex revealed that interfacial activation is achieved by the displacement of the lid structure, which exposes the catalytic groups and creates a hydrophobic surface that stabilizes the contact between the lipase and the lipid interface (5). *Humicola lanuginosa* lipase displays a pronounced interfacial activation with *p*-nitrophenyl acetate, indicating that no substantial aggregation of substrate molecules occurs below the solubility limit of the substrate (6). We previously observed that, when the concentration of PUFA salts is near the critical micelle concentration (CMC), the PUFA salts are in equilibrium between monomeric and aggregate states (7). The PUFA salts exist as aggregates at much higher concentrations than the CMC (7).

PUFA are also important because of their numerous biological functions and the low-temperature adaptation of biological membranes. The competition factor has been used for kinetic resolution of PUFA and less common fatty acids catalyzed by lipases from different sources (8,9). Thus, DHA has been enriched in the acyl glycerol fraction after *Geotrichum candidum* lipase-catalyzed reaction, and this procedure is now industrialized (10). Hydrolysis of rapeseed oil at low temperature by lipase from *Candida rugosa* provides a high concentration (approximately 95%) of erucic acid in the diacyl glycerol fraction (11). The foregoing examples indicate that, for kinetic resolution of a particular fatty acid, it is important to select the lipase and physical conditions. Although the explanation of the PUFA state in the ester substrate is only speculation from the results of PUFA salt, this study investigated the enzymatic recognition of PUFA in different aggregation states in water to identify how lipase recognizes lipid diversity in aqueous solution. The hydrolysis specificities of lipase from *R. miehei* for PUFA were

measured from hydrolysis rate or initial velocity and the competition factor (12,13), which is a comparison of catalytic power (i.e., maximum velocity/Michaelis constant) near the CMC and at much higher than CMC of fatty acyl salts.

MATERIALS AND METHODS

Materials. Lipase (Lipozyme 10,000 L) from *R. miehei* was provided by Novo Nordisk (Chiba, Japan) as a liquid that contained 10,000 LU/g, as specified by the manufacturer. Ethyl docosahexaenoate was a gift from Harima Kasei Co. (Osaka, Japan). DHA ethyl arachidonate, ethyl linolenate, ethyl linoleate, ethyl oleate, and fatty acid sodium salts were products of Nu-Chek-Prep, Inc. (Elysian, MN). Ethyl eicosapentaenoate was a product of Larodan Fine Chemicals AB (Malmo, Sweden). Eicosapentaenoic acid was obtained from Funakoshi Co. (Tokyo, Japan). Arachidonic acid, linolenic acid, and linoleic acid were products of Serdary Research Laboratories Inc. (London, Canada). Spherosil C, a weakly acidic cation exchange carrier with carboxylic acid groups, was provided by Rhône-Poulenc Industry (Paris, France). Water-soluble carbodiimide of 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide-*p*-toluenemetho-sulfonate was obtained from Sigma Chemical Co. (St. Louis, MO). Dowex MWA-1, a macroporous weakly basic anion exchange resin with tertiary amine groups, was obtained from The Dow Chemical Co. (Midland, MI).

Immobilization of lipase. The flexible lipase was prepared as follows. One gram of Spherosil C was activated with the water-soluble carbodiimide as described previously (14). The activated Spherosil C and 2 g of Lipozyme 10,000L were combined with 1 mL of 0.067 M McIlvaine buffer at pH 5, and the resultant mixture was shaken overnight at 4. The lipase immobilized on the cation exchange carrier was washed with water and dried by suction. The adsorbed lipase was prepared as follows. One gram of Dowex MWA-1 was washed with water and mixed with 2 g of Lipozyme 10,000L and 1 mL of 0.067 M McIlvaine buffer at pH 5. The mixture was shaken at 4°C overnight and dried as described above. The rigid lipase was prepared from the above adsorbed lipase by treatment with glutaraldehyde as previously described (15).

Hydrolysis reaction. The reaction mixture, containing 1 mM ethyl ester, was composed of 60.0 g water, 10–18 mg ethyl ester, and 30 mg immobilized lipase or 75 mg Lipozyme 10,000L. The reaction mixture, containing 100 mM ethyl ester, was composed of 1 g water, 20–30 mg ethyl ester, and 30 mg immobilized lipase or 75 mg Lipozyme 10,000L. Each composition in the reaction mixture was accurately weighed. Before the reaction was initiated, argon gas was bubbled for 2–3 min through the mixture. During the reaction, the mixture in a test tube (21 × 200 mm) was vigorously agitated with a small magnetic stirrer (3 × 6 mm). The reaction was carried out at 45°C for 1–2 h to measure the initial velocity. The relationship of product amount and reaction time within 3 h to the final degree of hydrolysis at 20–30% was a straight line that coincided with the origin. No buffer and metal ion were used in the reaction mixture to eliminate soap formation. The pH of the reaction mixture was weakly acidic, but the pH was not so low as to inhibit the reaction. These conditions were also used for our previous hydrolysis studies (16).

Assay methods for lipolysis product. The hydrolysis product was extracted with hexane two times, and the upper phases were collected. The solvent was evaporated by bubbling with argon gas. The product was redissolved in acetone. The concentrated hydrolysate was analyzed by high-performance liquid chromatography (GL Science, Tokyo, Japan) with a refractive index detector (RI Model 504; GL Science) and a chromatointegrator (Shimadzu CR-4A chromatopac; Shimadzu, Kyoto, Japan). The fixed phase column was a Superspher RP-18 (4 × 25 mm; Merck, Darmstadt, Germany), and the mobile phase was a mixture of acetone and acetonitrile (1:1, vol/vol, at a flow rate of 0.8 mL/min and a pressure of 120 kgf/cm^2). The retention times of $C_{12:0}$ acid, $C_{12:0}$ ethyl ester, $C_{18:1}$ acid, $C_{18:1}$ ester, $C_{18:2}$ acid, $C_{18:2}$ ester, $C_{18.3}$ acid, $C_{18:3}$ ester, $C_{20:4}$ acid, $C_{20:4}$ ester, $C_{20:5}$ acid, $C_{20:5}$ ester, $C_{22:6}$ acid, and $C_{20:6}$ ester were 4.19, 5.06, 5.20, 6.70, 4.60, 5.67, 4.07, 4.64, 4.19, 4.81, 3.86, 4.50, 3.92 and 4.36 min, respectively. The quantitative factors of integrator area by weight of $C_{12:0}$ for the above compounds were 1.00, 1.10, 0.82, 1.26, 0.79, 0.89, 0.71, 0.85, 0.71, 0.77, 0.64, 0.70, 0.55, and 0.69, respectively.

Kinetic studies. Hydrolysis rates were calculated from the composition of the reaction products as micromoles of fatty acid formed per gram per minute, and the competition factor was obtained as follows: In the competitive reaction of two substrates (Ac1X and Ac2X, with the same leaving group X and two different acyl groups Ac1 and Ac2) at the same catalytically active site on the enzyme molecule, the ratio of the hydrolysis rates for each substrate (v_1 and v_2) is given (17) by:

$$v_1/v_2 = [(V_{Ac1X}/K_{Ac1X})/(V_{Ac2X}/K_{Ac2X})](Ac1X)/(Ac2X) \quad [1]$$

where (Ac1X) and (Ac2X) are the concentrations of the two acyl donors (two fatty acyl ethyl esters), V_{Ac1X} and V_{Ac2X} are the maximum velocities, and K_{Ac1X} and K_{Ac2X} are the Michaelis constants for each substrate. The competitive factor α is then defined as the ratio of the catalytic powers according to the following equation (12,13):

$$(V_{Ac1X}/K_{Ac1X})/(V_{Ac2X}/K_{Ac2X}) = \alpha \quad [2]$$

The competitive factor α is calculated from the integral form of Equation 1:

$$\log([Ac1X]_0/[Ac1X]) = \alpha\log([Ac2X]_0/[Ac2X]) \quad [3]$$

Then, the specificity constant $1/\alpha$ (13) can be expressed from Equations 2 and 3:

$$(V_{Ac2X}/K_{Ac2X})/(V_{Ac1X}/K_{Ac1X}) = \log([Ac2X]_0/[Ac2X])\ /\log([Ac1X]_0/[Ac1X]) \quad [4]$$

The specificity constant $1/\alpha$ is the apparent catalytic power as (V_{Ac2X}/K_{Ac2X}), based on (V_{Ac1X}/K_{Ac1X}) being equal to 1. In this paper, Ac1X is lauric acid ethyl ester.

Nuclear magnetic resonance (NMR) and surface tension measurement. ^{1}H NMR was performed at room temperature (25°C) with 0.1–10 mM of PUFA sodium salt in deuterium oxide (D_2O) in a JEOL α-500 NMR spectrometer (499.45 MHz; Tokyo, Japan). The sampling was done under an argon atmosphere. The standard compound was sodium 3,3-dimethyl-3-silapentanoate-d_8 in a D_2O capillary system. The CMC was obtained from the curves of the surface tension vs. the logarithm of the sodium salt of fatty acid concentration. The surface tension was measured with an Automatic Surface tension meter (CBVP-Z; Kyowa Interface Science Co., Asaka, Saitama, Japan).

RESULTS AND DISCUSSION

PUFA salts. Amphipathic lipids display an extensive range of phenomena and structural patterns that depend on chemical structure, concentration, temperature, etc. PUFA ethyl esters did not dissolve in water, but PUFA salts dissolved in water easily to analyze their NMR spectrum. The CMC in water, measured by surface tension plots of DHA sodium salt, was 0.35 mM (7). Figure 1 shows the ^{1}H NMR spectra of the methylene regions of DHA sodium salt in D_2O. Below the CMC, DHA sodium salt is present in the monomeric state. The chemical shift of the bis-allylic protons (*e* in Fig. 1) in the monomeric 0.1 mM DHA sodium salt was 2.8–2.9 ppm. The chemical shifts of the bis-allylic protons became 2.8–2.9 and 2.7–2.8 ppm for 1 mM DHA sodium salt. Above the CMC, 10 mM DHA sodium salt is present in the micelle or aggregate state. The chemical shift of the bis-allylic protons in 10 mM DHA aggregate was 2.7 ppm. The two similar signals were also observed in the chemical shifts of the olefinic protons (*f* and *f** in Fig. 1). However, these chemical shifts are at 5.3–5.5 ppm, which are not illustrated in Figure 1. These results indicate that DHA salts exist as monomers and as aggregates at lower and higher concentrations, respectively. When the concentration is near the CMC, DHA salts exist in an equilibrium between the monomeric and aggregate states. The same tendency mentioned above was observed in sodium linoleate solution (7).

Immobilization of lipase. Lipase from *R. miehei* was immobilized as described in the Materials and Methods section. Generally, lipase with an isoelectric point of 4.3 (according to the manufacturer) does not adsorb on a cation exchange carrier under the pH 5–6 conditions used here, owing to electrostatic repulsion, but it is fixed on the carrier by covalent bonding and is called flexible lipase. The lipase can be fixed on an anion exchange resin at pH 5–6 through ionic bonding and hydrophobic bonding and is called adsorbed lipase. When treated with glutaraldehyde, the adsorbed lipase can be fixed on the anion exchange resin through covalent bonding, ionic bonding, and hydrophobic bonding, and it is called rigid lipase. Native lipase and flexible lipase can undergo structure changes easily, but rigid lipase can achieve only little structure change with changes in the physical conditions. The specificities of the lipase were markedly affected by immobilization of the lipase, as shown in Figures 2–5.

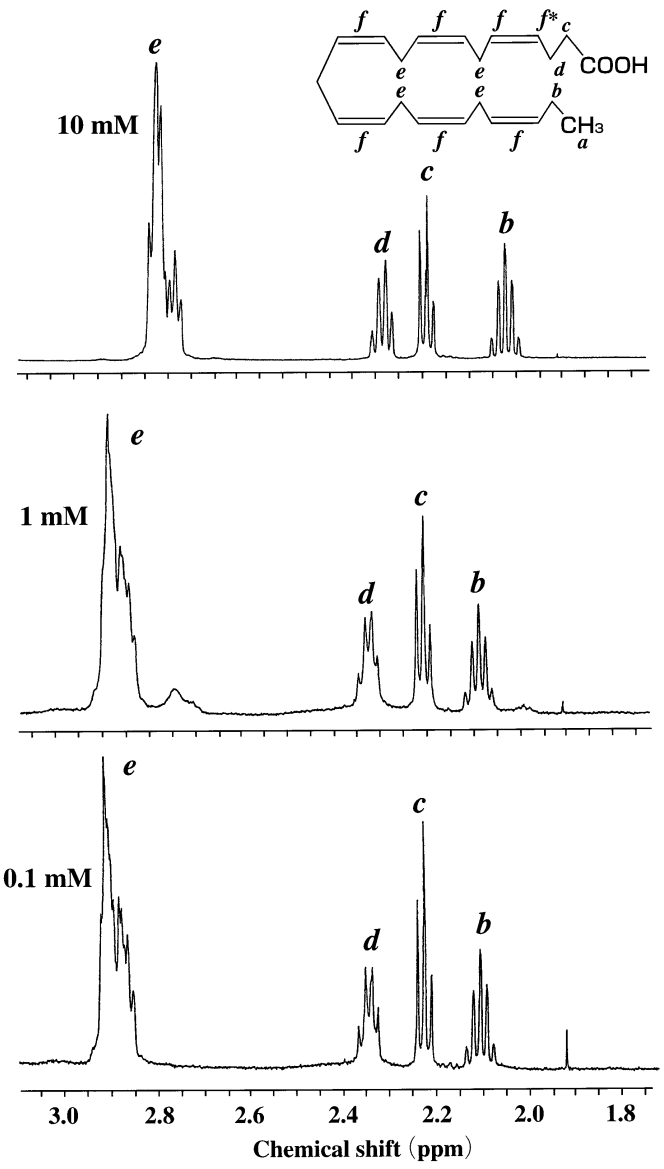

FIG. 1. ^{1}H nuclear magnetic resonance spectra of the methylene regions of sodium docosahexaenoate. The labeled peaks (*b, c, d,* and *e*) correspond to the functional groups indicated in the structure of sodium docosahexaenoate.

Activity yields (ratios of immobilized lipase activity and lipase activity before preparation) of immobilized lipases, measured with 1 mM substrate, were more than 100% (Fig. 4). Native lipase amount (75 mg) for the hydrolysis reaction was more than the lipase amount (60 mg) used for preparing the immobilized product (30 mg). However, hydrolysis rates of immobilized lipases were higher than those for the native lipase. The activity yield of immobilized lipase is low because the emulsion substrate diffuses inside an immobilized carrier at a notably low speed (18). However, the high activity yields obtained may be due to a better mass transfer effect of 1 mM substrate. The activity yield of adsorbed lipase, measured

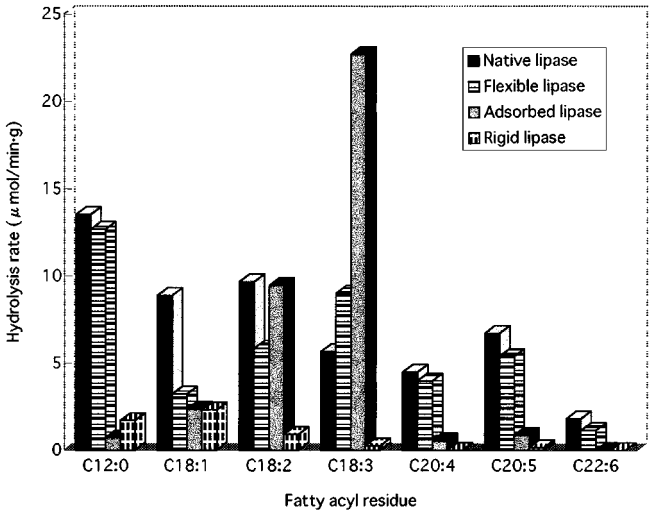

FIG. 2. Reaction rates for the hydrolysis of 100 mM fatty acyl ethyl ester. The columns show the reaction rates for native lipase, flexible lipase, adsorbed lipase, and rigid lipase from *Rhizomucor miehei.*

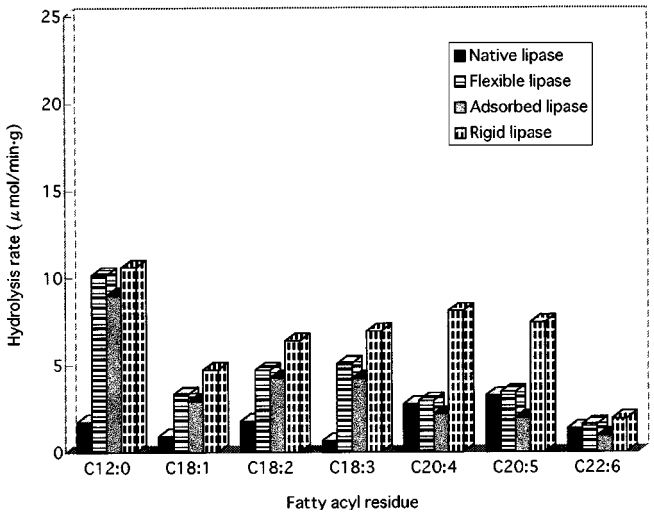

FIG. 4. Reaction rates for the hydrolysis of 1 mM fatty acyl ethyl ester.

with 100 mM ethyl linolenate ($C_{18:3}$), was also typically high, as shown in Figure 2, indicating that immobilization results in a suitable conformation and microenvironment (19) for *R. miehei* lipase to hydrolyze the substrates.

Interfacial activation. Higher hydrolysis rates for 1 mM substrates were observed by rigid lipase, fixed on anion exchange resin with glutaraldehyde, and for flexible lipase, fixed on cation exchange carrier with carbodiimide (Fig. 4). The glutaraldehyde and the carbodiimide attack basic amino acids, such as arginine, histidine, and lysine. An arginine (Arg-86) is present in the lid of *R. miehei* lipase, and the C-terminal fragment of the lid region 93–96 is stabilized by interactions of the main-chain atoms of Val-95: N to N σ 1 of His-108 and O to N of Lys-109 (20). Therefore, some modification may have occurred in the basic amino acids related to the lid, resulting in some activation of lipolysis of the 1mM substrate. As is evident from Figure 2, the higher hydrolysis rates for 100 mM substrates were not observed by the rigid lipase and the flexible lipase, indicating that interfacial activation always occurred by the aggregates of the 100 mM substrates, and that modifications of the basic amino acid related to the lid had no effect on the hydrolysis rate for 100 mM substrate. These chemical modifications were not achieved *via* site-directed mutagenesis (21); however, the above speculation proposes interesting targets for mutagenesis, such as Arg-86.

Specificity spectrum for PUFA. The highest hydrolysis rate for 100 mM fatty acyl ethyl ester was observed for linolenic acyl ester when immobilized lipase adsorbed on anion exchange resin was used (Fig. 2). The foregoing result is clearer in the comparison of the specificity constants (Fig. 3). The native lipase showed higher catalytic power for PUFA substrates at 1 mM fatty acyl ester (Fig. 5). Higher hydrolysis rates for 1 mM fatty acyl ethyl ester were observed (Fig. 4) for rigid and flexible lipases, as mentioned in the previous

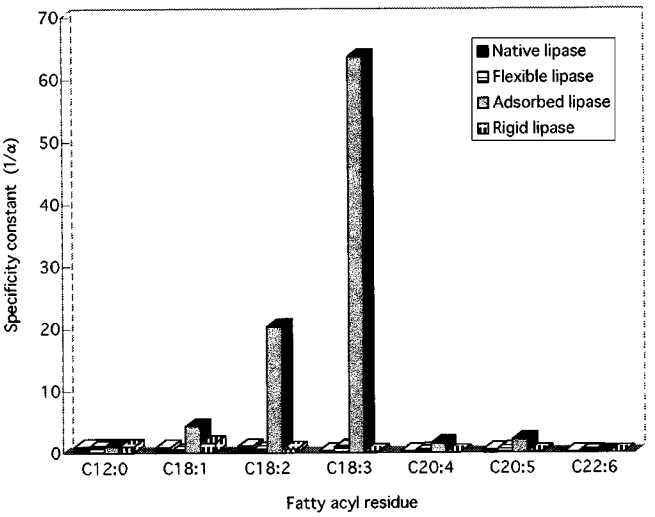

FIG. 3. Specificity constants for the hydrolysis of 100 mM fatty acyl ethyl ester.

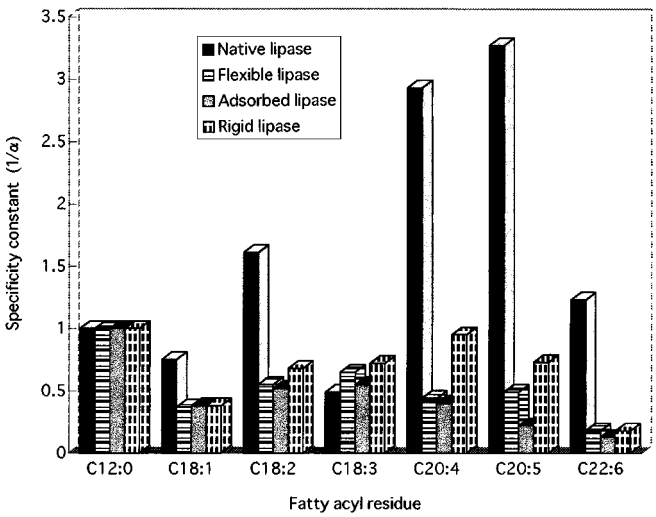

FIG. 5. Specificity constants for the hydrolysis of 1 mM fatty acyl ethyl ester.

section. Differences in hydrolysis rate and affinity between lipase preparations were typically higher during hydrolysis of the substrate at 100 mM than at 1 mM, as shown in Figures 2–5. The PUFA monomer may be present in an aqueous 1 mM solution. Such monomer would easily diffuse into the immobilized carriers. However, mostly aggregates are present in the 100 mM substrate. These would diffuse less readily to the immobilized carriers. The above observations show that various changes occur in the substrate specificities of the lipase from *R. miehei* at different aggregation states. The specificity spectrum of fatty acids for the lipase from *C. rugosa* is also changed at different concentrations of fatty acids (22).

The use of competitive factors is a simple and easy way of describing the kinetics of the lipase-catalyzed reaction with two substrates, but this is not true in a biphasic system, where the kinetics are usually much more complex (12). We used reaction rate and the competition factor to determine the fatty acid specificity of the lipase in a biphasic system. We observed various changes in the fatty acid specificities (i.e., different competition factors) in 1 mM and 100 mM fatty acyl ethyl esters as described above. However, competition factors are not affected by changes in the physical conditions of the reaction (water content, substrate concentration, nature of nucleophiles, etc.) (13). One simple explanation for these contradictory phenomena is that the assembling structures of the fatty acyl ethyl ester were greatly different between the 1 mM substrate and 100 mM substrate concentrations, and therefore, these aggregates behave as different entities.

According to the induced fit theory of enzyme action, the substrate induces conformational changes in the protein and stabilizes a new structure of the protein because of the new hydrophobic, hydrophilic, and electrostatic interactions formed in the binding process. The conformational flexibility of proteins appears to be a universal property that enables these molecules to be adapted to their widespread functions in the living system (23). The conclusion of our observation is that lipase from *R. miehei* recognizes the molecular aggregation of lipids, and various changes occur in the hydrolytic characteristics and fatty acid specificity as a result.

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