Enantioselectivity in the Hydrolysis by Lipase: A Study of MALDI TOF-MS Analysis

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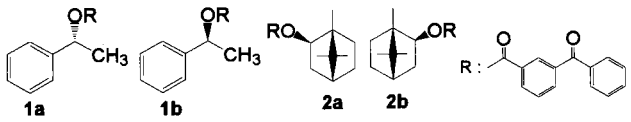
The mechanism of the enantioselectivity of lipase has been investigated by MALDI TOF-MS with photoaffinity probes. When 1-phenylethyl *m*-benzoylbenzoate was used as a photoaffinity probe, both the enantiomer that reacts as a substrate of lipase and the non-reactive enantiomer combined with lipase. However, in the case of isobornyl *m*-benzoylbenzoate, only the reactive photoaffinity probe combined with lipase.

Studies on the enantioselective hydrolyses of esters by using lipases have a long history,[1–3] and nowadays lipase-catalyzed reactions are widely applied to many chemical syntheses.[4,5] These applications rely on the property of lipase of hydrolyzing esters having the *R* configuration. As regarding the mechanism of the enantioselectivity, it has been believed that "the pocket that is unique to lipase regulates the enantioselectivity of lipase (Binding model)". In other words, the *R*-enantiomer, which is "reactive enantiomer", can enter into the pocket of lipase, while the *S*-enantiomer (i.e., non-reactive enantiomer) cannot. On the other hand, recently, Cygler et al. proposed that the enantioselectivity of lipase was determined by the stabilization of the lipase-substrate complex.[6,7] Ema and his co-workers showed that the high enantioselectivity of lipase did not depend on the stabilization induced by "Binding" the reactive substrate (*R*-enantiomer) with lipase, but rather on the destabilization caused by the non-reactive substrate (*S*-enantiomer) in the transition states.[8,9] This epoch-making explanation suggests that lipase binds with both *R*- and *S*-enantiomers. To clarify the mechanism of the enantioselectivity of lipase, we have analyzed the changes in the molecular mass of lipase caused by photolabeling. If Ema's theory is correct, the molecular mass of the lipase after photoreaction with both *R*- and *S*-enantiomers must become larger than that of native lipase, because both enantiomers should enter into the pocket. On the other hand, if the binding model is correct, the molecular mass of the lipase after photoreaction with the non-reactive enantiomer must be same as the molecular mass of native lipase, since the non-reactive enantiomer cannot enter into the pocket. To confirm the working hypothesis, the molecular mass of the lipase-photoaffinity probe complex was determined by using MALDI TOF-MS.

Photoaffinity probes **1a, 1b, 2a** and **2b** were synthesized by esterification of the corresponding chiral alcohols, (*R*)-(+)- and (*S*)-(–)-1-phenylethanols and (1*R*,2*R*,4*R*)-(–)- and (1*S*,2*S*,4*S*)-(+)-isoborneols, with *m*-benzoylbenzoyl chloride.[10,11] For the photolabeling, lipase (*Pseudomonas sp.*; 0.1 mg/mL) in 50 mM phosphate buffer, pH 7.0, was incubated with the photoaffinity probes (**1a**–**2b**; 0.1 mg/mL) in sample tubes. After a 5-min incubation at room temperature, the mixture was irradiated with UV light (254 nm, 25 μW/cm^2) from a distance of 10 cm for 5 min at 4 °C. After the irradiation, the reaction mixture was quenched by addition of ice-cold trichloroacetic acid (final concentration 7%), kept on an ice bath for 30 min, and then centrifuged at 10000 *G* for 15 min at 4 °C. The pellet was suspended with the matrix solution and MALDI TOF mass spectra[12] were measured.



The MALDI TOF-MS of native lipase (Figure 1-A) gave a major peak at *m/z* 33248. The mass spectrum of the lipase photolabeled with the *R*-enantiomer **1a** exhibited a major peak at *m/z* 33541 (panel B). The difference in molecular mass between these two peaks is 293, which corresponds well to the molecular weight of **1a** (MW 330). The mass spectrum of the lipase photolabeled with the *S*-enantiomer **1b** also exhibited a major peak at *m/z* 33545 (panel C). This result indicates that not only the *R*-enantiomer but also the *S*-enantiomer can enter into the lid and/or pocket of lipase. Therefore, the "Binding model" does not seem appropriate to explain the enantioselectivity of lipase.

Next, we examined the mass spectra of the lipase labeled with the isobornyl derivatives, **2a** and **2b**, which bear bulky alcohol moieties. The mass spectrum of lipase modified with the reactive photoaffinity probe **2a** exhibited a major peak at

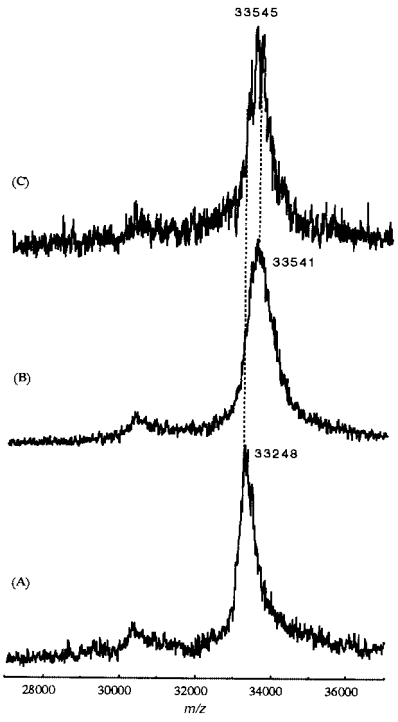


Figure 1. MALDI TOF mass spectra of lipases before and after photoreaction with 1-phenylethyl *m*-benzoylbenzoate. (A) Native lipase. (B) Lipase treated with photoaffinity probe **1a**. (C) Lipase treated with photoaffinity probe **1b**.

m/z 33530 (Figure 2-A). This molecular mass is 282 mass units larger than that of native lipase, the increase corresponding to the molecular weight of **2a**. However, as shown in Figure 2-B, a major peak at *m/z* 33245 was observed in the case of the *S*-enantiomer **2b**. This result let us think that the non-reactive photoaffinity probe **2b** cannot bind with lipase.

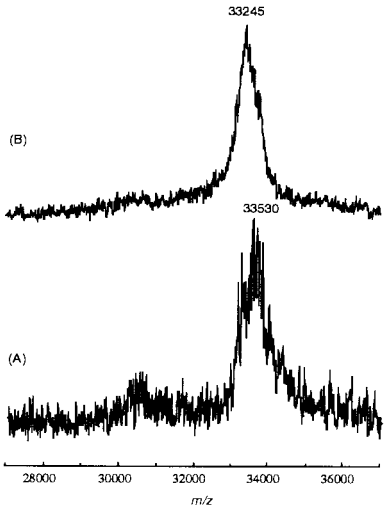


Figure 2. MALDI TOF mass spectra of lipases after photoreaction with isobornyl *m*-benzoylbenzoate (A) Lipase treated with photoaffinity probe **2a**. (B) Lipase treated with photoaffinity probe **2b**.

To investigate whether these photoaffinity probes were bound to the active site of lipase, the activities of the lipases treated with **1a**–**2b** were measured by using 4-nitrophenyl acetate as substrate.[13, 14] The reaction rate of the hydrolysis of 4-nitrophenyl acetate with native lipase was 45 μM s^{-1} mg^{-1} lipase. The activity (reaction rate) of the lipase labeled with **1a** decreased to 16 μM s^{-1} mg^{-1} lipase. The activity of the lipase labeled with **1b** (reaction rate; 18 μM s^{-1} mg^{-1} lipase) was almost equal to that of the lipase labeled with **1a**. These results support the notion that both *R*- and *S*-enantiomers (**1a** and **1b**) can enter into the lid and/or pocket of lipase. On the other hand, the activity of the lipase treated with **2b** (reaction rate; 48 μM s^{-1} mg^{-1} lipase) was almost equal to the activity of native lipase, while the activity of the lipase labeled with **2a** (reaction rate; 27 μM s^{-1} mg^{-1} lipase) decreased to a half of the activity of the non-modified lipase. This indicates that only the photoaffinity probe **2a** (*R*-enantiomer), and not **2b** (*S*-enantiomer), can enter into the pocket of lipase.

Therefore, the enantioselectivity of lipase should be explained by the stereo-sensing mechanism (Ema's theory),[8] because both (*R*)-(–)- and (*S*)-(+)-1-phenylethyl *m*-benzoylbenzoates (**1a** and **1b**) can enter into the lid and/or pocket of lipase. Nakamura and his co-workers showed that the K_m values in the transesterification of (*R*)-(–)- and (*S*)-(+)-1-phenylethanol with lipase are similar.[15] This observation also indicates that even the non-reactive substrate can enter into the pocket of the lipase. However, in the case of the bulky esters **2a** and **2b**, the non-reactive substrate seemed to be unable to enter into the pocket of lipase. Namely, there is still room for the possibility to explain the enantioselectivity of lipase by using the "Binding model", when the substrate bears a bulky alcohol moiety.

Finally, we propose the combined mass spectrometry and chiral-photolabeling method applied in the present work as a useful approach for the elucidation of the mechanisms of the enantioselectivity in the enzymatic reactions.

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10 (*R*)-(–)- and (*S*)-(+)-1-Phenylethyl *m*-benzoylbenzoates (**1a** and **1b**) were prepared from the corresponding alcohols, (*R*)-(+)- and (*S*)-(–)-1-phenylethanol (Wako Pure Chemical Ind.), by esterfication with *m*-benzoylbenzoyl chloride. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.68 (3H, d, *J* = 6.6 Hz), 6.15 (1H, q, *J* = 6.6 Hz), 7.30 (1H, tt, *J* = 7.5 and 1.8 Hz), 7.36 (2H, brt, *J* = 7.5 Hz), 7.43 (2H, brd, *J* = 7.5 Hz), 7.49 (2H, ddd, *J* = 8.2, 7.6 and 1.5 Hz), 7.57 (1H, t, *J* = 7.7 Hz), 7.61 (1H, tt, *J* = 7.6 and 1.4 Hz), 7.80 (2H, dd, *J* = 8.2 and 1.4 Hz), 7.98 (1H, dt, *J* = 7.7 and 1.5 Hz), 8.29 (1H, dt, *J* = 7.7 and 1.5 Hz), 8.49 (1H, t, *J* = 1.5 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 22.4, 73.5, 126.1, 128.0, 128.4, 128.5, 128.6, 130.1, 130.9, 131.1, 132.8, 133.2, 134.0, 137.0, 137.9, 141.5, 165.0, 195.7. IR (neat) 1720, 1668 cm^{-1}; (*R*)-form $[\alpha]_D^{25}$ –27.8° (*c* 11.3, EtOH), (*S*)-form $[\alpha]_D^{25}$ +29.0° (*c* 10.3, EtOH).
11 (1*R*,2*R*,4*R*)-(–)- and (1*S*,2*S*,4*S*)-(+)-Isobornyl *m*-benzoylbenzoates (**2a** and **2b**) were synthesized from the corresponding alcohols with *m*-benzoylbenzoic chloride. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.87 (3H, s), 0.91 (3H, s), 1.05 (3H, s), 1.14 (1H, ddd, *J* = 12.3, 9.2 and 4.0 Hz), 1.23 (1H, ddd, *J* = 12.3, 9.2 and 4.0 Hz), 1.74 (1H, tt, *J* = 12.3 and 4.0 Hz), 1.61 (1H, td, *J* = 12.3, 4.0 Hz), 1.80 (1H, t, *J* = 4.0 Hz), 1.87–1.95 (2H, m), 4.94 (1H, dd, *J* = 7.3 and 4.3 Hz), 7.49 (2H, ddd, *J* = 8.2, 7.5 and 1.8 Hz), 7.59 (1H, t, *J* = 7.8 Hz), 7.61 (1H, tt, *J* = 7.5 and 1.2 Hz), 7.80 (2H, dd, *J* = 8.2 and 1.2 Hz), 8.03 (1H, dt, *J* = 7.8 and 1.5 Hz), 8.23 (1H, dt, *J* = 7.8 and 1.5 Hz), 8.40 (1H, t, *J* = 1.5 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 11.6, 20.0, 20.1, 27.0, 33.7, 38.0, 45.0, 47.0, 49.0, 82.0, 128.4, 128.6, 130.0, 131.0, 131.1, 132.7, 133.1, 133.8, 137.0, 137.8, 165.1, 195.8; IR (neat) 1736, 1670 cm^{-1}; (*R*)-form $[\alpha]_D^{25}$ –45.9° (*c* 5.0, EtOH), (*S*)-form $[\alpha]_D^{25}$ +48.2° (*c* 5.2, EtOH).
12 The MALDI TOF mass spectra were measured with a Voyager Elite-DE Mass Spectrometer (Perseptive Biosystems) in the linear and reflectron modes using delayed extraction. The sinnapic acid matrix was prepared by dissolving 10 mg sinnapic acid in 1 mL acetonitrile/0.1% TFA (7:3 v/v).
13 The enzyme assay was performed with 4-nitrophenyl acetate as substrate by use of Ubest-55 spectrophotometer (JASCO). The reaction rate of the hydrolysis of 4-nitrophenyl acetate was measured at 25 °C with 50 mM phosphate buffer (pH 7.0) as reported by Ihara et al.[14]
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