

## Binding site of acyl moiety in ester hydrolysis by *Candida rugosa* lipase

Michimasa Goto<sup>a,\*</sup>, Masashi Kawasaki<sup>b</sup>, Tadashi Kometani<sup>a</sup>, Takamasa Nonaka<sup>c</sup>,  
Yukio Mitsui<sup>c</sup>

<sup>a</sup> Department of Chemical and Biochemical Engineering, Toyama National College of Technology, Hongo 13, Toyama 939-8630, Japan

<sup>b</sup> Faculty of Engineering, Toyama Prefectural University, 5180 Kurokawa, Kosugi-Machi, Toyama 939-0398, Japan

<sup>c</sup> Faculty of Engineering, Nagaoka University of Technology, 1603-1 Kamitomioka-Machi, Nagaoka 940-2188, Japan

### Abstract

The sizes of the large, medium, and small substituent recognition sites (L, M, and S pockets, respectively) in *Candida rugosa* lipase (CRL) were roughly estimated by measuring the specific hydrolytic activity against several *p*-nitrophenyl esters. These relative sizes were assessed as L pocket > phenyl, ethyl > M pocket > methyl > S pocket > hydrogen. The hydrolysis of a series of *p*-nitrophenyl esters of  $\omega$ -substituted fatty acids was also examined. In this series, *p*-nitrophenyl esters having one methylene between the ester-carbonyl carbon and cyclohexyl, phenyl, or isopropyl moiety largely demonstrated decreases in hydrolytic activity. © 2001 Elsevier Science B.V. All rights reserved.

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### 1. Introduction

Enzymatic resolution of racemic compounds is widely used by organic chemists today. Many hydrolytic enzymes, especially lipases, are commercially available and are used to resolve racemic acids and alcohols. Several empirical rules about faster reacting stereoisomers have been proposed [1–3]. Furthermore, in order to elucidate the active sites of these enzymes, structural studies of enzymes by X-ray analysis have been reported.

Among the hydrolytic enzymes, *Candida rugosa* lipase (CRL) is one of the most studied. Its stereose-

lectivity towards racemic esters having a stereocenter in the carboxylic acid moiety [1,4–10] and in the alcohol moiety [3,9,11,12] and the stereostructure of its active site [11,13–15] have been reported. Although many successful enantioselective hydrolysis reactions of racemic esters have been reported in the literature, no substrate inert to CRL-catalyzed hydrolysis have been described. Therefore, we investigated several model compounds that we expected to be highly resistant to CRL-catalyzed hydrolysis and tried to better understand the nature of the active site. In order to elucidate the sizes of the medium and small pockets of CRL, we first examined the hydrolyses of phenylacetic acid esters having methyl, ethyl, and/or phenyl groups at the  $\alpha$ -position (**1**). Because the large substituent recognition site in the active site of this enzyme has a unique long, bent, tunnel-like

\* Corresponding author. Tel.: +81-76-493-5459; fax: +81-76-493-5459.

E-mail address: goto@toyama-nct.ac.jp (M. Goto).

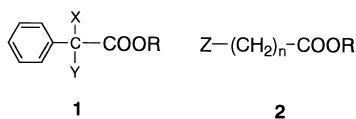


Fig. 1. X and/or Y represent either hydrogen, methyl, ethyl or phenyl group. Z represents phenyl, cyclohexyl, and isopropyl groups.

structure [15], the hydrolysis of a series of  $\omega$ -substituted fatty acids, such as cyclohexyl, phenyl, or *iso*-type substituent (**2**) was also examined (Fig. 1).

## 2. Materials and methods

### 2.1. Materials

CRL (lipase OF) and *C. antarctica* lipase (CAL, CHIRAZYME L-2) were obtained from Meito Sangyo and Roch Diagnostics K.K., respectively. *Pseudomonas fluorescense* (PFL, lipase AK), *P. cepacia* (PCL, lipase PS), and porcine liver esterase (PLE) were obtained from Amano Pharmaceutical. Porcine pancreatic lipase (PPL) were purchased from Sigma. All esters were synthesized by condensing the corresponding carboxylic acid (commercially available) and *p*-nitrophenol in the presence of dicyclohexylcarbodiimide in dichloromethane at room temperature and purified by passage through a silica

gel column. All esters were fully characterized by a spectral analysis.

### 2.2. Preparation of CRL solution

Thirty milligrams of lipase was suspended in 3 ml of phosphate buffer (pH 7.5, 10 mM) and stirred for 24 h at 4°C. The resultant viscous solution was centrifuged at 10000 rpm for 20 min at 4°C. The supernatant was used for the hydrolysis as a lipase solution. The protein concentration of the lipase solution was determined by Lowry's method [16] with bovine serum albumin as the protein standard.

### 2.3. Determination of the specific activity of CRL

The activity of lipase against each *p*-nitrophenyl ester was determined by measuring the rate of production of the *p*-nitrophenol. Before performing the hydrolysis, all the solutions, the 50 mM acetonitrile solution of each *p*-nitrophenyl ester (substrate solution), the lipase solution, and the phosphate buffer (pH 7.5, 0.1 M), were kept at 25°C in the water bath. Ten microliters of the substrate solution was then added to 2 ml of the phosphate buffer at 25°C and the enzymatic reaction was initialized by the addition of 10  $\mu$ l of the lipase solution to the phosphate buffer. The mixture was shaken vigorously for a few

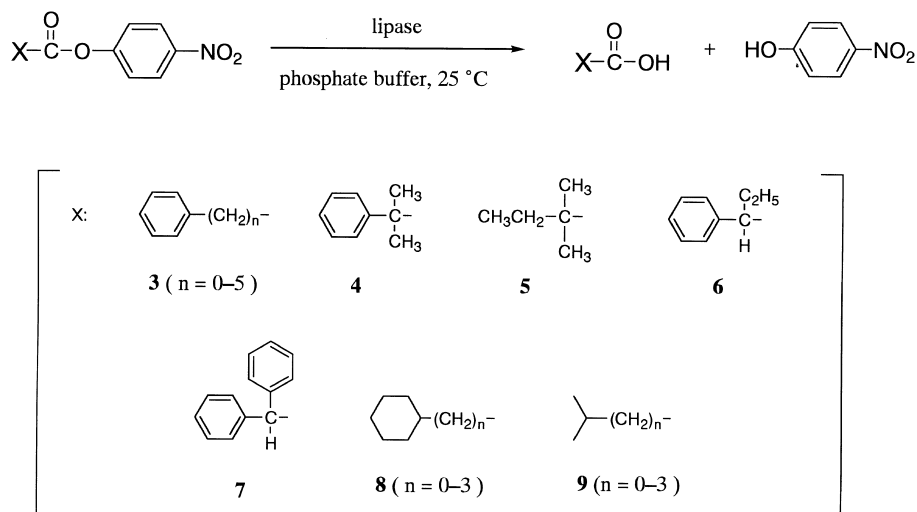


Fig. 2. CRL-catalyzed hydrolysis of synthesized *p*-nitrophenyl esters.

seconds. The absorbance was immediately monitored on a spectrophotometer (U-2000, HITACHI, Japan or V-530, JASCO, Japan) at 404 nm. The absorbance was recorded for 120 s and the CRL solution was so diluted in order to be displayed as linear as possible. Activity was calculated by using the change in the initial linear position of the absorbance between 12 and 18 s. One unit was defined as the amount of enzyme that catalyzed the hydrolysis of 1  $\mu$ mol of substrate per min at 25°C. Specific activity was calculated from both the activity obtained above and the concentration of protein and expressed by U/mg. The specific activity was judged to be zero when the absorbance of the reaction mixture was not changed for 30 min.

### 3. Results and discussion

We estimated the rough limitations of the large, medium, and small substituent recognition sites in the CRL (L, M, and S pockets, respectively) by measuring the specific activity against each ester (**3**–**7**) as shown in Fig. 2. Whether or not the enzymatic reaction proceeds at all rather than the relative values of the specific activity might well be the important fact. All the values of specific activity toward the esters are listed in Table 1.

Ester **3** ( $n = 1$ ) was hydrolyzed with 0.89 U/mg of specific activity. This was interpreted in terms of the phenyl group being recognized as a large substituent and accommodated in the L pocket. The two

protons at the benzylic position would be incorporated one into the M and the other into the S pocket. Because, as reported, [4] 2-phenyl-propanoic acid chloroethyl ester was stereoselectively hydrolyzed, it was concluded that the L, M, and S pockets accommodated the phenyl, methyl, and hydrogen, respectively [4]. *p*-Nitro-phenyl 2-phenylpropanoate was hydrolyzed at a reasonable rate with 0.30 U/mg of specific activity. However, the hydrolysis of esters **4**, **6**, and **7** did not proceed at all (see Table 1). The fact that ester **7** was inert to CRL implied that the phenyl group was larger than the M pocket and this group could be accommodated only by the L pocket. The inactivity of ester **4** indicated that the methyl group was larger than the S pocket. This was confirmed also by the absence of any CRL-catalyzed hydrolysis of compound **5**. Because ester **6** was not hydrolyzed at all, the ethyl group was judged to be larger than the M pocket. From these results, the relative size of each substituent recognition site of CRL was assessed as follows; L pocket > phenyl, ethyl > M pocket > methyl > S pocket > H.

We also examined the hydrolyses of these model esters with five commercially available hydrolytic enzymes. The hydrolyses of esters **4**–**7** did not proceed with CAL, PFL, PCL, and PPL but did proceed with PLE. The specific activities of the PLE to esters **4**, **5**, **6**, and **7** were 0.03, 0.41, 0.09, and 0.02 U/mg, respectively. Thus, PLE was able to hydrolyze these esters, though the specific activities were very low. From these results, it was judged that PLE would have a slightly larger active site than the other hydrolytic enzymes studied.

An X-ray structure of a CRL-inhibitor complex demonstrates that in the active site, CRL has a tunnel as a pocket for discriminating a large substituent and that the scissile fatty acyl chain can be bound in the long, narrow, hydrophobic tunnel [11,15]. Therefore, we tried to hydrolyze a series of *p*-nitrophenyl esters of  $\omega$ -substituted fatty acids, **3**, **8**, and **9**, and to elucidate the structure of the tunnel from the observed specific activities (see Fig. 3) [15,17].

In this series, *p*-nitrophenyl esters having one methylene between the ester-carbonyl carbon and phenyl, cyclohexyl, and iso-type moieties demonstrated large drops in hydrolytic activity. In particular, the cyclohexylacetic acid ester **8** ( $n = 1$ ) was not hydrolyzed at all by CRL.

Table 1  
Specific activity of CRL-catalyzed hydrolysis toward *p*-nitrophenyl esters

Ester	Specific activity <sup>a</sup>	Ester	Specific activity <sup>a</sup>
<b>3</b> ( $n = 0$ )	21	<b>7</b>	0
( $n = 1$ )	0.89	<b>8</b> ( $n = 0$ )	17
( $n = 2$ )	5.0	( $n = 1$ )	0
( $n = 3$ )	9.5	( $n = 2$ )	3.9
( $n = 4$ )	24	( $n = 3$ )	5.4
( $n = 5$ )	5.4	<b>9</b> ( $n = 0$ )	12.8
<b>4</b>	0	( $n = 1$ )	0.95
<b>5</b>	0	( $n = 2$ )	18
<b>6</b>	0	( $n = 3$ )	3.4

<sup>a</sup>Specific activity expressed as U/mg.

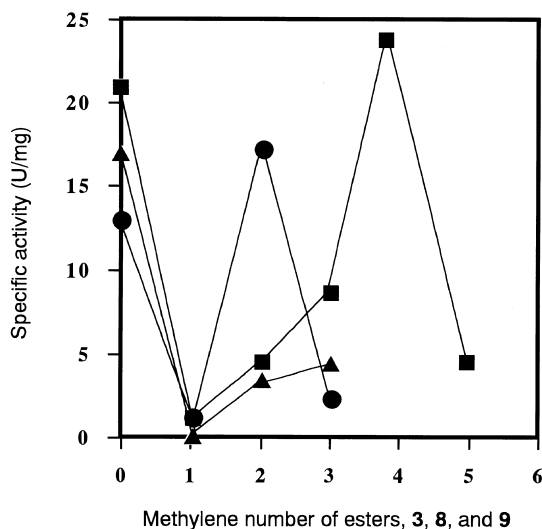


Fig. 3. Specific activity against *p*-nitrophenyl esters of  $\omega$ -substituted fatty acids. Symbols: ■ ester 3; ▲, ester 8; and ●, ester 9.

The reason for this could be explained by molecular modeling. The X-ray structure of the CRL *O*-methyl hexylphosphonochloridate (MPC) complex is

known [15]. This is a model of the transition state of the acylating step. By superimposing the cyclohexylmethyl moiety of ester 8 ( $n = 1$ ) instead of the hexyl group, the energy of the CRL–cyclohexylmethyl phosphonate complex was minimized using the QUANTA/CHARM program [15] revealing that the cyclohexane ring could not avoid the conflict with the amino acid residues, Leu 304, Phe 415, 125, and Met 213, in the tunnel (Fig. 4) explaining why the ester 8 ( $n = 1$ ) was not hydrolyzed by the CRL catalyst.

Because the hydrolysis proceeded with 3 ( $n = 1$ ) and 9 ( $n = 1$ ) albeit with extremely slow reaction rates (0.89 and 0.95 U/mg specific activities, respectively), the  $\omega$ -substituted part in these esters could not be in complete conflict with the amino acid residues contrary to the case of 8 ( $n = 1$ ). Thus, the reason for the large drops in specific activity of compounds 3 ( $n = 1$ ) and 9 ( $n = 1$ ) could be explained by their  $\omega$ -moieties being positioned so that they interact unfavorably with the amino acid residues in the tunnel in the acylating step. On the other hand, esters 3 ( $n = 2–5$ ), 8 ( $n = 2, 3$ ), and 9 ( $n = 2, 3$ ) were hydrolyzed with CRL, but they might leave the

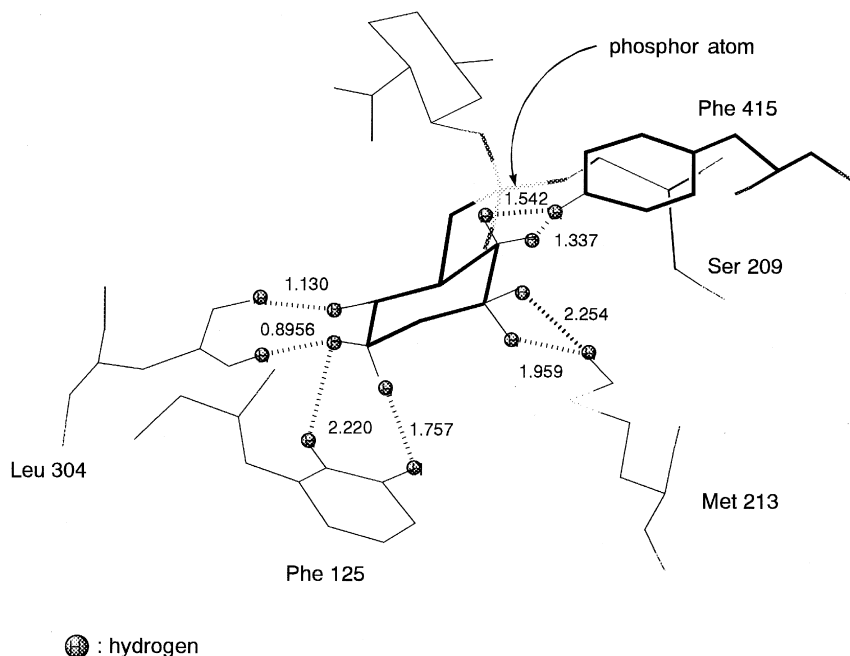


Fig. 4. QUANTA plot of MPC in CRL replaced by cyclohexylmethyl group instead of the hexyl group at phosphor atom. Distance under 2.1 Å means each hydrogen's confliction.

tunnel empty and be bound outside the tunnel in the active site according to the “Hairpin binding mode” proposed by Berglund et al. [18]. From our results and from the known structure of the tunnel in the CRL, it can be concluded that the entrance of the tunnel is narrow relative to the inside of the tunnel.

In this paper, the relative size of the substituent recognition site and a feature of the tunnel part in the CRL were clarified to some degree. We will further investigate the detailed size of each pocket through enzymatic reactions and the relationship between the relative size of the substituents and both the reactivity and stereoselectivity.

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