CL-150667 Received: July 14, 2015 | Accepted: July 24, 2015 | Web Released: October 5, 2015

Editor's Choice

Chemical Modification of Lipase for Rational Enhancement of Enantioselectivity

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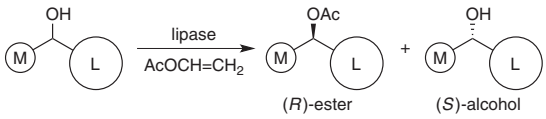
Chemical modifications of the I287C mutant of a *Burkholderia cepacia* lipase afforded various I287C-X conjugates, among which I287C-PAA bearing an *N*-phenylacetamide (PAA) moiety showed excellent enantioselectivity and catalytic activity for secondary alcohols. Site-directed chemical modifications are powerful tools to control enantioselective biocatalysis.

Lipases are useful biocatalysts that show high catalytic activity and enantioselectivity especially for secondary alcohols under mild reaction conditions (Scheme 1).[1] Unlike other enzymes, lipases can work in both aqueous and non-aqueous media. Obviously, lipases are one of the most useful biocatalysts. However, they do not always give satisfactory results toward unnatural substrates. In such cases, the solvent,[2] temperature,[3] acylating agent,[4] and enzyme structure[5] are changed to gain better outcomes. Figure 1 shows a transition-state model proposed to rationalize the high enantioselectivity (*R*-preference) of lipases for a wide range of secondary alcohols (Scheme 1).[6] Based on this transition-state model, we have rationally altered the structure of a lipase. For example, the I287F variant of a *Burkholderia cepacia* lipase showed higher enantioselectivity for 1-phenylethanol than the wild-type enzyme.[7a] Further studies



Scheme 1. Lipase-catalyzed kinetic resolution of secondary alcohols.

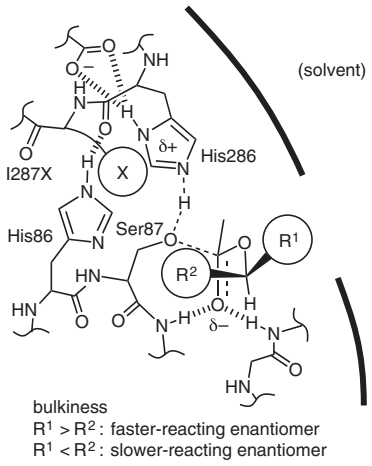


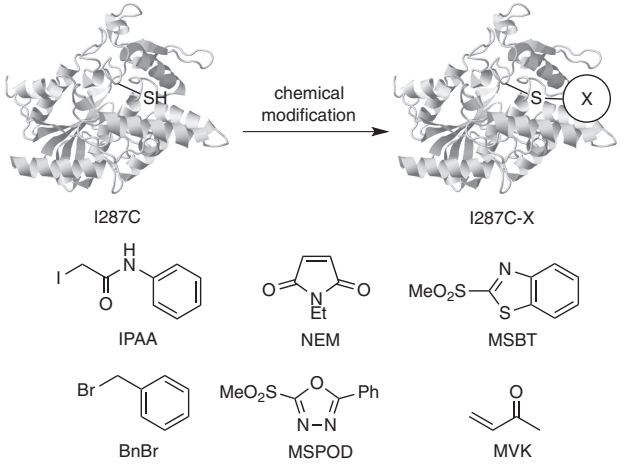
Figure 1. Transition-state model for lipases toward secondary alcohols, where residue 287 is added to the original version.

also allowed us to create variants that are superior to the wild-type enzyme.[7b,7c]

Random or site-directed mutagenesis is certainly the most effective method for the alteration of enzyme structures, leading to the improvement of enzymatic functions such as catalytic activity and/or enantioselectivity.[5,7] However, only twenty natural amino acids can be introduced by mutagenesis, which restricts diversity. With this limitation in mind, we decided to investigate the potential of chemical modifications. Chemical modifications can introduce various organic groups into enzymes to give a variety of hybrid biocatalysts, some of which may fit a particular type of substrate. A number of methods for chemical modifications have been developed.[8,9] Among them, we focused on cysteine-specific chemical modifications.[9] The thiol group of the cysteine residue is reactive for nucleophilic aromatic substitution (S_NAr),[10] conjugate addition,[11] the radical reaction,[12] disulfide bond formation,[8a,9] and S_N2 reaction.[9,13] The high selectivity of the thiol group in these reactions should be useful for the creation of hybrid biocatalysts with improved enantioselectivity. It is a challenge to rationally improve or control the enantioselectivity of enzymes by chemical modifications because successful examples of rational alterations are limited.[8a,14] Here we employed an I287C variant of a *Burkholderia cepacia* lipase because position 287 is a hot spot for the rational control of enantioselectivity for secondary alcohols (Figure 1).[6,7] The single I287C mutant was used to prepare several chemically modified lipases (I287C-X), and the effects of chemical modifications on catalytic activity and enantioselectivity were examined. Among them, I287C-PAA with an *N*-phenylacetamide (PAA) moiety showed excellent enantioselectivity and catalytic activity for secondary alcohols.

We newly prepared the I287C variant of the lipase from *Burkholderia cepacia* (NBRC 14595) via site-directed mutagenesis, heterologous expression, in vitro refolding, and chromatographic purification.[7] The catalytic activity of this I287C variant was slightly lower than that of the wild-type enzyme but was comparable to that of the I287A variant.[7] This result strongly suggests that an activator protein, which was produced and mixed with the denatured lipase according to the previously reported protocol,[7] achieved the successful refolding of the lipase including a disulfide bond formation. Therefore, among the three cysteine residues in the I287C variant, Cys287 bears a free thiol group while the other two cysteine residues (Cys190 and Cys270) are properly connected to each other via a disulfide bond.

The I287C variant was subjected to chemical modifications with six agents (IPAA, NEM, MSBT, BnBr, MSPOD, and MVK) (Scheme 2). Except for MVK, a solution of the chemical modification agent in DMSO was added to a solution of the I287C mutant in 10 mM phosphate buffer (pH 7.0), while MVK,



Scheme 2. Preparation of I287C-X with chemical modification agents.

Table 1. ESI-MS of altered lipases

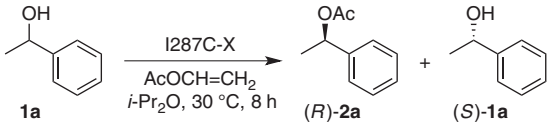
Entry	I287C-X	Expected value[a]	Observed value[b]
1	I287C	33277	33277
2	I287C-PAA	33410	33410
3	I287C-NEM	33402	33404
4	I287C-BT	33410	33410
5	I287C-Bn	33367	$33277 + 90n$ ($n = 1$–6)
6	I287C-POD	33421	$33277 + 144n$ ($n = 1$–5)
7	I287C-MVK	33347	$33277 + 70n$ ($n = 19$)

[a]The values expected for the protein without the N-terminal Met. [b]The values obtained by the deconvoluted mass spectra.

which is water soluble, was directly added to the lipase solution. The mixture was stirred at 4 °C or room temperature for several hours. After a small amount of sample was taken for the ESI-MS analysis, the chemically modified lipase was immobilized on Toyonite-200M, a porous ceramic support with an organic group at the surface, according to the previously reported procedure.[7] The analytical sample was desalted, concentrated, and then analyzed by the ESI-MS measurement. The results are summarized in Table 1. The I287C mutant showed an expected peak at 33277 (Entry 1), while I287C-PAA had an expected peak at 33410 (Entry 2), which clearly indicates the successful formation of the I287C-PAA conjugate. I287C-NEM and I287C-BT exhibited an expected peak indicating the formation of the corresponding conjugate (Entries 3 and 4) although a peak of the unreacted I287C mutant was also observed at 33277. On the other hand, I287C-Bn showed peaks suggesting that several (one to six) benzyl groups were attached (Entry 5), while I287C-POD also exhibited peaks suggesting that several (one to five) 5-phenyl-1,3,4-oxadiazole (POD) moieties were attached (Entry 6). Interestingly, I287C-MVK had a peak that was greater than expected by 1260, which suggests that nineteen MVK moieties were attached (Entry 7). It is most likely that BnBr, MSPOD, and MVK were not cysteine-selective but reacted nonselectively with several amino acid residues.

We next investigated the abilities of the chemically modified lipases (I287C-X) as biocatalysts. The lipase-catalyzed kinetic resolution of 1-phenylethanol (**1a**) was conducted with vinyl acetate in dry *i*-Pr_2O at 30 °C for 8 h. The I287C variant was

Table 2. Kinetic resolution of **1a** with altered lipases[a]



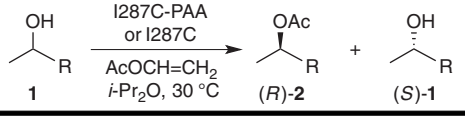
Entry	I287C-X	c/%[b]	E
1	I287C	20	5
2	I287C-PAA	41	29
3	I287C-NEM	24	20
4	I287C-BT	36	16
5	I287C-Bn	47	11
6	I287C-POD	34	6
7	I287C-MVK	34	38

[a]Conditions: lipase I287C-X (200 mg, 0.5% (w/w) enzyme/Toyonite-200M), **1a** (0.50 mmol), vinyl acetate (1.0 mmol), molecular sieves 3A (3 pieces), dry *i*-Pr_2O (5 mL), 30 °C, 8 h. [b]Conversion.

used for comparison. The enantioselectivity was evaluated by the E value.[15] The results are shown in Table 2. The I287C mutant gave a 20% conversion and an E value of 5 (Entry 1). This low E value is reasonable because the steric hindrance that is important for enantioselectivity (Figure 1) is decreased by the replacement of Ile287 with less bulky cysteine. In contrast, to our delight, I287C-PAA gave an E value of 29, which is almost six times higher than that of the I287C mutant (Entry 2). This change of the E value from 5 to 29 amounts to an energetic difference of 1.0 kcal mol^{-1},[7c] clearly indicating the effectiveness of the chemical modification. Furthermore, I287C-PAA showed a twofold conversion (41%) as compared with the I287C mutant (20%), which indicates the enhanced activity of I287C-PAA for **1a**. The bulky organic group at position 287 may suppress the fluctuation of the catalytic residue His286 to enhance the catalytic activity (Figure 1). I287C-NEM and I287C-BT also showed three to four times greater E values than the I287C mutant (Entries 3 and 4). I287C-Bn showed twofold E value (Entry 5), whereas I287C-POD showed no enhancement in the E value (Entry 6). I287C-MVK with nineteen MVK moieties exhibited seven times higher E value than the I287C mutant, which is an unexpected but interesting result although the effect of each MVK moiety attached to the protein is unclear (Entry 7). All the results strongly suggest that the organic groups introduced into Cys287 acted as stereochemical controllers.

In view of the above results, we selected I287C-PAA as the best hybrid biocatalyst and examined the substrate scope of I287C-PAA. The results are shown in Table 3. Surprisingly, in all cases for **1a**–**1g**, I287C-PAA gave higher E values than the I287C mutant (Entries 1–7). In particular, I287C-PAA exhibited excellent enantioselectivity ($E > 200$) for **1b**, **1d**, **1f**, and **1g**. These results clearly demonstrate that I287C-PAA is an excellent hybrid biocatalyst although it was created by a simple chemical modification. Although I287C-PAA exhibited a higher E value for **1c** than the I287C mutant, I287C-PAA showed a lower conversion than the I287C mutant. I287C-PAA also showed good enantioselectivity for **1e**. The fact that I287C-PAA showed higher enantioselectivity for **1b**–**1g** than for **1a** can be explained by an increased steric hindrance between the PAA moiety of lipase and the more bulky substituent of the (*S*)-enantiomer of **1b**–**1g** (Figure 1). The reaction time for **1b** was shorter than that for **1a** probably because the nucleophilicity of the hydroxy

Table 3. Comparison between I287C-PAA and I287C in the kinetic resolution of **1**[a]



Entry	1	R	Time /h	I287C-PAA		I287C	
				c/%[b]	*E*	*c*/%[b]	*E*
1	**1a**	Ph	8	41	29	20	5
2	**1b**	4-Me-C_6H_4	5	44	>200	34	34
3	**1c**	3-Me-C_6H_4	19	18	102	50	8
4	**1d**	4-Br-C_6H_4	10	50	>200	47	88
5	**1e**	3-Br-C_6H_4	9	43	111	36	7
6	**1f**	2-naphthyl	13	35	>200	30	79
7	**1g**	$(CH_2)_2$Ph	5	44	>200	34	126

[a]Conditions: I287C-PAA or I287C (200 mg, 0.5% (w/w) enzyme/Toyonite-200M), **1** (0.50 mmol), vinyl acetate (1.0 mmol), molecular sieves 3A (3 pieces), dry *i*-Pr_2O (5 mL), 30 °C. [b]Conversion.

group of **1b** was increased by the electron-donating methyl group. It was also found that **1c** and **1e** with the substituent at the meta position were less enantioselective than **1b** and **1d** with the substituent at the para position. In addition, I287C-PAA took a longer reaction time for **1c** than for **1a**. Probably, the PAA moiety in I287C-PAA has a bad contact with the substituent at the meta position of the (*R*)-enantiomers. **1f** with the more bulky substituent (naphthyl group) took a longer reaction time probably because of steric repulsion with the lipase. On the whole, I287C-PAA showed higher enantioselectivity and catalytic activity than the I287C variant, and the enantioselectivity was well controlled by the chemical modification of the hot spot residue at position 287. Although I287C-PAA was less enantioselective for **1a** ($E = 29$) than the wild-type enzyme ($E = 68$),[7c] the enzymatic function of I287C-PAA for **1b**–**1g** reached a practical level ($E > 100$).

In summary, we have chemically modified a *Burkholderia cepacia* lipase to create various hybrid biocatalysts. Among them, the I287C-PAA conjugate, which was prepared by the treatment of the I287C variant with 2-iodo-*N*-phenylacetamide, showed excellent enantioselectivity for secondary alcohols. Site-directed chemical modifications are powerful tools to control enantioselective biocatalysis. This method will be useful even when it is combined with directed evolution using random mutagenesis.

This work was partly supported by JSPS KAKENHI Grant Number 25288078. We are grateful to the SC-NMR Laboratory of Okayama University for the measurement of NMR spectra. We are also grateful to Ms. Tsugumi Shiokawa and Dr. Hiroko Tada at Division of Instrumental Analysis for the ESI-MS measurements.

Supporting Information is available electronically on J-STAGE.

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