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Enantioselectivity and catalysis improvements of *Pseudomonas cepacia* lipase with Tyr and Asp modification†

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A concise strategy to improve the *p*-NPP (*p*-nitrophenyl palmitate) catalytic activity and enantioselectivity towards secondary alcohols of *Pseudomonas cepacia* lipase (*Pc*L) has been described. The *Pc*L was modified by I_3^-, *N*-acetyl imidazole (NAI), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) and ethylenediamine (EDA) in the absence or presence of *n*-hexane, respectively. After being modified by the four modification reagents, the enantioselectivity (*E* value) of the *Pc*L towards secondary alcohols was enhanced by 2- to 4-fold. The catalytic activity of EDA-*Pc*L was increased by about 6-fold. The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis of modified *Pc*L showed that Tyr^4, Tyr^{29}, Tyr^{45}, Tyr^{95}, Asp^{36} and Asp^{55} were the modified sites. When Tyr^{29} was modified, the *E* value of *Pc*L towards secondary alcohols was largely improved. MALDI-TOF-MS characterization and molecular dynamics simulation of the lipase indicated that Tyr^{29} located inside the catalytic cavity had a significant impact on the *E* value. The strong steric hindrance of acetyl and iodine ion to the groups on the chiral center of the substrates is responsible for the improvement. In addition, the enhancement of hydrophobicity on the surface of the lipase due to the sidechain replacement of Asp with uncharged hydrophobic groups also improved the *E* value.

Introduction

Enantiomerically pure chemicals, such as secondary alcohols, play significant roles in pharmaceuticals, agrochemicals, flavors and fragrances.[1,2] Biocatalysis is an economic and green approach for chiral resolution of a racemic mixture,[3] in which lipase (triacylglycerol ester hydrolases, EC 3.1.1.3) is among the most attractive targets.[4–7] Kazlauskas *et al.* investigated lipase-catalyzed resolution towards 94-pair enantiomers of secondary alcohols and proposed the well-known empirical law of lipase's enantiopreference: the enzyme's stereospecific catalytic cavity contains large and small pockets, and the preferred enantiomer's large and small groups on the chiral center fit better into the corresponding pocket.[8] However, when the substituents on the chiral center are not suitable for the corresponding pocket, the yields of optically pure compounds are usually not optimal.[4,6]

Chemical modification is an alternative choice besides DNA-based techniques, which are typically limited to the 20 primary proteinogenic amino acids. Chemical modification of enzymes allows an almost unlimited variety of groups to be introduced with easily obtained reagents and succinct operations.[9,10] Therefore, chemical modification of proteins has long been utilized to investigate proteins and is a rapidly expanding area in chemical biology. Previous studies showed that chemical modification could change substrate preference and specificity,[11–13] affect the activity,[14] identify key amino acid residues[15–17] and improve stability and resistance to proteases.[18–20] The enantioselectivity (*E* value) of lipases from *Candida rugosa* and *Pseudomonas cepacia* (*Cr*L and *Pc*L) increased by 2- and 1.3-fold, respectively, as Kim *et al.*[21] and Ueji *et al.*[22] described, with no significant improvement or negative effect on the catalytic activity of lipases. In another case, Bianchi *et al.* decreased the *E* value of *Pc*L by free amino group acetylation coupled with an enhanced catalytic activity.[23] A recent work improved the *E* value by 1.9- and 2.7-fold through immobilization of *Cr*L on two types of costly nanoparticles with the catalytic activity improved subtly.[24]

We previously proposed a mechanism of enantioselectivity towards primary alcohols by lipase from *Pseudomonas cepacia*. Chemical modification on the lipase and molecular dynamics simulations indicated that Tyr^{29} located within the catalytic cavity affected the enantioselectivity significantly *via*

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the hydrogen bond with the $O^{non\text{-}\alpha}$ of the substrate.[25] In the present study, a combination of chemical modification, molecular dynamics simulation and MALDI-TOF-MS was employed to shed light on effects of chemical modification on the *p*-NPP catalytic activity and enantioselectivity towards secondary alcohols of *Pc*L. Enantioselectivity catalyzed by modified *Pc*L towards 5 pairs of different chiral secondary alcohol esters was improved by 2- to 4-fold and the catalytic activity of EDA-*Pc*L increased by about 6-fold. Since lipases are widely known to be activated at the aqueous–hydrophobic interface, *n*-hexane was employed as the activator of *Pc*L in this study.[26,27]

Experimental

Materials

Butan-2-ol, hexan-2-ol, octan-2-ol, NAI, NaI, I_2, EDA and EDC (Fig. 1) were purchased from Tokyo Chemical Industry Co. Ltd. Trypsin and *Pc*L were obtained from Amano Enzyme Inc. Other chemical reagents were of analytical grade.

Synthesis of substrates

Esters of secondary alcohols were synthesized from their corresponding alcohol in our lab following the procedure given.[25] Alcohol and triethylamine (molar ratio = 1 : 1.5) were mixed in an ice bath. Acyl chloride was slowly added next. The final products were washed with saturated sodium bicarbonate solution several times. The pure products were obtained by reduced pressure distillation.

Chemical modification of Tyr and Asp/Glu

Modification of Tyr was carried out using NAI and I_3^- in the procedure described by Cacace *et al.*[28] Asp/Glu modification was performed by employing EDA and EDC following the strategies of Barbosa.[29] According to the standard reaction conditions of the chemical modification methods, 10% (*V*/*V*) of *n*-hexane was added to the reaction mixture as the activator.

Lipase assay in aqueous solution

The activity of native and modified *Pc*L was assayed by employing the well-known *p*-nitrophenyl palmitate (*p*-NPP).[30] The basis of this assay protocol was the estimation of *para*-nitrophenol (*p*-NP) released as a result of enzymatic hydrolysis of *p*-NPP at 410 nm. The enzyme activity was defined as the amount of protein liberating 1 μmol of *p*-nitrophenol per minute.

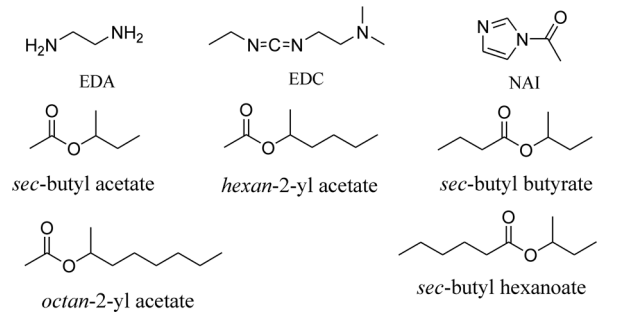

Fig. 1 Structures of modification reagents and substrates.

Hydrolysis of racemic esters

A mixed solution of *Pc*L (10 mg mL^{-1}), substrates (esters of secondary alcohols, 100 mM) and PBS (100 mM, pH 7.0, including 10% *V*/*V* isopropanol as co-solvent) was incubated at 27 °C under 200 rpm stirring. All the conversion ratios were controlled between 10% and 20%.[31] The substrate and the product were extracted from the reaction mixture with equal volume of *n*-hexane and dried with sodium sulfate (Na_2SO_4), then analyzed by gas chromatography (GC).

Gas chromatography

All the samples extracted from the hydrolysis reaction were injected into an Agilent 6890® GC instrument (Agilent Inc.) equipped with a CP-cyclodextrin-β-2,3,6-M-19, FS 50 m × 0.25 mm column with a film thickness of 0.25 μm (Varian Inc.) and a flame-ionization detector for analysis. Nitrogen was utilized as the carrier gas. Both of the injection and detector temperatures were 260 °C.

One-dimension SDS-PAGE and lipase digestion with trypsin in a gel

SDS-PAGE was performed according to the method of Das.[32] After recovery and decolorization of the lipase, 40 ng of trypsin was added. Digestion was performed at 37 °C overnight. The peptides were extracted by water with 5% TFA, then lyophilized using a Labconco FreeZone 18 freeze drier (Labconco Corporation, Kansas City, MO, USA) vacuum concentrator centrifuge.

MALDI-TOF-MS analysis of modified *Pc*L

All MALDI-TOF-MS analyses were performed using a Bruker ultrafleXtreme™ TOF/TOF system mass spectrometer equipped with a modified Nd:YAG laser in positive ion mode, with data acquisition using the Bruker flexControl 3.4 (Germany). Saturated α-cyano-4-hydroxycinnamic acid (HCCA) was chosen as the matrix. The samples were dissolved in 40 μL of water with 0.1% TFA. A portion (1.5 μL) of the sample and matrix (1.5 μL) were spotted on a MALDI target plate sequentially and dried at room temperature prior to MALDI-TOF-MS analysis. Ions were extracted into the mass spectrometer in reflection mode using an extraction potential of 20 kV with a low-mass detection method.

Docking of the substrate and binding free energy calculation

The crystal structure of *Pc*L was obtained from the work of Luis (PDB entry 2NW6).[33] The secondary alcohol esters' enantiomers were docked into the catalytic cavity of *Pc*L using the CDOCKER protocol in Discovery Studio 3.0 software and the complex conformation with the highest score was recorded. The hydroxyl group of Ser87 was dehydrogenated

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manually in the Discovery Studio molecule window. The carboxyl C of the docked molecule was changed from the sp^2 to sp^3 hybridization state and covalently bonded to OG of Ser87. The C═O bond was changed to a C–O single bond, and the carbonyl O was charged with −1. All other parameters were set to the standard values.

All the binding free energies were obtained from the results of molecular dynamic calculations (MD) which were carried out using the Discovery Studio 3.0 program and the CHARMM force field parameters. The simulation was performed over 5 ns at constant temperature and pressure using the Berendsen algorithm with a coupling constant of 2 ps for both parameters. Electrostatic interaction was calculated using the particle mesh Ewald (PME) method with a non-bonded cutoff of 12 Å. The conformations of the system were recorded every 10 ps for further analysis.

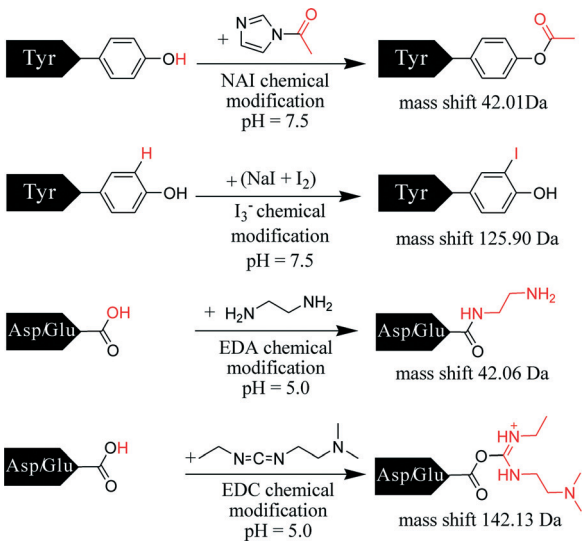
Results and discussion

Chemical modification of *Pc*L

Scheme 1 shows the modification strategies of *Pc*L at Tyr, Asp or Glu residues. NAI reacted with the hydroxyl group of Tyr selectively. The hydrogen atom of the hydroxyl group was substituted by the acetyl and a mass shift of 42.01 Da was detected. When I_3^- was used as the modification reagent, the hydrogen atom at the *ortho* site of the hydroxyl group was replaced by the iodine ion. The mass shift was identified by 125.90 Da. The carboxyl group reacted with EDA to form the respective amide. The mass shift was 42.06 Da. When carboxyl group was reacted with EDC, the hydrogen atom was replaced by CH_3–$N(CH_3)$–CH_2–CH_2–NH–CH═NH–CH_2–CH_3 and the mass shift was 142.13 Da.

Characterization of native and modified *Pc*L by MALDI-TOF-MS

These reactions of chemical modification are typically non-specific in nature and direct identification of modified sites is not convenient.[34,35] As a powerful tool, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) can be used to solve the problem. MALDI-TOF-MS has become a popular method to study peptides, proteins and other organic molecules because of its high mass accuracy, fast analysis speed and simplicity of operation.[36–39] Recently, MALDI-TOF-MS was applied to determine the modification sites of congII and the affinity binding sites of boronic acid-decorated lectins.[40,41]



Scheme 1 Modification strategies of *Pc*L at Tyr, Asp or Glu.

Here MALDI-TOF-MS was used to verify that Tyr and Asp residues were reactive towards NAI, I_3^-, EDC and EDA. Native and modified *Pc*L samples were treated with trypsin before undergoing peptide mass fingerprinting. The theoretical peptide masses taken from native *Pc*L should appear at *m*/*z* 881.411, 1020.572, 1047.507, 1211.638, 1424.760, 1519.746, 1527.858, 1706.897, 2124.994, 2138.160 and 2231.077 (Table S4†). The peptide fragments of residues 1–8, 23–40, 41–61 and 95–115, which contained Tyr^4 (ADNYAATR), Tyr^{29} (YAGVLEYWYGIQEDLQQR), Tyr^{45} (GATVYVANLSGFQSDDGPNGR) and Tyr^{95} (YVAAVAPDLVASVTTIGTPHR), were detected at *m*/*z* 881.416, 2231.074, 2124.995 and 2138.162, in line with the theoretical peptide masses of native *Pc*L (Fig. S2†). The analysis of the solvent accessible surface (ASA) showed that the potential modification sites were Tyr^4, Tyr^{23}, Tyr^{29}, Tyr^{31}, Tyr^{45}, Tyr^{95}, Asp^{36}, Asp^{55}, Asp^{102}, Asp^{303}, Glu^{63} and Asp^{302} (Fig. S1†). As expected, a mass shift of 42.02 Da (corresponding to the weight of the fragment of NAI covalently bonded to Tyr) for peptide fragments of modified *Pc*L with *n*-hexane was found at *m*/*z* 923.436 (Tyr^4), 2273.094 (Tyr^{29}), 2167.015 (Tyr^{45}) and 2180.212 (Tyr^{95}) (Fig. 2). When *n*-hexane was absent, only Tyr^4, Tyr^{45} and Tyr^{95} were modified and the peptide fragments were detected at *m*/*z* 923.429, 2167.027 and 2180.181 (Fig. S7†). Tyr^{45} and Tyr^{95} were modified by I_3^- without *n*-hexane as the activator and a mass shift of 125.90 Da (corresponding to the weight of the iodine atom covalently bonded to Tyr) for the peptide fragments of modified *Pc*L was found at *m*/*z* 2250.897 and 2264.051 (Fig. S3†). After addition of *n*-hexane, one more peptide fragment of modified *Pc*L by I_3^- showed up at *m*/*z* 2356.964 (Fig. S4†), which indicated that Tyr^{29} was modified. In Fig. S5,† Asp^{36} (YAGVLEYWYGIQEDLQQR) of *Pc*L was identified as the site modified by EDA. A mass shift of 41.93 Da for the peptide fragment of modified *Pc*L was detected at *m*/*z* 2273.009. When EDC was the modifier, Asp^{55} (GATVYVANLSGFQSDDGPNGR) was modified and a mass shift of 142.10 Da appeared at *m*/*z* 2267.097 (Fig. S6†). These data confirmed that NAI, I_3^-, EDA and EDC were covalently bonded to Tyr and Asp residues.

Effect of chemical modification on *p*-NPP catalytic activity of *Pc*L

The hydrolysis of *p*-NPP was examined to assess the effect of chemical modification on the catalytic activity of *Pc*L. By varying the molar ratio of the reagent over the lipase, the maximum concentration of the modifier was determined. All the degrees of modification were obtained under the maximal

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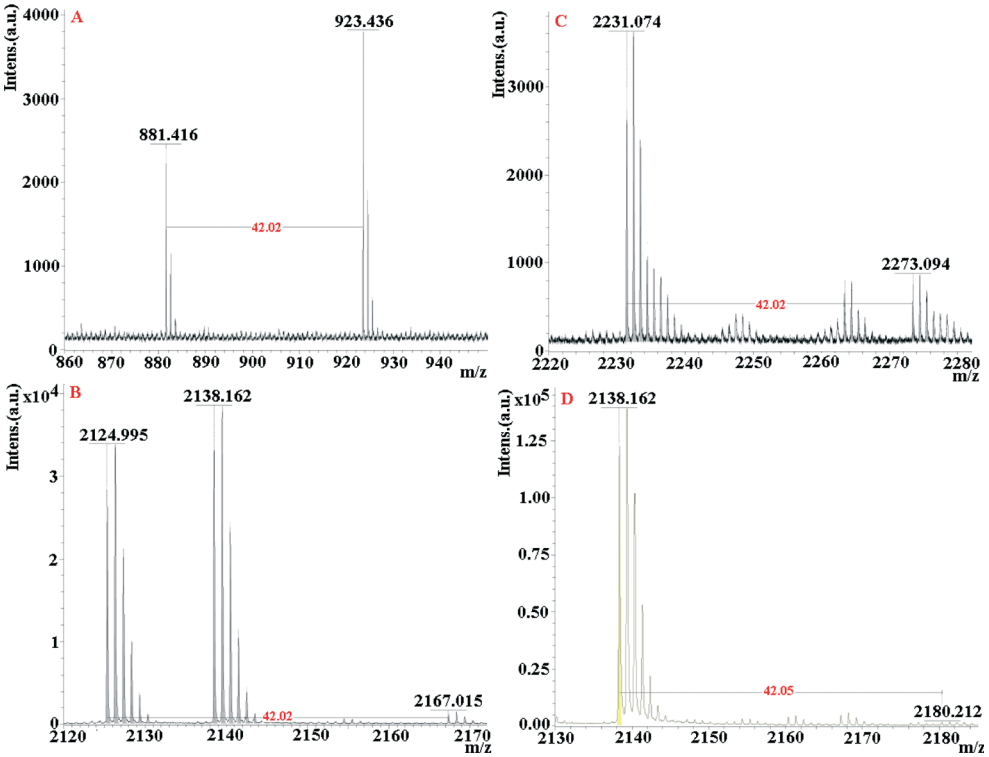

Fig. 2 Expanded MALDI-TOF mass spectra by tryptic digestion of NAI-*Pc*L in the presence of *n*-hexane. (A) Tyr^{4} modification, ADNYAATR, 1–8, *m*/*z* change from 881.416 to 923.436. (B) Tyr^{45} modification, GATVYVANLSGFQSDDGPNGR, 41–61, *m*/*z* change from 2124.995 to 2167.015. (C) Tyr^{29} modification, YAGVLEYWGIQEDLQQR, 23–40, *m*/*z* change from 2231.074 to 2273.094. (D) Tyr^{95} modification, YVAAVAPDLVASVTTIGTPHR, 95–115, *m*/*z* change from 2138.162 to 2180.212.

concentration of the reagents and were calculated according to the strategy described by Pelton *et al.*[42] For all modification reagents, the maximal degree of modification was reached at 50–70% (Table S3†). Fig. 3 shows that *Pc*L is quite sensitive to the four modifiers and the reagents have no effect on the hydrolysis of the substrate when *Pc*L was absent. For NAI-*Pc*L in the absence of *n*-hexane, the relative activity was reduced to about half, whereas for modification in the presence of *n*-hexane, the relative activity of NAI-*Pc*L was almost lost (Fig. 3A). The modification of *Pc*L with I_3^- (Fig. 3B) reduced both of the relative activities in the presence and absence of *n*-hexane. The EDC modification (Fig. 3C) also decreased the relative activity. By contrast, *Pc*L modified by EDA enhanced the relative activity by about 6-fold (Fig. 3D).

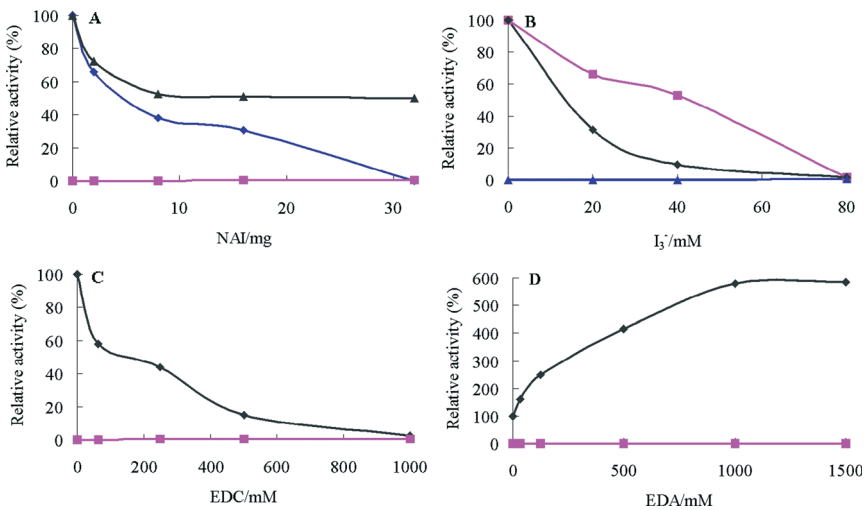

Fig. 3 Effect of chemical modification on relative activity of *Pc*L in the absence or presence of *n*-hexane (100 μL). (A) NAI for (■), NAI + *Pc*L for (▲) and NAI + *Pc*L + *n*-hexane for (♦); (B) I_3^- for (▲), I_3^- + *Pc*L for (♦) and I_3^- + *Pc*L + *n*-hexane for (■); (C) EDC for (■) and EDC + *Pc*L for (♦); (D) EDA for (■) and EDA + *Pc*L for (♦).

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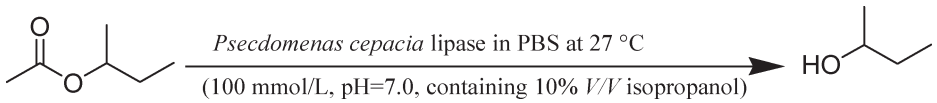
Table 1 The initial rate, enantioselectivity, and the binding free energy for the hydrolysis of *sec*-butyl acetate catalyzed by the native and modified *Pc*L



Entry	Modification reagent	Initial rate (μmol min^{-1})v_R	*E* value	Enantiopreference	Binding free energy (kJ mol^{-1}) $R(\Delta G)$	$S(\Delta G)$	$\lvert\Delta(\Delta G)\rvert$ (kJ mol^{-1})
1	Native *Pc*L	14.0	1.2	*R*	−107.37	−106.74	0.6
2	NAI + *n*-hexane	12.0	3.2	*R*	−113.91	−80.04	33.9
3	NAI alone	13.0	1.2	*R*	−22.27	−21.74	0.5
4	I_3^- + *n*-hexane	8.0	2.0	*R*	−110.91	−93.95	17.0
5	I_3^- alone	9.0	1.8	*R*	−102.57	−97.39	5.2
6	EDA alone	16.0	2.3	*R*	−110.27	−102.26	8.0
7	EDC alone	5.0	3.1	*R*	−87.55	−65.13	22.4

All initial rates of native and modified *Pc*L were given towards the five substrates (Table 1 and S1†). Table 1 and S1† show the initial rate of EDA-*Pc*L, which is higher than the native one.

As shown in Fig. 3A and B, the reduction tendency of the relative activity of NAI- and I_3^--*Pc*L in the absence and presence of *n*-hexane is diverse which indicated that *Pc*L was activated by *n*-hexane and the amino acid residue in the cavity of the catalytic center was modified by NAI and I_3^-. Preliminary studies showed that introduction of more hydrophobic modifiers or the change in conformation was the major cause of hydrolytic activity diversity.[43,44] The latter factor might result in the different catalytic activities of modified *Pc*L in our experiments, especially for the hydrolytic activity increase of EDA-*Pc*L.

Enantioselectivity improvement towards secondary alcohol by chemical modification

*Pc*L-catalyzed resolution of five pairs of different chiral secondary alcohols *via* hydrolysis of their corresponding esters was investigated. Hydrolysis of *sec*-butyl acetate was chosen as a model (Table 1). Both native and modified *Pc*L's enantiopreference was the (*R*)-configuration. Enantioselectivity of the native *Pc*L was poor which was around one (entry 1). When modified by NAI in the absence of *n*-hexane, the *E* value was not improved (entry 3). After addition of *n*-hexane, the modification of *Pc*L with NAI enhanced the *E* value by 3-fold (entry 2). The *E* value was enhanced from 1.2 to 1.8 in the absence of *n*-hexane (entry 5), and 2.0 in the presence of *n*-hexane by I_3^- modification (entry 4). The modification with EDA and EDC also favored an improvement in the *E* value by about 2- and 3-fold (entries 6 and 7), respectively.

NAI- and I_3^--*Pc*L in the presence of *n*-hexane had more influence on the *E* value towards *sec*-butyl acetate because of the modification of the Tyr[29] in the catalytic cavity. From the data listed in Table S3,† the *E* value of *Pc*L depended not only on the nature of the modification group but also on the degree of the modification. The modification degrees of Tyr[29] of NAI- and I_3^--*Pc*L were 19.2 and 12.3 (Table S3†), respectively. It demonstrated that the *E* value increased with an increase in the modification degree. The enhancement of the *E* value catalyzed by EDC- and EDA-*Pc*L is attributed to the replacement of the charged amino acid (aspartic acid residue) on the lipase surface with the uncharged hydrophobic group.[22]

Molecular dynamics study

The complex of *Pc*L and *sec*-butyl acetate was simulated by using molecular dynamics (MD) computation. For native and modified *Pc*L, all binding free energies of the (*R*)-configuration were lower than the (*S*)-configuration (Table 1). After modification, $\lvert\Delta(\Delta G)\rvert$ between (*R*)- and (*S*)-configurations was much higher than that of the native one. That is to say substrates of the (*R*)-configuration were liable to be hydrolyzed. The correlation between $\lvert\Delta(\Delta G)\rvert$ and enantioselectivity corresponding to native and modified *Pc*L at Tyr was given in Fig. 4. The tendency of $\lvert\Delta(\Delta G)\rvert$ was consistent with the enantioselectivity. The $\lvert\Delta(\Delta G)\rvert$ and enantioselectivity were almost a linear relation (R^2 = 0.9566). It indicated that the enantioselectivity was highly correlated with $\lvert\Delta(\Delta G)\rvert$. Towards the other four substrates, *sec*-butyl butyrate, *sec*-butyl hexanoate, *hexan*-2-yl acetate and *octan*-2-yl acetate, the laws

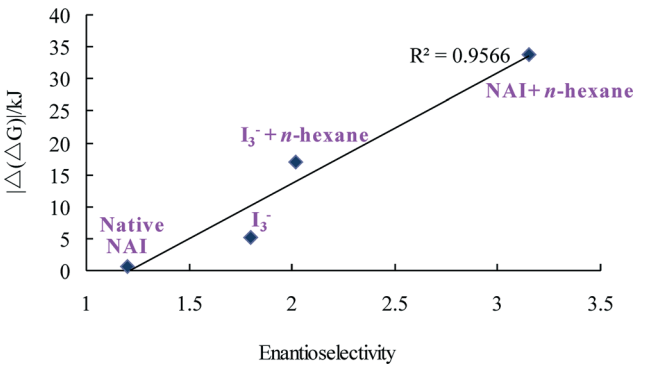


Fig. 4 Correlation of $\lvert\Delta(\Delta G)\rvert$ and enantioselectivity corresponding to native and modified *Pc*L at Tyr (substrate: *sec*-butyl acetate).

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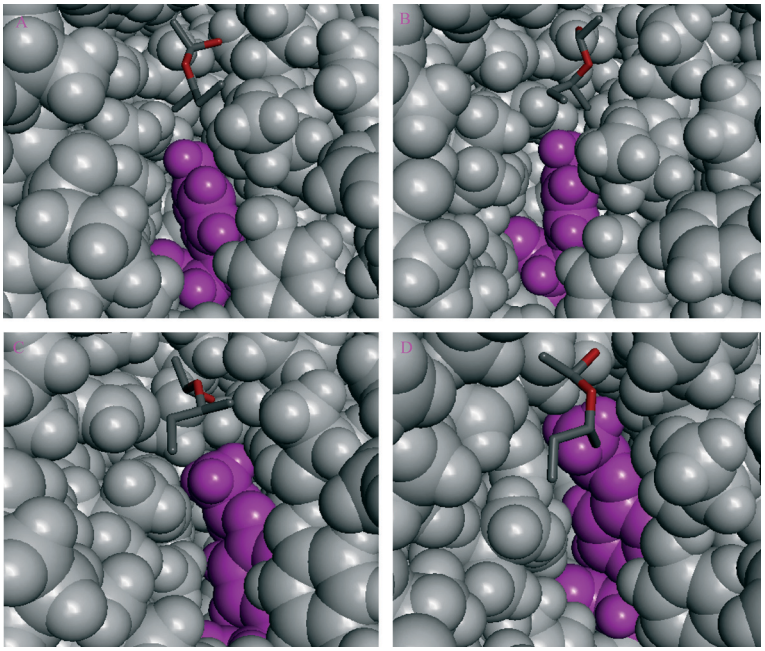

Fig. 5 Conformation of a *sec*-butyl acetate enzyme complex resulting from MD simulation. In the figure, all non-polar hydrogen atoms have been hidden to avoid visual disturbance. (A) (*R*)-*sec*-Butyl acetate + native *Pc*L (purple for unmodified Tyr^{29}). (B) (*S*)-*sec*-Butyl acetate + native *Pc*L (purple for unmodified Tyr^{29}). (C) (*R*)-*sec*-Butyl acetate + modified *Pc*L (purple for Tyr^{29}-*O*-acetylated by NAI). (D) (*S*)-*sec*-Butyl acetate + modified *Pc*L (purple for Tyr^{29}-*O*-acetylated by NAI).

of *E* values (Fig. S8†) and binding free energies were similar to that of the model one (Fig. 4). The experimental results were approved by the MD data.

Mechanism discussion of enantioselectivity improvement

Our experimental results are well in line with Kazlauskas's law and indicated that chemical modification was an effective method to improve the enantioselectivity of *Pc*L. According to Kazlauskas's work, enantioselectivity was related to the size difference between groups directly connected to the chiral center of the substrate.[8]

Before modification, the size difference between methyl and ethyl was not so big that (*R*)-configuration enantioselectivity was poor (Table 1). The (*R*)-*sec*-butyl acetate enantiopreference was confirmed by the lower binding free energy of the (*R*)-*sec*-butyl acetate (Table 1). MD simulation revealed that the small substituent on the chiral center, methyl, and the large substituent, ethyl of the (*R*)-*sec*-butyl acetate, fit better into the corresponding pocket (Fig. 5A and B). Because the groups on the chiral center were the same with *sec*-butyl acetate, the results above can be applied to interpret the phenomena of the other two substrates, *sec*-butyl butyrate and *sec*-butyl hexanoate catalyzed by native *Pc*L (Tables S1 and S2†). When the substrates were *hexan*-2-yl acetate and *octan*-2-yl acetate, (*R*)-configuration enantioselectivity was enhanced with the size difference of the groups on the chiral center increase (Tables S1 and S2†).

The enantioselectivity of modified lipase depended on the nature of the modification group. When the hydrogen atom at the hydrophobic side chain of Tyr^{4}, Tyr^{45} and Tyr^{95} was replaced by a larger hydrophobic group, acetyl, on the surface of *Pc*L in the absence of *n*-hexane, the *E* value was not improved. The reason was that the hydrophobic side chains of Tyr were folded back on to the modified *Pc*L's surface in aqueous conditions and could not extend fully. The conformation or flexibility of modified *Pc*L slightly changed so that the *E* value was not enhanced. On the other hand, the *E* value can be improved by the replacement of the charged amino acid on the lipase surface with the uncharged hydrophobic group in most cases. However, Tyr was the uncharged amino acid. Instead, in the presence of *n*-hexane, Tyr^{29} in the catalytic cavity was acetylated which led to an increase in the *E* value. The modification group, acetyl, facilitated a much stronger steric hindrance to methyl than ethyl (Fig. 5C and D). It made the (*R*)-*sec*-butyl acetate combination to the enzyme more convenient and hydrolysis reaction much faster. Because iodide ion (diameter: 440 pm) was smaller than acetyl (diameter: 893 pm), its steric hindrance to the methyl group was not as strong as that to the acetyl group. Thus, whenever the modification process was in the presence or absence of *n*-hexane, the *E* value changed slightly (Table 1 and Table S1†).

Conclusions

In conclusion, this study demonstrated that *Pc*L was modified through a one-step reaction by NAI, I_3^-, EDA and EDC. This method successfully improved the *p*-NPP catalytic activity and enantioselectivity towards secondary alcohols of

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*Pc*L. The modified sites were identified by MALDI-TOF-MS and it was indicated that Tyr^{29} was critical for the improvement of enantioselectivity catalyzed by *Pc*L. Chemical modification *in vitro* is an effective method to modify a protein and further to modulate its function. In addition, the investigation confirms the crucial roles of a steric hindrance. This cost-effective and environmentally friendly method is promising for the resolution of chiral compounds in many industrial fields.

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