Journal of Molecular Catalysis B: Enzymatic 68 (2011) 286–291

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Improving the catalytic activity of lipase LipK107 from *Proteus* sp. by site-directed mutagenesis in the lid domain based on computer simulation

Bei Gao[a], Tao Xu[a], Jinping Lin[a], Lujia Zhang[a], Erzheng Su[a], Zhengbing Jiang[b], Dongzhi Wei[a,*]

[a] State Key Laboratory of Bioreactor Engineering, New World Institute of Biotechnology, East China University of Science and Technology, Shanghai 200237, PR China
[b] College of Life Science, Hubei University, Wuhan 430062, PR China

ARTICLE INFO

Article history:
Received 21 July 2010
Received in revised form 1 December 2010
Accepted 1 December 2010
Available online 9 December 2010

Keywords:
LipK107
1-Phenylethanol
Lid
Site-directed mutagenesis
Computer simulation

ABSTRACT

The capacity of lipase LipK107 from *Proteus* sp. catalyzing the kinetic resolution of racemates was investigated. The resolution of racemic 1-phenylethanol in organic medium was selected as model reaction. The conversion was dramatically dependent on the water content and the LipK107 showed high activity in a wide range of water content without appreciable loss of enzyme enantiodiscrimination. Besides, the chain length of acyl donor also had a significant effect on the conversion, and the highest enantioselectivity was achieved when methyl palmitate was used. Based on the analysis of computer model structure of LipK107, different mutations were introduced into the lid region. Each derivative of LipK107 was expressed, purified, and assessed of the activity. According to the prediction, using mutants E130L + K131I and T138V as catalyst, respectively, the conversions of 1-phenylethanol improved greatly with a slight increase of enantiodiscrimination. In addition, the effects of hydrophobicity and electrostatic of the lid on lipase activity were determined. This work indicated that the modification of the lid might considerably enhance the activity and improve the yield of catalytic reactions, which could apply to other lipases. The computer simulations would make the process of identifying amino acids for substitution efficiently.

1. Introduction

Lipase is a diverse and ubiquitous family of enzymes catalyzing both the hydrolysis and synthesis of various ester compounds [1,2]. Since lipase usually displays exquisite chemoselectivity, regioselectivity and stereoselectivity in nonaqueous reactions, more and more researches have focused their attention on developing lipase into ideal tool for industries [3,4]. Although the organic solvents are the main component in reaction, some water is needed and proved to be crucial [5]. However, the exact role of water is still not very clear so far, and one common hypothesis is that water retains the fundamental role of enzyme in controlling its three-dimensional structure [6,7].

It is well-known that most lipases are activated by the presence of a water–lipid interface. It associates with a conformational change that the lid domain, consisting of at least one α-helix, opens up by rotating around its hinge regions [8,9]. Fluorescence experiments have suggested that the lid open conformation is stabilized by a combination of hydrophobic and electrostatic interactions between the lid and the lipid assemblies [10,11]. Therefore, the specific residues of the lid might be important for activity and specificity of lipases [12,13]. However, to our knowledge, only a few attempts were made to demonstrate the influence of specific amino acids of the lid on lipase activity, and even fewer studies were focus on modifying the lid to improve the outcome of reactions [14]. One possible reason is that the characterization of the lid and such large conformational transitions remain challenging for experimental methods. Thus, molecular modeling techniques are of utmost interest to access dynamic information on macromolecular systems [15,16].

Molecular modeling is a collective term that refers to theoretical methods and computational techniques to model or mimic the behaviors of molecules. And it has been widely used in the area of biology. For lipases of microbes, a rather simple enzyme without any coenzymes or cofactors, molecular modeling becomes the alternatives to investigate the structure–function relationship [17].

Previously, we cloned the lipase gene *lipK107* from *Proteus* sp. It consisted of 864 bp, and belonged to the Group I Proteobacterial, but showed low identities with other known lipases [18]. In this work, we utilized the LipK107, into the resolution of racemic 1-phenylethanol. Some key parameters were explored. Based on the analysis of computer model structure of LipK107, we introduced mutations into the lid domain and improved the yield. The effect

* Corresponding author. Tel.: +86 21 64252981; fax: +86 21 64250068.
E-mail addresses: beigao@mail.ecust.edu.cn (B. Gao), robertxutao@mail.ecust.edu.cn (T. Xu), jplin@ecust.edu.cn (J. Lin), ljzhang@ecust.edu.cn (L. Zhang), ezhsu@mail.ecust.edu.cn (E. Su), zhbjiang@hubu.edu.cn (Z. Jiang), dzhwei@ecust.edu.cn (D. Wei).

1381-1177/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2010.12.001

Table 1
Oligonucleotides used in this experiment.

Function	Names	Sequences (5′ → 3′)
Primers for the 5′ and 3′ ends of gene *lipK107*	Up	ccggaattcatgtcaacgaaatatcctat
	Down	cccaagcttctttaaagttgcttactcgcta
Primers for mutant E130L + K131I	Mu-1a	catttaatactattaggacaatatattctg
	Mu-1s	atatattgtcctaatagtattaaatgcatt
Primers for mutant T138V	Mu-2a	gcgtgttgagataataacaccaaatgc
	Mu-2s	gcggcatttggtgttattatctcaaca
Primers for mutant I128E + V129D	Mu-3a	gcgtacttttttcgtcttcatattctggg
	Mu-3s	gcgcccagaatatgaagacgaaaaagta
Primers for mutant R120P + K121P	Mu-4a	gcggatactgtctggagccataatgcgt
	Mu-4s	gcgacgcattatggctccagacagtatc
Primers for mutant H146S + R147T	Mu-5a	gcgaggatcgcctgtactgccagaaaat
	Mu-5s	gcgattttctggcagtacaggcgatcct

of the characteristics of the lid on the LipK107 activity was also determined.

2. Materials and methods

2.1. Materials

Plasmid pMD19-T (Takara, Dalian, China) and pET-28a (Novagen, Madison, WI) were used as TA cloning vector and expression vector respectively. *Escherichia coli* strains DH5α and BL21 (DE3) were used as the recipient strains for recombinant plasmids. DNA polymerase and restriction enzymes were purchased from Takara. All molecular techniques were performed essentially as described by Sambrook et al. [19].

Racemic 1-phenylethanol, methyl acetate, methyl butyrate, methyl caprylate, methyl dodecanoate, methyl palmitate and methyl arachidate (Sigma, USA) were used for resolution as substrate and acyl donors, respectively. All other chemicals and reagents were obtained commercially and were of analytical grade.

2.2. Site-directed mutagenesis and construction of the recombinant plasmids

Lipase gene *lipK107* (GenBank accession no. EU600201) was cloned from a strain of *Proteus* sp. as reported previously [18]. Five *lipK107* derivatives: E130L + K131I, T138V, I128E + 129D, R120P + K121P, H146S + R147T were constructed using the PCR overlap extension technique [20] with two internal primers carrying the specific mutations, and another two external primers corresponding to the 5′ and 3′ ends of *lipK107*, respectively (Table 1). Purified PCR products were sequenced. Primers Up and Down (Table 1), with *Eco*RI and *Hind*III restriction sites respectively, were used to amplify the complete ORF of *lipK107* gene and its derivatives. The purified PCR products were digested and cloned into plasmid pET-28a, then expressed in *E. coli* BL21.

2.3. Preparation of enzymes

Isopropylthiogalactoside (IPTG) (0.8 mM) was used to induce the expression of LipK107 and its derivatives at 30 °C. Cells were harvested and disrupted ultrasonically. After centrifugation (10,000 × *g*, 4 °C for 10 min), the supernatants were loaded on Ni-NTA sepharose columns. The proteins were eluted with imidazole and desalted with desalting columns (Amersham Pharmacia Biotech, Sweden). The purified proteins were lyophilized in a lyophilizer (Christ, Germany), and these dry powders were used as catalysts.

2.4. General procedure with LipK107 and its derivatives for the resolution reaction

Resolution reactions were carried out with 0.5 mmol racemic 1-phenylethanol and 1.5 mmol methyl palmitate. LipK107 (1% based on 1-phenylethanol weight) was used as catalyst. Various amounts of water (0–400% by weight of the 1-phenylethanol) were added to examine the water effect. Isooctane was added to make up a 5-ml system.

To test the influence of acyl donor on the resolution, starting mixtures were 0.5 mmol racemic 1-phenylethanol and 250% water (based on 1-phenylethanol weight). Methyl acetate, methyl butyrate, methyl caprylate, methyl dodecanoate, methyl palmitate and methy arachidate were added as acyl donor, respectively.

Mutants E130L + K131I, T138V, I128E + 129D, R120P + K121P, H146S + R147T were used as catalysts, respectively, for the resolution reaction. Starting mixtures were 0.5 mmol racemic 1-phenylethanol, 1.5 mmol methyl palmitate, and 250% water (based on 1-phenylethanol weight). Isooctane was added as described above. All these reactions were performed at 20 °C with shaking at 180 rpm.

Enantiomeric excesses (*ee*), conversions (*c*), and enantiomeric ratios (*E*) were calculated using the corresponding peak areas:

- $ee = 100 \times (A - B)/(A + B)$, where ($A$ and B) were the peak areas of enantiomers, and (A) > (B);
- $c = ee_s/(ee_s + ee_p)$, where s and p represented substrate and product;
- $E = \ln[1 - c(1 + ee_p)]/\ln[1 - c(1 - ee_p)] = \ln[(1 - c)(1 - ee_s)]/\ln[(1 - c)(1 + ee_s)]$.

The conversion and the enantiomeric excess of products (ee_p) were examined by HPLC according to the method of Tamalampudi et al. [21]. All values are an average of three independent experiments.

2.5. Hydrophobic and electrostatic mapping of the lid

The hydrophobic and electrostatic mapping of the lids of LipK107 and its derivatives were modeled using the SYBYL molecular modeling package version 7.3 (Tripos Inc.) on a SGI Octane workstation. The hydrophobicity of lids was depicted according to the Ghose method [22]. The partial charges of all atoms were assigned according to the Gasteiger–Marsili rules.

3. Results

3.1. Effect of water and acyl donor

In the resolution of racemic 1-phenylethanol with methyl palmitate, the effect of water was investigated first. As presented

Table 2
The effect of water content on the resolution of racemic 1-phenylethanol.

Water content[a]	0	10	30	50	70	100	150	200	250	300	350	400
ee_p (%)	0	92.2	92.8	93.0	93.9	95.4	95.9	95.8	95.8	95.4	95.3	95.2
E	0	25.2	27.4	28.4	37.3	59.4	70.1	70.4	70.7	63.9	61.8	58.6

[a] The water content was expressed as the percentage of the weight of 1-phenylethanol.

in Fig. 1, LipK107 displayed low activity in the presence of less than 50% water (based on 1-phenylethanol weight). However, when more than 50% water was added, the conversion of 1-phenylethanol increased sharply, and reached a plateau of nearly 30% after 24 h. This result indicated that water played a crucial role in the organic reaction catalyzed by LipK107. When water was added more than 300%, the conversion decreased, mainly due to the fact that high water content induced the diffusive limitations of substrate and promoted the hydrolysis of product. It was worth mentioning that with the increasing of water, a considerable increase of activity was towards the R-isomer (preferred isomer) without appreciable loss of enzyme enantiodiscrimination (Table 2).

Different acyl donors were screened to improve the yield. As shown in Fig. 2, the conversion of 1-phenylethanol was dramatically dependent on the chain length of acyl donor, and the highest conversion of 29.9% was achieved when methyl palmitate was used. The decrease of conversion with methyl arachidate might be ascribed to the large steric hindrance, which could block the enzyme active site. It could be further observed that the enantiomeric excess of product (ee_p) also crept up with the increasing of chain length of acyl donors (Table 3).

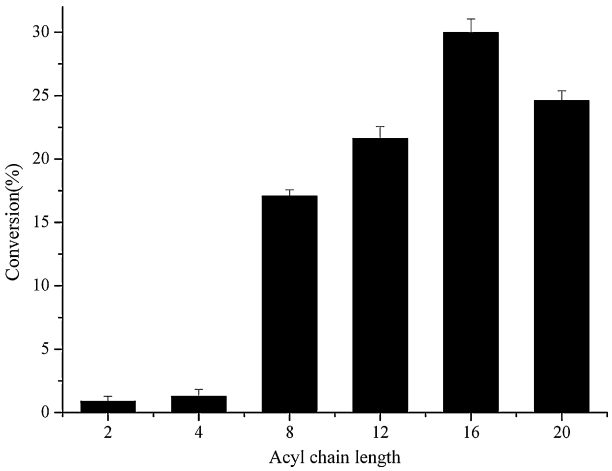

Fig. 2. Effect of different acyl donors on the resolution of racemic 1-phenylethanol. Reaction conditions: 20 °C, 180 rpm, 1% (based on 1-phenylethanol weight) dry powder of purified LipK107 used as catalyst, 250% (based on 1-phenylethanol weight) water added, methyl acetate, methyl butyrate, methyl caprylate, methyl dodecanoate, methyl palmitate and methy arachidate used as acyl donors respectively. Reaction time: 24 h. The values shown are the means of three independent experiments and the error bars represent the standard deviations.

3.2. Mutation design in the lid domain

It was reported that the hydrophobic and electrostatic interactions between the lid and the lipid assemblies contributed to the stabilization of the lipase open conformation [23]. In this work, state-of-the-art of molecular modeling was used to give a visual description of the lid of LipK107. Then several mutations were designed to change the hydrophobicity and electrostatic of the lid domain. Fig. 3 shows the whole structure of the LipK107. Fig. 4 displays the hydrophobicity mapping of the lid helixes of all three mutants as well as the native LipK107. Compared with the LipK107 (Fig. 4a), there was an additional hydrophobic region (yellow circled region) in mutant E130L + R131I (Fig. 4b). A little increase of hydrophobicity was also observed in mutant T138V (Fig. 4c). However, in mutant I128E + V129D (Fig. 4d), an originally existing hydrophobic region was changed to hydrophilic region (yellow circle). Fig. 5 demonstrates the electrostatic mapping of the lid regions of LipK107 and its derivatives. As we can see in Fig. 5a, positive residues (R120 and K121 in upstream coil, H146 and R147 in downstream coil) existed in the corresponding flank-

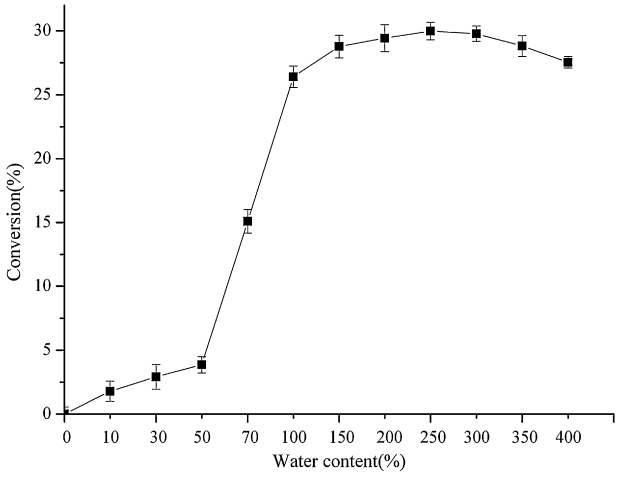

Fig. 1. Effect of water content on the resolution of racemic 1-phenylethanol. Reaction conditions: 20 °C, 180 rpm, methyl palmitate used as acyl donor, 1% dry powder of purified LipK107 (based on 1-phenylethanol weight) used as catalyst. Water contents from 0 to 400% by weight of the 1-phenylethanol were added. Reaction time: 24 h. Data presented are an average of three independent experiments with the standard deviation, indicated by the error bar.

Fig. 3. The whole structure of the lipase LipK107. It consisted of 9 α-helix and 6 β-sheet. The catalytic residues were Ser[79], Asp[232], and His[254].

Table 3
The effect of different acyl donors on the resolution of racemic 1-phenylethanol.

Chain length[a]	2	4	8	12	16	20
ee_p (%)	90.1	90.5	92.4	94.1	95.8	94.3
E	19.3	20.4	30.5	42.4	71.0	46.3

[a] The chain length represented the alkyl part of the acyl donor.

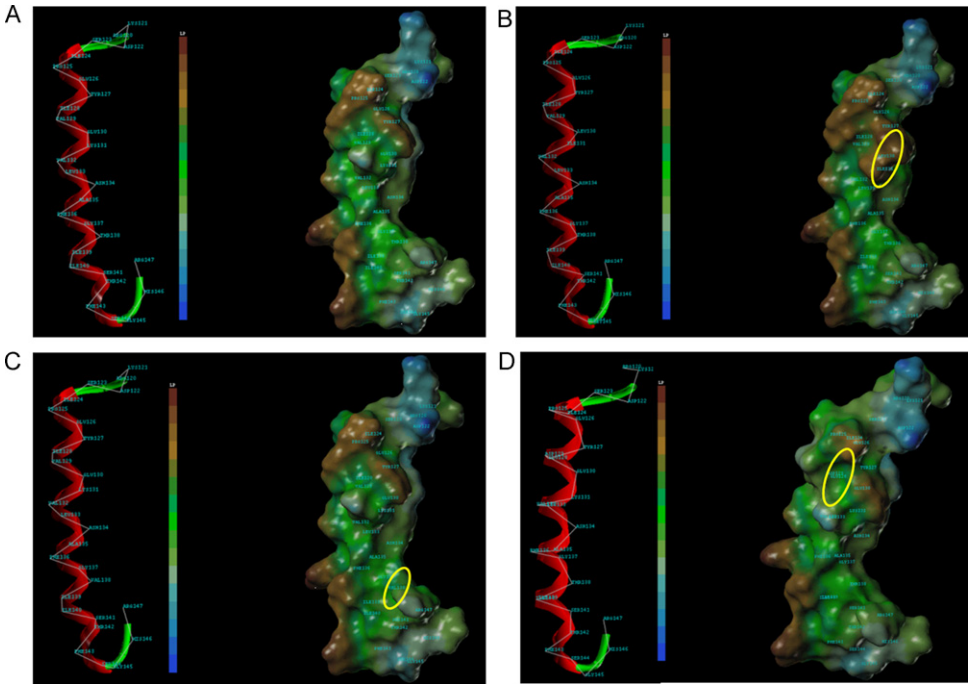

Fig. 4. Hydrophobicity mapping of the lid regions of LipK107 and its mutants. A black and white ramp was displayed for measuring the hydrophobicity. As the hydrophobicity increase, the color of the surface changed from light grey to dark grey. The circle gives prominence to the site of the mutation. (a) Lid of LipK107; (b) lid of mutant E130L + R131I; (c) lid of mutant T138V; (d) lid of mutant I128E + V129D.

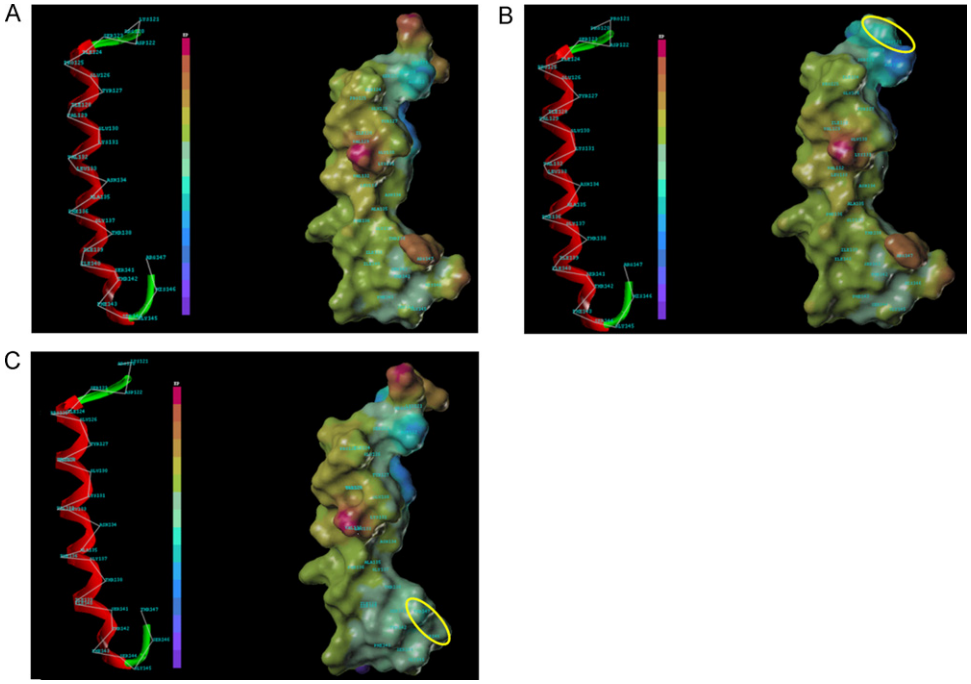

Fig. 5. Electrostatic mapping of the lid regions of LipK107 and its mutants. A black and white ramp was displayed for measuring the electrostatic. As region become more positively charged, the color of the surface changed from light grey to dark grey. The circle gives prominence to the site of the mutation. (a) Lid of LipK107; (b) lid of mutant R120P + K121P; (c) lid of mutant H146S + R147T.

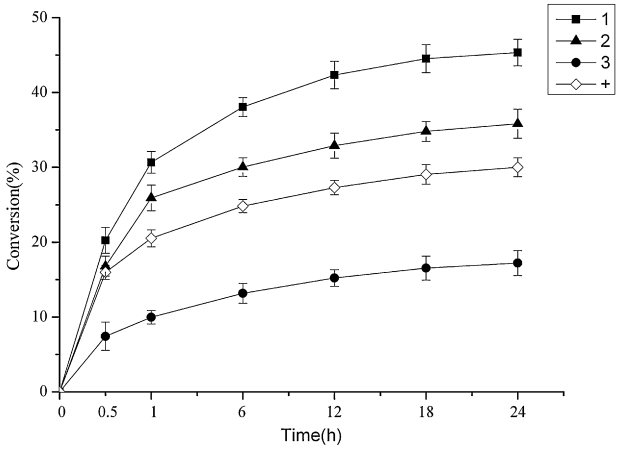

Fig. 6. Effect of hydrophobicity of the lid domain on the resolution of racemic 1-phenylethanol. Reaction conditions: 20 °C, 180 rpm, methyl palmitate used as acyl donor, 250% (based on 1-phenylethanol weight) water added. (□) native LipK107 as catalyst; (■) mutant E130L + K131I as catalyst; (▲) mutant T138V as catalyst; (●) mutant I128E + V129D as catalyst. Reaction time: 24 h. Data presented are an average of three independent experiments with the standard deviation, indicated by the error bar.

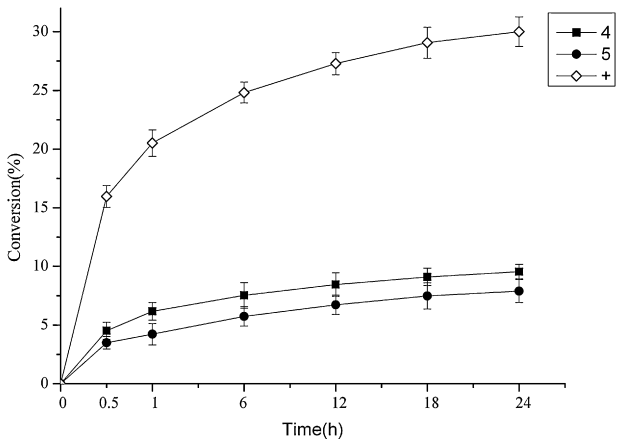

Fig. 7. Effect of electrostatic of the lid domain on the resolution of racemic 1-phenylethanol. Reaction conditions: 20 °C, 180 rpm, methyl palmitate used as acyl donor, 250% (based on 1-phenylethanol weight) water added. (□) native LipK107 as catalyst; (■) mutant R120P + K121P as catalyst; (●) mutant H146S + R147T as catalyst. Reaction time: 24 h. The values shown are the means of three independent experiments and the error bars represent the standard deviations.

ing regions of the lid of LipK107, while these positively charged region disappeared in mutants R120P + K121P and H146S + R147T (Fig. 5b and c).

3.3. Construction, expression and purification of the mutants

Mutants were constructed by the method of site-directed mutagenesis as described in Section 2.2. The presence of the correct mutation and the absence of PCR-generated random mutations were verified by DNA sequencing. The hydrophobicity of the lid domain was increased in mutants E130L + K131I and T138V, and decreased in mutant I128E + V129D. The electrostatic of the lid domain was decreased in mutants R120P + K121P and H146S + R147T. The gene of LipK107 and its derivatives were expressed in *E. coli* BL21 (DE3), and the recombinant lipases protein were purified by a Ni-NTA sepharose column.

3.4. Resolution of 1-phenylethanol catalyzed by LipK107 and its derivatives

The purified proteins LipK107 and its derivatives were lyophilized and these dry powders were used as catalysts for the resolution of racemic 1-phenylethanol with methyl palmitate. As Fig. 6 illustrates, the conversions of 1-phenylethanol catalyzed by mutants E130L + K131I and T138V were much higher than that catalyzed by LipK107. This result suggested that increasing the hydrophobicity of the lid could greatly increase the lipase activity. A further demonstration of the role of the hydrophobicity of the lid was obtained when mutant I128E + V129D was used as catalyst. As expected, the conversion of 1-phenylethanol was lower due to a reduced ratio of hydrophobic residues of the lid. Noteworthy, the ee_p and E (enantiomeric ratio) of the resolution changed slightly, indicating that the lid played a role in dictating the selectivity in nonaqueous media (Table 4) [24].

The effect of electrostatic property of the lid on the lipase activity is presented in Fig. 7. It can be seen that the conversions of 1-phenylethanol catalyzed by mutants R120P + K121P and H146S + R147T were much lower than that catalyzed by LipK107. In addition, the ee_p and E (enantiomeric ratio) of the resolution edged down (Table 4), which in agreement with the view that lid affected the enantioselectivity of lipase. These observed changes indicated that the electrostatic property of lid was another factor that played a key role in the function of lipases, and decreasing the electrostatic property of lid could result in a great loss of lipase activity.

4. Discussion

Lipase is one of the most versatile enzymes in bioconversion processes. During the last decade, there has been an increasing interest in the development of lipases for asymmetric synthesis and kinetic resolution to obtain pure enantiomers for pharmaceuticals and agrochemicals [25]. Here we widened the application of LipK107 into the resolution of 1-phenylethanol, the chiral derivatives of which were important building blocks for drug substrates and agricultural products.

Water effect was investigated first and the activity of LipK107 was dramatically dependent on the amount of water added in the reaction. The shape of the curves obtained in Fig. 1 was quite similar to that presenting the kinetic behavior of lipase [26,27], which reminded us of the fact that the oil–water interface was required for most lipases to unmask and restructure of the active site through conformational changes. In this work, LipK107 was purified and freeze-dried without a covalently bound inhibitor or detergent occluding the active site, in what was assumed to be closed conformation when used as catalyst in organic reaction. Therefore, the water added might increase the flexibility of lid and form a well-defined interface to help the lid open. The open lid rendered the active site accessible for substrate binding and significantly increasing the activity of LipK107.

Table 4
The effect of different catalysts on the resolution of racemic 1-phenylethanol.

Mutant[a]	1	2	3	4	5	6
ee_p (%)	97.1	96.3	92.6	92.3	93.0	95.8
E	167.5	92.1	31.5	27.6	30.0	70.5

[a] 1, 2, 3, 4, 5, 6 represented the mutants E130L + K131I, T138V, I128E + 129D, R120P + K121P, H146S + R147T, and the LipK107 respectively.

On the other hand, excessive water in non-aqueous system may stimulate the competing hydrolysis reaction. Thus the optimum water content is a compromise between minimizing hydrolysis and maximizing synthesis. For most lipases, the resolution activities are inhibited at high water content, so many industrial substrates could not be used, or they need to be pre-prepared. Even the extensively used Novozym435 (lipase B from *Candida antarctica*) could catalyze the resolution of secondary alcohols when no water was added [28]. However, LipK107 showed high activity with wide range of water (50–300% by weight of the substrate), which would make it a potential effective enzyme to eliminate substrate availability limitations and distinctly simplify operational procedures.

According to our knowledge, most lipases showed a preference of short chain acyl donor in the resolution of racemic 1-phenylethanol [29,30]. But this was not true for LipK107, which displayed high activity when methyl palmitate was used. It might be related to the extremely large and hydrophobic lid of LipK107. The hydrocarbon chains of methyl palmitate might exhibit the optimal size to be accommodated along the hydrophobic face of the amphipathic lid helices, thus inducing and stabilizing the open conformation of LipK107 [31].

Some reports have indicated that the specific residues of the lid might be of importance for the activity of lipases. But only a few attempts were made to demonstrate the influence of amino acids of the lid on the specific catalytic reactions [32]. On the other hand, the importance of molecular modeling in mutant design has been uncovered [33,34]. Therefore, we analyzed the 3D structure of LipK107 and introduced some mutations based on the hydrophobicity and electrostatic mapping of the lid. Section 3.4 showed that these mutants did work according to our prediction and the conversion of 1-phenylethanol increased dramatically. Additionally, the effect of hydrophobic and electrostatic interactions between the lid and substrate on the activity of lipase was determined. These findings provided evidence for the idea that the characteristics of lid were crucial for the activity of lipase. For most other lipases, the modification of the lid region may also greatly enhance the activity and improve the yield of reactions. Furthermore, the computer simulation made the process of identifying amino acids for substitution more efficiently. Therefore, the current work in our lab might shed some light on the site-directed mutagenesis to enhance the lipase activity and develop highly performing biocatalysts for industrial needs.

In conclusion, we successfully improved the activity of lipase LipK107 by combining the computer simulation with the site-directed mutagenesis. The biocatalyst with high activity can distinctly simplify operational procedures and improve the production of chiral derivatives, which make it a potential viable biocatalyst in industry.

Acknowledgements

This work was supported by the following grants: National Basic Research Program of China (973 Program): 2009CB724703; National Special Fund for State Key Laboratory of Bioreactor Engineering: 2060204; Nation Natural Science Foundation of China: 50808069.

References

[1] M.T. Reetz, Curr. Opin. Chem. Biol. 6 (2002) 145–150.
[2] K.E. Jaeger, T. Eggert, Curr. Opin. Biotechnol. 13 (2002) 390–397.
[3] U.T. Bornscheuer, R.J. Kazlauskas, Hydrolases in Organic Synthesis- Regio- and Stereoselective Biotransformations, Wiley-VCH, Weinheim, 1999.
[4] M.Y. Lee, J.S. Dordick, Curr. Opin. Biotechnol. 13 (2002) 376–384.
[5] L. Ma, M. Persson, P. Adlercreutz, Enzyme Microb. Technol. 31 (2002) 1024–1029.
[6] A. Zaks, A.M. Klibanov, J. Biol. Inorg. Chem. 263 (1988) 8017–8021.
[7] N.M. Micaelo, V.H. Teixeira, A.M. Baptista, C.M. Soares, Biophys. J. 89 (2005) 999–1008.
[8] A.M. Brzozowski, U. Derewenda, Z.S. Derewenda, G.G. Dodson, D.M. Lawson, J.P. Turkenburg, F. Bjorkling, B. Huge-Jensen, S.A. Patkar, L. Thim, Nature 351 (1991) 491–494.
[9] R. Verger, Trends Biotechnol. 15 (1997) 32–38.
[10] Y. Cajal, A. Svendsen, J. De Bolos, S.A. Patkar, M.A. Alsina, Biochemie 82 (2000) 1053–1061.
[11] A. Thomas, M. Allouche, F. Basyn, R. Brasseur, B. Kerfelec, J. Biol. Chem. 280 (2005) 40074–40083.
[12] Y.B. Ali, H. Chahinian, S. Petry, G. Muller, F. Carriere, R. Verger, A. Abousalham, Biochemistry 43 (2002) 9298–9306.
[13] V. Belle, A. Fournel, M. Woudstra, S. Ranaldi, F. Prieri, V. Thome, J. Currault, R. Verger, B. Guigliarelli, F. Carriere, Biochemistry 46 (2007) 2205–2214.
[14] C.A. Godoy, B. de las Rivas, M. Filice, G. Fernandez-Lorente, J.M. Guisan, J.M. Palomo, Process Biochem. 45 (2010) 534–541.
[15] K. Henzler-Wildman, M. Lei, V. Thai, S.J. Kerns, M. Karplus, D. Kern, Nature 450 (2007) 913–916.
[16] K. Henzler-Wildman, D. Kern, Nature 450 (2007) 964–972.
[17] R.J. Kazlauskas, Curr. Opin. Chem. Biol. 4 (2000) 81–88.
[18] B. Gao, E. Su, J. Lin, Z. Jiang, Y. Ma, D. Wei, J. Biotechnol. 139 (2009) 169–175.
[19] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989.
[20] R. Higuchi, Using PCR to Engineer DNA in PCR Technology: Principles and Applications for DNA Amplification, Stockton, New York, 1992, 61–70.
[21] S. Tamalampudi, S. Hamac, T. Tanino, M.R. Talukder, A. Kondo, H. Fukuda, J. Mol. Catal. B: Enzym. 48 (2007) 33–37.
[22] A.K. Ghose, V. Viswanadhan, J.J. Wendoloski, J. Phys. Chem. A 102 (1998) 3762–3772.
[23] D.M. Lawson, A.M. Brzozowski, S. Rety, C. Verma, G.G. Dodson, Protein Eng. 7 (1994) 543–550.
[24] M. Skjot, L. Maria, R. De Chatterjee, A. Svendsen, S.A. Patkar, P.R. Tergaard, J. Brask, Chem. Biochem. 10 (2009) 520–527.
[25] M. Persson, D. Costes, E. Wehtje, P. Adlercreutz, Enzyme Microb. Technol. 30 (2002) 916–923.
[26] H. Chahiniana, L. Ninib, E. Boitardc, J. Dubèsc, L. Comeau, L. Sarda, Lipids 37 (2002) 653–662.
[27] A.B. Bacha, Y. Gargouri, Y.B. Ali, N. Miled, J. Reinbolt, H. Mejdoub, Enzyme Microb. Technol. 37 (2005) 309–317.
[28] R.N. Patel, A. Banerjee, V. Nanduri, A. Goswami, F.T. Comezoglu, JAOCS 77 (2000) 1015–1019.
[29] M. Habulin, Z. Knez, J. Mol. Catal. B: Enzym. 58 (2009) 24–28.
[30] M. Singh, R.S. Singh, U.C. Banerjee, Process Biochem. 45 (2010) 25–29.
[31] M.C. Bano, H. Gonzalez-Navarro, C. Abad, Biochim. Biophys. Acta 1632 (2003) 55–61.
[32] S. Ranaldi, V. Belle, M. Woudstra, J. Rodriguez, B. Guigliarelli, J. Sturgis, F. Carriere, A. Fournel, Biochemistry 48 (2009) 630–638.
[33] R.R. Klein, G. King, R.A. Moreau, M.J. Haas, Lipids 32 (1997) 123–130.
[34] P. Trodler, J. Pleiss, BMC Struct. Biol. 8 (2008) 9.