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Enhancing lipase-catalyzed hydrolysis by adding macrocyclic tetraamines

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ABSTRACT

Macrocyclic tetraamines (MTs) were employed as novel additives to improve the enzyme's performance in *Pseudomonas cepacia* lipase (PCL)-catalyzed enantioselective hydrolysis of *N*-(2-ethyl-6-methylphenyl)alanine methyl ester (NEMPA-ME). Our results showed that the activity of PCL was significantly enhanced and the higher enantioselectivity of the reaction was maintained when some MTs were added to the reaction media. The acceleration of the initial reaction rate greatly depended on MT structure and additive concentration. Among the 21 MTs we tested, 10-(2-hydroxydecyl)-2, 6-dioxo-1,4,7,10-tetraazacyclododecane (MT#20) was found to be the best enzyme activator as 11-fold increase in the initial reaction rate was seen when an optimal concentration of 9.6 mmol/L of the amine was added. Kinetic analysis indicated that the affinity of lipase PCL toward the substrate was modified in the presence of MTs. Molecular modeling suggested that MTs could lead to a more native and stable conformation of lipase, thereby enhancing its enzymatic activity. This is the first systematic study of enhancement of an enzymatic reaction by MT-type additives.

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1. Introduction

(*R*) and (*S*)-*N*-(2-ethyl-6-methylphenyl)alanine ((*R*) and (*S*)-NEMPA) are important chiral building blocks for the synthesis of most widely used herbicides [1]. We have previously described a biocatalytic two-step approach to obtain both (*R*) and (*S*)-NEMPA with high optical purity [2]. Among the lipases screened, the lipase from *Pseudomonas cepacia* (PCL) was found to give excellent enantioselectivity for (*R*)-NEMPA, but the reaction activity was rather low – it needed 48 h to reach 48.2% conversion. Therefore, developing a new method to accelerate this reaction is desirable.

Many methods have been reported to improve the performance of enzymes, such as pretreatment of enzymes with polar solvents [3], chemical modification of enzymes [4,5], medium engineering [6], substrate engineering [7], protein engineering [8] and usage of additives [9]. Among these approaches, the addition of a compound to the reaction medium is the simplest way to enhance enzyme reactivity. However, only a few compounds have been reported to enhance selectivity or reactivity so far. Guo et al. have reported an improvement in the *Candida cylindracea* lipase-catalyzed enantioselective hydrolysis of alkyl 2-(aryloxy)propanoates or 2-arylpropanoates using amines as additives [10]. Griebenow et al. introduced methyl-β-cyclodextrin as an additive to simultaneously enhance the activity and enantioselectivity of dehydrated subtilisin [11]. Similarly, some crown ethers and novel imidazolium salt ionic liquids have been reported to enhance the reaction rate and/or enantioselectivity of lipase-catalyzed reactions [12,13].

As part of our continuing interest in strategies for improving the performance of enzymes, we recently found that macrocyclic tetraamines (MTs) could enhance the reaction rate in PCL-catalyzed hydrolysis of (*R*, *S*)-NEMPA-ME. MTs were selected as additives in this work because MTs have similar chemical structure as crown ethers and the chemical properties of amine compounds. Crown ethers as well as simple amines are well-known additives in lipase-catalyzed reactions [9,13].

Here, we examined 21 MTs as additives in PCL-catalyzed enantioselective hydrolysis of (*R*, *S*)-NEMPA-ME. The values of the initial rates V_S were obtained for the substrate, and the kinetic data K_m and K_{cat} were analyzed. On the basis of these experimental data, the mechanism of MT-induced regulation of lipase-catalyzed reactions was investigated by molecular modeling.

2. Materials and methods

2.1. Materials

P. cepacia lipase (PCL, 2500 U/g) was purchased from Amano Pharmaceutical Co., Ltd. (Japan). (*R*, *S*)-NEMPA-ME and macrocyclic tetraamines (MTs) were prepared as previously reported [14–16] and confirmed by spectroscopic analysis including 300 MHz NMR (Mercury-300B, VARIAN, USA) and GC–MS (Saturn 220, VARIAN,

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USA). All organic solvents used in our experiments were of reagent grade and used without further purification. Other reagents were all of analytical grade or better.

2.2. Analysis

The hydrolysis of (*R*, *S*)-NEMPA-ME was monitored by HPLC (Acme 9000, Young Lin Instrument Co., Ltd.) using Daicel Chiralpack AD-H column capable of separating the internal standard of benzoic acid. The retention times of benzoic acid, (*R*)- and (*S*)-NEMPA were 9.3, 6.7 and 7.5 min, respectively. The mobile phase was a mixture of *n*-hexane/isopropanol/trifluoroacetic acid (97/3/0.05, v/v/v) at a flow rate of 1.0 mL/min. UV detection at 254 nm was employed for quantification at the column temperature of 25 °C. Enantiomeric ratio (E) of hydrolysis of (*R*, *S*)-NEMPA-ME was calculated from the conversion (c) and enantiomeric excess (e.e.$_p$) of (R)-NEMPA, using the equation: $E = \ln[1 - c(1 + \text{e.e.}_p)]/\ln[1 - c(1 - \text{e.e.}_p)]$, where $\text{e.e.}_p = (c_R - c_S)/(c_R + c_S)$, while c_R and c_S are the concentrations of (*R*)- and (*S*)-NEMPA, respectively [17].

2.3. Kinetic analysis

PCL-catalyzed hydrolysis was generally carried out in a non-buffered aqueous solution to exclude the effect of complexation between MT and metal cations. Typically, (*R*, *S*)-NEMPA-ME (34 mmol/L) and MT additive (0–25 mmol/L) were added to 2.0 mL aqueous solution containing 1.5 mg/mL of PCL. The resulting mixture was stirred at 40 °C, and samples were removed and injected into the HPLC system at different time intervals for analysis. From the time-course conversions, the initial rates for (*R*, *S*)-NEMPA-ME were estimated. Similar experiments were carried out with the substrate concentrations varied from 10 to 50 mmol/L. The kinetic constants were estimated from the variation in initial rates with changes in substrate concentration. In order to exclude non-enzymatic MT mediated hydrolysis, blank experiments with only MT and no enzymes added to the reaction mixture were conducted. No effects of the additive were observed on the initial rates and enantioselectivity in these experiments.

2.4. Molecular modeling

All modeling was done on SGI fuel station (Silicon Graphics, Inc.) using Insight II (Accelrys) package, and the CVFF force field was used.

The structure of the open form of PCL was taken from the PDB data bank (entry 3lip) and the water molecules were removed. The catalytic histidine, His286, was defined as protonated. Modeling of the substrate–PCL tetrahedral intermediate was guided by the crystal structure of PCL complexed with triacylglycerol analogue (PDB entry 5lip) [18]. The substrate (*R*)-NEMPA-ME was manually modeled into the binding site of PCL and covalently linked to side-chain oxygen Oγ of catalytic Ser87. The carbonyl carbon atom and its associated carbonyl oxygen atom, hydroxyl oxygen atom and chiral carbon atoms in (*R*)-NEMPA-ME corresponded with the phosphorus atom P_1 and its associated oxygen atoms O_4, O_1, C_4 of the triglyceride analogue in tetrahedral intermediate form. Then the superimposition was performed, and the dihedral angle of substrate molecules was adjusted to eliminate steric hindrance. Finally, energy minimization of the whole system was performed, alternatively using the steepest descent and conjugated gradient methods until the energy was converged at 0.01 kcal mol^{-1} Å^{-1}, obtaining a best-structured model for subsequent investigation [19,20].

MT#20 was selected and docked into the substrate (*R*)-NEMPA-ME–PCL complex. The conformation of MT was randomly searched by the Monte Carlo method. First, the binding modes were screened based on van der Waals force, and the modes thus found were optimized by a series of short dynamics simulations and energy minimizations. Subsequently, the modes were further screened based on the electrostatic and van der Waals force. The structure obtained in this way was optimized by the method of annealing simulation, and the annealing temperature was lowered from 500 K down to 300 K. The energy-minimized PCL–substrate–MT system was selected and analyzed [21,22].

Table 1
Effects of MTs on PCL-catalyzed hydrolysis.[a]

Amine	Time (h)	Conversion (%)	V_S (μmol/h)	e.e.$_p$ (%)	*E* value
None	0.5	0.4	0.5	99	200
1	0.5	0.4	0.5	99	200
2	0.5	0.5	0.7	99	200
3	0.5	2.1	2.8	99	203
4	0.5	2.8	3.8	99	205
5	0.5	2.6	3.6	99	204
6	0.5	2.4	3.3	99	204
7	0.5	3.7	5.0	99	207
8	0.5	1.6	2.2	99	202
9	0.5	2.1	2.8	99	203
10	0.5	3.3	4.5	99	206
11	0.5	3.9	5.3	99	207
12	0.5	3.9	5.3	99	207
13	0.5	1.6	2.1	99	202
14	0.5	2.3	3.1	99	204
15	0.5	2.4	3.2	99	204
16	0.5	2.9	3.9	99	205
17	0.5	3.4	4.6	99	206
18	0.5	1.3	1.8	99	202
19	0.5	2.6	3.5	99	204
20	0.5	4.1	5.6	99	208
21	0.5	3.7	5.0	99	207
Triethylamine	0.5	30.5	41.4	1.3	1.0
Diethylamine	0.5	40.8	55.4	0.8	1.0

[a] Reactions were carried out in 2.0 mL deionized water containing 1.5 mg/mL of PCL, (*R*, *S*)-NEMPA-ME (34 mmol/L), amine (10 mmol/L). The mixture was stirred at 40 °C.

3. Results and discussion

3.1. Effects of MT structure and additive concentration

Five types and a total of 21 MTs (Fig. 1) were selected as potential regulators of the catalytic properties of PCL in the hydrolysis of (*R*, *S*)-NEMPA-ME. Initial reaction rates (V_S) were calculated using data after letting the reaction run for 0.5 h in each case and reported in Table 1. It is noteworthy that the initial rates of PCL-catalyzed hydrolysis were enhanced and higher enantiomeric ratios (*E*-values) were maintained in the presence of most additives tested.

The length of the side chain of MTs had great influence on the activity of lipase PCL. Increase in the length of hydrophobic alkyl side chain was favorable for the improvement of lipase performance. Generally, MTs bearing C_6–C_{14} alkyl chains were better, and acceleration of the initial rate ranged from 4-fold to 11-fold. However, the reaction rates decreased when the side chain length increased to C_{16}. The catalytic activity of PCL was also affected by MTs containing a hydroxyl group on the side chain (#6, #7, #19, #20, #21). In particular, MTs bearing hydroxyl groups on longer alkyl chains such as #7, #20 and #21 were effective for improvement in PCL activity. The highest initial reaction rate we recorded was 5.6 μmol/h with the use of 10-(2-hydroxydecyl)-2, 6-dioxo-1,4,7,10-tetraazacyclododecane (#20), which was 11-fold higher than in reactions without any additives. Other MTs bearing a hydroxyl pendant also gave satisfactorily high initial reaction rates: 3.3 μmol/h for #6, 5.0 μmol/h for #7, 3.5 μmol/h for #19 and 5.0 μmol/h for #21.

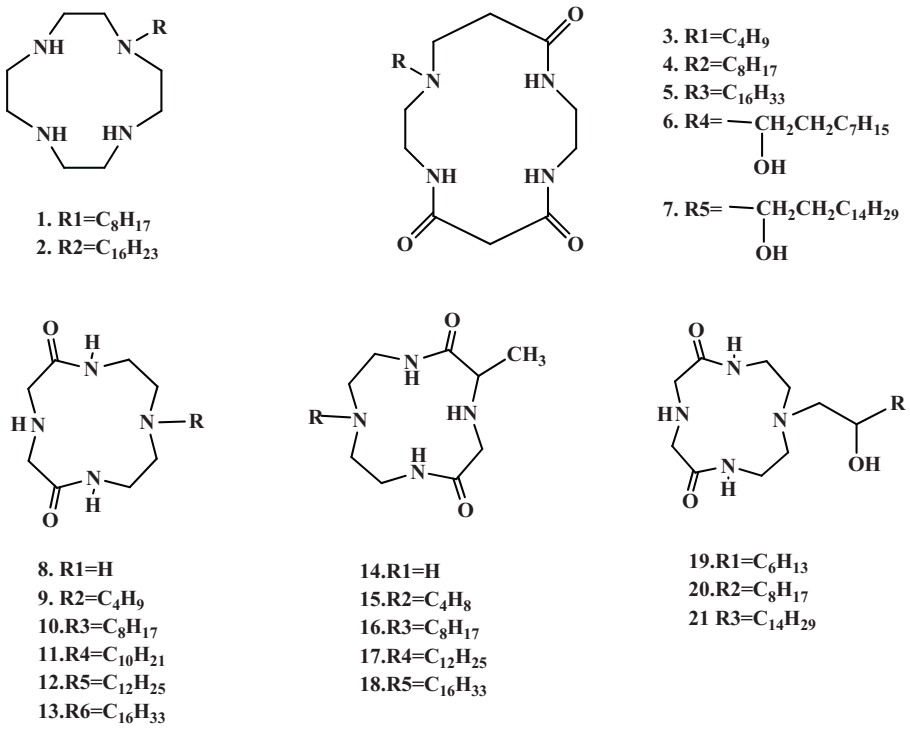

Fig. 1. The chemical structure of MTs.

In all the MT-induced reactions we tested, we found that MT seriously improved the hydrolysis rate of the optimum enantiomer (*R*)-NEMPA-ME, whereas the hydrolysis of the incorrectly binding *S* enantiomer was almost completely suppressed ($V_{S(S)} \approx 0$). The increase in the initial rate of reaction of the *R* enantiomer resulted in up to 43% conversion during PCL-catalyzed hydrolysis in 8 h.

How does MT affect lipase-catalyzed reactions? Some studies have suggested that some amines such as triethylamine can improve the selectivity or reactivity in lipase-catalyzed reactions due to the formation of a soluble ion-pair between the acid generated in the reaction and triethylamine, thus shifting the equilibrium in the direction of the product [23,24]. In order to investigate if MTs worked similarly, we added triethylamine and diethylamine to the reactions. We observed that triethylamine and diethylamine significantly enhanced the reaction rate but, unlike MTs, significantly decreased enantioselectivity (Table 1). These results suggest that MTs possibly affect lipase-catalyzed hydrolysis by a different mechanism than triethylamines have been proposed to. The large enhancement in reaction rate was observed only in the presence of both lipase PCL and MT, which could be ascribed to a synergistic cooperation between MT and the enzyme. Such cooperation could modify the conformation of the enzyme, thus improving its catalytic properties. Additionally, the observation that MTs bearing C_6–C_{14} alkyl side chains enhanced the reaction rate, but further increase in the length of alkyl chain decreased enzyme performance could be attributed to spatial hindrance caused by very long alkyl chains. We verified this idea by molecular modeling, which we discuss later in this report.

It has been reported that the extent of enhancement in enzyme activity produced by additives depends on the ratio of additives to enzymes [9]. For this reason, we decided to investigate the effects of MT additive concentration on the activity of the enzyme using the five MTs that we had found to significantly enhance reaction rates (#7, #16, #17, #20, #21). Fig. 2 illustrates the effect of additive concentration on the relative initial rate $V_S/V_{S,0}$ for various MTs, where $V_S/V_{S,0}$ was the initial rate of hydrolysis of (*R*, *S*)-NEMPA-ME with PCL in the additive system compared with that without additive. The results showed that the activity of PCL increased with increase in the concentration of MTs, and different MTs had different optimum additive concentration. For MTs bearing alkyl chains (#16, #17), lower additive concentration favored the highest reaction rates of PCL, with the optimum concentration for #16 being 3.6 mmol/L, and that for #17 being 2.4 mmol/L. Among MTs containing a hydroxyl group on the alkyl side chain (#7, #20, #21), the optimum concentration was 9.6 mmol/L for #20, 18.0 mmol/L for #7 and 14.4 mmol/L for #21. Increasing the additive concentration beyond the optimal concentration led to decrease in reaction rates.

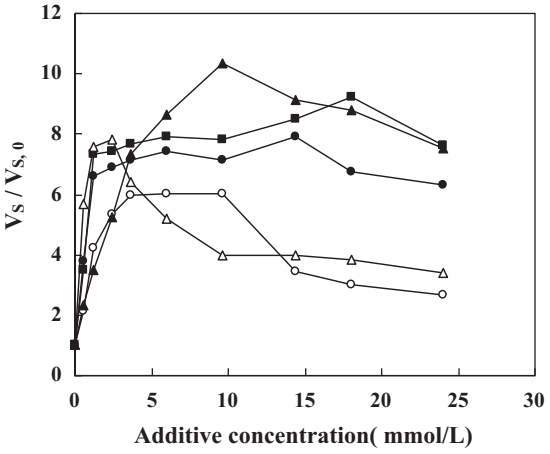

Fig. 2. Relative initial rates $V_S/V_{S,0}$ varied with MT additive concentration for #7 (■), #16 (○), #17 (△), #20 (▲) and #21 (●).

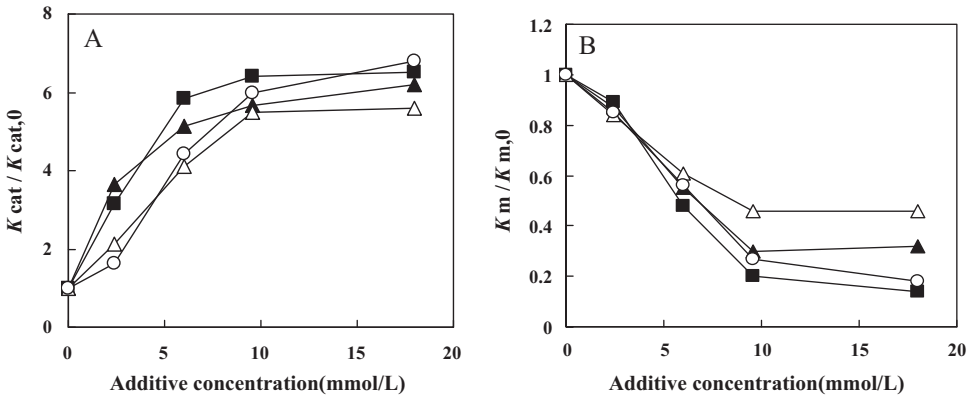

Fig. 3. (A) Relative kinetic contants $K_{cat}/K_{cat,0}$ varied with MT additive concentration for #7 (▲), #16 (△), #20 (■) and #21 (○). (B) Relative kinetic contants $K_m/K_{m,0}$ varied with MT additive concentration for #7 (▲), #16 (△), #20 (■) and #21 (○).

3.2. Kinetic analysis

In order to elucidate the kinetic behavior of PCL in the presence of MTs, the kinetic constants (K_{cat} and K_m) of the hydrolysis of (*R*, *S*)-NEMPA-ME were determined from the Lineweaver–Burk plots and compared with those without additive ($K_{cat,0}$ and/$K_{m,0}$). The relative values of $K_{cat}/K_{cat,0}$ and $K_m/K_{m,0}$ are listed in Fig. 3. The increase in $K_{cat}/K_{cat,0}$ values that we observed imply that the rates of reaction catalyzed by PCL with the addition of MT were increased. K_m values for PCL with addition of MT were lower than that of free enzyme ($K_m/K_{m,0} < 1$), and the values of $K_m/K_{m,0}$ decreased with increase in additive concentration, which indicates that the structure in the active cavity of the enzyme might be induced to change by the additive, thereby modulating its affinity toward the substrate.

3.3. Molecular modeling

In order to gain insight into the mechanism by which the enzyme activity is enhanced by MT, the interactions between lipase and MT#20 were studied by molecular modeling.

In the three-dimensional structure of triacylglycerol complexed to lipase PCL (PDB entry 5lip), four binding pockets for the triacylglycerol could be detected: an oxyanion hole (25 Å × 15 Å × 10 Å) and three pockets (HA, HH and HB). The HA pocket binds the *sn*-3 fatty acid chain of triacylglycerol deeply buried in the enzyme's surface; the HH pocket accommodates the *sn*-2 fatty acid chain; and the HB pocket loosely binds the *sn*-1 chain [18]. However, it could be clearly observed that the pockets were too "massive" for the substrate (*R*)-NEMPA-ME (Fig. 4), and a large hydrophobic area (HH pocket) was exposed to the solvent, which would obviously affect the stability of PCL's "open" conformation. In addition, the larger pocket might easily lead to the incorrect orientation and pattern for NEMPA-ME binding to PCL.Molecular docking showed that

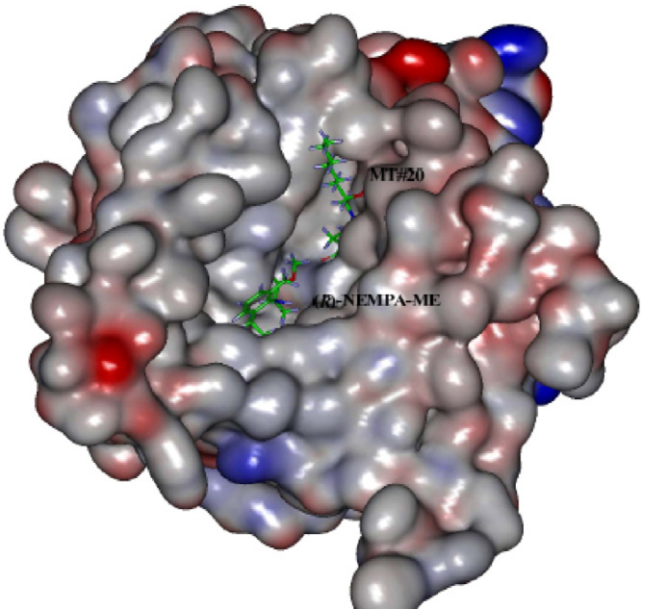

Fig. 4. The orientation and location of (*R*)-NEMPA-ME and MT#20 in PCL active cavity. The PCL molecular surface is colored according to the charge, where the red is negative, the blue is positive and the white is neutral. The (*R*)-NEMPA-ME and MT#20 are represented in sticks with carbon in green, oxygen in red, nitrogen in blue and hydrogen in white. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

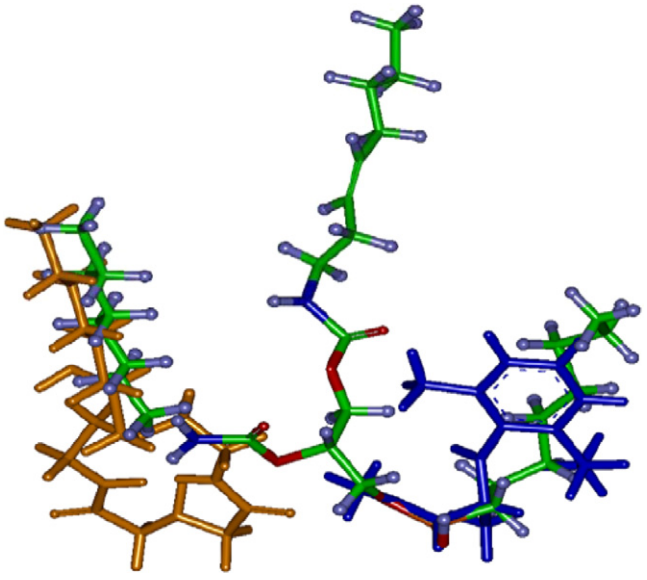

Fig. 5. The superposition of the modeled (*R*)-NEMPA-ME and MT#20 on the triacylglycerol. The (*R*)-NEMPA-ME and MT#20 are represented in blue and orange sticks, respectively. The triacylglycerol is represented in ball and stick type, with carbon in green, oxygen in red, nitrogen in blue and hydrogen in white. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

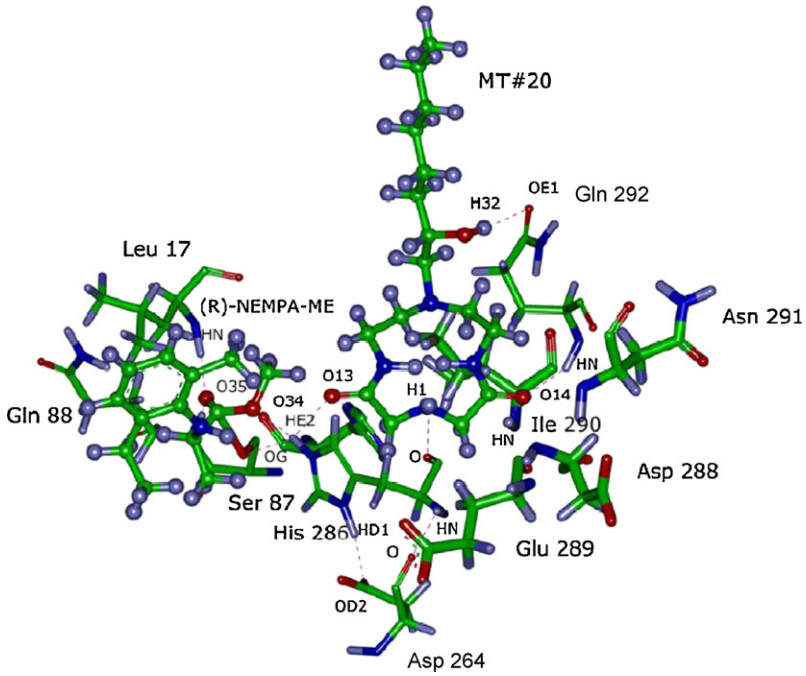

Fig. 6. Schematic representation of the hydrogen bonding formed between the active-site residues of PCL and MT#20. The (*R*)-NEMPA-ME and MT#20 are represented in ball and stick type, and the active-site residues of PCL are presented in sticks, with carbon in green, oxygen in red, nitrogen in blue and hydrogen in white. The hydrogen bonds are described in purple dotted lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

MT#20 could effectively bind to the part of the cavity which was exposed to the solvent, namely the HH pocket, as shown in Fig. 4. In this molecular docking model, the interaction between MT#20 and PCL can be divided into two parts. The macrocyclic part of MT#20 was in the more hydrophilic regions in the HH pocket while the side chain of MT#20 was in the stronger hydrophobic regions, vertically and tightly close to the wall of HH pocket, forming stronger van der Waals forces. This might effectively eliminate the active site of PCL exposed to the solvent, stabilize the PCL "open" conformation, and prevent incorrect binding of the substrate. The docking model also predicted that the MTs bearing C_6–C_{14} alkyl chain are beneficial, which is consistent with the results of our experimental data. If the alkyl side chain of MT was further extended, it would be exposed to the solvent. Conversely, if the chain was much shorter, it could only produce weak van der Waals force between the HH pocket wall and the MT molecular. Both cases would lead to lower binding strength and lower effectiveness in regulating the activity of PCL.

In order to further clarify the rationality of molecular docking, the triacylglycerol in crystal structure (PDB entry 5lip) was selected as a reference to compare the spatial arrangement of (*R*)-NEMPA-ME, MT#20 and triacylglycerol in PCL, as shown in Fig. 5. We found that the binding sites of (*R*)-NEMPA-ME and MT#20 overlapped with the triacylglycerol *sn*-2 chain and *sn*-3 position, respectively. Based on this, we think that MT might lead to a more native-like complex structure of the lipase and NEMPA-ME compared with that achieved without any additives.Multiple hydrogen bonds were observed between the macrocyclic part of MT#20 and the active part of PCL (Fig. 6). Information on those hydrogen bonds is listed in Table 2. The active-site His286 of the lipase was seen to be hydrogen-bonded to the carbonyl oxygen atom and N_1 hydrogen atoms of MT#20. Perturbation of the added MT on the hydrogen bonding of the active site might result in decrease in pK_a of the catalytic imidazole, thereby accelerating the reaction [23]. A hydrogen bond also connected another carbonyl oxygen atom in the MT ring to the main-chain hydrogen atom of Ile290 and Gln292. These hydrogen bond allow the enzyme to maintain its "open" conformation. In addition, we noticed that a hydrogen bond was formed between the branched chain hydroxyl of MT and the side chain

Table 2
The hydrogen bonding information.

Name	Donor atom	Acceptor atom	Distance	Angle DHA[d]
His286:HN-Asp264:O	HN	O	2.034	112.74
His286:HD1-Asp264:OD2[c]	HD1	OD2	1.796	169.80
His286:HE2-(*R*)-NEMPA-ME:O34	HE2	O34	1.823	137.49
His286:HE2-Ser87:OG[c]	HE2	OG	2.080	125.34
His286:HE2-MT#20:O13	HE2	O13	2.072	95.51
MT#20:H1-His286:O	H1	O	1.852	86.10
Leu17:HN-(*R*)-NEMPA-ME:O35[b]	HN	O35	1.895	144.70
Gln88:HN-(*R*)-NEMPA-ME:O35[a,b]	HN	O35	1.823	137.50
Ile290: HN -MT#20:O14	HN	O14	2.339	133.89
MT#20:H32-Gln292:OE1	H32	OE1	2.398	175.63
Gln292:HN-MT#20:O14	HN	O14	1.844	151.13

[a] Hydrogen bond formed by Gln88: HN and (*R*)-NEMPA-ME: O35 is on the other side and not shown in Fig. 6.
[b] Hydrogen bonds which contributed to the oxygen hole stabilization.
[c] Catalytic triad side-chain hydrogen bonding.
[d] Angle DHA means the angle formed by donor, hydrogen and acceptor.

oxygen atom of Gln292, which might explain why MTs containing hydroxyl groups on the side chain were more effective activitors than MTs without hydroxyl groups.

4. Conclusions

Initial rates of reaction in PCL-catalyzed hydrolysis of (*R*, *S*)-NEMPA-ME were significantly improved by the addition of some macrocyclic tetraamines (MTs). The structure and concentration of MTs had significant effect on their ability to enhance the hydrolysis reaction. Among the MTs that we selected for our study, MTs bearing C_6–C_{14} alkyl side chain or those containing hydroxyl groups on longer alkyl chains were found to be the best activators. Kinetic analysis showed that the affinity of PCL toward the substrate was strengthened when MTs were added in the reaction medium. Additionally, we did molecular modeling to investigate the possible mechanism by which MTs enhance the enzymatic activity of lipase. We found that MTs could change the conformation or flexibility of lipase and affect the active cavity of PCL, thereby leading to an enhancement of the reaction rate. This work not only reveals a new way to regulate the activity of lipase but also shows a novel use of MTs.

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