Journal of Molecular Catalysis B: Enzymatic 74 (2012) 83–88

Contents lists available at SciVerse ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

Enhancing lipase-catalyzed hydrolysis by adding macrocyclic tetraamines

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ARTICLE INFO

Article history:
Received 20 May 2011
Received in revised form 4 September 2011
Accepted 4 September 2011
Available online 10 September 2011

Keywords:
Pseudomonas cepacia lipase
Resolution
N-(2-ethyl-6-methylphenyl)alanine
Macrocyclic tetraamines

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.

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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doi:10.1016/j.molcatb.2011.09.002

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.