

Modeling and Experimental Analysis of Cephalosporin C Acylase and Its Mutant

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Abstract: 7-amino cephalosporanic acid (7-ACA) is the crucial intermediate for the synthesis of semi-synthetic antibiotics, which is currently prepared by two-step biocatalysis using D-amino acid oxidase and glutaryl-7-amino cephalosporanic acid acylase (GL-7-ACA acylase) starting from cephalosporin C (CPC). Compared with the two-step enzymatic method, one-step method is more efficient and economical. But, the available Cephalosporin C acylase (CPC acylase) always take glutaryl-7-amino cephalosporanic acid (GL-7-ACA) as their primary substrate, and have low catalytic activities towards CPC to be used in industry. We investigated the catalytic mechanism of CPC acylase by the sequence alignment, homology modeling, and active site analysis to a series of CPC acylases from *Pseudomonas* where some effective mutations have been reported for activity enhancement. Two CPC acylases coded by the genes *acyII* and *S12* are studied intensively for the interaction between the amino acid residues in the activity region and the substrate CPC based upon the complex structure obtained from the homology modeling and molecular docking. Furthermore, the catalytic parameters of the two CPC acylases were measured experimentally in order to corroborate the modeling analysis and propose potential designing strategy for improvement of enzymic activity.

Keywords: 7-amino-cephalosporanic acid, Cephalosporin C, Cephalosporin C acylase, Homology modeling, Molecular docking, Protein designing.

1. INTRODUCTION

The semi-synthetic cephalosporins became the popular antibiotics due to their excellent characteristics such as broad spectrum, low toxicity, and resistance to the β -lactamase and made tremendous contribution to fight with bacterial infection [1]. The semi-synthetic cephalosporin is synthesized from the intermediate 7-amino cephalosporanic acid (7-ACA), which shares more than 40% of the global anti-infective market [2].

Currently, 7-ACA used for the semi-synthetic cephalosporin antibiotics is produced from Cephalosporin C (CPC) by either the chemical or the enzymatic methods. Among them, the two-step enzymatic method, is becoming dominant gradually [3, 4] because of its friendship to environment. However, this process is expensive and can't completely satisfy the industrial production. In comparison, the one-step enzymatic method is efficient and has been studied intensively [2]. Researchers separated CPC acylase from the micro-organisms which could convert CPC into 7-ACA directly [5-9]. But, the application of the wild strain was inconvenient [3] and the question was that CPC acylases

used glutaryl-7-amino cephalosporanic acid (GL-7-ACA) as their primary substrate normally and their specificity towards CPC was too low to be used in industry [10, 11]. Some mutations with improved activity towards CPC have been developed By protein engineering of the CPC acylases Oh *et al.* [12] found that the deacylation activity of the mutation Q50 β M-Y149 α K-F177 β G toward CPC was improved by 790%. Pollegioni *et al.* [2] used the approach of the the homology modeling combined with the site-directed mutagenesis to produce the A215Y-H296S-H309S mutation which had slightly higher activity towards CPC (3.8U/mg protein) than to GL-7-ACA (2.7 U/mg protein). Ishii *et al.* [13] found that the mutation M269W caused the 1.6-fold increase of the specific activity against CPC and observed that the minor change of conformation induced by the mutation increased the stability of the enzyme-substrate complex. Saito *et al.* [14] suggested that Met164 was located in the binding region in the interior surface of the CPC acylase for recognition of the substrate and found that the mutation M164L enhanced CPC acylase activity.

In this paper, the homology modeling and the structural analysis have been applied into a series of CPC acylases on which some mutations have been reported for improved activity (Table 1). The two genes named *acy II* and *S12* coding the CPC acylase *Acy II* and its mutation named *S12* respectively. *acy II* and *S12* were constructed into pET28a and expressed in the *E.coli* BL21(DE3) for experimental analysis.

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Table 1. Reported CPC Acylases and Mutants from *Pseudomonas* sp.

Author, Year	Modified Amino Acids	Enzyme Activity
Ishii <i>et al.</i> , 1994	Y270F	Decreased activity
Nobbs <i>et al.</i> , 1994	Y270F	Decreased activity
Ishii <i>et al.</i> , 1995	M269Y or F	1.6-fold and 1.7-fold increase
Saito <i>et al.</i> , 1996 ^b	M164L	Enhance activity
Saito <i>et al.</i> , 1996 ^a	A271Y	Increase 1.2-fold
	Y270A or Y270F or Y270L or Y270S	decrease
Yamada <i>et al.</i> , 1996	C199S,C277S,C305S,C391S,C496S	decrease
	C305S-M269S	1.6-fold increase

^a[14]; ^b[26].

With the approach of the homology modeling and the experimental analysis, we established the preliminary knowledge about discovering the potentially efficient CPC acylase *in silico*, which played the important role for the one-step preparation of 7-ACA for CPC enzymatically.

2. MATERIALS AND METHODS

2.1. Homology Modeling

A series of CPC acylases originated from *Pseudomonas* sp. were chosen for modeling analysis in this work. Six protein templates from PDB [15] were selected to build the homology model of AcyII, i.e., Penicillin G acylase from *Escherichia coli* (PDB code 1e3a) sharing 36.3% sequence similarity with AcyII; Penicillin acylase complexed with 3, 4-dihydroxyphenylacetic acid (PDB code 1ai4) sharing 37.5% similarity; Cephalosporin acylase in complex with glutaryl-7-aminocephalosporanic acid (PDB code 1jvz) sharing 37.9% similarity; Penicillin amidohydrolase (PDB code

1pnm) sharing 36.5% similarity; Glutarylamidase (PDB code 1gk1) sharing 38.5% similarity, and Penicillin G acylase from *Alcaligenes faecalis* (PDB code 3k3w) sharing 39.4% similarity with AcyII. The result of sequence alignment was obtained by using Discovery Studio 2.1 (Accelrys, v2.0, 2009).

The model of AcyII was constructed by using the Homology Modeling Module in Accelrys Discovery Studio 2.1. The quality of the predicted model was evaluated by the Discrete Optimized Protein Energy (DOPE) by running the Verify Protein module. By the CHARMM force field [16], the conformation of amino acid residues in AcyII structural model was further modified by a standard dynamics cascade created by joining a set of steps of minimization and equilibration including minimization with steepest descent, minimization with conjugate gradient, dynamics with heating, equilibration dynamics and production dynamics. Next, the potential binding region on which the CPC was docked were identified by the Dock Ligands Module in Discovery Studio.

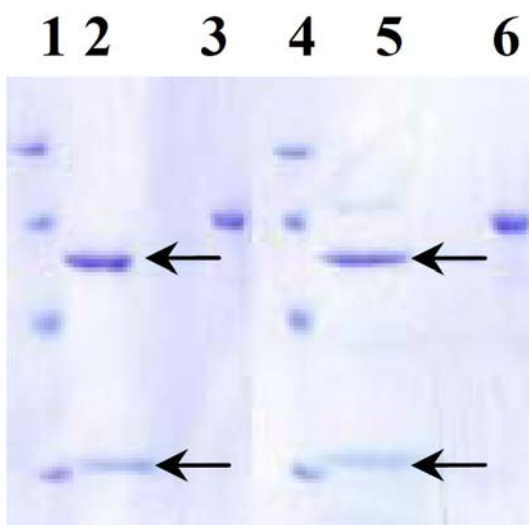


Fig. (1). SDS-PAGE of purified CPC acylase Acy II and S12. Lane 1, Molecular weight marker (from above to below: 97.2kDa, 66.4kDa, 44.3kDa, 29.0kDa); lane 2, Acy II; lane 3, BSA; lane 4, Marker; lane 5, S12; Lane 6, BSA.

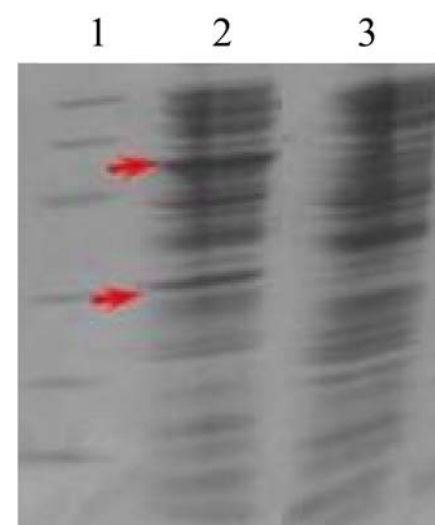


Fig. (2). Expression of CPC acylase Acy II and S12. Lane 1, Molecular weight marker (from above to below: 97.2kDa, 66.4kDa, 44.3kDa, 29.0kDa, 20.1kDa, 14.3kDa); lane 2, Acy II; lane 3, S12.

After obtaining the preliminary model of AcyII-CPC complex, the PRODA, a PROtein Design Algorithmic software [17, 18], was applied to place the CPC on the active region under the catalytic constraints between the CPC and the four catalytic residues, i.e., Ser β 1, His β 23, His β 70 and Asn β 242.

2.2. Experimental Procedures

2.2.1. Mutagenesis

The gene *acy II* [19] was synthesized. Its mutant named *S12* was obtained by the overlapping primer PCR with the substituted amino acid residues V121 α A-G139 α S-F58 β N-I75 β T-I176 β V-S471 β C. The two genes were cloned into the pET28a(+) plasmids, sequenced and transformed into the *E. coli* BL21(DE3). For all primers, mutant positions were denoted in lowercase and the restriction sites are underlined. Backward primers designed with completely complementary role are marked with asterisk.

5'-GAGCTCATGACCATGGCGGCGAA-3' *CTCGAGTACTGGTACCGCCGCTT

5'-CAGGAAGTGGTGCCGGCGCTCGAG-3'
*GTCCTTGACCACGGCCGCGAGCTC

5'-GCGTATgcgGCTGGAGTTAA-3'
*CGCATAcgcCGACCTCAATT

5'-CGAATATagcCTGCT-3' *GCTTATAtcgGACGA

5'-GCTTTCCGCATaatGCGCA-3'
*CGAAAGGCGTAttaCGCGT

5'-CGTTTATGGATaccCAT-3'
*GCAAATACCTAtggGTA-3'

5'-GGCCTGgttGATCAT-3' *CCGGACcaaCTAGTA

5'-CGCGCTGtgcCGTTAT-3'
*GCGCGACacgGCAATA

2.2.2. Expression and Purification of AcyII and S12

E. coli BL21(DE3) carrying the gene *acy II* was grown in LB medium containing 50 μ g/mL kanamycin with shaking at 37°C overnight. A quantity of 100ml of fresh LB medium was inoculated with 1ml overnight culture and incubated with shaking to an O.D._{600nm} of 0.6. Then expression was induced by addition of 1mmol L⁻¹. The cell pellet of 100ml induced BL21(DE3) containing *acy II* was suspended in 100mM Tris-HCl buffer (pH8.0) and was sonicated for 20 \times 10s with 10s pause at 200-300w. The supernatant was loaded to 2ml Ni-NTA and eluted with 0mM imidazole, 50mM imidazole, 100mM imidazole, 200mM imidazole and 500mM imidazole in a succession. The target fractions were pooled and analyzed by SDS-PAGE.

2.2.3. Assay of AcyII and S12 Activity

Enzymic activity was determined for conversion of CPC to 7-ACA. 500 μ l S12 (approximately 1 μ M for CPC) was added to 500 μ l CPC (20 mg/ml in 0.1 M Tris/HCl, pH 8.0), and the mixture was incubated at 37°C for 8 min. The reaction was stopped by addition of 5% acetic acid. After centrifugation (10,000 rpm, 5 min), the formed 7-ACA in the supernatant was determined by HPLC. One unit was defined as the amount of the enzyme liberating 1 μ mol 7-ACA/min.

3. RESULTS AND DISCUSSION

3.1. Analysis of AcyII Model

The Ramachandran diagram for the model of AcyII shows that there were 92.1% of residues falling in the allowed region, and 6.3% of residues in the marginal region. The remaining 1.6% of residues in the disallowed region was mostly far from the active region. In addition, the Verify Score of the model predicted by Accelrys Discovery Studio 2.1 was 178.74 while the Verify Expected High and Low Scores were 214.58 and 96.56, respectively. (The Verify Expected High Score is the score that would be expected for a correct structure having this sequence length, based on a statistical analysis of high-resolution structures in the Protein Data Bank. The Verify Expected Low Score is 45% of the first and is a score that is typical of grossly misfolded structures having this sequence length. The higher the Verify Score is and the more correct the structure is). These two types of data confirmed the reliability and correctness of the model of AcyII.

The homologous model and the active region of AcyII shown in Fig. (3a,b) and Fig. (4) were consistent with those of other acylases described by Fritz-Wolf *et al.* [20]. These acylases used conserved Ser1 β as catalytic residue which was characteristic of the N-terminal hydrolase family. For Ser1 β , its hydroxyl group was fixed by the conserved His23 β and its NH group formed a hydrogen bond with His23 β . The NH groups from the backbone of His70 β and side chain of Asn242 β formed the oxyanion hole for carboxyl group of CPC. The sequence alignment (Fig. 5a) for different acylases, i.e., AcyII, CAD [21], and PGA [22] implied that those catalytic residues were conserved, just His70 β was variable. The role of His70 β was to stabilize the hydrogen bond by using its backbone, which was consistent with the corresponding residues in PGA and CAD whose crystal structures were known. In the binding region, oxygen atoms from the carboxylate group of CPC interacted with Arg24 β , Tyr32 β and His57 β . The amino adipyl moiety of CPC was stabilized

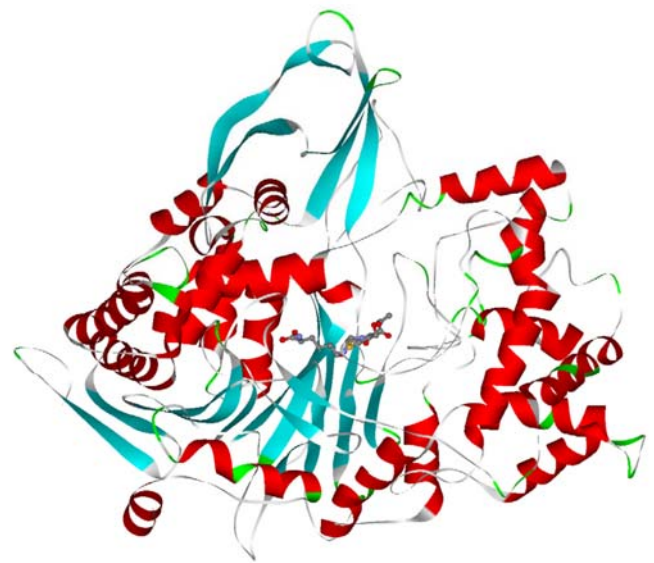


Fig. (3a). The model of AcyII, in which the substrate CPC is shown in ball and stick mode.

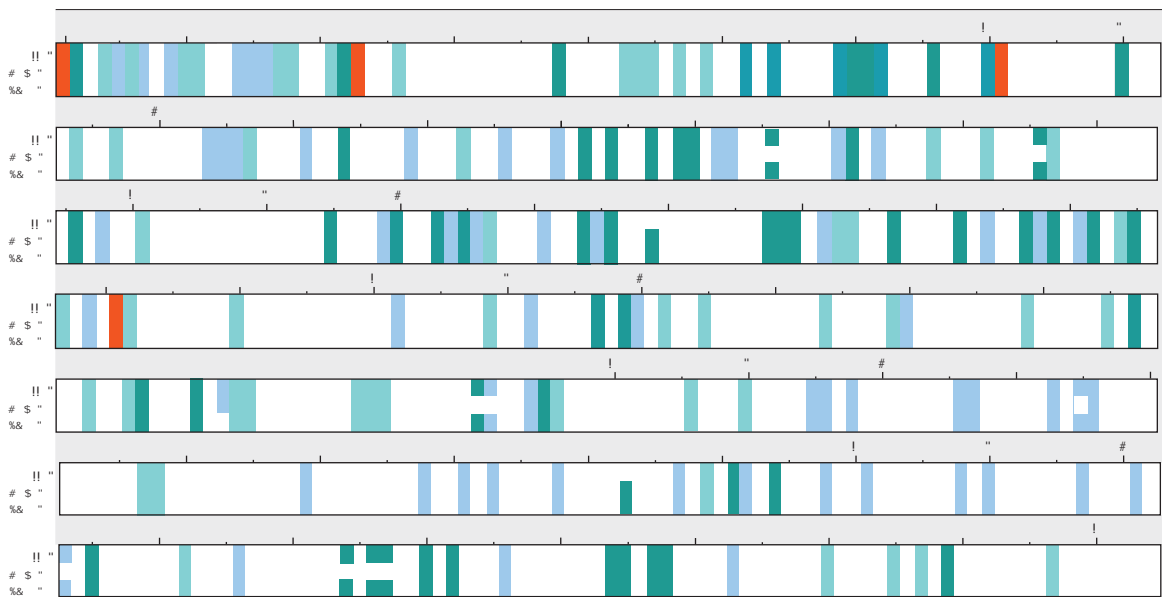


Fig. (5a). Sequences alignment of β -chains from AcyII, CAD and PGA

The four catalytic sites, i.e., Ser1 β , His23 β , His70 β and Asn242 β in AcyII, and their corresponding sites in CAD and PGA are shown in red.



Fig. (5b). Sequence alignment of N176 and Acy II, where different residues are indicated.

I75 β T and I176 β V were close to the active region according to the model. For the mutation F58 β N firstly, Asn58 β had the polar carboxyl group which was different from the original hydrophobic Phe58 β , thus could form hydrogen bond with the glyoxaline group on His 57 β and stabilize the car-

boxyl group of CPC by the N-O hydrogen bond, as that shown in Fig. (6a). Secondly, the phenyl group from Phe58 β would clash with CPC because of its larger side chain and lead to increasing energy and decreasing stability compared with the mutation Asn58 β .

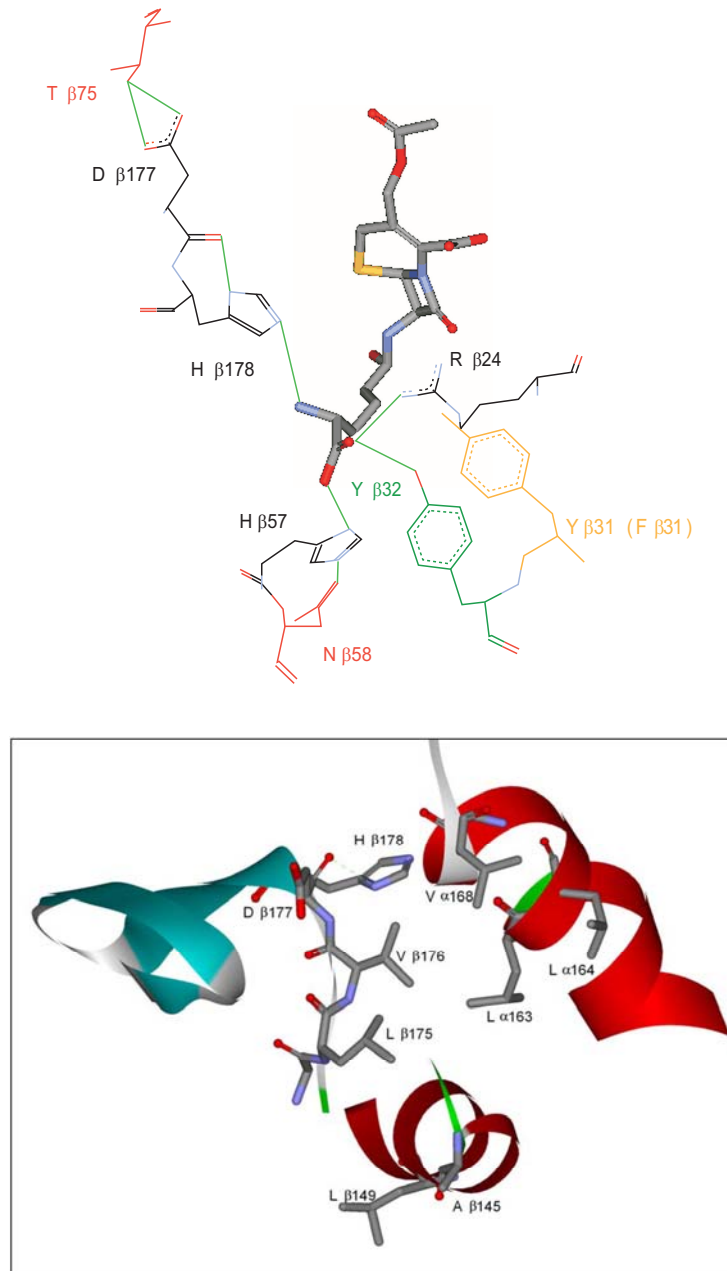


Fig. (6). View of the mutations at active sites of AcylII

(a). The two residues, i.e., Asn58 β and Thr75 β , are colored by red. The residues Tyr31 β or Phe 31 β is colored by orange. The important binding residue Tyr32 β which interacts with the CPC is colored by dark green. The other residues are shown in line mode while CPC is in stick mode; (b). The residue Val176 β with its neighboring non-polar residues

As to the mutation I75 β T, the non-polar side residue Ile75 β was replaced by the polar residue Thr75 β which was located in a loop area in the binding region shown by Fig. (6a). The carboxyl group on Thr75 β could stabilize the neighboring Asp177 β by forming two hydrogen bonds. The C-O group on Asp177 β interacted with ND1 on His178 β which fixed the amino adipyl moiety in CPC. These structural interactions implied that the mutation from Ile75 β to Thr75 β was more favorable because the polar side chain contributed to the stability of the binding region by supplying additional hydrogen bonds.

For the mutation I176 β V, Val176 β had shorter side chain than that of Ile176 β , which could avoid the side chain clashes with the neighboring residues, such as Leu163 α and Leu175 β , as that shown in Fig. (6b). And Val176 β was located in a loop region near the two important binding residues, i.e., Asp175 β and His176 β , which interacted with the amino adipyl moiety in CPC, so the effect of reducing spatial clashes was beneficial to stabilize the important interactions for binding.

In addition, the three residues, V121 α A, G139 α S and S471 β C were far from the active region. The mutations V121 α A and G139 α S were related to expression level as

Table 2. Catalytic Kinetic Parameters for CPC Acylase Acy II and S12

Protein	K_m (mM)	k_{cat} (sec ⁻¹)	k_{cat}/K_m (sec ⁻¹ (μ M) ⁻¹)
Acy II	23.71	7.622	0.321
S12	15.26	14.03	0.919

Specific activities for Acy II and S12 are measured to be 2.868 unit/mg proteins and 6.011 units/mg proteins for CPC, respectively (pH8.0). Kinetic parameters were calculated from Lineweaver-Burk plots of the primary velocity of 7-ACA transformed directly from CPC (2.1, 4.2, 6.3, 8.4, 10.5 and 20.9mM) in the presence of Acy II and S12 (1 μ M) at 37°C for 8 min.

shown in Fig. (2) [26]. The mutation S471 β C gave S12 the product inhibition as Pollegioni's report [2].

3.4. Experimental Analysis About Mutation of AcyII

From the SDS-PAGE results shown in Fig. (1), we observed that both of the CPC acylase AcyII and S12 were expressed with the MW approximately 87 kDa and was composed of two subunits, the 58kDa α -subunit and the 25kDa β -subunit, which was consistent with that reported by Mstsuda *et al.* [27]. The expression levels of AcyII and S12 were measured to be 322 U/L and 291 U/L, respectively, shown in Fig. (2). The specific activities of AcyII and S12 were measured and the results were shown in Table 2. And we could see that the specific activity of S12 was 2-fold higher than that of AcyII and it reaches 6.011 U/mg protein. The catalytic parameters K_m , k_{cat} and k_{cat}/K_m of AcyII and S12 were determined by Lineweaver-Burk plot method. The k_{cat}/K_m of S12 was higher than that of AcyII, which indicated that the mutation of six amino acid residues increased the catalytic efficiency. The result was similar to that of N176 [14]. Combined with the theoretical analysis based on structural modeling presented in Section 3.3, it was implied that the mutations around the active region, i.e., F58 β N- I75 β T- I176 β V, enhanced the binding capability between the enzyme and the transition state of the substrate instead of substrate itself since S12 had larger K_m but higher k_{cat} . By virtue of the transition state theory for enzyme catalysis [28], the enhancement of binding between enzyme and the transition state of substrate reduced the activation energy and led to the increase of turnover number, i.e., k_{cat} . Because the structures between substrate and its transition state was different, the strong binding capability between enzyme and transition state of the substrate increased the dissociation reaction between enzyme and the substrate, which could be certificated by the Michaelis constant, i.e., K_m , shown in Table 2.

4. CONCLUSION

In this work, Acy II from *Pseudomonas sp.* and the mutation named S12 were structurally modeled and experimentally characterized in order to investigate their catalytic mechanism for further designing highly efficient enzyme to the one-step preparation of 7-ACA from CPC. With the methods of sequence alignment, homology modeling, and molecular docking, the structures of the active region of CPC acylase and the complex were obtained and the mutations around active site were analyzed based on intermolecular binding interaction, including steric hindrance, hydrogen bonding stabilization, solvation, and electrostatic contribution. The further experimentally measured catalytic param-

eters, i.e., k_{cat} and K_m , for CPC acylase AcyII and S12 confirmed the predicted model and provided strong evidence that the mutations around active region for increased activity would contribute to binding reaction between enzyme and the transition state substrate and decreasing activation energy for reaction. This implied that further designing of highly efficient CPC acylase *in silico* should focus on the amino acid sites for stronger transition state binding capability.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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