Modeling and Experimental Analysis of Cephalosporin C Acylase and Its Mutant

Ren Yu*

Key Laboratory of Ecology and Biological Resources in Yarkand Oasis at Universities under the Education Department of Xinjiang Uygur Autonomous Region, Department of Kashi Normal College, No.29. Xueyuan Road, Kashi Prefecture, Xinjiang Uygur Autonomous Region, China

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 Q50βM-Y149αK-F177βG 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 mutantion M164L enhanced CPC acylase activity.

In this paper, the homology modeling and the structural analysis have been applied into a series of CPC acylyses 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.

*Address correspondence to this author at the Key Laboratory of Ecology and Biological Resources in Yarkand Oasis at Universities under the Education Department of Xinjiang Uygur Autonomous Region, Department of Biological Science, Kashi Normal College, No.29. Xueyuan Road, Kashi Prefecture, Xinjiang Uygur Autonomous Region, China;
Tel: 0086-15739952831; E-mail: renyu1020@yahoo.com.cn

1874-0707/13

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

Author, Year	Modified Amino Acids	Enzyme Activity
Ishii *et al.*, 1994	Y270F	Decreased activity
Nobbs *et al.*, 1994	Y270F	Decreased activity
Ishii *et al.*, 1995	M269Y or F	1.6-fold and 1.7-fold increase
Saito *et al,* 1996[b]	M164L	Enhance activity
Saito *et al.*, 1996[a]	A271Y	Increase 1.2-fold
	Y270A or Y270F or Y270L or Y270S	decrease
Yamada *et al.*, 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 *Alcaligence 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.

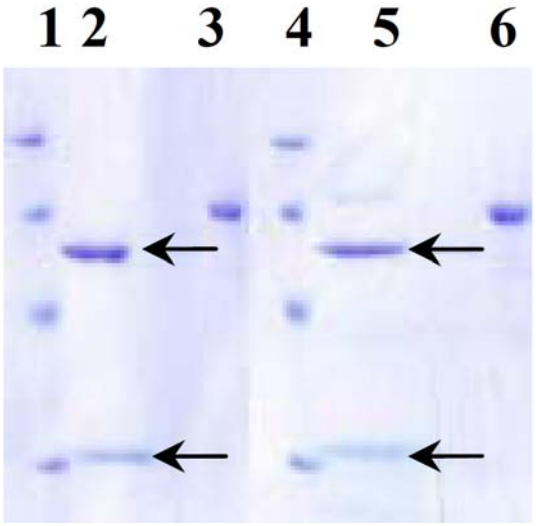

Fig. (1). SDS-PAGE of purified CPC acylase AcyⅡ and S12
Lane 1, Molecular weight marker（from above to below: 97.2kDa, 66.4kDa, 44.3kDa, 29.0kDa）; lane2, AcyⅡ; lane 3, BSA; lane 4, Marker; lane 5, S12; Lane 6, BSA.

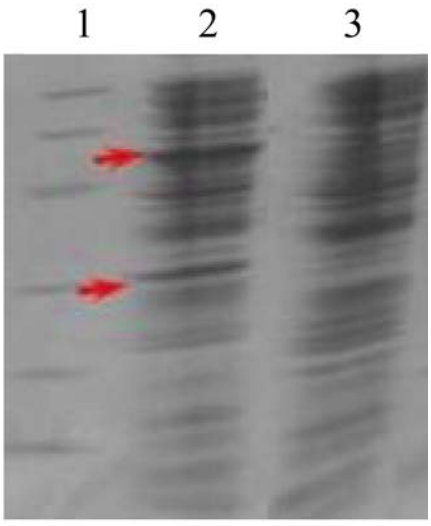

Fig. (2). Expression of CPC acylase AcyⅡ and S12
Lane 1, Molecular weight marker（from above to below: 97.2kDa, 66.4kDa, 44.3kDa, 29.0kDa, 20.1kDa, 14,3kDa）; lane2, AcyⅡ; lane 3, S12.

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 β1, Hisβ23, Hisβ70 and Asnβ242.

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 V121αA-G139αS-F58βN-I75βT-I176βV-S471βC. The two genes were cloned into the pET28a(+) plasmids, sequenced and transformed into the *E.coli* BL21(DE3). For all primers, mutant positions were donoted in lowercase and the restriction sites are underlined. Backward primers designed with completely complementary role are marked with asterisk.

5'-GAGCTCATGACCATGGCGGCGAA-3'*CTCGAGTACTGGTA-CCGCCGCTT

5'-CAGGAACTGGTGCCGGCGCTCGAG-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 ug/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 20×10s with 10s pause at 200-300w. The supernatant was loaded to 2ml Ni-NTA and eluted with 0mM inidazole, 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 u1 S12 (approximately 1 uM for CPC) was added to 500 ul 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 umol 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 Ser1β as catalytic residue which was characteristic of the N-terminal hydrolase family. For Ser1β, its hydroxyl group was fixed by the conserved His23β and its NH group formed a hydrogen bond with His23β. The NH groups from the backbone of His70β and side chain of Asn242β 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 His70β 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 Arg24β, Tyr32β and His57β. The amino adipyl moiety of CPC was stabilized

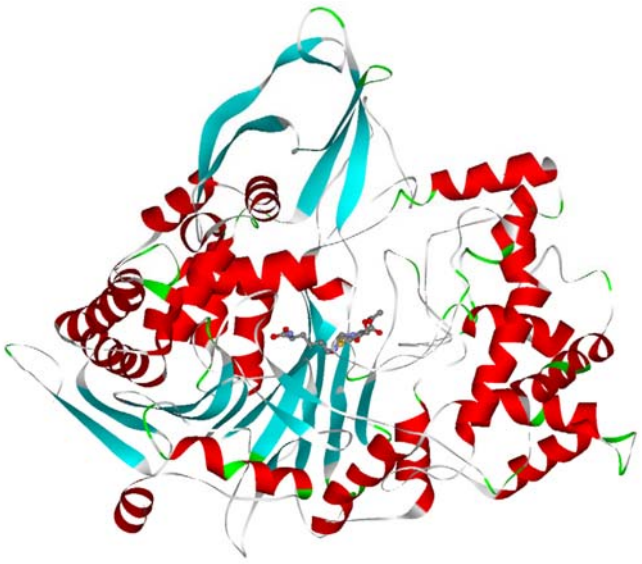

Fig. (3a). The model of AcyII, in which the substrate CPC is shown in ball and stick mode.

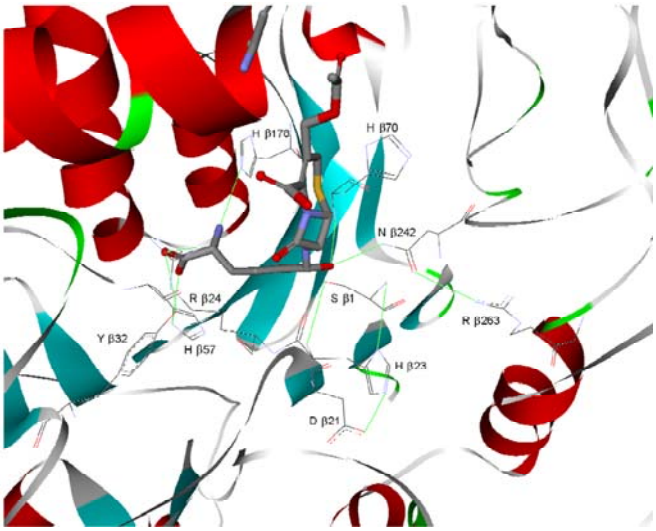

Fig. (3b). View of the active region with substrate for AcyII based on model.
The key catalytic and binding residues are shown in thin line model while the substrate CPC is shown in stick model. The hydrogen bonds are shown in green lines.

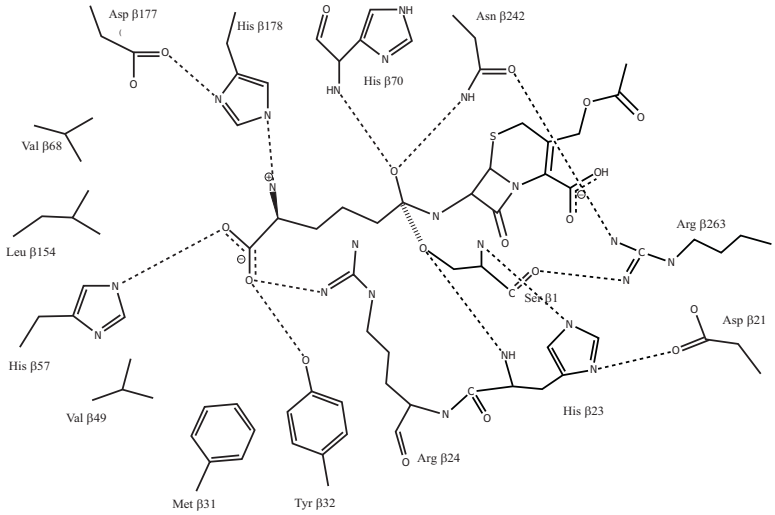

Fig. (4). Schematic drawing of the active amino acid residues for AcyII based on model.

by the formed hydrogen bond with His178β which simultaneously interacted with Asp177β stated by Fig. (**4**).

N176 was found active to CPC. Since AcyII and N176 shared a sequence similarity up to 93.5%, shown by Fig. (**5b**) for sequence alignment, it was reasonable to consider that N176 and S12 shared the high structural similarity. So the analysis of mutation of N176 could be based on the same model of AcyII.

3.2. Structural Analysis of Active Site Mutations of N176

Ishii *et al*. [23] reported that the mutation of W32βF led to the decreasing of k_{cat} to about 50%. Nobbs *et al.* [24] reported the mutation of W32βL whose k_{cat} was reduced by 32.2%. Another effort was made by Saito *et al.* [25] who changed Tyr32β to Ala/ Ser, which resulted in the decreasing of specific activity. These three studies confirmed the key binding effect of Tyr32β which interacted with the carboxyl group of CPC shown in Fig. (**6a**). Either of the mutations to Phe32β or Leu32β demolished the original hydrogen bond with the carboxyl group on CPC, thus led to the decreasing of binding effect with CPC. The decreased activity from mutation W32βS was possibly that the distance between the polar groups on Ser32β and CPC was too short to form hydrogen bond.

The mutations from Met31β to Phe31β or Tyr31β reported by Ishii *et al.* [13] enhanced catalytic activity by 1.6-fold and 2.5-fold. The effect of these mutations is rational as the phenyl group from either phenylalanine or tyrosine could form π-π conjugation with that from the neighboring Tyr32β and this would help the neighboring residue Tyr32β to maintain the suitable conformation in order to form the hydrogen bond with CPC, as that shown by Fig. (**6a**).

3.3. Structural Analysis About the Mutations for AcyII

In this paper, the mutation V121αA-G139αS-F58βN-I75βT-I176βV-S471βC named S12 was investigated. F58βN,

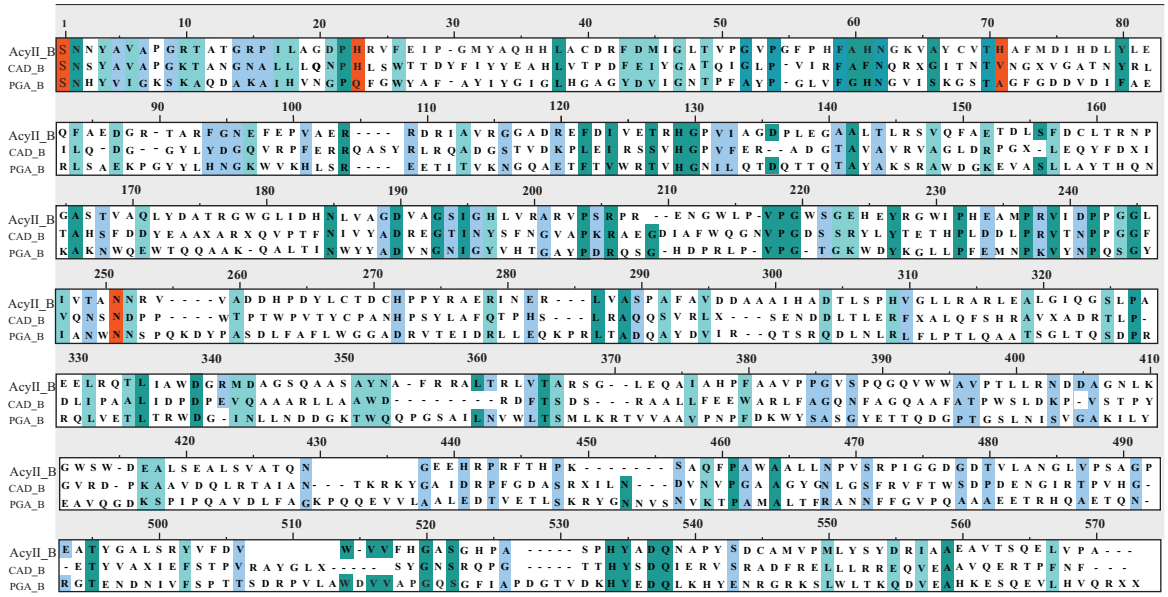

Fig. (5a). Sequences alignment of β-chains from AcyII, CAD and PGA
The four catalytic sites, i.e., Ser1β, His23β, His70β and Asn242β in AcyII, and their corresponding sites in CAD and PGA are shown in red.

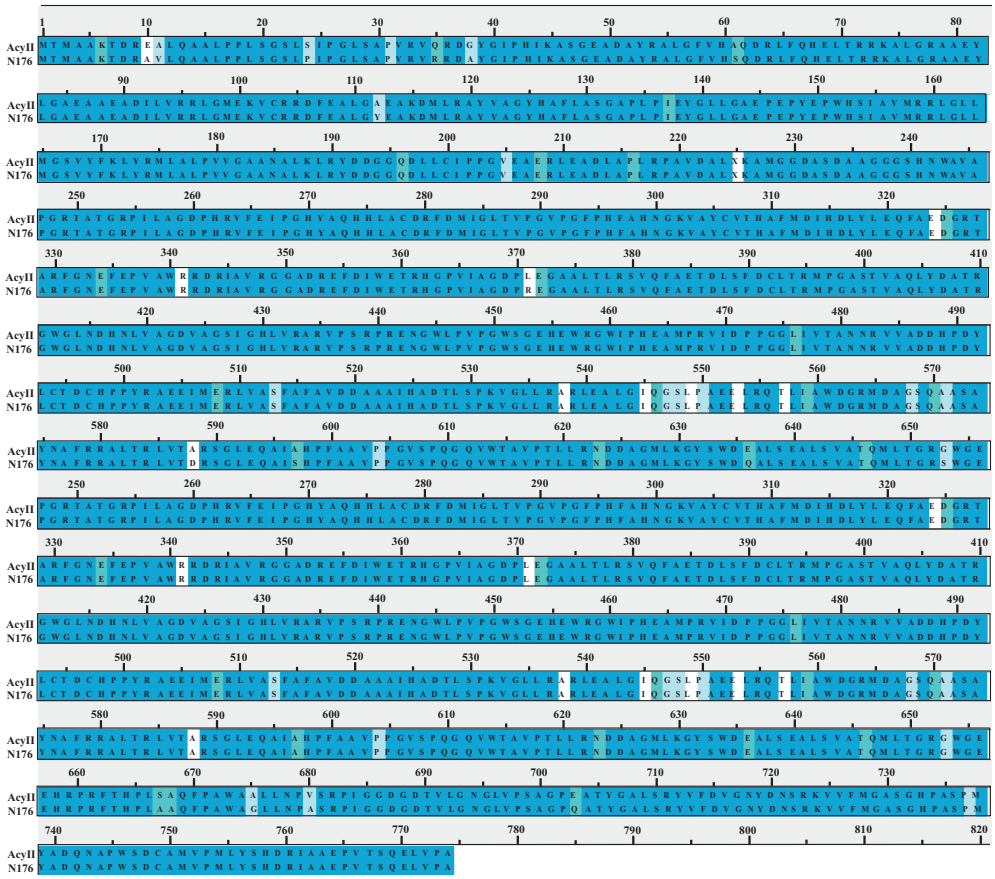

Fig. (5b). Sequence alignment of N176 and Acy Ⅱ , where different residues are indicated.

I75βT and I176βV were close to the active region according to the model. For the mutation F58βN firstly, Asn58β had the polar carboxyl group which was different from the original hydrophobic Phe58β, thus could form hydrogen bond with the glyoxaline group on His 57β and stabilize the carboxyl group of CPC by the N-O hydrogen bond, as that shown in Fig. (**6a**). Secondly, the phenyl group from Phe58β would clash with CPC because of its larger side chain and lead to increasing energy and decreasing stability compared with the mutation Asn58β.

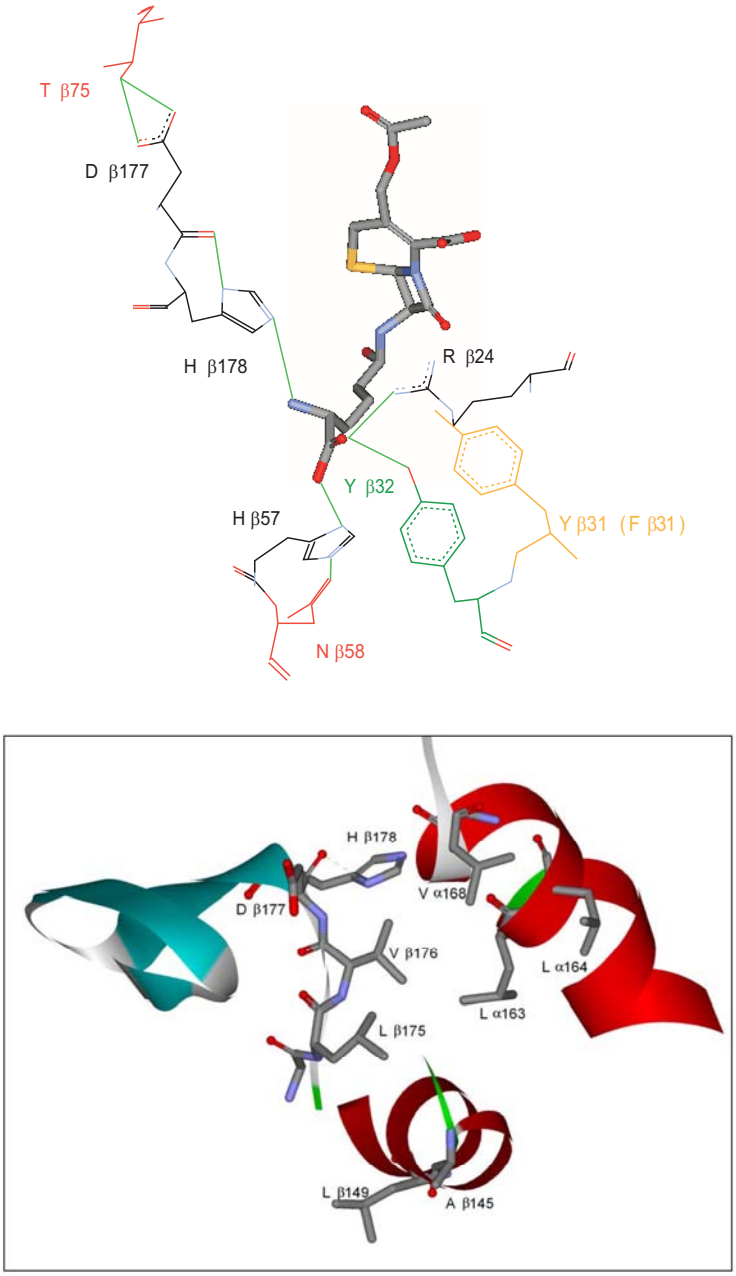

Fig. (6). View of the mutations at active sites of AcyII
(**a**). The two residues, i.e., Asn58β and Thr75β, are colored by red. The residues Tyr31β or Phe 31β is colored by orange. The important binding residue Tyr32β which interacts with the CPC is colored by dark green. The other residues are shown in line mode while CPC is in stick mode; (**b**). The residue Val176β with its neighboring non-polar residues

As to the mutation I75βT, the non-polar side residue Ile75β was replaced by the polar residue Thr75β which was located in a loop area in the binding region shown by Fig. (**6a**). The carboxyl group on Thr75β could stabilize the neighboring Asp177β by forming two hydrogen bonds. The C-O group on Asp177β interacted with ND1 on His178β which fixed the amino adipyl moiety in CPC. These structural interactions implied that the mutation from Ile75β to Thr75β was more favorable because the polar side chain contributed to the stability of the binding region by supplying additional hydrogen bonds.

For the mutation I176βV, Val176β had shorter side chain than that of Ile176β, which could avoid the side chain clashes with the neighboring residues, such as Leu163α and Leu175β, as that shown in Fig. (**6b**). And Val176β was located in a loop region near the two important binding residues, i.e., Asp175β and His176β, 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, V121αA, G139αS and S471βC were far from the active region. The mutations V121αA and G139αS 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℃ for 8 min.

shown in Fig. (**2**) [26]. The mutation S471βC 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., F58βN- I75βT-I176βV, 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 stronge 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 parameters, 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.

ACKNOWLEDGEMENT

Y.R sincerely appreciates the financial support from the National Science Foundation of China and the National High Technology Research and Development (863) Program of China. Y.R thanks to Dr. Yushan Zhu and Dr. Jianan Zhang for their support and helpful discussion throughout the experimental course of this work.

REFERENCES

[1] Stefanie S, Christian MD, Jeffrey S, *et al*. The cephalosporin antibiotics. Prim Care Update for Ob Gyns 1997; 4: 168-74.

[2] Pollegioni L, Lorenzi S, Rosini E, *et al*. Evolution of an acylase active on cephalosporin C. Protein Sci 2005; 14: 3064-76.

[3] Shibuya Y, Matsumoto K, Fuji T, *et al*. Isolation and properties of 7β-(4-carboxy butanamido) cephalosporanic acid acylase producing bacteria. Agric Biol Chem 1981; 45: 1561-7.

[4] Ichikawa S, Murai Y, Yamamoto S, *et al*. The isolation and properties of *Pseudomonas* mutants with all enhanced productivity of 7β-(4-carboxybutanamido) cephalosporinic acid acylase. Agric Biol Chem 1981; 45: 2225-9.

[5] Zhu SC, Yang YL, Zhao GP, *et al*. A rapid and specific method to screen environmental microorganisms for cephalosporin acylase activity. J Microbiol Methods 2003; 54: 131-5.

[6] Kim DW, Yoon KH. Cloning and high expression of glutaryl 7-aminocephalosp- oranic acid acylase gene from *Pseudomonas diminuta*. Biotechnol Lett 2001; 23: 1067-71.

[7] Aramori I, Fukagawa M, Tsumura M. Cloning and nucleotide sequencing of a novel 7β-(4-carboxybutanamido) cephalosporanic acid acylase gene of *Bacillus laterosporus* and its expression in *Escherichia coli* and *Bacillus subtilis*. J Bacteriol 1991[a]; 173: 7848-55.

[8] Aramori I, Fukagawa M, Tsumura M, *et al*. Isolation of soil strains producing new cephalosporin acylases. J Ferment Bioeng 1991[b]; 72: 227-31.

[9] Aramori I, Fukagawa M, Tsumura M, *et al*. Cloning and nucleotide sequencing of new glutaryl 7-ACA and cephalosporin C acylase genes from *Pseudomonas* strains. J Ferment Bioeng 1991[c]; 72: 232-43.

[10] Zhang QJ, Xu WX. Morphological, physiological and enzymatic characteristics of cephalosporin acylase-producing *Arthrobacter* strain 45-8A. Arch Microbiol 1993; 159: 392-5.

[11] Deshpands BS, Ambedkar SS, Shewale JG. Cephalosporin C acylase and penicillin V acylase formation by *Aeromonas* sp.ACY95. World J Microbiol Biotechnol 1998; 12: 373-8.
[12] Oh B, Kim M, Yoon J, *et al*. Deacylation activity of cephalosporin acylase to cephalosporin C is improved by changing the side-chain conformations of active-site residues. Biochem Biophys Res Commun 2003; 310: 19-27.
[13] Ishii Y, Saito Y, Fujimura T, *et al*. High-level production, chemical modification and site-directed mutagenesis of a cephalosporin C acylase from *Pseudomonas* strain N176. Eur J Biochem 1995; 230: 773–8.
[14] Saito Y, Ishii Y, Fujimura T, *et al*. Protein engineering of a cephalosporin C acylase from *Pseudomonas* strain N176. Ann NY Acad Sci 1996[b]; 782: 226-40.
[15] Berman HM, *et al.* The Protein Data Bank. Acta Crystallogr D Biol. Crystallogr 2002; 58: 899–907.
[16] Brooks BR, Bruccoleri RE, Olafson BD, *et al*. CHARMM: A Program for Macromolecular Energy, Minimization, and Dynamics Calculations. J Comput Chem 1983; 4: 187-217.
[17] Zhu Y. Mixed-integer linear programming algorithm for a computational protein design problem. Ind Eng Chem Res 2007; 46: 839-45.
[18] Luo W, Pei J, Zhu Y. A fast protein-ligand docking algorithm based on hydrogen bond matching and surface shape complementarity. J Mol Model 2010; 16: 903-13.
[19] Matsuda A, Toma K, Komatsu K. Nucleotide sequences of the genes for two distinct cephalosporin acylases from a *Pseudomonas* strain. J Bacteriol 1987[b]; 169: 5821-6.
[20] Fritz-Wolf K, Koller K-P, Lange G, *et al*. Structure-based prediction of modifications in glutarylamidase to allow single-step enzymatic production of 7-aminocephalosporanic acid from cephalosporin C. Protein Sci 2002; 11: 92-103.
[21] Kim Y, Yoon K.-H, Khang Y, *et al*. The 2.0Å crystal structure of cephalosporin acylase. Structure 2000; 8: 1059-68.
[22] Duggleby HJ, Tolley SP, Dodson EJ, *et al*. Penicillin acylase has a single amino-acid catalytic center. Nature 1995; 373: 264-8.
[23] Ishii Y, Saito Y, Sasaki H, *et al*. Affinity labelling of cephalosporin C acylase from *Pseudomonas* sp. N176 with a substrate analogue, 7β-(6-bromohexanoylamido) cephalosporanic acid. J Ferment Bioeng 1994; 77: 598-603.
[24] Nobbs TJ, Ishii Y, Fujimura T, *et al*. Chemical modification and site-directed mutagenesis of tyrosine residues in cephalosporin C acylase from *Pseudomonas* strain N176. J Ferment Bioeng 1994; 77:604-9.
[25] Saito Y, Fujimura T, Ishii Y, *et al*. Oxidative Modification of a Cephalosporin C Acylase from *Pseudomonas* Strain N176 and Site-Directed Mutagenesis of the Gene. Appl Environ Microbiol 1996[a]; 62: 2919–25.
[26] Saito Y, Ishii Y, Fujimura T, *et al.* Protein engineering of a cephalosporin C acylase from *Pseudomonas* strain N176. Ann N Y Acad Sci 1996; 15:226-40.
[27] Matsuda A, Matsuyama K, Yamamoto K, *et al*. Cloning and characterization of the genes for two distinct cephalosporin acylases from a *Pseudomonas* strain. J Bacteriol 1987a; 169: 5815-20.
[28] Fersh F. Structure and mechanism in protein science. New York: W. H. Freeman and Company 1999.

Received: August 18, 2013 Revised: August 22, 2013 Accepted: August 22, 2013