Türk Biyokimya Dergisi [Turkish Journal of Biochemistry–Turk J Biochem] 2014; 39 (1) ; 51–56
doi: 10.5505/tjb.2014.48569

Research Article [Araştırma Makalesi]

Yayın tarihi 30 Mart, 2014 © TurkJBiochem.com

[Published online 30 March, 2014]

Site-directed mutagenesis of cephalosporin C acylase and enzymatic conversion of cephalosporin C to 7-aminocephalosporanic acid

[Sefalosporin C açilazın bölge-hedefli mutasyonları ve sefalosporin C'nin 7-aminosefalosporonik aside enzimatik dönüşümü]*

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Registered: 6 April 2013; Accepted: 23 September 2013

[Kayıt Tarihi: 6 Nisan 2013; Kabul Tarihi: 23 Eylül 2013]

ABSTRACT

Objective: A cephalosporin C acylase catalyzes hydrolysis of cephalosporin C to 7-aminocephalosporanic acid directly. This work was considered helpful for the further study of the cephalosporin C acylase and also useful for the strain improvement.

Methods: Its mutant (G139αS/F58βN/I75βT/I176βV/S471βC) named A12 was cloned into pET-28a (+) vector and expressed in *E.coli* BL21 (DE3). The three dimentional structure of A12 was constructed by the homology modeling and its' catalytic sites was analyzed by the DOCK software.

Results: The mutant A12 was expressed in *E.coli* BL21 (DE3) with the molecular weight 87kDa containing two subunits of 58kDa α-subunit and 25kDa β-subunit. The activity of A12 was 291 U/L which was lower than that of AcyⅡ (322 U/L) because of the low expression level. The specific activity of A12 was 6.011 U/mg which was higher than that of the AcyⅡ (2.868 U/mg). Catalytic analysis suggested that A12 had the improved catalytic efficiency (*kcat*/Km) to convert cephalosporin C to 7-ACA at the beginning of the reaction. These results combined with the model analysis indicated that Phe58β、Ile75β and Ile176β were involved in the catalysis from CPC to 7-ACA.

Conclusion: In this work, the gene of cephalosporin C acylase AcyⅡ was synthesized, mutated and expressed successfully in the *E.coli* BL21 (DE3). The specific activity and the catalytic efficiency of A12 increased 2-fold and 3-fold respectively. Compared with the study of cephalosporin C acylase in N176, this work was considered helpful for the further study of the catalytic mechanism of cephalosporin C acylase and also useful for the strain improvement for the cephalosporin C acylase production.

Key Words: Cephalosporin C, site-directed mutagenesis, 7-amino-cephalosporanic acid, cephalosporin C acylase

Conflict of Interest: None

ÖZET

Amaç: Sefalosporin C açilaz, sefalosporin C'nin 7-aminosefalosporonik aside hidrolizini direkt katalizler. Bu çalışmanın, sefalosporin C ile ileride yapılacak çalışmalara faydalı olmasının yanısıra, suş gelişimi için de faydalı olacağı düşünüldü.

Gereç ve Yöntemler: A12 olarak isimlendirilen mutant (G139αS/F58βN/I75βT/I176βV/S471βC), vektör pET-28a (+)'a kopyalandı ve *E.coli* BL21 (DE3) de ifade edildi. A12'ye ait üç boyutlu yapı homoloji modelleme ile oluşturuldu ve katalitik bölgeleri DOCK yazılımı ile analiz edildi.

Bulgular: *E.coli* BL21 (DE3)'de A12 olarak isimlendirilen mutant, 87kDa moleküler ağırlıklı 58kDa α- ve 25kDa β içeren 2 altbirim ile ifade edildi. 291 U/L olan A12 aktivitesi, zayıf ifade edilme düzeyine bağlı olarak AcyⅡ (322 U/L)'den de düşüktü. Ancak A12 spesifik aktivitesi (6.011 U/mg) AcyⅡ (2.868 U/mg) spesifik aktivitesinden yüksekti. Katalitik analizler sonucunda reaksiyonun başında A12'nin sefalosporin C'yi 7-ACA'ya dönüştürebilecek gelişmiş katalitik verime (kcat/Km) sahip olduğu öne sürüldü. Bu sonuçlar model analizler ile birleştirildiğinde CPC'den 7-ACA'ya katalizde Phe58β, Ile75β ve Ile176β'nın da bulunduğu gösterildi.

Sonuç: Bu çalışmada, sefalosporin C açilaz Acy II geni *E.coli* BL21 (DE3) de başarıyla sentezlendi, mutasyona uğradı ve ifade edildi. A12 spesifik aktivitesi ve katalitik verimi sırasıyla 2 ve 3 kat arttı. N176 da bulunan sefalosporin C açilaz çalışması ile kıyaslandığında bu çalışmanın sefalosporin C açilazın katalitik mekanizmalarına yönelik çalışmalarda ve sefalosporin C açilaz üretimi için suş geliştirilmesinde faydalı olacağı düşünülmüştür.

Anahtar Kelimeler: Sefalosporin C, bölge-hedefli mutagenez, 7-amino-sefalosporonik asit, sefalosporin C açilaz

Çıkar Çatışması: Yazarlar herhangi bir çıkar çatışması bildirmemiştir.

Introduction

As the world's best-sold antibiotics parallel to the penicillin, Semi-synthetic cephalosporins made tremendous contributions for the human to resist bacterial infection [1] with the characteristics of broad spectrum, low toxicity, and resistance to the β-lactamase.

Semi-synthetic cephalosporin antibiotics produced through 7-amino cephalosporanic acid (7-ACA) shared more than 40% of the global anti-infective market. As the intermediate for the synthesis of semi-synthetic cephalosporin, 7-ACA was mainly prepared with the cephalosporin C (CPC).

7-ACA could be produced with CPC through chemical and enzymatic method. The two-step enzymatic method was used widely [2-3] because of its environmental friendship. However, this process was expensive and time-consuming. So, one-step enzymatic method was developed. Researchers tried to separate cephalosporin C acylase from the micro-organisms that could convert CPC into 7-ACA directly [4]. One-step enzymatic method achieved an annual product value 400 million U.S dollars in the global market [5].

It was the biggest obstacle that the cephalosporin C acylase catalyzed the CPC to 7-ACA directly in a very low efficiency. So in recent years, protein engineering of cepholoporin C acylase were concerned to develop the enzyme suitable to one-step enzymatic method. Oh *et al.* developed the mutants by saturate site-directed mutagenesis to increase the deacylation activity to CPC [6]. Otten explored the importance of the site Asn266 for the substrate specificity and obtained mutants N266H, N266Q and N266M, which had a significant increase in hydrolysis activity of CPC [7]. Ishii mutated Met269 and Ala271 of the cephalosporin C acylase and the activities increased 1.6-fold and 1.7-fold respectively [8]. Saito studied the effect of Met164 on the enzymatic character and found that Leu could enhance cephalosporin C acylase activity [9].

In this research, we synthesized the gene *acyII* (GenBank: M18278.1) cloned by Matsuda [10], constructed its model by the method of homology modeling and found that Ser1β, His23β, His70β, Asn242β, Arg24β, Tyr32β, His57β, His178βand Asp177β were the active sites for forming the catalytic pocket in which CPC was fixed to them through a series of hydrogen bonds. The analysis above helped us to choose the F58βN, I75βT and I176βV close to them as the target in order to strengthen the interaction or release more space for the enzymatic catalysis from CPC to 7-ACA. The mutant named A12 was expressed in *E.coli* BL21 (DE3). The activity of A12 was 291 U/L which was lower than AcyⅡ (322 U/L). But the catalytic and converting efficiency of A12 from CPC to 7-ACA were enhanced at the beginning of the catalytic reaction. This work was considered helpful for the study of cephalosporin C acylase and also useful for the strain improvement by the site-directed mutagenesis.

Materials and Methods

Mutation of Cephalosporin C Acylase

The gene *acyII* was synthesized. Its mutant named *A12* was obtained by the overlapping primer PCR with the substituted amino acid residues G139αS-F58βN-I75βT-I176βV-S471βC. For all primers, mutant positions were donated in lowercase and the restriction sites were underlined. Backward primers designed with completely complementary role were marked with asterisk. The gene *acyII* and its mutant *A12* were cloned into the pET-28a vector and sequenced using an automatic DNA sequencer (Perkin–Elmer, USA) to confirm the correctness of the mutagenesis. The recombinant plasmids were used to transform *E. coli* BL21 (DE3).

5'-GAGCTCATGACCATGGCGGCGAA-3'
*CTCGAGTACTGGTACCGCCGCTT

5'-CAGGAACTGGTGCCGGCGCTCGAG-3'
*GTCCTTGACCACGGCCGCGAGCTC

5'-CGAATATagcCTGCT-3' *GCTTATAtcgGACGA

5'-GCTTTCCGCATaatGCGCA-3'
*CGAAAGGCGTAttaCGCGT

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

5'-GGCCTGgttGATCAT-3' *CCGGACcaaCTAGTA

5'-CGCGCTGtgcCGTTAT-3' *GCGCGACacgGCAATA

Preparation of A12

Single colony of *E. coli* transformant was precultured at 37°C for 9 hr in the flask and then 1 ml medium was transformed to 100 ml fresh LB medium and cultured at 37°C for 4 hr. IPTG was added into the culture in the final concentration of 0.5mM with shaking 150 rpm at 28°C for 12 hr. Cells were harvested by centrifugation at 12,000 rpm for 5 min, washed twice with 10 ml cold 0.1 M Tris-HCl buffer, pH 8.0, suspended in 0.1M Tris-HCl and disrupted by an ultrasonic crusher (Ningbo Scientz Research Institute of Instruments, Ningbo, China) with 5s pulse on and 5s pulse off for 5min. Then cell were centrifuged (12,000 rpm for 15 min) and the supernatant was used as the crude enzyme solution. The crude enzyme extract of A12 was loaded on to a chelating affinity column (GE, China). The bound protein was eluted with 100 mM Tris-HCl buffer (pH 8.0), containing 0 mM-500 mM imidazole. The purified enzyme was analyzed by 12 % SDS-PAGE.

Conversion of CPC and Formation of 7-ACA

Enzymatic activity was determined for conversion from CPC to 7-ACA. 500 µ1 A12 (approximately 1 µM for CPC) was mixed with 500 µl CPC (20 mg/ml in 0.1 M Tris/HCl, pH 8.0), and incubated at 37°C for 8 min. At intervals, an aliquot of the reactive mixture was withdrawn, stopped by addition of 5% acetic acid and analyzed by HPLC (Shimadzu 20A HPLC system,

Shimadzu, Japan; Eluent was 15% methanol, 15% acetonitrile, 7.5% acetic acid; detection was at 280 nm) to determine the amount of the remaining CPC and the formed 7-ACA. One unit was defined as the amount of the enzyme liberating 1 μmol 7-ACA/min.

Homology Modeling

The model of AcyII was constructed by using the Homology Modeling Module in Accelrys Discovery Studio 2.1. Next, the potential binding region on which the CPC was docked was identified by the Dock Ligands Module in Discovery Studio. After obtaining the preliminary model of AcyII-CPC complex, the PRODA, a PROtein Design Algorithmic software [12, 13], 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 Asnβ24.

Results and Discussion

Mutation of Cephalosporin C Acylase

The gene *acyII* was changed by the site-directed mutagenesis and the mutant gene was named *A12*. The nucleotide sequences showed the large open reading frames of 2.3 kb coding the expression product A12 approximately 87k Da. A12 expressed in BL21 (DE3) (Fig.1b) was composed of two subunits, the 58 kDa α-subunit and the 25 kDa β-subunit. Compared with the fermentation activity 322 U/L of AcyⅡ, the activity of A12 was decreased to 291 U/L.

Cho [14] and Saito [9] discovered respectively that the mutation of residues in α subunit had an impact on the activity because the mutants could not be expressed in the supernatant or expressed clearly. In this paper, The Gly139αwas the only one amino acid located in the α subunit, so we speculated that the low expression level of A12 (291 U/L) was probably related to the mutation of Gly139α (Fig.1a).

Determination of Catalytic Kinetics Parameters

A12 was purified by the Ni-affinity chromatography. The purity was over 90% analyzed by the software Gel-Pro analyzer (TRANSILLUMINATOR, Gel-Pro 4400) (Fig.1b). The catalytic parameters of A12 were analyzed and the results were shown that the specific activity of A12 was increased 2-fold than that of AcyⅡ and reached 6.011 U/mg (Table 1). The catalytic parameters K_m, k_{cat} and k_{cat}/K_m of A12 were determined. As table 1 suggested, the k_{cat}/K_m of A12 was higher than that of AcyⅡ, which indicated the catalytic efficiency of A12 was increased by the site-directed mutation in 8 minutes. The specific activity of the cephalosporin C acylase from *Pseudomonas* N176 [11] was 3.8 U/mg [5] and the k_{cat}/K_m was 0.4 $sec^{-1}(\mu M)^{-1}$ [8]. While the k_{cat}/K_m of A12 was 0.9 $sec^{-1}(\mu M)^{-1}$ that was higher compared with N176. The A12 obtained in this paper showed the improved activity for catalyzing CPC to 7-ACA through the one-step enzymatic process (Table 1).

7-ACA Productivity with One-step Process Catalyzed by A12

The experiment about the conversion of CPC and formation of 7-ACA by A12 was carried out (Fig. 2). A12 had the improved production efficiency of 7-ACA within 15 min, but kept in a lower level with the extension of reaction time.

We speculated that the enzymatic reaction was the multi-stage process. That was, the outputs catalyzed by the cephalosporin acylase from CPC included 7-ACA, intermediate and the byproduct. F58βN/I75βT/I176βV promoted accumulation of 7-ACA and decrease of the byproduct. The A12 converted CPC to 7-ACA more quickly than AcyⅡ in the 15 minutes. But the 7-ACA, intermediate and byproduct probably inhibited the catalysis with the extension reaction time.

Pollegioni found that the mutant H296S/H309S of cephalosporin C acylase from N176 had the improved product inhibition [5]. In this research, after 15 minutes, A12 perhaps was inhibited by the 7-ACA or byproduct produced numerously due to the mutation of S471β.

Catalytic Sites Analysis of A12

The three-dimensional structural model of the A12 was set up to analyze the catalytic sites clearly (Figure 3 and Figure 4). Ser1β was the catalytic residue. 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 on CPC. For the binding sites, oxygen atoms from the

Table 1. Catalytic Parameters of AcyⅡ, A12 and Cephalosporin acylase N176

	Specific Activity (U/mg)	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($sec^{-1}(\mu M)^{-1}$)
AcyⅡ	2.868	23.71	7.622	0.321
A12	6.011	15.26	14.03	0.919
Cephalosporin C Acylase in N176 [11]	3.8	—	—	0.4

Catalytic parameters of AcyII and A12 were calculated from Lineweaver-Burk plots of the primary velocity of 7-ACA formed from CPC (2.1, 4.2, 6.3, 8.4, 10.5 and 20.9 mM) in the presence of acylases (1μM) at 37°C for 8 min.

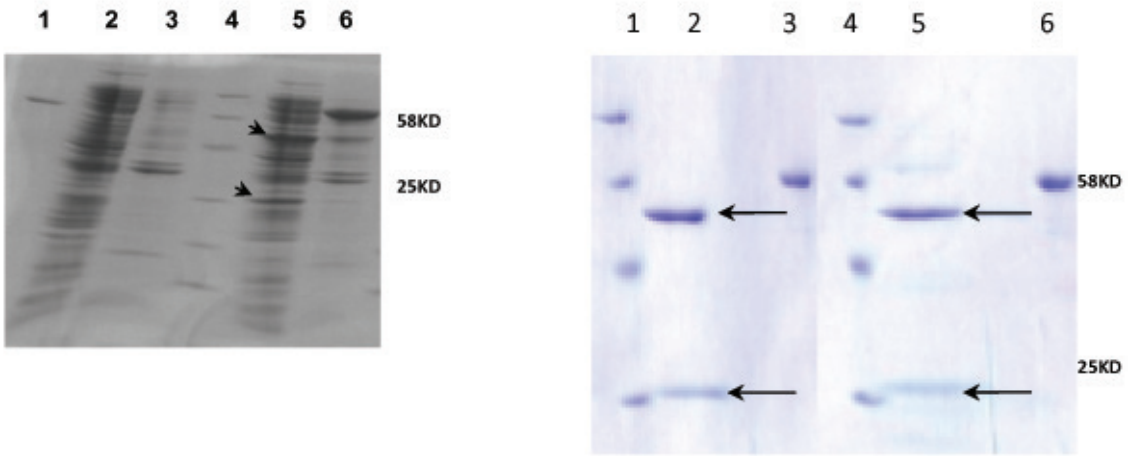

Fig 1. The expression level of AcyⅡ and A12.
(a) SDS-PAGE of AcyⅡand A12 preparations from the *E. coli.* Lane 1，Bovine serum albumin; Lane 2，Supernatant of A12; Lane 3, Cell drbris of A12; Lane 4, Protein low molecular weight marker (97.2 kDa, 66.4 kDa, 44.3 kDa, 29.0 kDa, 20.0 kDa, 14.4 kDa); Lane 5, Supernatant of AcyⅡ; Lane 6, Cell drbris of AcyⅡ.
(b) The purification of AcyⅡ and A12 preparations from the *E. coli* . Lane 3 and 6, Bovine serum albumin; Lane 1 and 4，Protein low molecular weight marker (97.2 kDa, 66.4 kDa, 44.3 kDa, 29.0 kDa); Lane 2 and 5, AcyⅡ and A12 purified by chelating affinity chromatography.

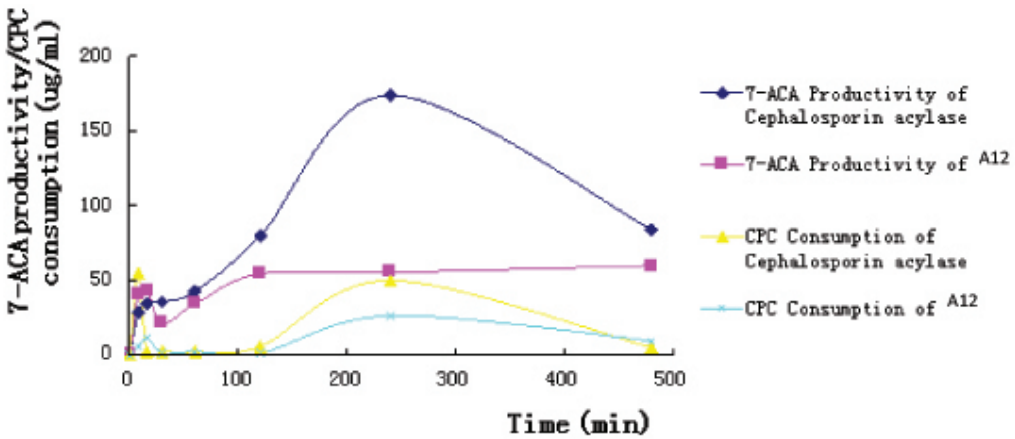

Fig 2. Conversion of CPC and formation of 7-ACA with AcyⅡ and A12.
(◆)**Acy**Ⅱ (7-ACA) ; (■) A12 (7-ACA); (▲)**Acy**Ⅱ (CPC) ; (×) A12 (CPC)

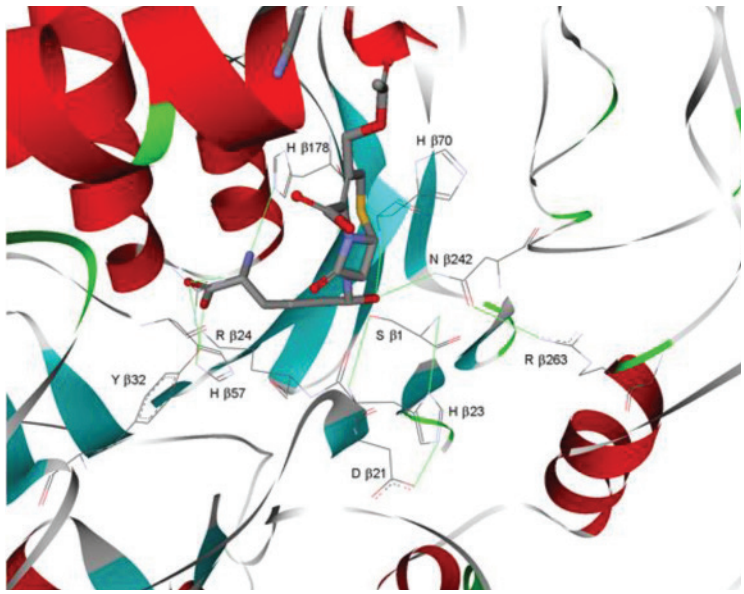

Fig 3. View of the active region with substrate for AcyII based on model.
The key catalytic and binding residues are shown in thin line mode while the substrate CPC is shown in stick mode. The hydrogen bonds are shown in green lines.

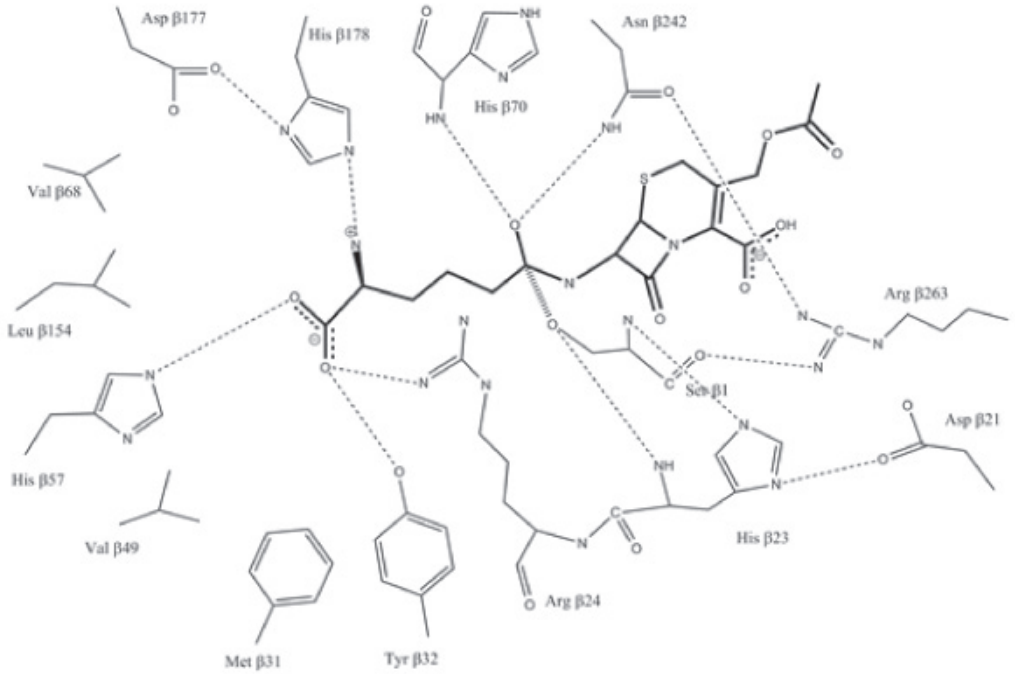

Fig.4. Schematic drawing of the active amino acid residues for AcyII based on model.

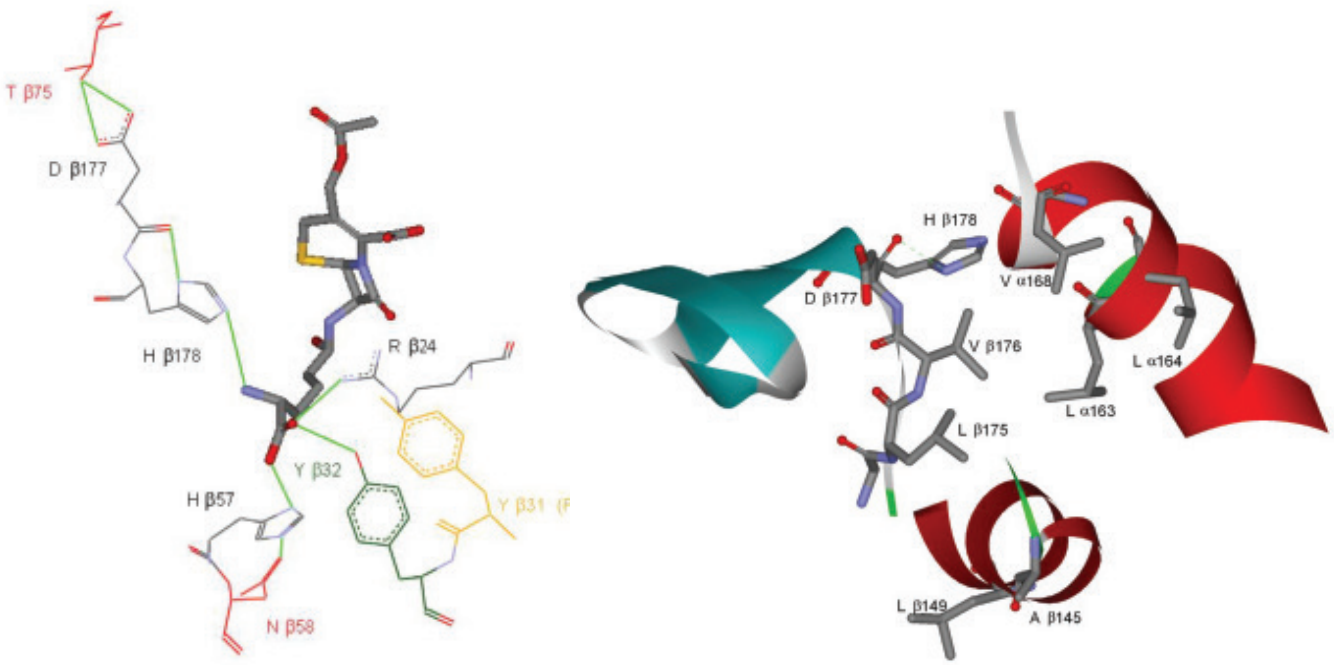

Fig 5. View of the mutations at active site of AcyII.
(a)View of the mutations at active site of AcyII.
The two residues, i.e., Asn58ß and Thr75ß, are colored by red. The residue Tyr31ß or Phe 31ß is colored by orange. The important binding residue Tyr32ß which interacts with the CPC is colored by dark green. The other residues are shown in line mode while CPC is in stick mode.
(b)View of the mutations at active site of AcyII.
The residue Val176ß with neighboring non-polar residues.

carboxylate group of CPC interacted with Arg24β, Tyr32β and His57β. The amino adipyl moiety of CPC was stabilized by the formed hydrogen bond with His178β which simultaneously interacted with Asp177β stated by Figure 4.

F58βN, I75βT and I176βV were close to the active sites of A12 according to the modeled structure. As to mutation F58βN, Asn58β had a polar carboxyl group which was different from the original hydrophobic residue Phe58β, and could form hydrogen bond with the glyoxaline group on His 57β to stabilize the carboxyl group of CPC by the N-O hydrogen bond, as that shown in Figure 5(a). As to mutation I75βT, the original non-polar side chain Ile75β was mutated to polar side chain. The carboxyl group from Thr75β could stabilize the neighbor 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 to Thr75β from Ile75β was more favorable due to its polar side chain which contributed to the stability of the binding pocket by supplying additional hydrogen bonds. For mutation I176βV, Val176β had shorter side chain compared with that of Ile176β, which was probably more favorable for avoiding side chain clashes with the neighboring residues. It was noted that Val176β was located in a loop region near the two important binding residues, i.e., Asp175β and His178β, which interacted with the amino adipyl moiety in CPC, the effect of reducing spatial clashes was also beneficial for stabilizing those important interactions for binding, as that shown in Figure 5(b). Both the specific activity and the catalytic efficiency had improved.

Conclusions

In this work, the gene of cephalosporin C acylase AcyⅡ was synthesized, mutated and expressed successfully in the *E.coli* BL21 (DE3). The specific activity and the catalytic efficiency of A12 increased 2-fold and 3-fold respectively. Catalytic sites analysis indicated that the Phe58β, Ile75β and Ile176βwere involved in the increased catalytic activity. Compared with the study of cephalosporin C acylase in N176, this work was considered helpful for the further study of the catalytic mechanism of cephalosporin C acylase and also useful for the strain improvement for the cephalosporin C acylase production.

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