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Engineering of a CPC acylase using a facile pH indicator assay

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Abstract Cephalosporin C (CPC) acylase is important for the one-step production of 7-aminocephalosporanic acid (7-ACA), a key intermediate for cephalosporin antibiotics. However, its application is hampered by the low activity, substrate inhibition, and product inhibition. In this study, two rounds of combinatorial active-site saturation testing (CASTing) were carried out on the CPC acylase acyII from *Pseudomonas* SE83, and one mutant H57βA/H70βY with no substrate inhibition was obtained. For further engineering to reduce the product inhibition, a quick pH indicator assay was developed, allowing for real-time monitoring of the product inhibition in the presence of added 7-ACA. The utility of the assay was demonstrated by screening six libraries of site-directed saturation mutagenesis libraries of H57βA/H70βY. A new mutant H57βA/H70βY/I176βN was obtained, which showed a k_{cat} 3.26-fold and a K_{IP} 3.08-fold that of the wild type, respectively. Given the commercial value of the enzyme, both this pH indicator assay and the triple mutant should be useful for further engineering of the enzyme to increase the specific activity and to decrease the product inhibition.

Keywords Cephalosporin C acylase · pH indicator · Product inhibition · Substrate inhibition

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Introduction

The chemical or enzymatic deacylation product of cephalosporin C (CPC) is 7-aminocephalosporanic acid (7-ACA), which is the main building block of semisynthetic cephalosporins. The chemical deacylation of CPC involves several steps using toxic reagents, which has been replaced by a more environmentally friendly two-step enzymatic deacylation of CPC using D-amino acid oxidase (DAAO) and glutaryl-7-aminocephalosporanic acid (gl-7-ACA) acylase [5]. CPC acylases that directly convert CPC into 7-ACA are more attractive for the industrial application [3, 13], and several gl-7-ACA acylases show activity on CPC [15]. However, the natural CPC acylases often suffer from low activity on CPC (in the range of 0–4 % of that on gl-7-ACA) [11, 15], significant substrate inhibition, and product inhibition [2, 21]. These factors limit the industrial application of CPC acylases.

Over the past few years, substantial efforts have been made in the protein engineering of CPC acylases to

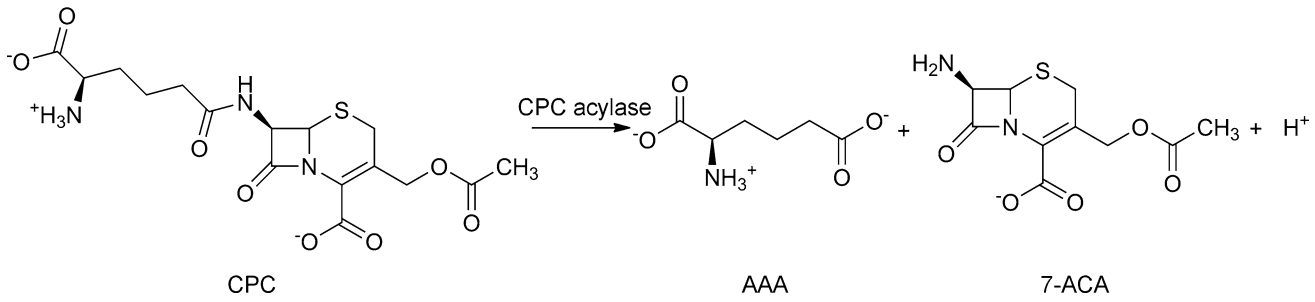

Fig. 1 Reaction catalyzed by CPC acylase. *CPC* cephalosporin C, *AAA* D-α-aminoadipic acid, *7-ACA* 7-aminocephalosporanic acid

overcome the above limitations. Several mutants have been obtained with remarkable improvements [15, 21, 27]. In particular, (1) two mutants of the CPC acylase from *Pseudomonas* N176, M31βF/H57βS/H70βS and A215αY/M31βF/H70βS, respectively, showed a 3.3- and 4.3-fold improvement in V_{max} on CPC compared with its starting template (M31βF), with eliminated substrate inhibition and reduced product inhibition [21]. For the latter, the product inhibition constant K_{IP} was improved by a factor of 1.1-fold compared with M31βF. (2) A mutant of the CPC acylase acyII from *Pseudomonas* SE83, V122αA/G140αS/F58βN/I75βT/I176βV/S471βC (also named S12) showed a 7.5- and 3.7-fold improvement in the specific activity and K_{IP}, respectively, compared with the wild type (WT) [27]. We have also found that this mutant was not sensitive to the substrate inhibition (data not shown). However, at the industrially relevant concentrations of 7-ACA (it can reach over 70 mM in the current two-step production route) [29], the product inhibition is still a significant issue [22].

Screening for CPC acylases with reduced product inhibition would be best performed in the presence of added 7-ACA [27]. However, a facile assay is lacking. The currently available endpoint assays using derivatizing reagents such as *p*-dimethylamino benzaldehyde (pDMAB) [19] or fluorescamine [25] are tedious, and often require a long reaction time (14–16 h) to generate enough 7-ACA over the background 7-ACA [27]. During the reaction 7-ACA degradation may also occur at the alkaline pH [2].Various pH indicator assays have already been used to monitor reactions catalyzed by anhydrase [9], nitrilase [4], esterase [12], dehalogenase [32], penicillin acylase [10], and many other hydrolases [6, 20, 31]. Since the CPC deacylation involves the release of one proton (Fig. 1), we set out to develop a quick and kinetic pH indicator assay for the CPC deacylation. This assay only required several minutes, and was independent of the added 7-ACA.

To test the utility of this pH indicator assay in screening, we used a novel mutant of CPC acylase SE83 acyII (H57βA/H70βY) that was created in our laboratory. This mutant showed no substrate inhibition. Six sites of H57βA/H70βY, which are also contained in the aforementioned S12 variant [27], were chosen for re-engineering by site-directed saturation mutagenesis (SDSM), and screened for mutants with reduced product inhibition. A best mutant containing one more novel mutation (I176βN) with reduced product inhibition, and increased specific activity was obtained. This new kinetic assay should facilitate further engineering of commercially important CPC acylases for decreased 7-ACA inhibition.

Materials and methods

Materials

Restriction enzymes, T4 DNA ligase, and Phusion DNA polymerase were obtained from New England Biolabs (Beverly, MA). Deoxyribonuclease I (DNase I) was purchased from Takara (Dalian, China). The kits for DNA purification, gel recovery, and plasmid miniprep were purchased from either Tiangen (Beijing, China) or Qiagen (Hilden, Germany). *Escherichia coli* strain BL21(DE3) and pET30a were obtained from Novagen (Madison, WI). Isopropyl-β-thiogalactopyranoside (IPTG) was from Amresco (Solon, OH). Lysozyme and tris(hydroxymethyl) aminomethane (Tris) were purchased from Dingguo (Beijing, China). CPC and 7-ACA were provided by North China Pharmaceutical Group Corporation (Shijiazhuang, China). Phenol red was from Sinopharm Chemical Reagent (Shanghai, China). Oligonucleotides were synthesized by Invitrogen (Shanghai, China). Sequence analysis was performed by Sunbiotech (Beijing, China).

Cloning of CPC acylase

The whole-length gene of CPC acylase SE83 acyII was codon-optimized and synthesized by GeneScript (Nanjing, China) based on the published amino acid sequence [GenBank: AAA25690.1] [14], with the insertion of an *Nde*I restriction site upstream the start codon and an *Xho*I

restriction site to replace the stop codon. The synthetic gene was digested with *Nde*I and *Xho*I and inserted into similarly digested pET30a vector backbone, and transformed into *E. coli* BL21(DE3). The His_6-tag downstream of the *Xho*I restriction site in pET30a was used for purification. The resulting plasmid was designated as pET30a-ca-his.

Comparative modeling and substrate docking

A homology model of the WT CPC acylase SE83 acyII was constructed by the Build Homology Models module in the MODELER application of Discovery studio 2.1 (DS 2.1; Accelrys Software Inc., San Diego, CA). The template was the recently solved structure of the substrate-free form of CPC acylase N176 (PDB entry 4HSR) [7], which shares a 90 % amino acid sequence homology with CPC acylase SE83 acyII. To identify the residues comprising the substrate binding sites of acylase SE83 acyII, the Dock Ligands module in the LibDock application of DS 2.1 was used with CPC as the substrate.

Construction and screening of the combinatorial active-site saturation testing (CASTing) or SDSM libraries

The CASTing libraries were constructed by overlapping PCR at specific sites with the outer primers CA-For (5′-ACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGG-3′) and CA-His-Rev (5′-CTCACGCTCGAGAGAAGCACCCGCCGGCACCAGTTCCTGG-3′), with the restriction sites *Xba*I and *Xho*I underlined, respectively. The primers used to introduce saturation mutagenesis at specific sites are listed in Online Resource 1, in which each given amino acid site was changed using the NNK degeneracy. The PCR products were digested with *Xba*I and *Xho*I and ligated into similarly digested pET30a vector, and transformed into *E. coli* BL21(DE3) cells. Single colonies of the transformants were randomly picked with toothpicks and inoculated into 200 μL Luria–Bertani (LB) medium supplemented with 50 μg/mL kanamycin in 96-well microtiter plates and simultaneously onto LB agar plates with 50 μg/mL kanamycin as replications. The aliquots of 25 μL overnight culture were re-inoculated into 1 mL LB medium containing 50 μg/mL kanamycin in a 96-deepwell plate. The 96-deepwell plates were incubated at 37 °C and 250 rpm for about 1.5 h. Expression at 30 °C and 250 rpm for 6 h was initiated with 0.5 mM IPTG induction. Cells were harvested and resuspended in 200 μL 100 mM Tris–HCl buffer (pH 8.0, containing 0.5 mg/mL lysozyme and 4 U/mL DNase I), and then incubated at 37 °C and 250 rpm for 1 h. After centrifugation, 15 μL cell lysis supernatant was transferred into a 96-well microtiter plate. The reaction system contained 68.6 mM CPC and 15 μL cell lysis supernatant in 100 mM Tris–HCl buffer (pH 8.0), in a total volume of 105 μL. For the CASTing libraries, the reaction was stopped by adding 100 μL 40 % acetic acid after 60 min incubation at 25 °C. The amount of 7-ACA generated was detected by the spectrophotometric assay using pDMAB [19, 21], in prescreening and by the HPLC assay in rescreening. For the pDMAB assay, 50 μL pDMAB (0.5 % (w/v) in methanol) was added, and the absorbance at 415 nm was measured using a Spectramax plate reader (Molecular Devices, Sunnyvale, CA) after 5 min of incubation at 25 °C. For the HPLC assay, samples were filtered for loading and assayed by HPLC (Shimadzu, Kyoto, Japan) using the following conditions: column, Diamonsil™ C18 (250 × 4.6 mm, 5 μm, Dikma Technologies, Beijing, China); flow rate, 1.0 mL/min; column temperature, 30 °C; detection, 254 nm; elution, 7.5 % acetonitrile, 15 % methanol, and 1 % acetic acid (v/v) [30]. The improved mutants were confirmed by growing the cells in the test tube, and assayed by HPLC using an identical procedure. For the SDSM libraries, a different pH indicator assay was used (see below).

The SDSM libraries were constructed similarly using the primers listed in Online Resource 1, and screened using the pH indicator assay as described below.

Protein expression and purification

For the purification of CPC acylase variants, *E. coli* BL21(DE3) cells harboring plasmid pET30a-ca-his carrying either the WT or mutant CPC acylase gene were grown overnight at 37 °C in LB medium containing 50 μg/mL kanamycin. The overnight culture was then inoculated into 400 mL LB medium with 50 μg/mL kanamycin, incubated at 37 °C and 250 rpm until the OD_{600} reached 0.4–0.6. The temperature was then lowered to 30 °C and the expression was induced with the addition of IPTG to a final concentration of 0.5 mM. The culture was allowed to grow for 6 h before harvest. Cells were resuspended in buffer A (20 mM sodium phosphate, pH 7.4, containing 500 mM NaCl and 30 mM imidazole) and then sonicated. After centrifugation, the supernatant was filtered through a 0.2-μm Acrodisc® Syringe Filter (Pall, Ann Arbor, MI), and loaded onto an Ni^{2+}-charged 1 mL/5 mL HiTrap™ Chelating HP column (GE Healthcare, Uppsala, Sweden) pre-equilibrated with buffer A on an ÄKTA Explorer™ station (GE Healthcare). The enzyme was eluted by buffer B (20 mM sodium phosphate, pH 7.4, containing 500 mM NaCl and 500 mM imidazole) with a linear gradient. The purified enzyme was exchanged into 5 mM Tris–HCl buffer (pH 8.0) with a 10 k Amicon® Ultra-4 centrifugal filter (Millipore, Billerica, MA). The purified enzyme was stored with 20 % glycerol (v/v) at −80 °C. The purity of the enzyme was more than 90 % as estimated by SDS-PAGE (12 % acrylamide resolving gel and 5 % acrylamide stacking gel). The concentrations of the purified enzyme were

determined by measuring the absorbance at 280 nm using an extinction coefficient calculated using the ProtParam tool from the ExPASy web site [8].

The pH indicator assay

For the screening of the SDSM library, the cells in 96-well plates were grown and harvested as described above. The pellet in each well was resuspended in 160 μL lysis buffer (5 mM Tris–HCl, pH 8.0, containing 0.0025 % phenol red, 0.5 mg/mL lysozyme and 4 U/mL DNase I) and then lysed by incubation at 37 °C and 250 rpm for 1 h. After centrifugation, the supernatants were transferred into two 96-well microtiter plates for the determination of CPC acylase activity in the absence and in the presence of 7-ACA. The reaction system contained 0.0025 % phenol red (w/v), 68.6 mM CPC, 5 mM 7-ACA if applicable, and 50 μL supernatant in 5 mM Tris–HCl (pH 8.0), in a final volume of 200 μL. The absorbance at 556 nm was measured for 4 min with a Spectramax plate reader. The standard curve for the proton release during the deacylation of CPC was mimicked by the addition of hydrochloric acid to the same reaction system [6]. Hydrochloric acid was added at varying concentrations from 0.1 to 0.9 mM. A quantitative linear relationship between the concentration of released proton and absorbance change was fitted using the Origin 8 software (Origin Labs., Northampton, MA).

The specific activities and kinetic parameters of purified CPC acylase variants were also determined with a similar procedure. The concentrations for the purified WT and Variant I was both 3.6 μM, for Variant II, 1.8 μM, and for Variant III, 0.9 μM. One unit of enzymatic activity was defined as the amount of the enzyme that catalyzes the reaction to produce 1 μmol proton per minute. To determine the values of k_{cat} and K_M, CPC was added to a final concentration of 5–120 mM. Data were fitted to a classical Michaelis–Menten equation (or Eq. (1), a substrate inhibition equation [21] for the WT). For the determination of the product inhibition constant K_{IP}, the concentration of CPC was fixed at 68.6 mM and 7-ACA was added to a final concentration of 1–5 mM. Data were fitted to Eq. (2) (or Eq. (3), a reaction rate equation [18] for the WT) to account for competitive product inhibition effects [2].

For comparison, Variant III was also characterized under the same conditions using the HPLC assay as outlined above, and the kinetic parameters were obtained by fitting data to the classical Michaelis–Menten equation or Eq. (2).

$$V = \frac{V_{\max}[S]}{K_M + [S] + \frac{[S]^2}{K_{IS}}} \quad (1)$$

$$\frac{1}{V} = \frac{K_M}{V_{\max}[S]K_{IP}}[P] + \frac{K_M + [S]}{V_{\max}[S]} \quad (2)$$

$$\frac{1}{V} = \frac{K_M}{V_{\max}[S]K_{IP}}[P] + \frac{K_M + [S] + \frac{[S]^2}{K_{IS}}}{V_{\max}[S]} \quad (3)$$

where $[S]$ is the substrate (CPC) concentration, V is the initial reaction rate, V_{max} is the maximum reaction rate, K_M is the Michaelis–Menten constant, K_{IS} is the substrate inhibition constant, $[P]$ is the product (7-ACA) concentration, and K_{IP} is the product inhibition constant. All the fitting was performed using the Origin 8 software.

Results and discussion

Engineering of CPC acylase SE83 acyII for reduced substrate inhibition

The CPC acylase SE83 acyII was chosen in this study as the starting template, because it is more active on CPC than most other CPC acylases [22]. In a previous report, H57β and H70β of CPC acylase N176 were identified to be located in the substrate binding pocket by docking, and for the mutant M31βF/H57βS/H70βS, the substrate inhibition was removed [21]. Since CPC acylase SE83 acyII and CPC acylase N176 share a 90 % homology, H57β and H70β in acylase SE83 acyII might also be important for eliminating the substrate inhibition. Additionally, F58β in CPC acylase SE83 acyII is spatially close to H57β in the substrate binding pocket, and similarly, V68β is close to H70β in the substrate binding pocket, with its side-chain oriented to CPC [21]. To better illustrate the positions of sites chosen for mutagenesis in this study, a homology model was constructed based on the recently published structure of CPC acylase N176 [7], as shown in Fig. 2. We applied two sequential rounds of combinatorial active-site saturation testing (CASTing) on H57β/F58β and V68β/H70β. CASTing has become a powerful tool in protein engineering that takes into consideration the synergistic interactions among residues [23, 28]. The libraries were constructed using overlapping PCR with degenerate primers (NNK). The screening was performed using 68.6 mM CPC in 100 mM Tris–HCl buffer (pH 8.0) at 25 °C, a condition similar to that used in the industrial production of 7-ACA [5]. The screening involved three steps: prescreening in microplates using the spectrophotometric assay using pDMAB, rescreening in microplates using the HPLC assay, and final confirmation in the test tube also using the HPLC assay. To achieve a 95 % probability of mutant coverage, in prescreening 3,000 clones were assayed for each pair of sites [24]. Variant I with one single amino acid alteration (H57βA) was obtained in the first round of CASTing on H57β/F58β. Variant II with one more amino acid alteration (H57βA/H70βY) was obtained in the second round of CASTing at V68β/H70β. When assayed with 15 μL crude extracts from cells (10 OD_{600}/mL), Variant I and

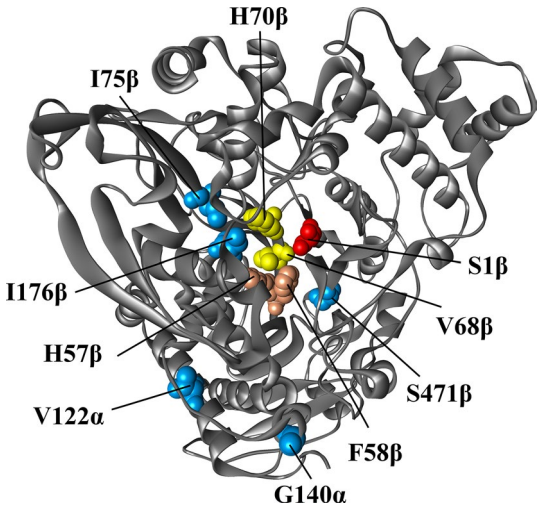

Fig. 2 Homology model of the WT CPC acylase SE83 acyII in a cartoon representation, as rendered by Discovery studio 2.1 (Accelrys Software Inc., San Diego, CA). Locations of the mutated residues in the first and second round of CASTing libraries are presented as spheres in *light brown* and *yellow*, respectively. Locations of the mutated residues in the SDSM libraries are presented as spheres in *light blue*

Variant II exhibited an accumulation of 7-ACA in 60 min of 0.97 ± 0.01 and 1.67 ± 0.06 mM, which was 2.1- and 3.6-fold that of the WT (0.46 ± 0.03 mM), respectively. The results suggest that the two additional sites (F58β or V68β) are however inconsequential in terms of substrate inhibition. Not surprisingly, even up to 80 mM CPC, we found no substrate inhibition for both Variants I and II (data not shown. See below for kinetic characterization). However, significant product inhibition still remained. For example, in the presence of 5 mM 7-ACA, the net accumulation of 7-ACA in 60 min for Variant II was 0.50 ± 0.06 mM (as determined by the HPLC assay), or 30 % of that seen in the absence of added 7-ACA (also see below for kinetic characterization).

Development of a pH indicator assay for CPC acylase

A facile assay in the presence of added 7-ACA can be useful in removing the product inhibition. Since various pH indicators have been used to monitor reaction processes catalyzed by hydrolases [4, 6, 9, 10, 12, 20, 31, 32], we wanted to devise such an assay for the CPC acylase for monitoring the deacylation reaction in a real-time fashion, independent of the added 7-ACA. To serve as a proper pH indicator for the assay, the dye should have a p*K*a similar to that of the buffer salt Tris–HCl [4]. Therefore, we selected phenol red, which has a p*K*a of 8.0 and transits from fuchsia to yellow in the pH range of 8.4–6.8. The buffer concentration was determined experimentally [4]. Using hydrochloric acid to mimic the proton release [6], three

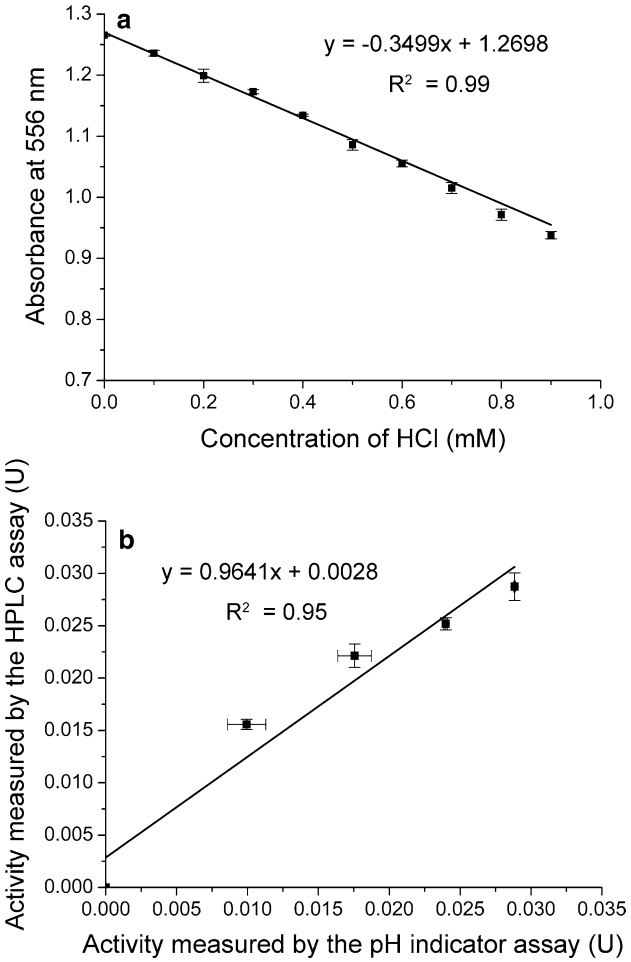

Fig. 3 **a** The standard curve for the absorbance change of phenol red, plotted against the concentration of HCl. The mixture contained 5 mM Tris–HCl, pH 8.0, 0.0025 % phenol red, and 68.6 mM CPC, and the absorbance at 556 nm was measured after addition of hydrochloric acid at varying concentrations from 0 to 0.9 mM. **b** Enzymatic activities measured by the HPLC assay and the pH indicator assay. The *solid lines* are the best liner fits of the data. All samples were measured in triplicate

different concentrations of Tris–HCl were tested (5, 10, and 20 mM), and 5 mM was found to generate sensitive and stable color changes. The optimal concentration of phenol red was also experimentally determined to be 0.0025 %.

A standard curve using His_6-tag purified Variant II was generated by adding hydrochloric acid (varying from 0.1 to 0.9 mM in the final concentration) into 5 mM Tris–HCl buffer (pH 8.0) containing 68.6 mM CPC in a final volume of 200 μL. The absorbance at 556 nm was recorded using a Spectramax plate reader [1]. As illustrated in Fig. 3a, the absorbance at 556 nm is linearly correlated with the hydrochloric acid concentration from 0 to 0.9 mM, which covers the range of the proton release in this study. Under the same condition, the 7-ACA generated was also assayed using HPLC. A reasonably good agreement was found for these

Table 1 The specific activities and relative residual activities of CPC acylase variants determined using the pH indicator assay or the HPLC assay

Variant	Specific activity (U[a]/mg)	Relative residual activity[b]
WT[c]	0.23 ± 0.01	0.23 ± 0.01
Variant I[c] (H57βA)	0.57 ± 0.01	0.27 ± 0.01
Variant II[c] (H57βA/H70βY)	1.00 ± 0.03	0.10 ± 0.01
Variant III[c] (H57βA/H70βY/I176βN)	1.80 ± 0.05	0.33 ± 0.01
Variant III[d] (H57βA/H70βY/I176βN)	1.59 ± 0.13	0.38 ± 0.24

[a] One unit was defined as the amount of the enzyme that catalyzes the reaction to produce 1 μmol proton per minute

[b] The relative residual activity was calculated as the initial reaction rate of enzyme in the presence of 5 mM 7-ACA (V_{5mM}) divided by the initial reaction rate of enzyme in the absence of 5 mM 7-ACA (V_{0mM})

[c] As determined using the pH indicator assay

[d] As determined using the HPLC assay

results, as a linear correlation of $y = 0.9641x + 0.0028$ was obtained (where x is the result from the pH indicator assay; y, the result from the HPLC assay), with a coefficient of determination (R^2) of 0.95 (Fig. 3b).

To demonstrate the utility of the pH indicator assay in quick screening of CPC acylase, the well-to-well variations were also determined using Variant II in the 96-well plate format (for each well, inoculation, cell growth, enzyme expression, cell lysis, and assay were all done independently). The initial reaction rates in the presence (V_{5mM}) and in the absence of 5 mM 7-ACA (V_{0mM}) were measured, respectively, and then the relative residual activity (V_{5mM}/V_{0mM}) was calculated. This relative number minimized the influence of cell density and expression level during the cell culture. These values were then plotted against the 96 clones in a descending order (Online Resource 2). The rate of false positives for screening was estimated by the coefficient of variance (CV), which equals to the standard deviation divided by the mean, assuming the activity profile follows a Gaussian distribution [26]. In our case, the CV of V_{0mM}, V_{5mM}, and V_{5mM}/V_{0mM} were 7.6 %, 12.3 %, and 8.9 %. This means for mutants with a 25 % improvement in any of the three parameters, from a library of 3,000 clones, approximately 1.5, 63.2, or 7.5 false positives would appear. Thus, V_{5mM}/V_{0mM} is a better measurement for screening for CPC mutants with reduced product inhibition than V_{5mM}.

Screening of mutants with decreased product inhibition using the pH indicator assay

The pH indicator assay was applied in the screening of SDSM libraries at sites of V122α, G140α, F58β, I75β, I176β, and S471β (Fig. 2) with Variant II as the template. These sites were previously identified in the CPC acylase variant S12 (V122αA/G140αS/F58βN/I75βT/I176βV/S471βC) from acylase SE83 acyII, which showed an improved activity on CPC and decreased product inhibition compared with the WT [27]. But the individual contribution for each mutation in terms of product inhibition was unknown. We re-screened these sites for reduced product inhibition by the current pH indicator assay. Thus, six libraries of single site mutagenesis were constructed using the primers listed in Online Resource 1. For each site, 100 mutants were screened, which covered all possible variants with a 95 % probability [24]. One mutant (Variant III) with both reduced product inhibition and increased activity was obtained. These results indicate that the pH indicator assay is suitable for quick screening of CPC acylase for decreased product inhibition toward 7-ACA as well as improved activity on CPC.

Characterization of CPC acylase variants

The three CPC acylase variants and the WT were expressed, purified, and characterized using the pH indicator assay (Table 1). The specific activities of Variants I and II were 0.57 and 1.00 U/mg, which were 2.48- and 4.35-fold that of the WT, respectively. The relative residual activities in the presence of 5 mM 7-ACA for Variants I and II were 0.27 and 0.10, which were 1.17- and 0.43-fold that of the WT, respectively, which suggested that product inhibition decreased for Variant I but increased for Variant II. For Variant III, the specific and relative residual activity was 1.80 U/mg and 0.33, or 1.80- and 3.30-fold that of its template, and 7.83- and 1.43-fold that of the WT, respectively. Thus this mutant improved both in terms of specific activity as well as product inhibition. For Variant III, the specific activity and the relative residual activity in the presence of 5 mM 7-ACA were also determined using the HPLC assay (Table 1), and the numbers were both in a good agreement with those obtained with the pH indicator assay developed in this study.

The kinetic parameters including k_{cat}, K_M, substrate inhibition constant (K_{IS}), and product inhibition constant (K_{IP}) were also determined using the pH indicator assay (Table 2). The initial reaction rates of CPC acylase variants were plotted against the CPC concentrations from 5 to 120 mM in the absence of 7-ACA, and against the 7-ACA concentrations from 1 to 5 mM (Online Resource 3) in the presence of 68.6 mM CPC, and fitted with either the classical or modified Michaelis–Menten equations [18, 21]. It should be noted that, the absorbance change of phenol red is also affected by the CPC concentration in the current assay condition, since the primary amine of CPC has a pKa of 9.8 and its ionization will influence the Tris–HCl

Table 2 The kinetic parameters of CPC acylase variants determined using the pH indicator assay or the HPLC assay

Variant	k_{cat} (s^{-1})	K_M (mM)	K_{IP} (mM)	K_{IS} (mM)
WT[b]	0.95 ± 0.02	19.07 ± 0.74	0.13 ± 0.01	58.59 ± 1.22
Variant I[b] (H57βA)	1.00 ± 0.02	8.45 ± 0.16	0.18 ± 0.01	[a]
Variant II[b] (H57βA/H70βY)	2.46 ± 0.10	38.15 ± 0.44	0.12 ± 0.05	[a]
Variant III[b] (H57βA/H70βY/I176βN)	3.10 ± 0.06	11.12 ± 1.94	0.40 ± 0.03	[a]
Variant III[c] (H57βA/H70βY/I176βN)	2.92 ± 0.03	10.13 ± 0.15	0.72 ± 0.78	[a]

[a] The substrate inhibition constants (K_{IS}) for these variants are in the range of 490−2.6×10^{26} mM, much higher than the concentration of CPC used in industrial production of 7-ACA (about 70 mM)

[b] As determined using the pH indicator assay

[c] As determined using the HPLC assay

buffer capacity at pH 8.0. Thus in this study, the standard curves for the assay were determined for the different CPC concentrations used (data not shown). As shown in Table 2, again, no substrate inhibition was observed for the three variants, while the WT showed a K_{IS} of 58.59 mM, suggesting significant substrate inhibition. Using the WT as the benchmark, the three mutants I, II, III showed a k_{cat} 1.05-, 2.59-, and 3.26-fold that of the WT, respectively. In terms of K_M and K_{IP}, Variant I showed a K_M and a K_{IP} 0.44- and 1.38-fold that of the WT. Variant II had a K_M 2.00-fold that of the WT and a slightly lower K_{IP}. Variant III showed a K_M and a K_{IP} 0.58- and 3.08-fold that of the WT.

For comparison, for Variant III, the kinetic parameters were also determined using the HPLC assay (Table 2). The k_{cat} and K_M were found to be 2.92 s^{-1} and 10.13 mM, respectively, which are similar to those obtained with the pH indicator assay. However, the K_{IP} determined by the HPLC assay is 0.72 mM, 1.80-fold that obtained with the pH indicator assay. We found that in the presence of added 7-ACA, the standard deviations for the reaction rates varied significantly, resulting in a large standard deviation for K_{IP} (0.78 mM). The reason might be that 7-ACA generated in the reaction was much lower compared with the added 7-ACA.

Therefore, the increased specific activity for all Variants I, II, and III largely came from the increased k_{cat} and eliminated substrate inhibition. On the other hand, the relative residual activity is determined by the ratio of K_M and K_{IP}, which is 147, 47, 318, and 28, for WT, Variants I, II, and III, respectively. The increased relative residual activity of Variants I and III came from the decreased K_M and increased K_{IP}, while the decreased relative residual activity of Variant II resulted from the increased K_M and unchanged K_{IP}.

Structural analyses of mutations

We subsequently built a docking model for CPC acylase SE83 acyII based on the homology model we constructed (Fig. 4), which is overall similar to that for CPC acylase

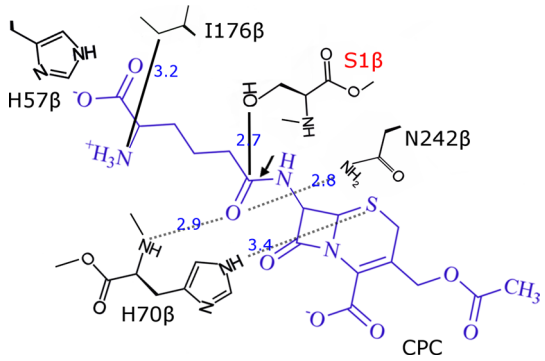

Fig. 4 Schematic diagram showing the predicted interactions of CPC (*blue*) with the CPC acylase SE83 acyII based on the docking model for CPC using the LibDock application of Discovery studio 2.1. S1β is the catalytic center (labeled in *red*). The NH groups of the main chain of H70β and the side-chain of N242β are involved in creating the oxyanion hole with the carboxyl group on CPC. The *broken lines* represent hydrogen bonds; the *solid lines* represent the distance between two atoms. The length of hydrogen bonds and the distance between two atoms are labeled in *blue*. The *arrow* indicates the cleavage site of CPC. Distances are not represented to scale

N176. Judging from this model, the substitution by alanine at H57β likely creates a larger binding pocket that would better accommodate CPC as compared with the native substrate gl-7-ACA. This is in a good agreement with the decreased K_M value. In acylase N176, the modeling predicted a similar effect for H57βS [7]. However, how this mutation removes the substrate inhibition remains unclear. Interestingly, in a different acylase SY-77, the corresponding site R255 was found to be conserved [16, 17]. This may be due to the fact that the substrate binding pocket for acylase SE83 acyII (a class III acylase) is more confined than that for acylase SY-77 (a class I acylase) [7], thus a smaller side-chain in H57βA in acylase SE83 acyII might be preferred for the CPC binding. For H70β, the NH group of the side-chain forms a hydrogen bond with the sulphur atom of CPC. The substitution by tyrosine in H70βY likely disrupts this hydrogen bond, which is consistent with the

increased K_M. However, the reason for the increase in k_{cat} is unknown. For I176β, the distance between the hydrophobic side-chain and the amino group of CPC is 3.2 Å. When it is replaced by asparagine in I176βN, the carbonyl group of the side-chain likely forms a hydrogen bond with the amino group of CPC, and increases the affinity between CPC and the enzyme, resulting in a lowered K_M. The underlying reasons for the changes in K_{IP} in three variants are however elusive.

In this work, two rounds of CASTing on CPC acylase SE83 acyII were carried out, resulting in a double mutant Variant II (H57βA/H70βY) with no substrate inhibition. A kinetic assay using the pH indicator phenol red was developed for this acylase, and further adopted in the 96-well plate format which can allow for detecting small improvements in the activity on CPC in the presence of added 7-ACA. The assay is quick as only 4 min are required, and uses fewer toxic or volatile reagents when compared with the conventional high-throughput assays (using pDMAB or fluorescamine) [17, 19]. A mutant Variant III carrying three new mutations (H57βA/H70βY/I176βN) was obtained with improvements in both the specific activity and the substrate or product inhibition, demonstrating the utility of this pH indicator assay in quick screening of this and likely other CPC acylases. The previously reported variant S12 still has significant product inhibition, with a relative residual activity of 0.68 in the presence of 5 mM 7-ACA (determined in this study). While Variant III has a lower performance compared to S12, given the commercial value of CPC acylases with reduced product inhibition, this new variant may serve as an alternative template for further evolving toward a better CPC acylase.

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