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Oriented Immobilization and Characterization of a Poly-Lysine-Tagged Cephalosporin C Acylase on Glyoxyl Agarose Support

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Abstract Cephalosporin C acylase (CCA), an important industrial enzyme for the production of 7-aminocephalosporanic acid, has very limited and scattered surface lysine residues. A mutant of cephalosporin C acylase (mCCA) has been designed to fuse a poly-lysine tag to the C-terminal of the β-subunit, which is far away from the active site. The free mCCA showed a near equal specific activity with the wild-type CCA, while a much higher activity recovery was obtained for the mCCA than its wild-type counterpart after immobilization on glyoxyl agarose support (73.3 versus 53.3 %). The mCCA's oriented immobilization enables it to obtain a higher substrate affinity and even a higher thermal stability than the wild-type enzyme. The improvement of stability might be attributed to the multipoint covalent attachment by the oriented enzyme immobilization via the adhered poly-lysine tag, which prevents the dissociation of the β-subunit of CCA from the support.

Keywords Cephalosporin C acylase · Characterization · Enzyme immobilization · Genetic modification · Glyoxyl agarose · Poly-lysine tag

Introduction

Cephalosporin C acylase (CCA) is a new enzyme that can directly convert cephalosporin C (CPC) to 7-aminocephalosporanic acid (7-ACA), the important intermediate required for the

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manufacture of a number of semisynthetic cephalosporin antibiotics [1, 2]. The CCAs from *Pseudomonas* sp. N176 and SE83 had been modified by directed evolution or rational design to substantially improve their activity toward CPC [3, 4]. The CCAs, comprising of two heterogeneous subunits, are classified into the family of serine proteinase because the N-terminal serine residue of the β-subunit serves as the nucleophile at the active site [5].

To ensure the enzymes' repeated uses, the enzymes are usually immobilized on diverse solid carriers [6, 7]. Among these carriers for enzyme immobilization, glyoxyl agarose-activated carriers (mainly agarose CL beads) are completely inert and hydrophilic, stabilizing the proteins by reacting with their surface lysine residues [8, 9]. Glyoxyl agarose supports favor the first covalent immobilization of enzymes through its surface regions having the highest amount of lysine residues, that is, where the highest likelihood of a further multipoint covalent attachment may be achieved. Generally, the kind of multipoint covalent attachment involving protein surface could have a significant impact on the enzyme stability [10, 11].

Besides the stability of the biocatalysts, diffusional limitation of the particles is also a very important issue to be concerned. Because of the undisciplined distribution of lysines on the surface of proteins, the covalent attachment of proteins on carriers are often considered randomly or unfavorably oriented, and thus the active site of some of the immobilized enzymes is not accessible for the substrate [12]. To improve the average substrate affinity of these immobilized enzymes, a lot of works concerning about oriented immobilization had been reported [13]. The superficial nature of the enzyme plays an important role in the immobilization of enzyme on solid carriers. And genetic manipulation of the enzyme to modify certain residues on its surface may subsequently change the immobilization of the enzyme, and often lead to an improved stability [13].

In the case of immobilization of a horseradish peroxidase, the protein was genetically modified by replacing arginine residues with lysines on the face of glycan-free side, which is opposite to the active site, and the stability of the immobilized mutated enzyme improved [14]. To induce a preferential orientation of the enzyme toward the support surface, Scaramozzino et al. [15] modified a penicillin G acylase (PGA) by adding a tag consisting of three lysines alternating with three glycines to the C-terminal end, a lysine-rich region of PGA surface. This modification improved the immobilization efficiency of PGA on glyoxyl agarose and the catalytic properties of the PGA derivative, although it impaired the posttranslational steps of protein maturation.

CCA is a very potential enzyme in 7-ACA industrial production, and a lot of efforts were taken to increase the enzyme's activity to meet the industrial requirement [3, 4]. However, the works about downstream process of this important biocatalyst were very limited. Previously, we had immobilized CCA on a commercial epoxy supports by a traditional strategy and further catalysis of the immobilisates was characterized [2]. Nidetzky's group recently reported a strategy for getting the immobilized CCA with high efficiency based on the intraparticle pH gradient detection technique [16]. Nevertheless, there still need more efforts to improve the properties of CCA immobilisate, e.g., stability and efficiency.

The aim of this work was to develop an effective method to prepare a stabilized-immobilized biocatalyst of CCA with good performance. To achieve this goal, the protocol for oriented immobilization was employed to permit the rigidification of the enzyme via multipoint covalent attachment in a certain direction. The distribution of its surficial lysine was discussed and a mutated CCA (mCCA) was designed by adding a peptide (LysGlyLysGlyLysGly) to the C-terminal end of β-subunit of the CCA. And the properties of free and immobilized enzyme on glyoxyl agarose support were investigated.

Materials and Methods

Strains and Reagents

The cephalosporin C acylase recombinant *Escherichia coli* BL21 (DE3)/pET28-CA was constructed and kept in this lab [17]. CPC and 7-ACA were kindly supplied by North China Pharmaceutical Co., Ltd. (Shijiazhuang, China). The agarose beads Sepharose CL-6B was purchased from GE Healthcare. Other reagents were obtained from different commercial sources.

Gene Mutation and Plasmid Construction

The mutagenesis of CCA gene was carried out by the method of PCR amplification with the plasmid pET28-CA as the template. The forward primer 5′-CGTATTGCAGTTCGCGGTGG T-3′ and the reverse primer 5′-TCCCAAGCTTTCACTTACCCTTGCCCTTACCCGCCGGC ACTAATTCTTGA-3′ were used and the amplified product was then cloned into the expression vector pET-28a. The obtained recombinant plasmid pET28-mCCA was thereafter transformed into *E. coli* BL21(DE3).

Preparation of CCA and mCCA

The recombinant *E. coli* BL21(DE3) with wild-type or mutated CCA gene was cultured with a lactose-containing medium [2]. The harvested cells were suspended in sodium phosphate buffer (20 mM, pH 8.0), and cell extracts were prepared by sonication and centrifuged. Then, the N-terminal hexa-histidine-tagged CCA was purified by means of immobilized metal affinity chromatography as described in our previous work [2].

Enzyme Immobilization

The glyoxyl agarose support and its immobilisate derivatives were prepared as reported [18]. And 2.4 g glyoxyl agarose support were added to 32.5 ml of enzyme solution in 0.1 M sodium bicarbonate, pH 10.05, at 25 °C and kept under gentle stirring. Enzymatic activities from the initial enzyme solution and from the final supernatant after 3 h of reaction were assayed. The initial enzyme solution had an activity of approximately 2.2 U ml^{-1}. After enzyme immobilization, the support was reduced by adding solid sodium borohydride to a final concentration of 1 mg ml^{-1}. After 30 min of reduction at 25 °C and under gentle stirring, the CCA-glyoxyl derivative was washed with an excess of phosphate buffer (20 mM, pH 8.0).

Assay of CCA Activity

The enzyme activity of CCA was calculated according to a previously characterized method [2]. One unit of CCA activity was defined as the amount of enzyme capable of producing 1 μmol of 7-ACA per minute at 37 °C, pH 8.5. The specific activity of the immobilized enzyme was determined by using the same method, and it was then defined as μmol of 7-ACA produced per minute and per gram of wet immobilisate under the previously described circumstances. All experiments were done in triplicate.

Thermal Stability and pH Stability Experiments of the Enzymes

To discuss the thermal stability of the enzymes, the free and immobilized CCA preparations were incubated at 45 °C and the samples were withdrawn at regular interval for assaying the residual activity.

To investigate the pH stability of the enzymes at low pH, the CCA preparations in soluble and immobilized form were incubated at pH 5.5, 25 °C. Samples were withdrawn at different times, and the residual activity was measured as previously described.

Kinetic Parameters of the CCA

The values of K_m and V_{max} for free enzyme and immobilized enzyme were measured according to the Lineweaver-Burk double reciprocal plots. K_m and V_{max} were calculated based on the substrate CPC-Na solution concentration of 4, 10, 15, and 20 mg ml^{-1}.

Enzyme Desorption from the Immobilisates by Boiling in SDS Solution

To check the stabilization of the enzyme on support, the immobilisates were boiled in the presence of 2 % (*w*/*v*) sodium dodecyl sulfate (SDS) for 10 min. This treatment releases from the support any protein molecule that was not covalently bound to the support. The desorbed protein from the immobilized samples and the free enzymes were analyzed by SDS-PAGE [19].

Results and Discussion

Characterization of the Free and Immobilized Enzyme of Wild-Type and Mutant CCA

The CCA in this work was originated from *Pseudomonas* SE83, sharing a homology about 93 % with the CCA from *Pseudomonas* N176. The protein structure of SE83 CCA could be obtained through the computational modeling with the recently determined X-ray crystal structure of N176 CCA (PDB accession code 4HST) as the template (Fig. 1) [20].

Since the ε-amino group of surface lysine residues plays an important role in the covalent binding of protein on a aldehyde activated support, the number and distribution of lysine residue on the protein surface is very crucial for a multipoint covalent binding and the orientation of enzyme immobilized as well [13]. There are nine lysine residues in CCA, and only two of them are located on the β-subunit, the larger one subunit. It was inferred that the very limited and uneven distribution of lysine residues would be a bottle neck to produce a stable immobilized CCA with good performance.

As shown in Fig. 1, the key residue of CCA (1Ser of β-subunit) is located in the active-site cavity, which is on the opposite side to the C-terminal of β-subunit. To favor the CCA's oriented immobilization on the carrier with the active site opening to the substrates in solution, the CCA was mutated by PCR method to add a 3G3K tag to the C-terminal of β-subunit. It was found that the wild-type and mutated enzyme have nearly equal specific activity, indicating that the adding tag has no negative effect on the CCA structure (Table 1).

After immobilization on glyoxyl agarose beads, both the mutated or wild-type enzyme acquired a high immobilization yield (nearly 100 %). However, the expressed activity for the two immobilisates exhibited a significant difference. The mCCA had a 20 % higher expressed activity than its wild-type counterpart (Table 1). Though the glyoxyl agarose support has been

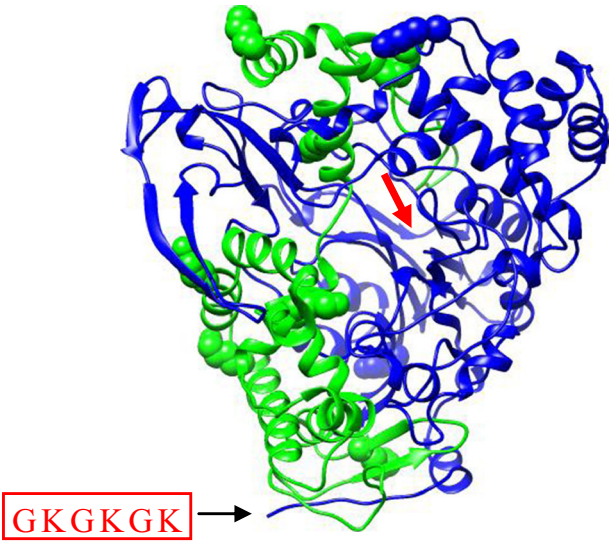

Fig. 1 Structure of cephalosporin C acylase. The 3D structure of CCA was obtained using SWISS-MODEL server (http://swissmodel.expasy.org) with 4HST.pdb as the template and displayed using UCSF Chimera vs. 1.8. The alpha-subunit is shown in *green* and the beta-subunit is colored *blue*. The lysine residues in both subunits are displayed as spheres. The additional tag GKGKGK was designed to adhere to the C-terminal of beta-subunit. The *red arrow* indicates the active site in the substrate binding cavity

utilized to highly stabilize many different enzymes with high activity recoveries values, enzyme structure distortion is still an inevitable problem during immobilization [21, 22]. The mCCA has a random-coil conformation at the lysine-rich C-terminal, which is opposite to the enzyme's active site and preferably attached to the support. Thus, the oriented immobilization induced by the poly-lysine tag would be favorable for the mCCA maintaining its dimensional structure, while the wild-type CCA was attached to the support at the surface with lysine residues where certain secondary structures might be influenced by the covalent immobilization.

Besides the less structure distortion of the mCCA, the reduced diffusional limitation of substrate by the oriented immobilization could partially explain why the mutant enzyme recovered higher activity than the wild-type counterpart. Since in CCA superficial lysine residues are also present near the active site, an immobilization that occurs through these residues may negatively affect the performance of the biocatalyst due to the difficult diffusion of the substrate into the active site. While the mCCA could be orientedly immobilized with the

Table 1 The specific activity of free enzymes and the recovered activity in the enzyme immobilization

	Free enzyme activity (U mg^{-1})	Immobilization yield (%)	Expressed activity (%)
CCA	9.3±0.1	98±2.5	53.3±2.3
mCCA	9.5±0.2	99±2.3	73.3±3.1

The activity was measured toward hydrolysis of CPC sodium salt at concentration of 20 mg ml^{-1}, pH 8.5, and 37 °C

Expressed activity is calculated as percent of activity expressed by the catalyst after immobilization. Enzyme load was 30 U g^{-1} of support and that was taken as 100 % of the expressed activity. Enzyme activity of the final supernatant after 3 h of reaction was assayed, and 0 U ml^{-1} was taken as 100 % of the immobilization yield

active site far from the support surface by the help of the 3G3K tag, which is preferable for the access of substrate to the active site.

Kinetic Parameters of CCA

The kinetic parameters, Michaelis constant (K_m), and the maximal initial reaction velocity (V_{max}) for free and immobilized CCA with CPC sodium salt as a substrate are presented in Table 2. Since the glyoxyl agarose supports were loaded with low amounts of enzyme (about 3.2 mg of protein per gram of support) in order to avoid mass transfer limitations when assaying the enzyme activity, the immobilized enzymes displayed slightly higher K_m values in comparison to their free counterparts, indicating that the diffusional limitation was not significantly existed in the immobilized enzymes.

However, the specific activity (V_{max}) of immobilisates for both CCA and mCCA were remarkably lower than that of free enzymes, suggesting that the enzyme structure distortion occurred during the immobilization. As discussed above, the immobilized mCCA that showed a higher specific activity than that of CCA might due to its less structure distortion in the immobilization. The K_m values of mCCA (for both the free and immobilized forms) were slightly lower than that of CCA, hinting that the fused poly-lysine tag enables the enzyme more feasible to bind the substrate.

Thermal Stability of the Enzymes

Being an enzyme with two heterogonous subunits, the CCA possesses a low thermal stability. As shown in Fig. 2, the free CCA and mCCA had very similar thermal stability, and both the two enzymes improved their stability after immobilization. As expected, the mutant enzyme immobilized on glyoxyl agarose support was clearly more stable than the wild-type preparation, exhibiting a twofold higher half-life time. These results suggested that the adhered 3G3K poly-lysine tag did not affect the property of enzyme in soluble form but led to a remarkable influence on the enzymes in immobilized form. While in Scaramozzino's previous work [15], a poly-lysine tag was added to the C-terminal end, a lysine-rich region of PGA surface, resulting in an impairment of posttranslational steps of protein maturation, and a lower activity and stability of the mutant PGA were obtained.

It was also found that the thermal stability of free CCA was correlated with the protein concentration (data not shown), suggesting that this multimeric protein is apt to dissociate its subunits in solution [19, 23]. Bearing in mind that β-subunit is the larger subunit and containing the active site in it, stabilization of β-subunit seems much important for the enzyme

Table 2 Kinetic constants of free and immobilized enzymes

	Free CCA	Free mCCA	Immobilized CCA	Immobilized mCCA
K_m (mM)	43.0±0.2	41.0±0.3	48.4±0.3	43.8±0.2
V_{max} (U mg^{-1} protein)	15.0±0.1	15.6±0.2	7.7±0.1	9.4±0.1
V_{max}/K_m	0.35	0.38	0.16	0.21

Michaelis-Menten kinetic constants (K_m and V_{max}) were calculated using the Lineweaver-Burk plot method from the experimentally obtained data by determining the reaction velocity in 0.1 M phosphate sodium buffer (pH 8.5) containing 4–20 mg CPC sodium salt ml^{-1} at 37 °C. Reciprocals of experimentally determined reaction velocity ($1/v$) were plotted against the reciprocal of substrate concentration (1/[S]) and linear regression analysis performed. V_{max} can be calculated from the reciprocal of the y-intercept, and K_m can be calculated from the slope of the line (slope=K_m/V_{max})

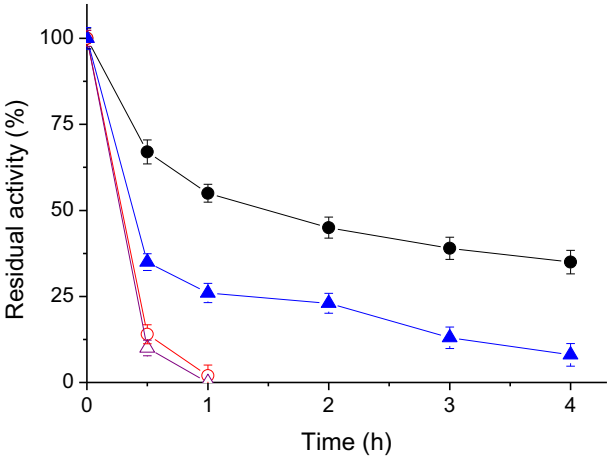

Fig. 2 Thermal stability of the free and immobilized CCAs. Soluble wild-type CCA (*open triangle*), soluble mCCA (*open circle*), immobilization of wild-type CCA (*solid triangle*), and mCCA (*solid circle*) on glyoxyl agarose support. The free and immobilized CCAs were incubated in 0.1 M phosphate buffer (pH 8.0) at 45 °C, and the thermal stability was determined by assaying the residual activity of the intervally taken samples. And 2.2 U ml^{-1} of the free CCAs activity was taken as 100 % activity of the free CCA. Then 16 and 22 U g^{-1} of the immobilized CCAs activity were taken as 100 % activity of the immobilized CCA and mCCA, respectively

immobilization. Since the high density of lysine in certain area rather than the total number of lysine residues on the protein surface are crucial for the adhesion of protein to glyoxyl agarose support [13], the wild-type CCA had less possibility to bind the support at the β-subunit for lacking of lysine residues on it. Obviously, the lysine-rich region in 3G3K tag could greatly increase the possibility of mCCA β-subunit attaching on the support and thereafter stabilize the mutated enzyme via multipoint covalent interaction. The rigidification of the enzyme arisen by the poly-lysine tag would be favorable for the enzyme's standing against the thermal treatment.

Stability of the Enzymes at Low pH Value

Since the CCA produces protons during the hydrolysis of CPC and the mass transfer limitation exists in the immobilized enzymes, a drastic pH gradient (sometimes as large as 2 units) could be formed in microenvironment of the immobilisate [16]. The performance of immobilized CCA is controlled (in the interplay between activity-stability) by the existence of internal pH gradients in the particles. Thus, it is necessary to assess the stability of the CCAs at low pH value.

As shown in Fig. 3, the enzyme activities of both free and immobilized enzymes were greatly reduced at pH 5.5, which could be derived from a high tendency to dissociation of the enzyme subunits under low pH conditions [19], suggesting that dissociation of enzyme subunits under low pH conditions might be one of the main cause of the enzyme inactivation in the bioconversion of CPC. The pH stability of the enzymes could be improved by immobilizing them on the support. Free CCA and mCCA displayed similar pH profiles at acidic pH, while the immobilized mCCA showed slightly higher pH stability. Though the conditions set for the low pH solution incubation (in this work) and the pH existing inside the particles (as mentioned in the work of Nidetzky group [16]) are different, the low pH stability test of these

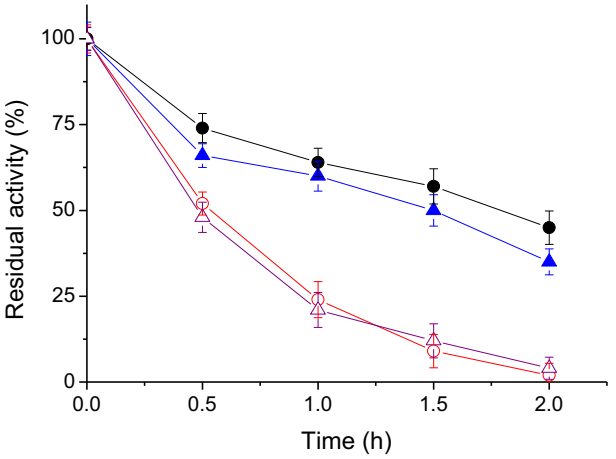

Fig. 3 Low pH profile of the wild-type and mutant at pH 5.5. Soluble wild-type CCA (*open triangle*), soluble mCCA (*open circle*), immobilization of wild-type CCA (*solid triangle*), and mCCA (*solid circle*). The free and immobilized CCAs were incubated in 0.1 M phosphate buffer (pH 5.5) at 25 °C, and the residual activities of the wild-type and the mutant were assayed in parallel. And 2.2 U ml^{-1} of the free CCAs activity was taken as 100 % activity of the free CCA. Then 16 and 22 U g^{-1} of the immobilized CCAs activity were taken as 100 % activity of the immobilized CCA and mCCA, respectively

immobilisates is valuable for the further application of CCA biocatalysts in the production of 7-ACA.

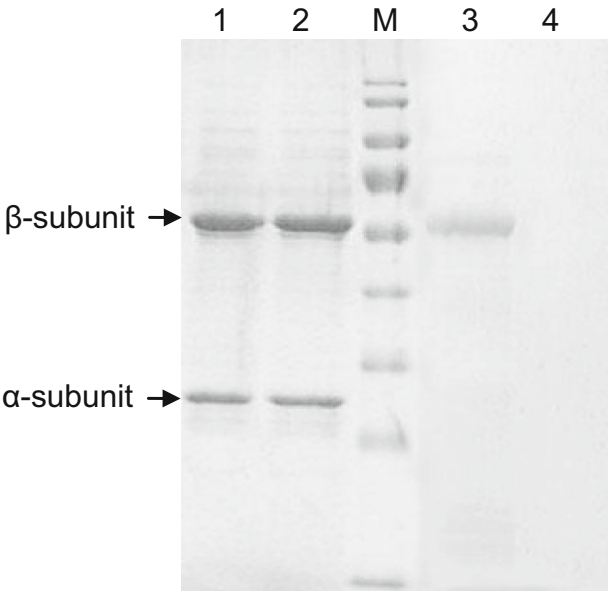

Fig. 4 SDS-PAGE analysis of the free or immobilized CCAs. *Lane 1* free wide-type CCA, *lane 2* free mCCA, *lane 3* protein molecular weight standards: 14, 26, 34, 43, 55, 72, 96, 130, and 170 kDa, *lane 4* the immobilized wide-type CCA sample after 10 min of treatment with 2 % SDS at 100 °C, *lane 5* the immobilized mCCA sample after 10 min of treatment with 2 % SDS at 100 °C

SDS-PAGE Analyses of the Binding of Proteins on the Support

CCA is a two heterogeneous subunits comprising enzyme, and there is no disulfide bond between the alpha- and beta-subunit of CCA. Then, the stabilization of CCA on support could be evaluated by a SDS desorption experiment to know which subunit is more preferable for the covalent attachment to the support [19, 20]. The immobilized preparations were boiled in 2 % SDS, and the proteins released to the supernatant were analyzed by SDS-PAGE (Fig. 4). This analysis revealed that none of the α-subunit of CCA was released, but some of its β-subunit was not covalently attached to the support. Based on the calculation of SDS-PAGE, the proportion of released β-subunit of wild-type CCA, which was uncovalently binding to the support, was estimated to be about 80 %. And to the extreme of the detection sensitivity by the Coomassie brilliant blue staining SDS-PAGE, the immobilized mCCA could not be found to release its β-subunit. Interestingly, the mCCA immobilisate did not release even its α-subunit, suggesting that the immobilization of this mutant preferentially occurs through the enriched lysine region at the C-terminal of β-subunit with the help of the lysine residues in the α-subunit.

Because the state of covalent or noncovalent attachment of protein subunits on support are to a large extent related to the stabilization of the enzyme [24, 25], the increment of stability of immobilized mCCA could be explained by a stronger binding of its β-subunit as well as α-subunit to the support than the wild-type CCA.

Conclusion

The number and distribution of surface lysine residues play pivotal role on enzyme stabilization by covalent immobilization on glyoxyl agarose support. CCA lacks surface lysine residues on its β-subunit, and it looks less likely that the several scattered lysine residues on the α-subunit could produce multipoint binding on glyoxyl agarose. In this work, to obtain the immobilized CCA with desired active site orientation and enzyme stability, the mutant enzyme was designed to add a poly-lysine tag to increase the density of lysine residues at the C-terminal of the β-subunit, a region far away from the active site. The free enzyme of mCCA showed very similar properties to the wild-type counterpart, while the activity and stability of the immobilized mCCA were significantly increased. The data of specific activity of the immobilized enzymes showed that mCCA might have less structure distortion than the wild-type CCA in the immobilization. By the help of the SDS releasing experiment, this improved stability of mCCA immobilisate could be explained by a more intense covalent attachment of the larger subunit of the enzyme on glyoxyl agarose support arisen from a directional immobilization introduced by the poly-lysine tag at the C-terminal of the β-subunit of CCA. In the case of genetic strategy of introducing poly-lysine tag at the C-terminal of CCA, the goals of less diffusional limitation by the enzyme-oriented immobilization, and multipoint rigidification induced by the increased lysine density, were achieved, simultaneously.

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Conflict of Interest The authors have no conflict of interest to declare.

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