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Facile Immobilization of Evolved *Agrobacterium radiobacter* Carbamoylase with High Thermal and Oxidative Stability

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Optically pure amino acids have been widely used as intermediates in the synthesis of antibiotics, antifungal agents, pesticides, and sweeteners. Of particular importance, D-*p*-hydroxyphenylglycine (D-HPG) can be produced from D,L-hydroxyphenly hydantoin (D,L-HPH) in a two-step reaction mediated by D-hydantoinase and *N*-carbamoyl-D-amino acid amidohydrolase (or carbamoylase). To make this production more industrially appealing, the carbamoylase gene from *Agrobacterium radiobacter* NRRL B11291 cloned in an *Escherichia coli* strain was intensively mutated to improve the thermal stability of carbamoylase by three rounds of DNA shuffling. After an extensive screening of the mutant library, the mutant *E. coli* strain M303 was obtained to produce variant carbamoylase, CBL303, with three critical mutated residues, including V40A, G75S, and V237A. Further characterization showed that in comparison with the wild-type counterpart the evolved carbamoylase exhibited more than 20-fold tolerance to heat and, in addition, hydrogen peroxide as a result of the synergistic effect caused by the three mutations. Moreover, with the fusion of the chitin-binding domain (ChBD) of Chitinase A1, the evolved carbamoylase CBL303 was specifically adsorbed on chitin beads. Subsequent analysis indicated that the linkage between the enzyme and the affinity matrix was substantially stable. The half-life of the immobilized carbamolyase CBL303 could reach 210 h at 45 °C, whereas its free form had that of 17 h. In particular, when applied to D-HPG production, the immobilized enzyme could be recycled 16 times with the achievement of 100% conversion yield. Along with the previous illustration of D-hydantoinase immobilization, the success achieved by immobilization of the evolved carbamoylase in this work apparently offers a promising way for the efficient production of D-HPG from D,L-HPH.

KEYWORDS: Directed evolution; carbamoylase; chitin-binding domain; enzyme immobilization; bioconversion

INTRODUCTION

Aided by the combined activity of stereoselective hydantoinase and *N*-carbamoylase, many enantiomerically pure D- or L-amino acids can be readily obtained from racemic D,L-5-monosubstituted hydantoins (*1*). This production process has received considerable interest by industry because of the broad use of optically pure amino acids as intermediates in the synthesis of antibiotics, antifungal agents, pesticides, and sweeteners (*2*). D-*p*-Hydroxyphenylglycine (D-HPG) is of particular interest and can be produced from D,L-hydroxyphenly hydantoin (D,L-HPH) using *Agrobacterium radiobacter* NRRL B11291 (*3*). The reaction starts with D-hydantoinase-mediated hydrolysis of D,L-HPH to *N*-carbamoyl-D-*p*- hydroxyphenylglycine (CpHPG). Subsequently, CpHPG is converted to D-HPG by *N*-carbamoyl-D-amino acid amidohydrolase (hereinafter, carbamoylase). The D-HPG production by such an approach is not appealing until the disclosure of feasibility in employing a recombinant *Escherichia coli* strain as the surrogate cell (*4*). As illustrated, it leads to a 5-fold increase in D-HPG productivity with the exploitation of the *E. coli* strain overexpressing *A. radiobacter* D-hydantoinase and carbamoylase.

In applied bioprocesses, the approach by confining enzymes on inert supports is particularly useful because it could facilitate product isolation, enzyme reutilization, and sometimes help to enhance the stability and activity of enzymes. Nevertheless, the conventional methods for enzyme immobilization are usually problematic and laborious to exploit. Prior to immobilization, purification of enzymes appears to be a prerequisite. To facilitate

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the protein purification, the most commonly method is to fuse target proteins with affinity tags. As a result, it endows the target proteins with the ability to bind specifically to their unnatural cognate ligands. This strategy is of great interest for simultaneous purification and immobilization of enzymes if the affinity ligands could serve as the immobilization matrix. Moreover, this immobilization method also offers several advantages including (i) strong and reversible binding of enzymes to the support, (ii) proper exposure of the enzyme active site, (iii) a mild condition for immobilization, and (iv) the lack of substrate diffusion barriers (*5*). Recently, we have explored the utilization of the chitin binding domain (ChBD) as an affinity tag to retain D-hydantoinase on chitin beads (*6*). In comparison with its unbound counterpart, D-hydantoinase immobilized in this way exhibits higher tolerance to heat and can be reused 15 times to achieve conversion yields exceeding 90% using 100-mM D,L-HPH as the substrate.

From our analysis, the flux control coefficient of carbamoylase over this two-step reaction is 0.8 (*7*). This indicates that the development of the D-HPG production process will be considerably limited by the carbamoylase-directed step. However, as compared to D-hydantoinase, much less effort has been directed toward immobilization of carbamoylase, probably due to the susceptibility of carbamoylase to heat and oxidation (*8–10*). In view of its important role, in this study the gene encoding *A. radiobacter* carbamoylase was intensively mutated for improvement of the thermal stability of carbamoylase by directed evolution. Given the fact that many factors may contribute to the varying thermostability of the protein, the method of DNA shuffling was implemented, and an evolved carbamoylase with high thermostability and oxidative stability was then scored. By fusion with ChBD, the mutant carbamoylase was specifically directed onto chitin beads. Consequently, the immobilized enzyme was illustrated with a great promise for repeated production of D-HPG.

MATERIALS AND METHODS

Materials. The enzymes required for gene cloning were obtained from New England Biolabs (MA, USA). For polymerase chain reaction (PCR), Pfu polymerase from Promega (WI, USA) was used. Chemicals were mainly from Sigma (MO, USA). Used as the immobilization matrix, chitin beads with the size ranging between 50 and 100 μm in diameter were acquired from New England Biolabs.

DNA Shuffling and Mutant Screening. The primers, plasmids, and strains used in this study are summarized in **Table 1**. *A. radiobacter* carbamoylase gene was amplified from pAH61 (*11*) by PCR using primers AL3 and AL5. The PCR DNA was digested by *Nde*I and *Hind*III and subsequently ligated into the corresponding sites of plasmid pET-20bI (*12*) to give plasmid pET-AL. As a result, the plasmid contained the carbamoylase gene with the His-tag fused at its C-terminus.

To generate a mutant library, DNA shuffling was carried out as described previously (*13*). In brief, the PCR DNA containing the cabamoylase gene was synthesized with primers AL3 and AL5 and subjected to digestion by DNase I. Fragments with the size of 50−200 bp were then purified and self-assembled by PCR. Finally, the shuffled DNAs were obtained by PCR upon the priming of the reassembled DNA with oligomers AL3 and AL5. By the *Nde*I-*Hind*III cleavage, the resulting DNAs and plasmid pET-AL were spliced together and subsequently used for transformation into *E. coli* strain BL21 (DE3) (Novagen, WI, USA) with electroporation. Transformants were grown on Luria−Bertani (LB) (*14*) agarose plates augmented with ampicillin overnight at 37 °C.

For preliminary screening, transformants were individually picked and inoculated in 96-wells culture plates containing LB medium with 10 mM sodium phosphate buffer (pH 6.7), 0.0012% phenol red, and 30 mM CpHPG (provided by WIDETEX Biotech. Co., Taiwan). After fermentation at 37 °C for 14 h, the cells exhibiting red color were recovered and reseeded into glass tubes containing LB medium (3 mL). The culture was then maintained in a water bath at 37 °C with gentle shaking for 20 h. By a brief centrifugation, cells were harvested and resuspended in 20 μL of 10 mM sodium phosphate buffer (pH 6.7). Followed by heat treatment at 60 °C for 45 min, the cells were mixed with 40-μL reaction solution consisting of 50 mM CpHPG, 10 mM sodium phosphate buffer (pH 6.7), and 0.0012% phenol red. Incubated at 37 °C for 90 min, the solution with the removal of cells by centrifugation was measured with spectrophotometer at 559 nm.

Table 1. Strains, Plasmids, and Primers Used in This Study

	relevant characteristics	source
Strain		
M12	bearing the evolved carbamoylase CBL12	this study
M203	bearing the evolved carbamoylase CBL203	this study
M303	bearing the evolved carbamoylase CBL303	this study
Plasmid		
pAH61	containing the wild-type carbamolyase	(*11*)
pET20bI	containing the T7 promoter with *lacI*	(*12*)
pET-AL	as pET20bI but carbamoylase positive	this study
pET-Chi	containing ChBD controlled by the T7 promoter	(*6*)
pChA203	as pET-Chi but carbamoylase CBL303 fused with ChBD	this study
Primer		
AL3	AGGCATATGACACGTCAGATGATAC	
AL5	TGACTCGAGGAATTCCGCGATCAGAC	
pM1	GCAGCCAGCCGGGGGCGCGAACTTCATCGTCTTTC	
pM2	GAAAGACGATGAAGTTCGCGCCCCGGCTGGCTGC	
pM3	GGAACTGCGCGAACACGTCTTCAATTTCAAAGC	
pM4	GCTTTGAAATTGAAGACGTGTTCGCGCAGTTCC	
pM5	CTGCGCGAACACGTCTTAAATTTCAAAGCCCATC	
pM6	GATGGGCTTTGAAATTTAAGACGTGTTCGCGCAG	

Furthermore, the cells with the determination of high absorbance were characterized by growing them in shake flasks containing 25 mL of LB medium and ampicillin. After growth for 8 h, cells were spun down and resolved in 100 mM sodium phosphate buffer (pH 7.0) to reach the cell density of 20 at OD_{550}. The harvested cells were disrupted by sonication and centrifuged to recover the supernatant as cell-free extract (CFX). CFX (250 μL) was removed and heated at 70 °C for 30 min, followed by addition into 1 mL of reaction solution composed of 50 mM CpHPG and 100 mM sodium phosphate buffer (pH 7.0). The reaction was carried out at 37 °C for 15 min and quenched by heating at 90 °C for 10 min. The reaction product, D-HPG, was measured with high performance liquid chromatography (HPLC) according to the previous method (*11*).

Enzyme Production and Purification. The carbamoylase-producing *E. coli* strain was cultured in shake flasks containing LB medium at 37 °C. Upon the cell density reaching 0.5 at OD_{550}, 100 μM IPTG was added into the culture broth to trigger protein production for 4 h. The induced cells were then harvested by centrifugation and resuspended in extract/wash buffer containing 03 M NaCl and 50 mM sodium phosphate (pH 7.0). After adjustment of cell density to reach 40 at OD_{550}, the cells were subject to disruption by sonication. CFX was then prepared to have a protein content of 2 μg/μL based on the Bradford assay with Bio-Rad dye reagent. Following the manufacturer's instruction, carbamoylase tagged with polyhistidine was purified using Metal Affinity Resins (NucleoSpin, Clontech). Finally, the purified protein was examined by dodecyl sulfate-polyacrylamide gel electrophoresis (SDS−PAGE).

Enzyme Immobilization. To create the C-terminal fusion of ChBD with the mutant carbamoylase, the DNA fragment carrying the CBL303 gene (**Table 2**) was obtained from the recombinant plasmid of strain M303 by *Nde*I-*Hind*III digestion. Subsequent ligation of the DNA into plasmid pET-Chi (*6*) gave plasmid pChA203. As described above, the shake-flask culture of the pChA203-bearing *E. coli* strain was carried out and induced for protein production. At the end of fermentation, cells were harvested and resuspended in 5 mL of adsorption buffer consisting of 500 mM NaCl, 1 mM EDTA, and 20 mM Tris (pH 8.0). Subsequently, CFX was prepared, and the protein content in CFX was adjusted to reach 10−15 mg/mL with the adsorption buffer.

Table 2. Thermal and Oxidative Stability of the Wild-Type (WT) and Evolved Carbamoylase[a]

	mutations	thermal stability (%)	oxidative stability (%)
WT		3.0 ± 1.0	5.0 ± 1.5
CBL12	V237A, C279S	22.4 ± 1.8	31.2 ± 2.5
CBL203	G75S, V237A	55.6 ± 3.2	42.8 ± 4.5
CBL303	V40A, G75S, V237A, I286V, F287A	72.3 ± 2.4	83.1 ± 5.2
CBL401	V40A, G75S, V237A	68.4 ± 3.0	80.3 ± 4.6
CBL404	I286 V, F287A	4.5 ± 2.1	4.8 ± 1.8

[a] The thermal and oxidative stability in terms of percentage were the residual enzyme activity after treatment with heat and hydrogen peroxide, respectively. The data were taken from three independent measurements. Refer to text for details. The mutation residues of the evolved carbamolyase CBL12, CBL203, and CBL303 were identified as indicated. The mutant proteins CBL401 and CBL404 were created by site-directed mutagenesis from CBL203 with primer pM1-pM2 and from WT using primers pM3-pM4 and pM5-pM6, respectively. Site-directed mutagenesis was performed on the basis of the method reported previously (*12*). All mutant carbamolyases were verified by DNA sequencing.

To immobilize enzyme, 1mL chitin beads were spun down and washed with a 20-fold volume of adsorption buffer. After centrifugation, chitin beads were soaked in 1 mL of adsorption buffer until use. By adding 10 mL of CFX to chitin beads, the adsorption reaction was carried out with occasional stirring at 4 °C for 6 h. Finally, chitin beads were gathered by centrifugation and rinsed by a 20-fold volume of the same buffer. The washing step was repeated three time,s and the recovered chitin beads were stored in 2 mL of adsorption solution at 4 °C until use.

Analytical Methods. The determination of carbamoylase activity essentially followed the previous report with slight modification (*11*). Unless stated otherwise, 10 μg of the free and immobilized enzyme was added to the 1-mL reaction solution containing 30 mM CpHPG and 100 mM sodium phosphate buffer (pH 7.0). The reaction took place at 37 °C for 10 min and was quenched by heating at 90 °C for 10 min. Subsequent determination of the D-HPG concentration was carried out by HPLC (*11*). The unit (U) of enzyme activity was defined as μmole of D-HPG produced per min.

Production of D-HPG by Immobilized Carbamoylase. Production of D-HPG was carried out in 10 mL of reaction solution consisting of 65 mM CpHPG and 0.5 M sodium phosphate buffer (pH 7.0). To initiate the bioconversion reaction, immobilized carbamoylase with 100-U activity was added, and the reaction was administrated at 45 °C for 1 h with shaking. The immobilized enzyme was collected by centrifugation, and the supernatant was removed for further analysis by HPLC. The subsequent run of the reaction was continued by augmenting the recovered enzymes into the solution. The cycle was then repeated as required.

RESULTS

Carbamolyase Evolution and Characterization. As described in Materials and Methods, the structural gene of carbamoylase was subjected to evolution by DNA shuffling for improving its thermostability. After an extensive screening of the mutant library generated, carbamoylase expressed by one variant cell (designated M12) was found to exhibit higher resistance to heat than its wild-type counterpart. Subsequently, the second round of DNA shuffling was carried out using the carbamoylase-born plasmid isolated from strain M12 as the DNA template. As compared to strain M12, one potential variant cell (called M203) was scored for showing improved thermostability of carbamoylase after the cyclical screening. In a similar fashion, the third round of mutation was made to the carbamoylase gene on the plasmid from strain M203. As a consequence of thorough screening, one positive cell with the exhibition of the highest thermostability of carbamoylase was isolated from the variant library and named M303.

For further characterization, the variant strains M12, M203, and M303 were cultured in shake flasks for the production of carbamoylase. As a result of gene construction, the protein was fused with the His tag and, therefore, purified using metal affinity resins. For parallel comparison, the wild-type carbamoylase was purified from strain BL21 (DE3) carrying plasmid pET-AL in a similar fashion. The result showed that all proteins with 95% purity were obtained as judged from SDS−PAGE (data not shown). To examine the thermal stability, purified proteins were treated with heat at 70 °C for 30 min and their residual activity was then assayed. Consequently, the wild-type carbamoylase almost completely lost its activity, whereas 20−70% of original activity remained for the mutant counterparts produced by the variant strains (**Table 2**). Among these mutants, the evolved carbamoylase (designated CBL303) produced by strain M303 displayed the highest thermostability.

To gain deeper insight, the evolved carbamoylase genes were analyzed by DNA sequencing (Tri Biotech. Co., Taiwan). It revealed 5 mutation residues for the mutant carbamoylase CBL303, including V40A, G75S, V237A, IZ86V, and F287A. With the help of site-directed mutagenesis, the mutant CBL401 possessing V40A, G75S, and V237A mutations was generated by the introduction of the V40A mutation into the evolved carbamoylase CBL203 of strain M203. Meanwhile, the mutant protein CBL404 was created with the recruitment of I286V and F287L mutations into the wild-type form. As indicated in **Table 2**, the thermal stability of carbamoylase CBL401 was similar to that of carbamoylase CBL303, while CBL404 and the wild-type protein exhibited the same degree of thermostability. The result suggests the main contribution of V40A, G75S, and V237A mutations to thermal stability of carbamoylase under study. In addition, the oxidative stability of purified proteins was investigated after treatment with 0.2 mM H_2O_2 for 30 min at room temperature. Interestingly, an increase in oxidative stability was positively correlated with improved thermostability for the evolved carbamoylase (**Table 2**). It led to the finding of the mutant carbamoylase CBL303, which exhibited the highest thermal and oxidative stability among the screened variants.

Immobilization of the Evolved Carbamoylase on Chitin Beads. As illustrated previously, directed immobilization of D-hydantoinase-ChBD fusion protein onto chitin beads was marked with high efficiency and stability (*6*). Therefore, a similar approach for immobilization of the evolved carbamoyolase CBL303 was attempted by construction of C-terminal fusion of the protein with ChBD. The carbamoylase CBL303-ChBD fusion protein was then produced using the recombinant *E. coli* strain grown in shake flasks. To perform the protein immobilization, CFX was prepared from the cultured cells and mixed with chitin beads as described in Materials and Methods. Upon the completion of the adsorption procedure, chitin beads were recovered by centrifugation. As depicted in **Figure 1**, over 98% of liberated proteins were identified as carbamoylase CBL303-ChBD after desorption of bounded proteins from chitin beads by heat. Moreover, chitin beads were washed with the buffer solution to remove unbounded proteins, and no trace of the fusion protein was found in the wash solution. Overall, it indicates the specific and strong association of the target protein with chitin beads.

Effect of pH and Temperature on Immobilized Carbamoylase CBL303. The effect of pH and temperature on immobilized carbamoylase CBL303 was further investigated. As indicated in **Figure 2A**, the optimal activity of the immobilized enzyme was obtained at pH 7.0, and the enzyme activity dropped rapidly as pH approached alkalinity (from 8 to 9). In parallel, the response of free enzyme to the pH change was examined

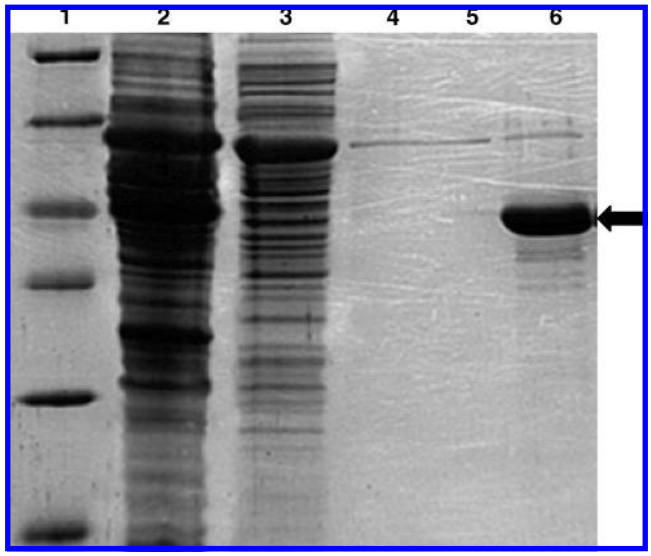

Figure 1. Affinity absorption of the ChBD-linked carbamoylase CBL303 fusion protein on chitin beads. CFX from IPTG-induced strain M303 was prepared and administrated to undergo the adsorption of protein onto chitins as described in Materials and Methods. At the end of adsorption, chitin beads were recovered and washed with the buffer solution. Subsequently, chitin beads were removed from the washing buffer by centrifugation and kept in 2% SDS solution. By heating in boiled water for 10 min, the bounded proteins released from chitin beads were analyzed by SDS−PAGE. Key: lane 1, protein marker; lane 2, CFX before adsorption; lane 3, CFX after adsorption; lanes 4 and 5, washing buffer; lane 6, released proteins from 3 μL of chitin beads by heating. The position of the target protein is indicated by the arrow.

and gave a trend similar to that exhibited by the immobilized counterpart. Moreover, the optimal temperature for the immobilized carbamoylase and its free complement was found at 65−70 and 70 °C, respectively (**Figure 2B**). Overall, it clearly indicates that the evolved carbamoylase CBL303 after immobilization on chitin beads could still retain its biological activity.

Indeed, the key for the practical application of immobilized enzymes resides in their thermal stability. Therefore, it was intriguing to see how the carbamoylase bound on chitin copes with heat. As shown in **Figure 3A**, constantly incubated at 45 °C and pH 7.0, the immobilized enzyme remained substantially stable and its half-life could reach 210 h. In sharp contrast, the free form of carbamoylase CBL303 had a half-life of 17 h. Nevertheless, the thermostability of immobilized carbamolyase CBL303 was found to be pH-dependent. Its half-life turned out to be shorter at higher pH and declined to 90 h when the pH was 8.0 (**Figure 3B**).

Repeated Production of D-HPG by Immobilized Carbamoylase CBL303. The impressive stability of the immobilized carbamoylase CBL303 prompted us to explore it as the biocatalyst for repeated production of D-HPG. The biotransformation reaction was carried out for 1 h. At the end of the reaction, the enzyme immobilized on chitin beads was recovered by centrifugation, and the concentration of CpHPG and D-HPG in the solution was then determined. Meanwhile, a new run of reaction was initiated upon the addition of the recycled enzymes into a fresh reaction solution. Such a reaction cycle was continued as required. As indicated in **Figure 4**, the immobilized enzyme could be reused 16 times to achieve a complete conversion of CpHPG to D-HPG. After that, the conversion yield gradually declined and dropped to 50% at the 22nd repeated run.

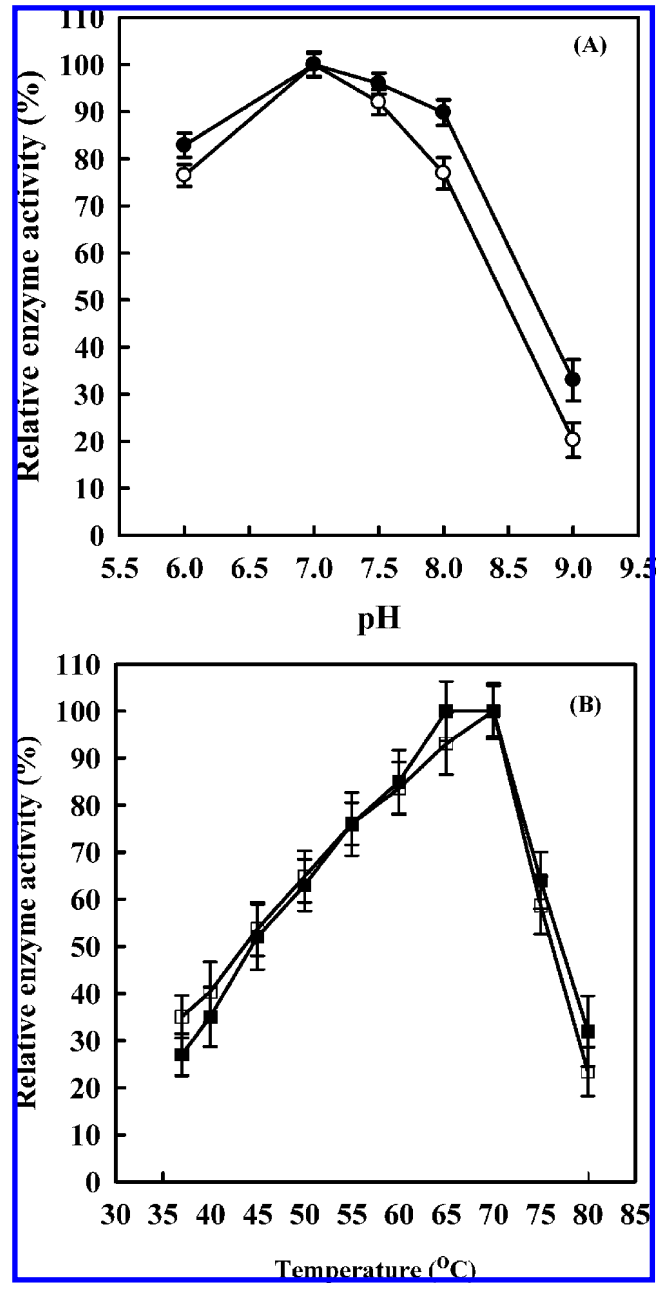

Figure 2. Effects of pH and temperature on the immobilized carbamoylase CBL303. (**A**) The effect of pH on the purified carbamoylase CBL303 in the free (○) and immobilized (●) form was investigated by determining their enzyme activity at 40 °C and various pH values as indicated. (**B**) The effect of temperature on the purified carbamoylase CBL303 in the free (□) and immobilized (■) form was examined by measuring their enzyme activity at pH 7.0 and various temperatures as indicated. The relative activity was defined as the respective enzyme activity relative to the maximum. The data were taken from two independent experiments.

The usefulness of the immobilized cabamoylase CBL303 was further evaluated by examining its shelf life (defined as 50% of the residual activity) when kept in stock. The result showed that the immobilized enzyme remained stable for at least 35 days during storage (**Figure 5**). After preserving for 90 days, it retained 50% of the initial activity.

DISCUSSION

Carbamoylase can be found in various microbes; however, its physiological function remains unclear (*3, 15–17*). Recently,

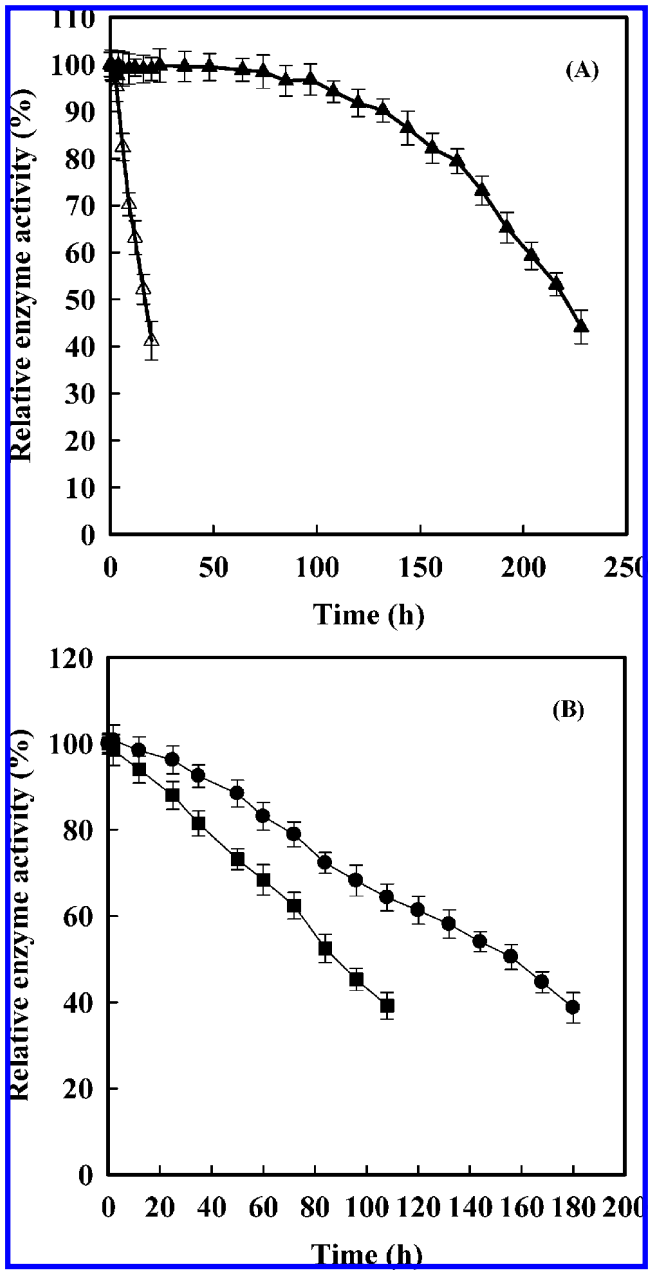

Figure 3. Effect of temperature on the stability of the immobilized carbamoylase CBL303. (**A**) The stability of the free (△) and immobilized carbamoylase CBL303 (▲) in response to heat was investigated by continuously incubating the enzyme at 45 °C and pH 7.0. Enzyme samples were withdrawn to determine their remaining activity during time intervals. Residual enzyme activity was calculated by dividing the remaining activity at each time point with the initial enzyme activity. (**B**) Similar to the procedure described in (**A**), thermostability of the immobilized carbamoylase CBL303 was characterized at pH 7.5 (●) and 8 (■). The data were taken from two independent experiments.

the crystal structure of *A. radiobacter* carbamoylase was solved and released (*18*). It reveals that a stable conformation of the catalytic cleft is ensured by the strict geometric requirement of His129, His144, and His215 residues. In particular, the protein possesses five cysteine residues where Cys172 locates in the active site, and Cys243 and Cys279 are in the proximity of external loops. It was proposed that the latter two cysteine residues might be responsible for the formation of intramolecular disulfide bonds at the oxidative state (*9*). As a result, this would render carbamoylase liable to oxidative inactivation. In addition, the finding of lower activity obtained for the expressed

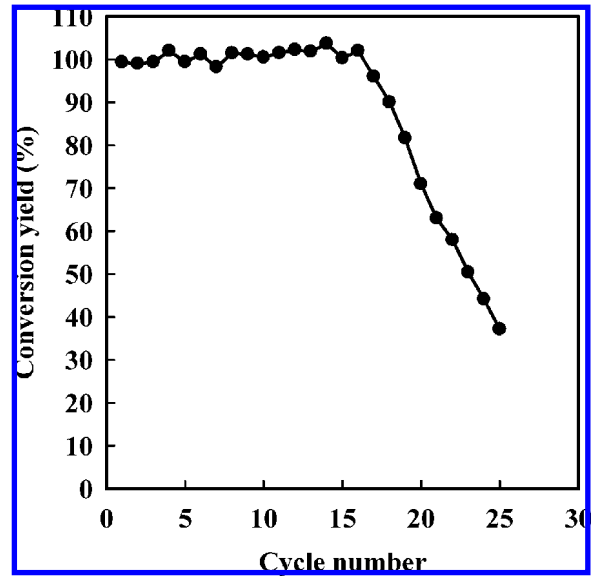

Figure 4. Repeated production of D-HPG by the immobilized carbamoylase CBL303. The reaction was carried out as described in Materials and Methods. The conversion yield was defined as the amount of D-HPG produced relative to that of CpHPG in mM.

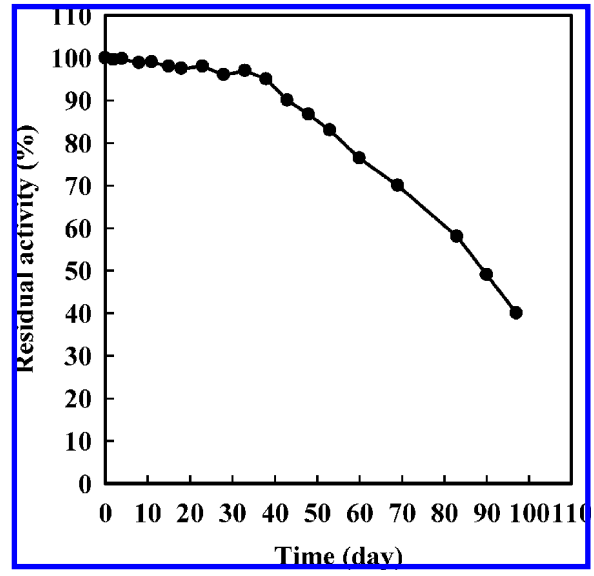

Figure 5. Effect of storage time on the stability of the immobilized carbamoylase CBL303. A large batch of the immobilized enzymes was prepared and kept in adsorption buffer at 4 °C. At each storage day as indicated in the figure, equal aliquots (10 μL) were withdrawn to determine the remaining enzyme activity.

carbamoylase at 37 °C than at 30 °C suggests high sensitivity of the protein to heat (*8*).

In this study, *A. radiobacter* carbamoylase was subjected to random mutations. By directed evolution of the protein toward thermal tolerance, over 10,000 independent variants were screened throughout the mutant libraries generated by three rounds of DNA shuffling. Consequently, the mutant carbamolyase CBL12 from the first-round of mutations was identified with altered residues of V237A and C279S (**Table 2**). As mentioned above, it is conceivable that the substitution of Cys279 for Ser would be favorable for the stabilization of carbamoylase. Meanwhile, the replacement of the Val237 with Ala seemed able to reduce the stereo-obstruction effect caused by the larger site chain of Val (**Figure 6A**). In agreement with the previous report (*19*), the substitute of the 237th residue with

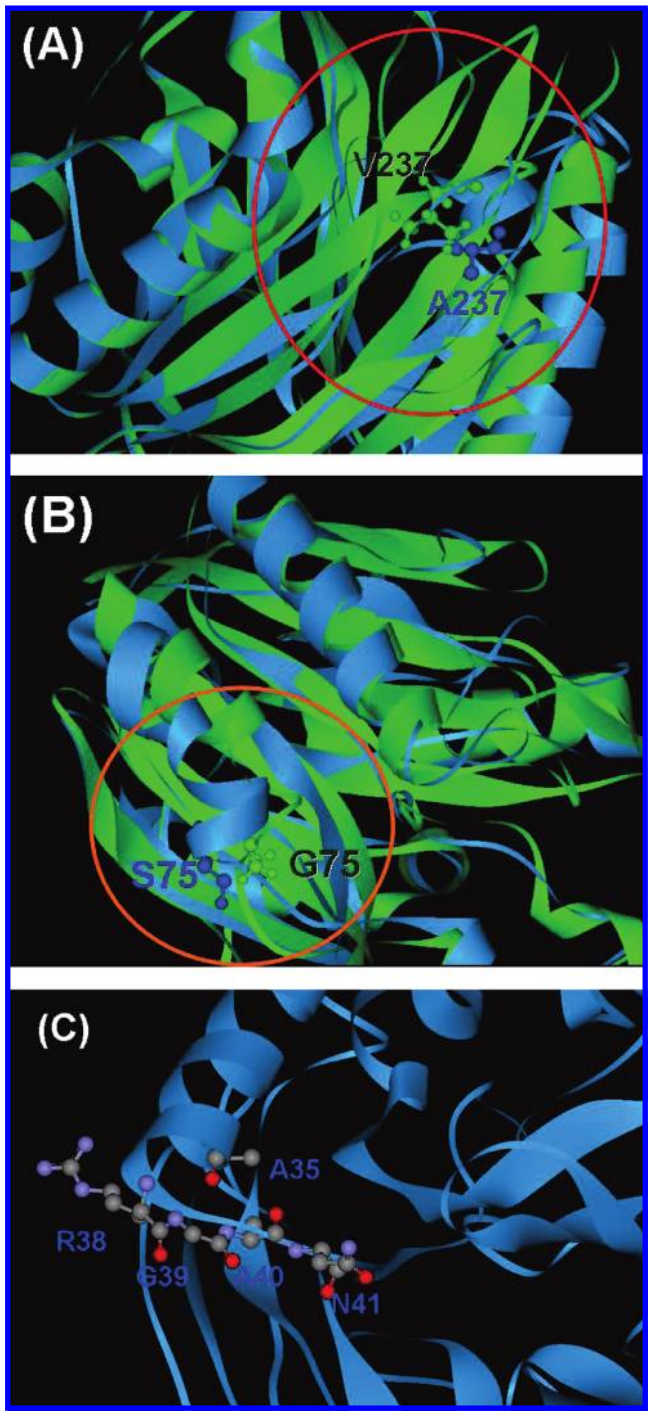

Figure 6. Structural analysis of three mutant residues as identified by directed evolution. The local molecular structure of the wild-type (green) and evolved carbamoylase (blue) was analyzed and rebuilt using Discovery Studio 2.0 (Accelrys, USA) with a cut radius of 15 Å. The local structure highlighting the V237A (**A**) and G75S (**B**) mutations are shown. (**C**) Location of the mutated residue A40 and the neighboring amino acids.

amino acids having smaller side chains (e.g., Ala) could lead to an increase in protein stability. However, the relationship between this conformational structure and protein stability needs further investigation. By continued mutation of CBL12, the mutant carbamoylase CBL203 was isolated with the finding of G75S mutation. Unexpectedly, the C279S mutation was absent in the mutant form CBL203 (**Table 2**). Nevertheless, the introduction of the G75S mutation increased the thermostability of the mutant protein CBL203 (**Table 2**). As revealed from the molecular model (**Figure 6B**), a helix structure consisting of Pro74, Ser75, and Pro75 was introduced and likely favored the stability of the protein. Moreover, the thermostability of CBL203 could be even enhanced with an additional mutation of V40A (i.e., CBL303 and CBL401). Indeed, Val40 is located in the water-proximal region surrounded by hydrophilic residues including Ala35, Arg38, Gly39, and Asn41 (**Figure 6C**). Therefore, the more water-loving residue Ala substituted for Val40 would be expected to increase the intermolecular interactions in this area, leading to the enhanced compactness of the protein. As recognized, simultaneous improvements in multiple properties of enzymes can rarely be achieved unless many constraints are imposed (*20*). Interestingly, the mutants, identified with one constraint of thermal stability in this study, conferred improved resistance to both heat and oxidation (**Table 2**). In particular, the modification of cysteine and methionine residues was reported to increase the oxidative stability of carbamoylase (*8, 21*). However, neither of them could be found in the most evolved carbamoylase CBL303. Taken together, the cumulative increase in thermostability of the evolved carbamolyase is apparently attributed to the synergistic act of multiple altered residues that provide different types of interactions within the protein. This might lead to the formation of a more rigid conformation, which is able to resist the small structural change caused by the oxidation of susceptible residues.

ChBD of Chitinase A1 from *Bacillus circulans* WL-12 is a small peptide consisting of 45 amino acids. The isolated ChBD assumes a tightly packed structure and binds very specifically to insoluble chitins (*22*). In addition, chitin is the most abundant naturally existing polysaccharide, and it will be appealing to use chitins as the adsorption matrix on the grounds of economics. In this work, we attempted to retain the evolved carbamoylase CBL303 on chitins by using ChBD as an affinity tag. As depicted in **Figure 1**, carbamoylase CBL303 tagged with ChBD could be exclusively targeted to chitins, and the adsorption capacity was estimated to account for 8 μg protein/μL chitin beads. The optimal pH and temperature for the enzyme activity of free and immobilized carbamoylae CBL303 remained roughly unchanged (**Figure 2A** and **B**). Nevertheless, the reactivity in terms of specific activity for the immobilized form was increased by 3-fold relative to its free form at various test conditions. Moreover, the thermostability of immobilized carbamoylase CBL303 was greatly enhanced (**Figure 3A**). It seems likely that the confinement of the protein on the support matrix would lead to its conformational reinforcement.

Without reductants, the immobilized carbamoylase CBL303 was exploited for the production of D-HPG from 65-mM CpHPG. The reaction was carried out in 1 h and repeated 16 times using recycled enzymes to achieve 100% conversion yield (**Figure 4**). It should be noted that the conversion of CpHPG to D-HPG is an alkalinity-prone reaction. As indicated in **Figure 3B**, the stability of the immobilized enzyme was conversely correlated with pH. Therefore, the solution pH was buffered during the reaction. However, it was found to have a slight increase in solution pH by 0.3 units at the end of the reaction. Previously, a study reported the immobilization of thermotolerant cabamoylase (with a single mutation) from *Agrobacterium* sp. KNK712 on a macroporous phenol formaldehyde resin, Duolite A-568 (*23*). After immobilization on Duolite A-568 by adsorption and cross-linking with glutaraldehyde, enzyme activity was greatly reduced. With 50-mM CpHPG, the conversion reaction by the immobilized enzyme was carried out at 40 °C for 23.5 h and under a stream of nitrogen gas. The use of nitrogen for keeping the reaction condition anoxic was reported to enhance the stability of the immobilized enzyme because of

the prevention of the protein from oxidative inactivation (*23*). Moreover, the immobilized carbamoylase CBL303 was sustained at a highly stable state during long-term storage in the absence of reductants (**Figure 5**). It was reported that 20% of enzyme activity for *A. radiobacter* carbamoylase was left after 14 days at 4 °C (*8*).

In conclusion, this work illustrates the success in directed evolution of carbamoylase for high thermal and oxidative stability and the utilization of ChBD for tight retention of the evolved protein on chitins. Like D-hydantoinase (*6*), the evolved carbamoylase immobilized by this approach without the need for prior purification of proteins is marked with high stability and facile operation, indicating its general use for enzyme immobilization. Apparently, the achievement in immobilization of the evolved carbamoylase and D-hydantoinase holds a great promise for the efficient production of D-HPG from D,L-HPH.

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