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Directed Evolution of *N*-Carbamyl-D-amino Acid Amidohydrolase for Simultaneous Improvement of Oxidative and Thermal Stability

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Directed evolution of *N*-carbamyl-D-amino acid amidohydrolase from *Agrobacterium tumefaciens* NRRL B11291 was attempted in order to simultaneously improve oxidative and thermal stability. A mutant library was generated by DNA shuffling, and positive clones with improved oxidative and thermal stability were screened on the basis of the activity staining method on a solid agar plate containing pH indicator (phenol red) and substrate (*N*-carbamyl-D-*p*-hydroxyphenylglycine). Two rounds of directed evolution resulted in the best mutant 2S3 with a significantly improved stability. Oxidative stability of the evolved enzyme 2S3 was about 18-fold higher than that of the wild type, and it also showed an 8-fold increased thermostability. The K_m value of 2S3 was comparable to that of wild-type enzyme, but k_{cat} was slightly decreased. DNA sequence analysis revealed that six amino acid residues (Q23L, V40A, H58Y, G75S, M184L, and T262A) were substituted in 2S3. From the mutational analysis, four mutations (Q23L, H58Y, M184L, and T262A) were found to lead to an improvement of both oxidative and thermal stability. Of them, T262A had the most significant effect, and V40A and G75S only increased the oxidative stability.

Introduction

Optically active D-amino acids are widely used as intermediate in the synthesis of antibiotics, antiviral agents, pesticides, peptide hormones, and sweetener (*1*, *2*). In the production process of D-amino acids developed by Yamada et al., chemically synthesized 5′-monosubstituted hydantoin is enantioselectively converted to *N*-carbamyl-D-amino acid by D-hydantoinase (*3*). The intermediate is further hydrolyzed to the corresponding D-amino acid either by a chemical method or by an enzymatic one. Chemical decarbamoylation process gives rise to a discharge of a large amount of waste, and much effort has been made to develop the enzymatic method employing *N*-carbamyl-D-amino acid amidohydrolase (*N*-carbamoylase). *N*-Carbamoylase catalyzes the hydrolysis of the *N*-carbamyl group of *N*-carbamyl-D-amino acid to D-amino acid, ammonia, and carbon dioxide. *N*-Carbamoylases from various sources have been isolated, characterized, cloned into *E. coli*, and employed for the production of D-amino acid (*4*−*9*). Recently, the three-dimensional structure of *N*-carbamoylases from *Agrobacterium* species was resolved, and a detailed reaction mechanism was demonstrated (*10*, *11*).

In an effort to develop a fully enzymatic process for the production of D-amino acid, we have attempted the enzyme reaction systems employing D-hydantoinase from *Bacillus stearothermophilus* SD-1 and *N*-carbamoylase from *A. tumefaciens* NRRL B11291 (*12*−*15*). However, oxidative and thermal stability of *N*-carbamoylase was found to be low compared to that of D-hydantoinase, which is considered one of the limiting factors in the process development. Over the past few years, directed evolution of enzymes has proved to be a powerful tool for improving and designing enzymes/proteins, and a great number of promising results have been reported (*16*−*18*). In this paper, we describe the bidirectional evolution of *N*-carbamoylase for enhancement of oxidative and thermal stability. A mutant library was generated by DNA shuffling, and positive clones were screened by using the activity staining method on a solid agar plate. Two rounds of directed evolution resulted in the best mutant 2S3 with simultaneously improved oxidative and thermal stability. Biochemical characteristics of 2S3 were investigated, and stabilizing effects of substituted amino acid residues were evaluated. Details are reported herein.

Materials and Methods

Materials. *N*-Carbamyl-D-*p*-hydroxyphenylglycine (NC-HPG) was synthesized as described in our previous work (*12*). Hydrogen peroxide solution (3%, w/w) was purchased from Sigma. Restriction enzymes and *Taq* DNA polymerase were from KOSCO (Sungnam, South Korea). *DNase*I was purchased from Boeringer Manheim

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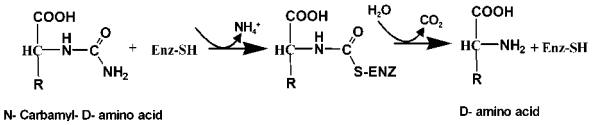

Figure 1. Reaction scheme for the hydrolysis of *N*-carbamyl-D-amino acid to D-amino acid by *N*-carbamoylase. R indicates the functional group.

(Manheim, Germany). All other reagents were of the analytical grade.

Mutant Library Construction. *N*-Carbamoylase gene from *A. tumefaciens* NRRL B11291 was amplified by using PCR with two primers, CAN (5′CAAAGGTC-CATGGCACGTCAGATG-3′) and CAC (5′-AAGGGATC-CTTATCGAATTCCGCGATCAG-3′) as N- and C-terminal primers, respectively. The corresponding gene was purified from 0.8% agarose gel using Bio101gene clean kit II (Qbiogene, Carlsbad, CA) and then digested with *DNaseI*. Fragments of 50−100 bp, purified from 2% agarose gel, were self-assembled by PCR (94 °C, 1 min; 50 °C, 1 min; 72 °C, 1 min; total 30 cycles). The reassembled DNA fragments were amplified by PCR (94 °C for 1 min; 50 °C, 1 min; 72 °C, 1 min; total 15 cycles) with two primers, CAN and CAC. All PCR procedures were performed using *Taq* polymerase under the conditions offered by the provider. The amplified DNA fragments (0.9 kb) were purified from agarose gel and then digested with *Nco*I and *BamH*I. The resulting genes were cloned into the expression vector pTrc99A and transformed into *E. coli* JM109 by electroporation (Gene Pulser II Electroporation System, Bio-Rad, Hercules, CA). Transformants were plated on Luria-Bertani (LB) plates containing ampicillin (100 μg/mL) and grown for overnight at 37 °C.

Screening of a Mutant Library. *N*-Carbamoylase variants with improved oxidative and thermal stability were primarily screened by using the activity staining method on a solid agar plate. Agar plate contained 5 g/L NC-HPG, 1 mM EDTA, 0.01% phenol red, and 1% bacto-agar. The final pH of the solution was adjusted to 6.3. *N*-Carbamoylase converts NC-HPG to D-*p*-hydroxyphenylglycine, producing ammonia and carbon dioxide, and the pH increases with product formation as depicted in Figure 1. Thus, the yellowish color changes to red around the colonies with *N*-carbamoylase activity.

Primary screening procedure of positive clones with an improved stability is described in Figure 2. Variants from a mutant library were tooth-picked to two LB plate with control colonies. The duplicated transformants were transferred onto the filter papers and incubated with lysozyme (2 mg/mL) at 37 °C for 30 min. The filters were washed with double distilled water and subjected to the enzyme inactivation steps. In the first round of evolution, one of the filters was incubated at 70 °C for 2 h in a dry oven for the thermal-inactivation step. The other was treated with 1.5 mM hydrogen peroxide at 37 °C for 30 min for the oxidative inactivation step. After inactivation steps, each filter paper was overlaid on the activity staining plate and incubated for 30 min at 37 °C, and colonies displaying the color change simultaneously on both agar plates were primarily selected from the master plate. Wild-type *N*-carbamoylase completely lost activity after the above-mentioned inactivation steps, and no color change occurred on the agar plates. In the second round, a mutant library was incubated under conditions where the best mutant selected in the first round lost activity completely: 70 °C for 4 h in dry oven and 2.5 mM hydrogen peroxide for 30 min, respectively. Selected

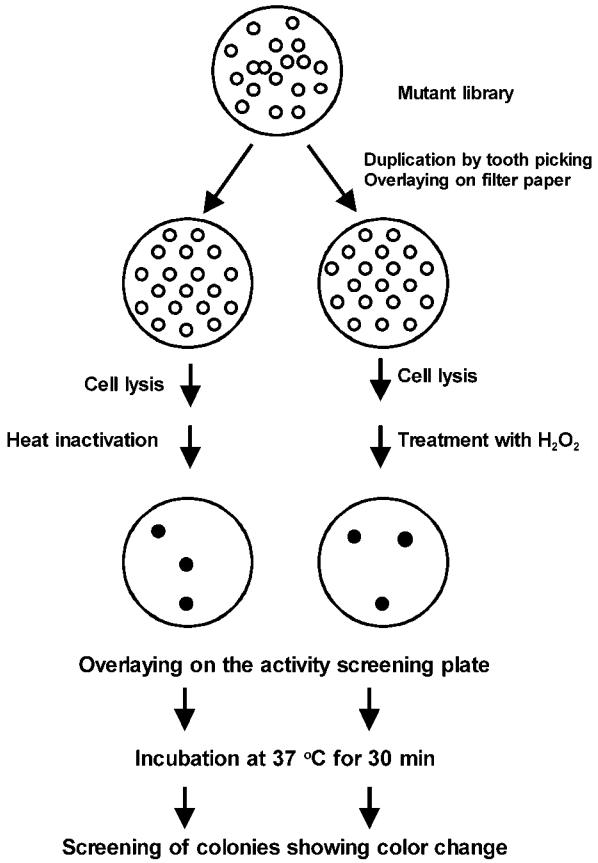

Figure 2. Procedure for primary screening of positive variants with improved oxidative and thermal stability on a solid agar plate.

variants were cultured in 3 mL of LB broth and then induced by addition of 1 mM IPTG when optical density reached about 0.6. The cells were further grown for 3 h and then separated by centrifugation. Collected cells were disrupted by sonication (VC 750, Sonics & Materials, Newtown, CT), and the soluble fraction was used for confirmation of oxidative and thermal stability.

Enzyme Purification. Wild-type enzyme and selected variants were purified as follows: Cells were grown in 300 mL LB broth, and induced by addition of 1 mM IPTG when the absorbance at 600 nm reached approximately 0.6. Cells were further grown for 3 h and collected by centrifugation. The pellet was resuspended in 20 mM of potassium phosphate buffer (pH 7.0) containing 1 mM phenylmethanesulfonyl fluoride and disrupted by sonication. After addition of 0.1% protamine sulfate and incubation on ice for 1 h, cell debris was removed by centrifugation at 12,000 × *g* for 30 min. The soluble fraction was collected and loaded onto a Sepharose Q resource column (Amersham Pharmacia Biotech, Uppsala, Sweden) preequilibrated with 20 mM potassium phosphate buffer (pH 7.0). The column was eluted with a linear gradient (0−1 M) of NaCl. The concentrated protein solution was loaded onto a Phenyl Superose column (Amersham Pharmacia Biotech) equilibrated with 20 mM of potassium phosphate buffer containing 4% sodium sulfate. The column was eluted with reverse linear gradient (4−0%) of sodium sulfate. Fractions exhibiting the *N*-carbamoylase activity were analyzed using SDS−PAGE, and samples showing a single band were used in further studies. All purification steps were carried out using FPLC system (Amersham Pharmacia Biotech).

Oxidative and Thermal Stability of Enzyme. Purified enzyme solution was incubated with 1 mM DTT for 1 h at 25 °C, and remaining DTT was removed by passing through a D-salting column (Pierce, Rockford, IL) prior to the enzyme inactivation steps. For determination of thermostability of enzyme, purified enzyme (0.25 mg/mL) was incubated at 70 °C, samples were taken at intervals, and residual activity was assayed. For oxidative stability, purified enzyme (0.25 mg/mL) was incubated with 0.2 mM hydrogen peroxide at 25 °C. Samples were taken at intervals, and residual activity was measured. Stability was estimated by measuring the ratio of the residual activity to the initial activity of enzyme. First-order inactivation constants of enzyme were determined by plotting the logarithmic residual enzyme activity as a function of time.

Enzyme Assay and Analysis. *N*-Carbamoylase was assayed in 100 mM potassium phosphate buffer (pH 7.0) containing 25 mM NC-HPG as substrate. The reaction mixture was incubated at 40 °C for 30 min, and produced D-HPG was analyzed by HPLC (Shimazu Co. Kyoto, Japan) as described in our previous work (*12*). Kinetic constants of enzyme were determined by double reciprocal plots of initial reaction rate and substrate concentration. Analytical SDS−PAGE was performed according to the method of Laemmli (*21*). Acrylamide gels were stained with Coomassie Brilliant Blue R250. The protein concentration was determined by the method of Bradford (*20*).

Results and Discussion

Screening of a Mutant Library. *N*-Carbamoylase hydrolyzes *N*-carbamyl-D-amino acid to D-amino acid, ammonia, and carbon dioxide, increasing the pH (Figure 1). The color of the pH indicator (phenol red) changes from yellow to red by enzyme reaction, which enables a rapid screening of colonies with residual enzyme activity on a solid agar plate. From the mutant library generated in the first round, about 10,000 colonies were subjected to inactivation steps as described in Figure 2. Under inactivation conditions, wild-type *N*-carbamoylase gave no color change on the activity screening plate. Thus, the variants with improved oxidative and thermal stability compared to the parental enzyme were easily screened by the activity staining method. Oxidative and thermal stability of selected mutants were confirmed by assaying the residual activity after treatment at various temperatures and hydrogen peroxide concentrations after partial purification. For the second round of evolution, the 10 best clones from the first round evolution were recombined and used for generation of a mutant library. Colonies were again subjected to the inactivation steps under conditions at which the best mutant of the first round completely lost its activity. Screening of 8,500 variants resulted in the best mutant 2S3 possessing the highest oxidative and thermal stability compared to that of other positive variants. By a similar procedure, a third round mutant library was generated and screened, but no variant with improved oxidative and thermal stability compared to that of 2S3 was found. In this work, 2S3 was finally selected for further study.

To validate the screening system used in this work, the oxidative and thermal stabilities of the primarily selected mutants from the first round were investigated. As shown in Figure 3, a large fraction (more than about 70%) of primarily selected variants displayed simultaneously improved oxidative and thermal stability compared to that of the wild-type counterpart. From this observation, it is likely that the activity staining method on a solid agar plate is effective for the screening of positive variants with improved oxidative and thermal stability. Recently, it was reported that *N*-carbamoylase belongs to the nitrilase superfamily (*21*). The superfamily enzymes catalyze the hydrolysis of the carbon−nitrogen bond (*21*, *22*), producing the corresponding acid and ammonia as *N*-carbamoylase. In this regard, the screening system developed in this work is expected to be useful for the directed evolution of nitrilase superfamily enzymes.

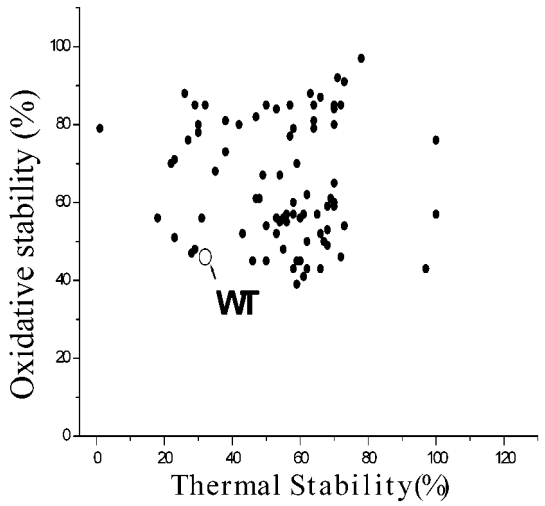

Figure 3. Oxidative and thermal stability of the primarily screened positive clones from the first round evolution. Oxidative and thermal stability were determined from residual activities after hydrogen peroxide (0.1 mM) and heat treatment (65 °C) for 30 min. Crude extract (1.0 mg/mL protein solution) was used for tests as described in Materials and Methods.

Characterization of the Evolved Mutant. The evolved mutant, 2S3, was purified as described in Materials and Methods, and its oxidative and thermal stability was determined. As shown in Figure 4A, first-order thermal inactivation constants of 2S3 and wild type at 70 °C were estimated to be 0.01 and 0.08 min^{-1}, respectively. As for oxidative stability, residual enzyme activity was traced in the presence of 0.2 mM hydrogen peroxide at 25 °C, and 2S3 exhibited an 18-fold increased oxidative stability compared to that of wild type (Figure 4B). Increment in both oxidative and thermal stability was distinctive for the evolved variant 2S3, which confirmed that oxidative and thermal stability of *N*-carbamoylase was simultaneously improved by directed evolution.

There has been an argument about the correlation between enzyme activity and thermostability. Stoichet et al. reported that improvement of thermostability is obtained at the cost of enzyme activity, such as increase in K_m or decrease in k_{cat} (*23*). On the other hand, recent study suggested that the thermostability of enzyme is not inversely correlated with the activity and that thermostability might be improved even without the expense of its activity (*24*). In the case of 2S3, the K_m value was almost the same as that of wild type, and the catalytic constant (k_{cat}) was lower than that of wild type (Table 1). The k_{cat}/K_m of the 2S3 maintained about 70% of the wild-type enzyme. On the basis of this result, it is plausible that thermal stabilization of 2S3 came at the cost of reduction in activity, even though it is not conclusive whether activity and thermostability are inversely correlated or not.

Amino Acid Substitutions in the Evolved Enzymes. Mutated amino acid residues in 2S3 were identi-

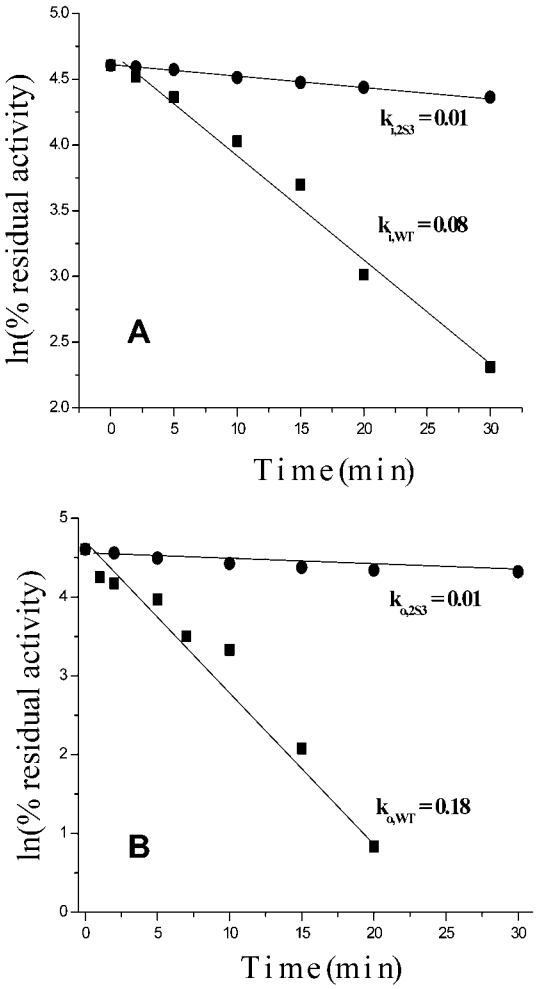

Figure 4. Time course inactivation of wild type and 2S3. (A) Incubation at 70 °C. (B) Incubation with 0.2 mM hydrogen peroxide at 25 °C. Samples were taken at intervals, and residual activities were assayed. $k_{i,WT}$ and $k_{i,2S3}$ represent the thermal inactivation constants of wild type and 2S3, respectively. $k_{o,WT}$ and $k_{o,2S3}$ are the oxidative inactivation constants of wild type and 2S3, respectively: (■) wild-type; (●) 2S3. Data represent the average of duplicated experiments.

Table 1. Kinetic Parameters of Wild Type and Evolved 2S3[a]

	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
WT	9.5 ± 0.2	1.1 ± 0.0	8.7 ± 0.2
2S3	6.9 ± 0.1	1.1 ± 0.1	6.3 ± 0.3

[a] Each value represents the average and mean of triplicate experiments.

fied by sequencing the corresponding gene. As a result, 2S3 was found to possess six mutations, Q23L, V40A, H58Y, G75S, M184L, and T262A. It was previously reported that the substitution of histidine at the position of 58 to tyrosine improved the thermostability of *N*-carbamoylase from *Agrobacterium* sp. KNK712 (*25*−*27*).

To evaluate the contribution of each amino acid change to an increase in oxidative or thermal stability, site-directed mutagenesis was conducted for incorporation of each mutation into wild-type enzyme, and the resulting mutants were subjected to stability test. As can be seen Table 2, both M184L and T262A led to an increase in oxidative and thermal stability, and these mutations were considered almost additive. In particular, T262A resulted in a significant improvement in both oxidative and thermal stability of 2S3. Q23L and H58Y also increased both stabilities to some extent. The mutation

Table 2. Oxidative and Thermal Stability of Wild Type and Mutants[a]

	thermal stability[b] (%)	oxidative stability[c] (%)
WT	10.0 ± 0.8	5.0 ± 1.2
M184L	19.7 ± 2.0	20.0 ± 2.3
T262A	50.5 ± 3.4	40.9 ± 2.2
Q23L	19.1 ± 1.4	63.9 ± 2.3
V40A	7.0 ± 2.3	20.6 ± 1.2
H58Y	20.0 ± 1.9	20.6 ± 1.2
G75S	3.5 ± 1.1	42.5 ± 0.8
2S3	78.5 ± 3.1	79.3 ± 1.2

[a] Each value represents the average and mean of triplicate experiments. [b] The residual activity after heat treatment at 70 °C for 30 min. [c] The residual activity after incubation with 0.2 mM hydrogen peroxide at 25 °C for 30 min.

Q23L led to the highest contribution to oxidative stability in the mutations found in 2S3. The other two mutations, V40A and G75S, mainly gave rise to increments in oxidative stability rather than thermostability. The thermostability of 2S3 was reinforced as these single thermostabilizing mutations accumulated. However, the cumulative effect on oxidative stability was not observed even though improvement of oxidative stability by single mutation was considerable.

It has been known that some amino acid residues such as cysteine, methionine, histidine, and tyrosine are liable for an oxidizing condition. The oxidation of methionine to sulfoxide is known to result in the generation of a larger and more polar side chain, causing disruption of the protein structure (*28*). Thus, the substitution of methionine 182 to leucine in 2S3 is likely to bring the resistance against oxidizing conditions. *N*-Carbamoylase from *A. tumefaciens* NRRL B11291 contains five cysteine residues, and of them, Cys172 was revealed to be involved in the active site (*4*). It was suggested that inactivation of *N*-carbamoylase arises from various degrees of thiol-oxidation including inter- or intramolecular disulfide bond formation and generation of a more oxidized product like cysteic acid (*4*). However, no change in cysteine residues was found in the evolved enzyme 2S3. It was reported that the substitution of oxidazible amino acid residues to non-oxidazible ones leads to an increase in oxidative stability of enzymes (*29*−*32*). In the case of peroxidases, on the contrary, replacement of non-oxidazible amino acid residues enhanced the oxidative stability (*33*, *34*). Therefore, it is noteworthy that the mutations on non-oxidizable amino acid residues in 2S3 (Q23L, V40A, H58Y, G75S, and T262A) made a significant contribution to an improvement of oxidative stability.

From the analysis of the mutant library by the activity staining method, we found that more than 50% of mutants with an increased thermostability also display improved oxidative stability (or vice versa) (data not shown). This observation strongly implies that oxidative stability is positively correlated with thermostability, if not directly correlated, and these properties can be simultaneously improved. It was found from the mutational analysis that four mutations (Q23L, H58Y, M184L, and T262A) in 2S3 simultaneously increase both the oxidative and thermal stability of *N*-carbamoylase, and this supports the above presumption.

Conclusion

We have demonstrated that oxidative and thermal stability of *N*-carbamoylase can be simultaneously improved by directed evolution. An activity staining method based on the pH change on a solid agar plate was

confirmed to be effective for primary screening of the variants with an improved stability. The second round of directed evolution resulted in the best mutant 2S3, and this evolved enzyme displayed significantly enhanced oxidative and thermal stability compared to that of the wild-type counterpart. Six amino acid residues were found to be changed in 2S3, and of them, four mutations (Q23L, V40A, H58Y, and T262A) led to an increase in both oxidative and thermal stability. The evolved enzyme 2S3 is expected to be employed for development of a fully enzymatic process for the production of D-amino acids in conjunction with D-hydantoinase.

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