Increase in Thermostability of N-Carbamyl-d-Amino Acid Amidohydrolase on Amino Acid Substitutions

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To improve the production of d-amino acids using an immobilized N-carbamyl-d-amino acid amidohydrolase, the enzyme gene of Agrobacterium sp. KNK712 was mutagenized randomly to increase its thermostability. The gene was inserted into M13mp19, mutagenized with hydroxylamine, ligated into pUC19 after restriction endonuclease digestion, and then used to transform Escherichia coli. The resultant transformants were screened by a newly developed colorimetric enzyme assay method, and the candidate colonies corresponding to red spots were separated from the master plates. Using cell-free extracts of these clones, the properties of the enzymes produced were investigated, it being proved that these enzymes had almost the same activity and improved thermostability by about 5°C compared with those of the native enzyme. As found on enzyme gene analysis of these mutants, the 57th amino acid, histidine, of the enzyme was changed to tyrosine, or the 203rd amino acid, proline, to leucine or serine.

Key words: N-carbamyl-d-amino acid amidohydrolase; thermostabilized enzyme; d-amino acid

D-Amino acids, which are used as intermediates of pharmaceuticals, are produced industrially, and one of conversion methods is derivation from N-carbamyl-d-amino acids. For an industrial process, an enzymatic method involving N-carbamyl-d-amino acid amidohydrolase (DCase) is of great advantage for converting N-carbamyl-d-amino acids to d-amino acids. Some DCase-producing strains have been found by screening and the enzyme proteins were purified.1,2,3,4,5 We found a high-producing strain, Agrobacterium sp. KNK712, by screening and cloned the enzyme gene.5,9 But all the enzymes from mesophiles seem not to be stable enough for repeated practical use in immobilized forms. And although we also screened for thermostolerant microorganisms producing stable DCases and producing a lot of enzyme by use of a cloned gene,5,9 these enzymes did not have high specific activity toward some necessary substrates, for example, N-carbamyl-d-p-hydroxyphenylglycine. A practical DCase that has both high reactivity and sufficient stability for a bioreactor has not been obtained yet. In the enzyme from Agrobacterium radiobacter, the role of the cysteine residues was investigated by a site-directed mutagenesis experiment, and a relationship was found between enzyme stability and activity.7,10 To produce a practical enzyme, we decided to improve DCase by amino acid substitutions using recombinant DNA technology.

In this paper, we report that the DCase gene from Agrobacterium sp. KNK712 was randomly mutagenized, thermostabilized enzymes were screened, and the nucleotide sequence of the gene was analyzed. The substituted amino acid residues of the enzymes were identified.

Materials and Methods

Media and methods. The culture medium for recombinant Escherichia coli was 2 × YT medium5,9 containing 100 μg/ml ampicillin, and the plate medium was the same with 15 g/l Bacto-agar (Difco). For cloning in M13mp19, a H-agar plate (10 g/l Bacto tryptone (Difco), 8 g/l NaCl, and 15 g/l Bacto-agar) was overlaid with H-top agar (same as H agar, except 8 g/l Bacto-agar was used) containing 0.02% 5-bromo-4-chloro-3-indolyl-β-d-galactoside and 1 mM isopropyl-β-d-thiogalacto-pyranoside. Positive color selection using pUC19 was done on 2 × YT agar plates with 0.02% 5-bromo-4-chloro-3-indolyl-β-d-galactoside and 1 mM isopropyl-β-d-thiogalacto-pyranoside. Vectors pUC19 and M13mp19, restriction endonuclease, T4 ligase, and the E. coli strains were from Takara Syuzo Co. d-Amino acid oxidase from porcine kidney and horseradish peroxidase were from Sigma Chemical Co. and Calzyme Lab. Inc. (USA), respectively. The filter paper for colony replica was from Toyo Roshi Kaisha, Ltd. (5C, 83 mm). The other chemicals used were all of guaranteed pure grade.

Mutagenesis. The DCase gene of Agrobacterium sp. KNK 712 was separated from recombinant plasmid pAD10857 by double digestion with EcoR I and Hind III. The resultant 1.8-kb DNA fragment was inserted into M13mp19, and then used to transform E. coli CJ236. The recombinant E. coli cells were cultivated and the phage particles generated were collected from the culture medium after precipitation by adding 0.2 volumes of PEG-NaCl (20% polyethylene glycol 6,000 and 2.5 mM NaCl). The recombinant phage particles that contained the DCase gene were treated with 0.25 M hydroxylamine hydrochloride (pH 6.0) for 1–8 h at 37°C,7,10 diluted tenfold with sterilized water, and then precipitated with

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Abbreviation: DCase, N-carbamyl-d-amino acid amidohydrolase.
PEG-NaCl. The precipitated phage was collected by centrifugation, dissolved in sterilized water, and then used to infect *E. coli* C1236. After preparation of double strand phage DNA, the 1.8-kb *EcoRI* *HindIII* fragment corresponding to the DCase gene was prepared, inserted into pUC19, and then transformed to *E. coli* JM109.

**Colorimetric enzyme assay. (a) Colony assay for first screening.** The recombiant *E. coli* colonies on the plate medium (the plates being called the master plates) were transferred to sterilized filter paper, and then lysed for 30 min at 37°C by adding 1.5 ml of a lysis solution (20 mM Tris·HCl (pH 7.5), 10 mM EDTA, 2 mg/ml lysozyme, and 1% Triton X-100). The filter paper was washed in water, incubated in hot water (65°C) for 5 min to inactivate heat sensitive DCases, cooled immediately in cold water, and then dried. A color reagent (1 ml), which comprised a mixture of two solutions (Sol. A: 30 mM potassium phosphate buffer (pH 7.4), 3 mg/ml N-carbamyl-d-phenylglycine, 2.5 mg/ml phenol and 0.8 units/ml β-amino acid oxidase; Sol. B: 70 units/ml horseradish peroxidase and 10 mg/ml 4-aminoantipyrine, Sol. A:Sol. B = 100:1, mixed just before use) was added to the filter paper. After 15 min of incubation at 37°C, the candidate colonies, which corresponded to red spots, were separated from the master plates.

(b) Cell-free assay for second screening. Recombinant *E. coli* cells were collected from 3 ml of an overnight culture, and then suspended in 1 ml of 100 mM potassium phosphate buffer (pH 6.9). After incubation with lysozyme (2 mg/ml) for 15 min at 37°C, the cells were disrupted by sonication (Tomy Seiko; UR-20P), and then centrifuged to remove cell debris. The cell-free extract was incubated for 10 min at 65°C to inactivate heat-sensitive DCases and denatured protein was removed by centrifugation. The crude enzyme sample was dispensed in 150-μl portions onto a 96-well microtiter plate (Corning; 43053), which was serially diluted two-fold with 100 mM potassium phosphate buffer (pH 6.9), and then 50 μl of modified color reagent, namely Sol. A:Sol. B = 19:1, was added. The plate was incubated at 37°C until the wells of the native enzyme as a control (not heat-treated) were colored sufficiently (30–120 min, maximal OD_{450} = 1–1.5), and then enzyme activity was measured as the absorbance at 505 nm (Labsystems; Multiskan Bichromatic).

**Effects of temperature and pH on the enzyme stability.** To evaluate the thermostability of the DCase, a cell-free extract (100 μl) was incubated at various temperatures (55–70°C) for 10 min, the denatured protein was removed by centrifugation, and the residual activity was measured.

To examine the effects of pH on the DCase stability, a cell-free extract (200 μl) was mixed with 800 μl of pH-adjusted buffers (pH 6–10, 0.1 M of each buffer as described in the legend of Fig. 2), and incubated 40°C for 4 h, and then the residual activity was measured.

**DNA sequencing and other analytical methods.** The nucleotide sequence of the mutagenized gene was analyzed using an automated DNA sequencer (Applied Biosystems; 373A).

The method used for measurement of DCase activity and other analytical methods were as described by Nanba et al. 

**Results**

**Mutagenesis of the DCase gene and screening of thermostable enzyme producers**

As a result of the first screening of 14,800 recombinant colonies, 17 clones were screened as thermostable-enzyme-producing strains by a colorimetric colony assay. These clones were then screened again, and 6 clones that produced thermostabilized enzymes having almost the same reactivity as that of the native enzyme were selected. The screening profile is shown in Table 1.

**Nucleotide sequence analysis of the mutagenized DCase gene**

The DNA sequence of the 1.8-kb *EcoRI* *HindIII* III

| Table 1. Screening Profile of Thermostable Enzyme-Producing Strains |
|--------------------------|-----------------|-----------------|-----------------|
| Mutagenesis (h) | Assayed strains | 1st screening | 2nd screening |
| 1 | 100 | 0 | 0 |
| 2 | 2,100 | 1 | 1 |
| 3 | 100 | 1 | 0 |
| 4 | 100 | 0 | 0 |
| 5 | 100 | 0 | 0 |
| 6 | 3,600 | 4 | 1 |
| 7 | 4,600 | 7 | 3 |
| 8 | 4,100 | 5 | 1 |
| (Total) | 14,800 | 17 | 6 |

The recombinant phage containing the DCase gene was mutagenized with hydroxylamine for 1–8 h. The mutagenized DCase gene was inserted into pUC19 and then used to transform *E. coli* JM109, followed by selection by a colorimetric colony assay for the 1st screening. The candidate clones were re-selected by a colorimetric cell-free assay (2nd screening). All numbers except those in the mutagenesis column represent those of the screened strains. The experimental conditions for mutagenesis and screening are described under Materials and Methods.

**Table 2. Nucleotide Analysis of the Mutagenized DCase Gene**

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Locations of nucleotide changes</th>
<th>Amino acid substitutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>401M</td>
<td>840 C→T</td>
<td>203 Pro→Leu</td>
</tr>
<tr>
<td>402M</td>
<td>401 C→T</td>
<td>57 His→Tyr</td>
</tr>
<tr>
<td>403M</td>
<td>177 C→T</td>
<td>(161 Val→Val)</td>
</tr>
<tr>
<td>404M</td>
<td>840 C→T</td>
<td>203 Pro→Ser</td>
</tr>
<tr>
<td>405M</td>
<td>839 C→T</td>
<td>203 Pro→Leu</td>
</tr>
<tr>
<td>406M</td>
<td>839 C→T</td>
<td>203 Pro→Ser</td>
</tr>
</tbody>
</table>

The mutagenized DCase genes of six thermostable enzyme-producing strains were analyzed and the locations of nucleotide changes were determined. The numbering of nucleotide was followed as described by Nanba et al. The amino acid substitutions resulting from the mutations were deduced.
fragment, which contained the mutagenized DCases gene, from these six clones was analyzed and is summarized in Table 2. One or some nucleotides of the DCases gene were changed and all the mutations were substitutions from cytosine to thymine. The mutations of the six mutant enzymes were classified into three types as to the deduced amino acid substitutions. The amino acid changes related to the thermostability increase proved to be that the 57th amino acid of DCases, histidine, was changed to tyrosine, and the 203rd proline was changed to serine or leucine.

**Evaluation of the thermostability of the mutagenized enzymes**

The cell-free extracts of the mutant DCases, which had almost the same activity as that of native enzyme, were incubated at various temperatures, and then the residual activities were calculated as relative activities in comparison with the activities of the non-heat-treated enzymes. The activities were plotted against temperature, as shown in Fig. 1. The thermostabilities of these three mutant enzymes increased about 5°C under these conditions compared to that of the native enzyme.

**Evaluation of the enzyme stability with various pHs**

The mutant DCases were incubated at various pHs, and then the residual activities were measured as the relative activity compared with the activity of each non-treated sample, and plotted against incubation pH (Fig. 2). The stabilities of these three mutant enzymes were increased in lower and higher pH regions in comparison with those of the native enzyme.

**Discussion**

In order to produce a stable DCases for a bioreactor, we improved the DCases of Agrobacterium sp. KNK712 using the random mutation technique, and produced three DCases mutants that had increased thermostability by about 5°C. On gene analysis of these mutants, it was proved that amino acid positions 57 and 203 of the enzyme are concerned with its thermostability. By use of a newly developed colorimetric enzyme assay, we screened for thermostabilized mutant enzymes efficiently.

From among 14,800 transformants, six mutants were screened for the production of thermostabilized enzymes. All the mutations were base substitutions from cytosine to thymine, and the base changes could be explained by the mutation mechanism of the mutagen, hydroxylamine. The total efficiency of isolation of thermostabilized mutants was about $4 \times 10^{-4}$, and the mutant DCases genes in these mutants had from one to three mutations. For these results, mutational treatment

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**Fig. 1.** Effects of Temperature on the Stability of the Mutagenized Enzymes.

The remaining activity was assayed under the standard conditions after a cell-free extract had been kept at various temperatures. The remaining activities of the mutant enzymes, 402M (●●●), 404M (▲▲▲), 406M (■■■), and the native enzyme (○○○) are expressed as a percentage of the activity of each non-heated enzyme.

**Fig. 2.** Effects of pH on the Enzyme Stability of the Mutagenized DCases, (A) 402M, (B) 404M, and (C) 406M, Respectively. A cell-free extract was incubated at 40°C for 4 h at the indicated pHs in 0.1 M K$_2$HPO$_4$-KH$_2$PO$_4$ (———), 0.1 M Tris·HCl (----), or 0.1 M Na$_2$CO$_3$-NaHCO$_3$ (⋯⋯⋯) buffers. The remaining activities were then assayed under the standard conditions, and presented as a percentage of the activity of each non-treated enzyme. As a control, the native enzyme (⋆) was treated in the same manner.
with hydroxylamine for 6–8 h under our conditions seems suitable.

These thermostabilized enzymes also showed increased stabilities in lower and higher pH regions and also improved resistance to oxidative inactivation (data not shown). From these facts, it seemed that the enzymes had increased stability in many aspects, and were probably stabilized by having a more rigid conformation. And considering the small changes of the enzyme reactivity and optimum pH (data not shown), conformation of the enzymatic active site seemed to have changed little.

In these DCase mutants, a single amino acid change stabilized the enzyme. This was reported for other enzymes previously. In thermolysin, the kinds of changes likely to increase thermostability which differed from the sequences of known mesophilic and thermophilic enzymes were compared, the changes being made by site-directed mutagenesis. The stabilization mechanism was assumed to comprise stabilization of the α-helix, which connected two domains. In glucose dehydrogenase, the enzyme gene was mutated randomly with a chemical mutagen, and then more stable mutants that had one or some amino acid substitutions were screened for. For the mechanism of the enzyme stabilization, it was assumed that the tetrameric structure of the enzyme was stabilized by the mutation.

The mechanism of the thermostability increase in our enzyme could be assumed in view of the conformational change. It seemed to be related to that in the mutant in which the 57th amino acid, histidine, was changed to tyrosine, the positive charge of the histidine residue was removed, and in the mutant in which the 203rd proline was changed to leucine or serine, the bend of the enzyme chain generated by proline was removed. We are going to investigate the conformational relationship between enzyme stability and structure of the enzyme molecule.

The mutated amino acid residues of these enzymes were different from the corresponding amino acid residues of thermostable DCase from Pseudomonas sp. KKN003A and the stability-related cysteine residues from Agrobacterium radiobacter. There seemed to be a possibility that other thermostability-related sites existed in the DCase. We are continuing to screen for thermostabilized mutant enzymes using other mutations to discover new thermostability-related sites. And for a further increase in the thermostability of DCase, we are trying to change these two thermostability-related sites to other amino acids. Because these improved enzymes will be valuable for D-amino acid production, the development of a production process is expected.

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