A Microbial D-Hydantoinase is Stabilized and Overexpressed as a Catalytically Active Dimer by Truncation and Insertion of the C-Terminal Region

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Abstract Previously, it was reported that the nonhomologous C-terminal regions of the D-hydantoinases are nonessential for catalysis, but affect the oligomeric structure of the enzyme [3]. In an effort to further confirm the above observation, the C-terminal region-inserted enzyme was constructed by attaching a peptide (22 residues) at the C-terminal of the D-hydantoinase from *Bacillus thermocatenulatus* GH2, and its structural and biochemical properties were compared with both the wild-type and C-terminal region-truncated enzymes. As a result, native tetrameric D-hydantoinase was dimerized as the truncated enzyme, and the inserted mutant with a new sequence was expressed as a catalytically active form in *E. coli*. Expression level of the inserted and truncated enzymes were found to be significantly increased compared to the level of the wild-type enzyme, and this appears to be due to the reduced toxic effect of the mutant enzymes on host cells. Dimerized enzymes exhibited increased thermo- and pH stabilities considerably when compared with the corresponding wild-type enzyme. Comparison of the substrate specificity between the mutant and wild-type enzymes suggests that the substrate specificity of the D-hydantoinase is closely linked with the oligomeric structure.

Key words: Hydantoinase, dimerization, stabilization, *Bacillus*

Microbial D-hydantoinase catalyzes stereo-specific hydrolysis of a variety of hydantoins, and is currently employed as an industrial biocatalyst for the production of optically pure D-amino acids that are intermediates for antimicrobial drugs, peptide hormones, and pyrethroids [20, 23].

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Based on the required substrate specificity toward the hydantoin derivatives, much attention has been paid to the isolation of the microbial D-hydantoinase retaining useful properties for practical application [13, 17, 18]. Some genes coding for the microbial D-hydantoinases were cloned and sequenced [1, 7, 11, 14], along with the detailed characterization of the enzymes. However, their structure-function relationships have not been well understood, especially concerning oligomerization, partly because most of the studies were focused on the typical characterization of enzymes and no structural data are available. In an effort to understand the structural and evolutionary relationships of microbial D-hydantoinase, a comparative study was recently reported on the cyclic amidohydrolase family, including D-hydantoinase, dihydropyrimidinase, allantoinase, and dihydroorotase, all of which are acting on the cyclic amide ring [4]. The enzymes belonging to the cyclic amidohydrolase family were found to share a number of conserved regions starting from the N-termini, and these genes are supposed to be derived from a common ancestor. On the other hand, the C-terminal regions have little homology among the enzymes. These findings were further confirmed by the identification of novel enzymes with high selectivity to useful substrates in *E. coli* [5].

From the comparative analysis of two microbial D-hydantoinases, the enzymes originating from two thermophilic *Bacilli* were observed to possess an identical organization that covered in the family of enzymes [3]. As an approach to understanding the structure-function relationship of the two enzymes, nonhomologous C-terminal regions were deleted, and the resulting mutant enzymes were partly characterized [6]. Interestingly, the C-terminal region was found to be nonessential for catalysis, but affected the oligomeric structure of the enzyme [3]. In this paper, we have attempted further to elucidate the involvement of the

C-terminal region in the oligomerization and catalytic property of the D-hydantoinase, by insertion of a peptide fragment at the C-terminal region. For a clear comparison, the resulting mutant was compared with wild-type and truncated enzymes, in terms of the oligomeric structure and catalytic properties. Based on the resulting properties, the potential of mutant (dimerized) enzymes was also evaluated.

MATERIALS AND METHODS

Enzymes and Reagents
Hydantoin, uracil, dihydrouracil, 2-thiouracil, and 2,4-dithiouracil were purchased from Sigma (St. Louis, MO, U.S.A.). Restriction enzymes and T4 DNA ligase were purchased from Boehringer Mannheim (Mannheim, Germany). A thermophilic DNA polymerase for PCR was purchased from New England Biolabs (Beverly, MA, U.S.A.). Oligodeoxynucleotides were obtained from DNA International (Lake Oswego, OR, U.S.A.). DL-Hydroxyphenyl hydantoin, DL-phenyl hydantoin, and DL-isopropyl hydantoin were chemically synthesized by the method of Suzuki et al. [19]. Prepacked Resource-Q (6 ml), Phenyl Superose (HR 5/5), and Superose 12 (HR 10/30) columns were purchased from Pharmacia Biotech (Uppsala, Sweden). All other solvents and chemicals were of analytical grade.

Construction of the C-Terminal Region-Inserted Enzyme
For the construction of the inserted enzyme, a thermophilic B. thermocatenulatus GH2 [16], previously isolated in the laboratory, was used as a source of the D-hydantoinase gene. Primer for the N-terminal region with promoter and a primer for the C-terminal region without stop codon, 5'-CGTGAGCTCTGTATTCAGTGCAATAAG-3' and 5'-CGAATTCCGCCCCATATTTCGCGCGTT-3', were used to remove the stop codon in the reading frame of the enzyme by PCR. The amplified fragment was introduced into the SacI-EcoRI site of pBluescript II SK, and the resulting vector used to express the mutant enzyme that additionally tagged a peptide of 22 residues, originated from the multicloning site of pBluescript II SK. The resulting construct was transformed into E. coli XL1-Blue by electroporation. E. coli strains were grown in Luria-Bertani medium at 37°C, and ampicillin (50 µg/ml) was added when needed. The colonies expressing the recombinant genes were screened on a selective plate as described elsewhere [8]. For the comparative study, C-terminal region-truncated enzyme was constructed as described in the previous report [3].

Purification of Enzymes
Wild-type and mutant enzymes were purified to homogeneity by a purification scheme consisting of two steps, ion-exchange column chromatography and hydrophobic interaction column chromatography, as described previously [4]. All purification steps were conducted at room temperature in the presence of 1 mM MnCl$_2$.

DNA Sequencing
The C-terminal region-inserted enzyme was confirmed by DNA sequencing. Nucleotide sequencing was carried out on a double-stranded template with appropriate primers by the use of an automatic DNA sequencer (ABI, Model 3700, Applied Biosystems, CA, U.S.A.). The complete nucleotide sequence was determined in both strands. Synthetic 20-mer oligonucleotides were used as primers when needed.

Structural Analysis of the C-Terminal Region-Inserted Enzyme
Analytical SDS/PAGE in slab gels was performed according to the method of Laemmli [10]. For native gel electrophoresis, the enzyme solution was loaded on an electrophoresis gel consisting of a stacking gel (5% acrylamide) and a running gel (12% acrylamide) without SDS and β-mercaptoethanol, and then activity staining was performed as described in the previous paper [8]. Acrylamide gels were stained with either Coomassie Brilliant Blue G250 or silver nitrate.

The oligomeric structure of the enzyme was determined by gel filtration chromatography. The gel filtration was performed by using a Superose 12 column (HR 10/30, Pharmacia, Uppsala, Sweden) equilibrated with 20 mM Tris-HCl (pH 8.0) containing 0.1 M NaCl and 1 mM MnCl$_2$. Standard proteins and their molecular masses were as follows: blue dextran, 2,000 kDa; ferritin, 440 kDa; catalase, 232 kDa; aldolase, 158 kDa; and Fab fragment, 50 kDa.

Circular dichroism measurement was carried out with a recording spectropolarometer (Jasco, J-700, Tokyo, Japan) at 25°C with a 1-mm pathlength cell. The CD spectra were obtained at the protein concentration of 2.5 µM in the Far-UV region (190–260 nm) under a nitrogen atmosphere. Fluorescence emission spectra were observed at 25°C using a spectrofluorometer (Jasco, FP770) with an excitation wavelength of 285 nm. The protein samples were identiculy prepared for the both measurements.

Assay of D-Hydantoinase Activity
D-Hydantoinase activity was determined by using either HPLC or color reagent as described in the previous paper [4]. One unit of hydantoinase activity was defined as the amount of enzyme producing one µmol N-carbamyl-D-amino acid from hydantoin derivative per minute under the specified conditions. The protein concentration was determined by using a protein assay solution (Bio-Rad, CA, U.S.A.).

Determination of Stability
For a clear comparison of the enhanced stability at acidic pH, and with considering the practical application, the

enzyme stability was determined by measuring the residual activity after incubating the enzyme at 50°C or 70°C for 1 h at pH 5.5. To determine the thermostability, the enzyme was heated at different temperatures for 30 min in 0.1 M Tris-HCl buffer (pH 8.0) containing 0.1 mM Mn^{2+}, and the residual activity was determined.

RESULTS

Construction and Oligomeric Structure of the C-Terminal Region-Inserted Enzyme

Wild-type D-hydantoinase from *B. thermocatenulatus* GH2 was expressed in *E. coli* as a tetramer, but in the case of truncation of the nonhomologous C-terminal region, its oligomeric structure was found to be a dimer [3]. This observation suggested that the C-terminal region was involved in the oligomerization of the enzyme. To further confirm the result, a mutant enzyme having an inserted peptide was constructed at the C-terminal end, as described in the experimental section. The resulting mutant enzyme (BT471F) was expressed in a catalytically active form under its native promoter of D-hydantoinase from *B. thermocatenulatus* GH2. The correct insertion at the C-terminal was confirmed by DNA sequencing, and retransformation of the construct into a fresh cell also showed an identical result.

Upon native gel electrophoresis of crude extracts, BT471F appeared at a different position than that of the wild-type enzyme. It was clearly shown by staining on native gel electrophoresis (data not shown). This result strongly suggested that the oligomeric structure and/or conformation of BT471F were different from that of the corresponding wild-type enzyme. For further detailed characterization and comparison of BT471F with the wild-type (BT471) and truncated (BT460) enzymes, three enzymes were purified to homogeneity via Resource Q column chromatography and Phenyl Superose column chromatography, as described elsewhere [4]. Fusion or tagging strategy for protein purification was intentionally avoided, because the possibility of oligomerization by additional fusion, although the method was simple and easy to handle. During the purification, chromatographic behavior of both the truncated and inserted enzymes was found to be similar to that of the wild-type enzyme.

Using the purified enzymes, whether the insertion at the C-terminal had an affect on the oligomeric structure of the enzyme was first analyzed. When BT471F was loaded on gel filtration chromatography, it was eluted at the position corresponding to a molecular mass of 110 kDa (Fig. 1A). The result was further confirmed by native gel electrophoresis and activity staining using the eluted fraction. As shown in Fig. 1B, the purified BT471F separated on native gel electrophoresis showed a different band position from that of BT471, as observed in the crude extract. These results
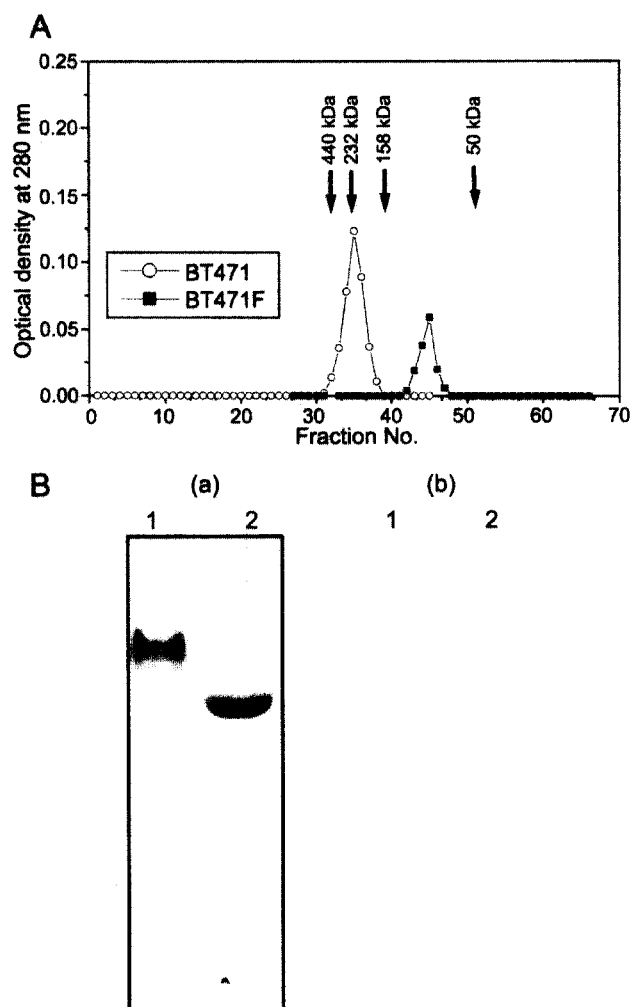
Fig. 1. Oligomeric structure of the mutant enzyme BT471F.
(A) The purified enzyme (50–75 µg) was analyzed on a Superose-12 gel filtration column. The molecular mass of the proteins was estimated from the elution profile of the standard protein markers: ferritin, 440 kDa; catalase, 232 kDa; aldolase, 158 kDa; MBP, 42 kDa. All experiments were repeated three times at different protein concentrations. The shift in elution time was negligible (<0.2 min). (B) Activity staining with purified enzymes on native gel electrophoresis. (a) Native gel: Lane 1, BT471; Lane 2, BT471F. (b) Activity staining: Lane 1, BT471F; Lane 2, BT471.

indicate that the inserted enzyme is dimerized as the truncated enzyme BT460 [3], and it also strongly supports that the C-terminal region is involved in the oligomerization of the enzyme. The cross-linking experiments, under similar conditions reported elsewhere [3,5], with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride also supported the results that the quaternary structure of the inserted enzyme was dimeric. Comparison of the secondary and tertiary structures based on circular dichroism and fluorescent emission spectra revealed no significant difference between the mutant and wild-type enzymes, supporting that BT471F maintained its favorable structure as the corresponding wild-type enzyme. The average contents of

Table 1. Substrate specificitiy of wild-type and mutant enzymes.

Substrate	Specific activity* (unit/μM enzyme)		
	BT471	BT460	BT471F
Hydantoin	11.41±0.21	4.05±0.09	5.82±0.13
Hydroxyphenylhydantoin	9.49±0.49	0.85±0.11	0.66±0.08
Phenylhydantoin	20.28±0.35	8.31±0.18	6.77±0.05
Isopropylhydantoin	1.24±0.04	1.38±0.03	0.82±0.04
Dihydrouracil	7.01±0.06	2.41±0.04	1.75±0.09
Uracil	ND	ND	ND

* ND: Not Detected.
ᵃAll substrates (50 mM) were preincubated for 20 min at 55°C under nitrogen flushing, and the enzyme activity (3 μg) was determined using standard reaction condition (55°C, 30 min) in 100 mM Tris-HCl buffer (pH 8.0).

α-helix, β-sheet, and loop based on CD spectra were calculated to be about 23±3, 34±2, and 42±4% for both enzymes, respectively (Figure not shown).

Catalytic Properties of the Mutant Enzymes

The specific activity of BT471F toward various hydantoin derivatives was compared with those of the wild-type BT471 and truncated BT460 (Table 1). Since oligomeric structures among the enzymes were different, the specific activity was calculated based on the molar concentration of the enzyme. As a result, the specific activity of BT471F was decreased toward most of the substrates tested compared to that of the wild-enzyme. In particular, the

specific activity of BT471F toward hydroxyphenyl hydantoin was less than 7% that of the wild-type enzyme, and one-fourth of the specific activity of the wild-type enzyme toward dihydrouracil was observed. The truncation enzyme BT460 exhibited a similar catalytic property with the inserted enzyme BT471F.

The dependency of enzyme activity on the pH was investigated, and we found that the activity profiles of the truncated and inserted enzymes were similar to that of the parent enzyme BT471 [16]. The stability of the truncated enzyme BT460 strongly improved in the acidic range compared to the wild-type BT471, and BT471F also exhibited a slight increase in stability at acidic pH (Fig. 2). Furthermore, the mutant enzymes retained longer half-life under more extreme conditions (even at the highly elevated temperature). The expected result was that the mutant enzymes were more efficient in the conversion of substrate, because the reaction pH was rapidly decreased by increasing the concentration of an acidic product, N-carbamyl D-amino acid, according to how the reaction proceeded. Furthermore, a sharp decrease in stability was observed around the isoelectric pH [12, 16], and this seems to be due to the aggregation of the enzymes at a high incubation temperature.

The effect of temperature on the activity and stability of the enzyme was also examined. The optimal temperature of the two mutant enzymes, BT460 and BT471F, was found to be about 65 and 60°C, respectively, which was similar to that of the wild-type BT471 (data not shown). Concerning the thermostability of the enzyme, BT471F
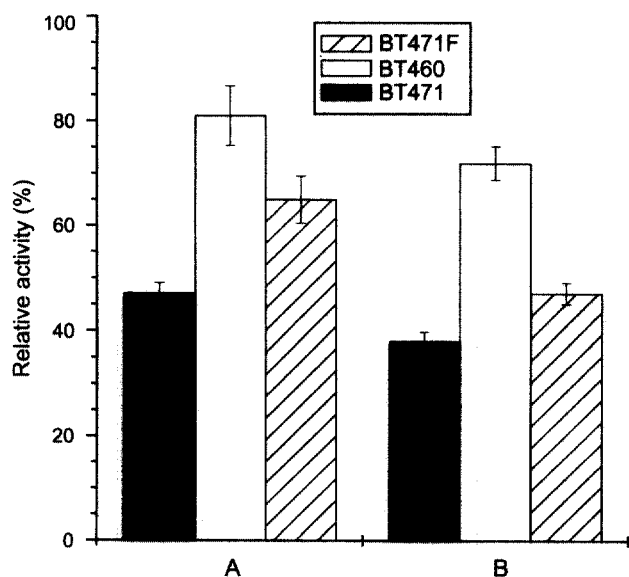
Fig. 2. pH stability of the wild-type and dimerized enzymes in an acidic condition.
The purified enzyme (0.1 mg/ml) was incubated at 50°C (A) or 70°C (B) in 20 mM sodium acetate buffer (pH 5.5) for 1 h, and residual activity was determined in standard reaction conditions as described in the experimental section. All experiments were repeated two times and the average values were represented.
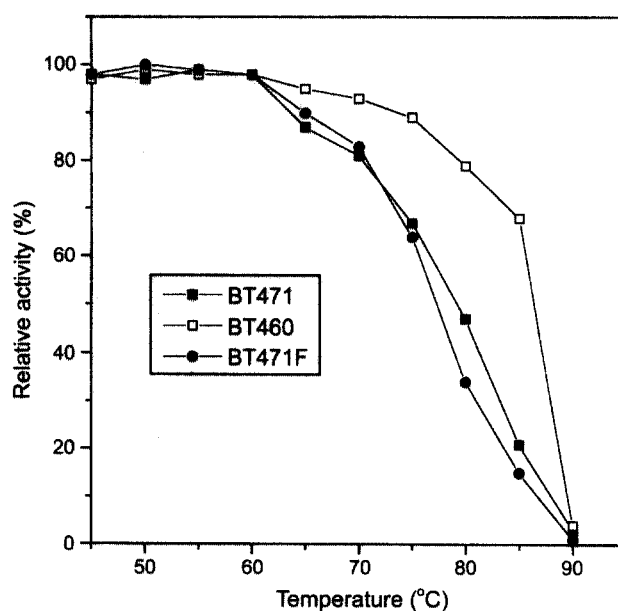
Fig. 3. Thermostability of the wild-type and mutant enzymes.
The enzyme (0.1 mg/ml) was incubated at different temperatures for 30 min in 20 mM Tris-HCl buffer (pH 8.0), and residual activity was determined under standard assay conditions. The average values calculated from repeated experiments were represented.

had a comparable stability to that of the wild-type BT471. In particular, the truncated enzyme BT460 exhibited a significantly enhanced thermostability compared with the wild-type BT471 (Fig. 3).

## High-Level Expression of the Dimerized Enzyme in E. coli

The D-hydantoinase of *B. thermocatenulatus* GH2 was expressed under the control of its own promoter on pBGK470 [3], and the expression level was relatively low (<0.5%). The high level expression of the enzyme from *B. thermocatenulatus* GH2 was attempted, but all cases resulted in very low expression levels due to the poor growth and lysis of the host cells. On the other hand, in the cases of the truncated and inserted mutant enzymes, expression levels were much higher than that of the wild-type enzyme as shown in SDS/PAGE of crude extracts of three enzymes (Fig. 4A). To exclude the effect of a foreign promoter originated from *B. thermocatenulatus* GH2 on the expression system in *E. coli*, three open reading frames coding for the wild-type and mutant enzymes were subcloned into pGEM 7f(+) under the control of SP6 promoter. As expected, analysis on SDS/PAGE also revealed that the expression level of both the truncated and inserted enzymes was significantly enhanced compared to that of the wild-type enzyme (Fig. 4B). Expression levels of the truncated and inserted enzymes were estimated to be increased about three- and five-fold, respectively, compared to that of the wild-type on the basis of enzyme assay and SDS/PAGE analyses. Although the mutant enzymes were highly expressed

KDa

214 -

116 -
98 -

66 -

40 -

30 -

1   2   3   1   2   3

Fig. 4. Expression level of the wild-type and mutant enzymes under the control of D-hydantoinase promoter (A) and SP6 promoter (B).
Crude extracts were separated by SDS/PAGE on 12% gels and scanned by a densitometer. Lanes were: 1, wild-type enzyme BT471; 2, truncated enzyme BT460; 3, inserted enzyme BT471F.

as shown in Fig. 4, the corresponding bands were further confirmed by activity staining, and then electro-eluted from the native gel under nondenaturing conditions. The resulting eluted enzymes also revealed a similar difference of activity that correlated well with the expression level determined by the corresponding band on SDS/PAGE and enzyme assay using the crude extracts.

DISCUSSION

The comparative study of the wild-type enzyme with mutant derivatives that share an almost identical primary structure but have a different catalytic and biochemical property is a useful approach to understand the structure-function relationship and functional importance of selected residues when no structural data are available [15]. In this context, the results reported here provide, not only an interesting structure-function relationship, but also some promising aspects regarding the practical application of an industrially useful enzyme D-hydantoinase.

An artificial sequence insertion at the C-terminal region of the enzyme from *B. thermocatenulatus* GH2 resulted in dimerization of the tetrameric enzyme. The identical result was also observed with the truncation mutant of the C-terminal end [3]. Although further elucidation in the more defined structure remains to be studied, these results strongly suggest that the region of wild-type enzyme might be closely linked with oligomeric structure. Additionally, the hydropathy profile and secondary structure prediction of this region revealed to be a hydrophobic and a helix, respectively, and thus it also provided a possibility that the C-terminal region is located at the interior or interface region of the enzyme. In contrast to this, the newly inserted sequence, EFDIKLIDTVDLEGGPGTQFAL, originating from the multicloning site of a vector sequence, revealed a high content of hydrophilic amino acids, and thus this might change the oligomeric structure. The inserted sequence used in this study was found unexpectedly from a serial attempt to construct a fusion or tagging protein. For this purpose, we intentionally deleted a stop codon at the C-terminal end, and the resulting construct amplified by PCR and subcloned into various vectors resulted in a fusion or tagging protein (peptides). The fusion protein BT471F was constructed based on the results when the PCR-amplified fragment was fused to the β-galactosidase domain coded in the multicloning site of pBluescript II SK. For further study, the inserted sequence is currently being randomized and analyzed to exclude the possibility of fortuitous results changing the oligomeric structure by a sequence and also to select the best mutant for high-level expression. Additionally, the two mutant proteins, BT460 and BT471F, will be also subjected to further improve the enzymes by random mutagenesis and molecular breeding [9].
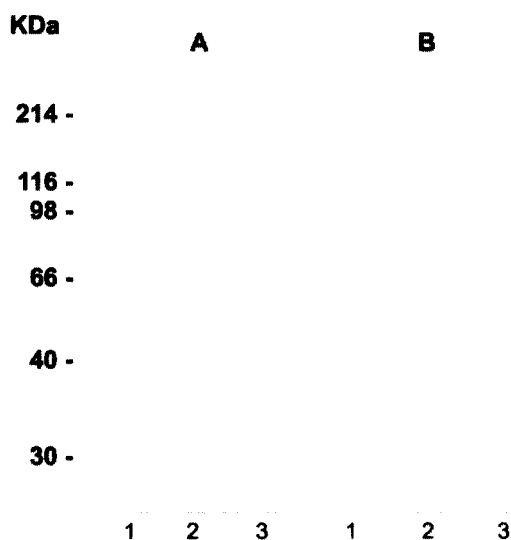
The expression of the mutant enzymes in *E. coli*, both fusion and truncated enzymes, were efficiently expressed in a catalytically active dimeric form. Differences in the expression level, even under the same promoter, might be due to either different biophysical stabilities (structural and folding property) or biochemical properties (substrate spectrum and toxic activity) of the enzymes. When analyzed by using CD and fluorescent emission spectra, no significant difference in secondary and tertiary structures was observed between the mutant and wild-type enzymes, and consequently, the former possibility was lowered. Microbial D-hydantoinase has been considered to be a microbial counterpart of animal dihydropyrimidinase that is involved in the catabolism of dihydrouracil and dihydrothymine [21]. When *E. coli* cells harboring the gene encoding the wild-type enzyme were cultivated, cell growth was very poor compared with those expressing the mutant enzymes, and consequently, the expression level was relatively low. The wild-type enzyme was found to exhibit the highest activity toward dihydrouracil. Therefore, high-level expression of this enzyme might be lethal to the microorganism because the degradation of dihydrouracil became serious with the expression of the enzyme, which led to a low expression level of the enzyme [1, 14]. Interestingly, most of the hydantoinases have been reported to be composed of four identical subunits and share similar structural property [1, 13, 16, 18] and higher activity toward the dihydrouracil, thus similar phenomena might be observed in other D-hydantoinases. In this regard, truncation or fusion strategy might be a useful approach to reducing the toxic effect of the enzyme on host cells, because the activity to dihydrouracil was decreased to be about 3 or 4 folds. In addition, a recombinant enzyme, such as tagging proteins at its C-terminal end, might be possible for simple purification and efficient production of the enzyme.

From the comparison of catalytic properties of the mutant enzymes with the wild-type enzyme, similar biochemical properties were revealed in terms of chromatographic behaviors, optimum pH and temperature, and CD spectra. However, deletion of the C-terminal resulted in an increase in both the thermal and pH stability compared with the corresponding wild-type enzyme. The C-terminal region of enzyme contains two negatively charged amino acid residues, and deletion of this region might reduce unfavorable electrostatic interactions by charged amino acid residues [2, 22], leading to high stability of the enzyme. From the analysis of the substrate specificity between wild-type and mutant enzymes, an interesting result was found in that the oligomeric structure of D-hydantoinase has an effect on its substrate specificity. The specific activities of both mutant enzymes were lowered toward most of the substrates tested, and interestingly, the specific activity of both enzymes was quite similar to that of the dimeric enzyme from *B. stearothermophilus* SD1 [12]. Therefore, it seems that the substrate specificity of the D-hydantoinase is closely linked with the oligomeric structure of the enzyme.

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