# Engineering the Thermostable D-Hydantoinases from Two Thermophilic *Bacilli* Based on Their Primary Structures

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## INTRODUCTION

Thermostable enzymes find wide applications to basic studies concerning protein stability as well as to development of industrial and specialty bioprocesses. In the process for the production of optically pure D-amino acids, obtained from the corresponding hydantoin derivatives using a microbial D-hydantoinase,[1] limited substrate solubility and enzyme stability were posed as problems. In this context, we have isolated and characterized a thermostable D-hydantoinase from *Bacillus stearothermophilus* SD1,[2] and the gene encoding the enzyme was cloned and its nucleotide sequence was determined.[3] However, the low specificity of the enzyme from *B. stearothermophilus* SD1 toward hydantoin derivatives with an aromatic group at the 5′-position prompted us to isolate an enzyme with improved substrate specificity from *B. thermocatenulatus* GH2.[4] The enzyme of *B. thermocatenulatus* GH2 was a tetramer and showed high specific activity toward aromatic substrates. On the other hand, previously isolated enzyme was composed of two identical subunits and had a low specific activity for aromatic substrates.

In order to get some insights into the difference in the biochemical characteristics of the two enzymes, the gene coding for the enzyme from *B. thermocatenulatus* GH2 was cloned and expressed in *E. coli*, and its nucleotide sequence was determined. Based on the primary structures of the two enzymes, hybrid and truncated mutant enzymes were constructed, and their catalytic properties were compared with wild-type enzymes. Details are reported here.

## SEQUENCE ANALYSIS AND CONSTRUCTION OF THE MUTANT ENZYMES

The nucleotide sequence of the open reading frame (ORF) of the gene encoding the D-hydantoinase from *B. thermocatenulatus* GH2 was determined and, surprisingly, its sequence was identical with that of *B. stearothermophilus* NS1122A.[5] *B. thermocatenulatus* GH2 was named on the basis of the 16S rRNA sequence in our previ-

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ous work.[4] As previously reported, the *Bacillus* D-hydantoinases share a long conserved region starting from the N-termini, and their genes are thought to be derived from a common ancestral gene.[3] The D-hydantoinase of *B. thermocatenulatus* GH2 shared an amino acid homology of 92% with that of *B. stearothermophilus* SD1, even though the biochemical properties of the two enzymes were quite different. However, despite the extremely high homology, the C-terminal regions of the enzymes possessed amino acid residues completely mismatched with each other. From this observation, it was supposed that the functional domain of the enzyme was dispensed with the nonhomologous C-terminal region. Analyses of average hydropathy and secondary structure prediction for the two enzymes also supported our presumption, which led us to investigate the characteristics of the C-terminal region–deleted mutant enzymes. We first constructed the C-terminal region–truncated mutant enzymes by the PCR method (FIGURE 1). Eleven and 12 amino acid residues were deleted from the C-termini of the two enzymes from *B. thermocatenulatus* GH2 and *B. stearothermophilus* SD1, respectively, and the resulting mutant enzyme from the former strain was designated BT460 and that from the latter one BS460. Corresponding wild-type enzymes were called BT471 and BS472, respectively. On the other hand, hybrid enzymes were constructed by an exchange of the conserved PvuII site of the two genes from thermophilic *Bacilli*. One hybrid enzyme (BTS162) contains an N-terminal segment (310 residues) derived from D-hydantoinase of *B. thermocatenulatus* GH2 followed by a C-terminal segment (162 residues) originating from *B. stearothermophilus* SD1. The other enzyme (BST161) derived from D-hydantoinase of *B. stearothermophilus* SD1 contains its own N-terminal segment followed by a C-terminal segment originating from *B. thermocatenulatus* GH2 (FIGURE 1).

The genes encoding the mutant enzymes, BT460, BS460, BST161, and BTS162, were subcloned in pGEM-7Zf(+) and overexpressed in *E. coli*. The wild and mutant enzymes were purified to homogeneity as determined in analytical electrophoresis.

## CATALYTIC PROPERTIES OF THE MUTANT ENZYMES

The D-hydantoinases described in this work were found to require manganese ions for activity.[2,4] The C-terminal region of each enzyme contains two negatively charged amino acid residues. Therefore, if the C-terminal region were involved in the metal binding domain, removal of this region might affect the metal binding property and consequently activity of the enzyme. When the C-terminal region–deleted enzymes, BS460 and BT460, were treated with EDTA and dialyzed against a metal-free buffer, activities of the enzymes were lowered to about 30–40% of the control. However, addition of manganese ions almost fully restored the activities of the enzymes (data not shown).

The specific activities of the wild and mutant enzymes were determined toward various hydantoin derivatives (TABLE 1). Since the oligomeric structures of the wild and mutant enzymes were different, specific activity was calculated based on the molar concentration of the enzyme. BS460 showed a similar substrate specificity to BS472, but the specific activity of BT460 was significantly decreased toward most of the substrates tested as compared to that of wild enzyme BT471. In particular, the specific activity of BT460 toward hydroxyphenylhydantoin was less than 10% of that

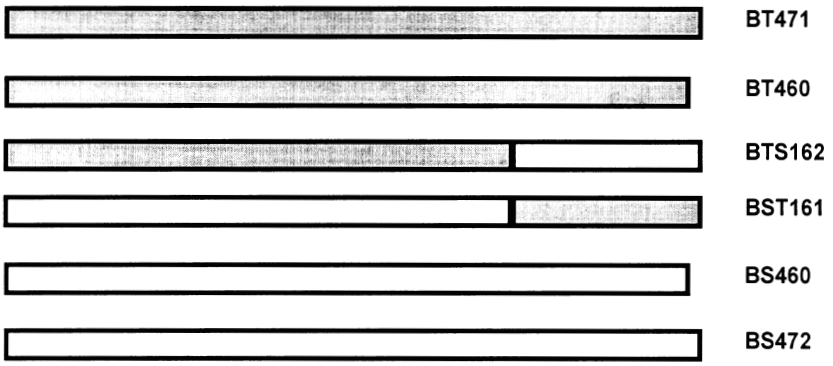


**FIGURE 1.** Schematic representation of the wild-type and engineered enzymes.

of the corresponding wild enzyme and similar to those of BS472 and BS460. The substrate specificity of the hybrid enzymes toward the various hydantoin derivatives could be classified as mainly depending on the origins of the N-terminal regions. Among the mutant enzymes, hybrid enzyme BTS162 was revealed to have different substrate specificity as compared to wild-type enzymes. Especially, the specific activities toward hydantoin, phenylhydantoin, and isopropylhydantoin were significantly increased.

The dependency of enzyme activity on the pH was also investigated and, as a result, the activity profiles of the mutant enzymes, BS460, BT460, BST161, and BTS162, were observed to be similar to those of the corresponding wild enzymes (data not shown). The stability of the truncated enzymes (BT460 and BS460) was slightly improved in the acidic range as compared to wild enzymes, and hybrid enzymes were determined to have a similar profile as revealed from the wild-type enzymes. However, a sharp decrease in the stability was observed around isoelectric

**TABLE 1.** Substrate Specificity of Wild-Type and Engineered Enzymes[a]

| | Specific Activity (units/μM enzyme) | | | | | |
|---|---|---|---|---|---|---|
| Substrate | BT471 | BT460 | BS472 | BS460 | BTS162 | BST161 |
| hydantoin | 11.41 | 4.05 | 5.28 | 5.01 | 23.12 | 5.88 |
| hydroxyphenylhydantoin | 9.49 | 0.85 | 0.59 | 0.38 | 8.30 | 0.71 |
| phenylhydantoin | 20.28 | 8.31 | 4.77 | 3.92 | 26.58 | 6.38 |
| isopropylhydantoin | 1.24 | 1.38 | 0.47 | 0.39 | 2.97 | 0.38 |
| dihydrouracil | 7.01 | 2.41 | 1.85 | 3.24 | 3.20 | 3.16 |
| uracil | ND | ND | ND | ND | ND | ND |
| 2-thiouracil | ND | ND | ND | ND | ND | ND |

[a]All substrates (50 mM) were preincubated for 20 min at 55 °C in nitrogen flushing conditions, and enzyme activity (3 μg) was determined using standard reaction conditions (55 °C, 30 min) in 100 mM Tris-HCl buffer (pH 8.0). ND: not detected.

pH, showing a characteristic feature of D-hydantoinases from two thermophiles, and this seems to be due to the aggregation of the enzymes at high incubation temperatures.

The effects of temperature on the activity and stability of the enzymes were also investigated. Concerning the heat stability of the enzymes, the C-terminal region–truncated enzymes were more thermostable than the corresponding wild enzymes (FIGURE 2). In particular, BT460 exhibited a significantly enhanced thermostability as compared with wild enzyme BT471. The optimal temperatures of BS460 and BT460 were shifted about 5 °C to higher temperatures as compared to those of the wild enzymes. The hybrid enzymes BST161 and BTS162 were very similar to those obtained from the two wild-type enzymes.

In conclusion, the truncated mutant enzymes displayed higher stabilities than those of the wild-type enzymes, while those of the hybrid enzymes were comparable to those of the wild-type enzymes. The hybrid enzymes were revealed to have improved specific activity toward some hydantoin derivatives. The approach attempted in this work might be applied to engineering the highly homologous proteins to improve the catalytic properties based on their primary structure.

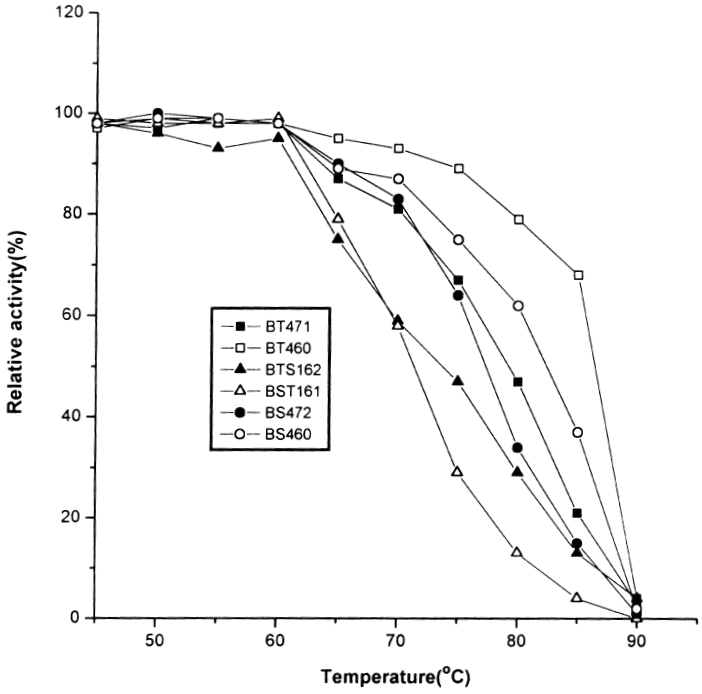


**FIGURE 2.** Thermostability of the wild-type and engineered enzymes. For the determination of thermostability, the residual activity was determined after incubation of the enzyme for 30 min at different temperatures.

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