0273-2289/99/81/0053/$13.25

Purification and Characterization of Thermostable D-Hydantoinase from *Bacillus thermocatenulatus* GH-2

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Received November 27, 1996; Revised March 11, 1997; Accepted March 12, 1997

Abstract

A thermostable D-hydantoinase was isolated from thermophilic *Bacillus thermocatenulatus* GH-2 and purified to homogeneity by using immunoaffinity chromatography. The molecular mass of the enzyme was determined to be about 230 kDa, and a value of 56 kDa was obtained as a molecular mass of the subunit on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, implying that oligomeric structure of the enzyme is tetrameric. Isoelectric pH of the enzyme was found to be approx 4.3. The enzyme required Mn^{2+} for the activity and exhibited its highest activity with phenylhydantoin as a substrate. The optimal pH and temperature for catalytic activity were about 7.5 and 65°C, respectively. The half-life of the enzyme was estimated to be about 45 min at 80°C.

Index Entries: Thermostability; D-hydantoinase; *Bacillus*; immunoaffinity.

Introduction

D-Hydantoinase is currently employed as a biocatalyst for the commercial production of optically pure D-amino acids that are intermediates for the synthesis of semisynthetic antibiotics, peptide hormone, pyrethroids, and pesticides. In the process developed by Yamada et al. *(1,2)*, DL-5-substituted hydantoin is asymmetrically hydrolyzed to the *N*-carbamoyl-D-amino acid by D-specific hydantoinase. This product is further chemically converted to the corresponding D-amino acid under acidic condition.

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During the last decade, much effort has been made toward the isolation of D-hydantoinase-producing microorganisms and the characterization of the enzyme *(3,4)*. From a practical standpoint, the operational stability of the enzyme is considered as one of the most important factors because the short half-life of the enzyme often limits the development of the enzymatic process. As an effective way of producing thermostable enzymes with great biotechnological potential, the isolation of an enzyme from thermophiles has attracted much attention *(5,6)*. However, studies regarding the thermostable D-hydantoinase have been rare so far. Jacob et al. *(7)* first reported the isolation of thermostable D-hydantoinase from two thermophiles—one strain Gram negative (CBS30380) and the other Gram positive (Lu1220)—but identification of the microorganisms and biochemical characteristics of the enzymes were not mentioned in detail. Recently, thermophilic *Bacillus stearothermophilus* NS1122A possessing both the hydantoinase and the *N*-carbamoyl-amino acid amidohydrolase was isolated for the production of L-amino acids *(8)*. The hydantoinase from *B. stearothermophilus* NS1122A was reported to hydrolyze D- and L-5-substituted hydantoins, showing much higher activity with D-form substrate than L-5-substituted hydantoins. Meanwhile, the *N*-carbamoyl-amino acid amidohydrolase was strictly L-specific.

We have been focusing on the screening of thermostable D-hydantoinase producing thermophiles, and have previously isolated and characterized the enzyme from *B. stearothermophilus* SD-1 *(9,10)*. The enzyme exhibited a comparable thermostability to that from *B. stearothermophilus* NS1122A, but the biochemical properties were quite different despite the similar microbial sources; typically the former was a homodimer with a molecular mass of about 120 kDa, and the latter homotetramer having a molecular mass of 200 kDa. However, from the characteristics of the D-hydantoinase of *B. stearothermophilus* SD-1, we found an inherent shortcoming. The activity of the enzyme toward the substrates with an aromatic group at the 5' position was relatively low. These hydantoin derivatives are the starting substrates for the synthesis of D-phenylglycine and D-*p*-hydroxyphenylglycine, which are of the highest demand among various D-amino acids.

In this article, we report the isolation of a thermostable D-hydantoinase with high affinity toward hydantoins with an aromatic group at the 5' position. The thermostable D-hydantoinase was isolated from *B. thermocatenulatus* GH-2 and purified to homogeneity by using immunoaffinity chromatography. Biochemical characteristics of the enzyme were investigated and compared with those from *B. stearothermophilus* SD-1 isolated in our previous work.

Materials and Methods

Chemicals

Hydantoin, glycine, *N*-carbamoyl-glycine, D- and L-hydroxyphenylglycine, dihydrouracil, *p*-dimethylaminobenzaldehyde, *p*-hydroxybenzal-

dehyde, benzaldehyde, and isobutylaldehyde were purchased from Sigma (St. Louis, MO). DL-Hydroxyphenylhydantoin (HPH) was obtained from Tokyo Kasei (Tokyo, Japan). Water and acetonitrile as an eluent of high-performance liquid chromatography (HPLC) were from Budick and Jackson (Muskegor, MI). Chiral thin-layer chromatography (TLC) plates to determine the stereospecificity of hydantoinase were also purchased from Sigma. DL-5-Phenylhydantoin and DL-5-isopropylhydantoin were synthesized with the corresponding aldehyde using the method of Suzuki et al. *(11)*. Standard proteins for isoelectric focusing and gel filtration were purchased from Pharmacia (Piscataway, NJ). All other chemicals used were of analytical grade.

Media

Medium I used for the isolation of hydantoinase-producing thermophiles consisted of 3 g of meat extract, 5 g of bactopeptone, 2 g of K_2HPO_4, 2 g of KH_2PO_4, 0.1 g of $MgSO_4$, 0.01 g of $MnSO_4$, and 1 g of hydantoin in 1 L of distilled water. The pH was adjusted to 7.2. The composition of medium II for the cultivation of isolated *B. thermocatenulatus* GH-2 was 5 g of glucose, 7 g of meat extract, 2 g of K_2HPO_4, 4 g of KH_2PO_4, 0.1 g of $MgSO_4$, 0.02 g of $MnSO_4$, and 5 g of hydantoin in 1 L of distilled water, and initial pH was adjusted to 7.2.

Isolation of Hydantoinase-Producing Thermophile

Soils from compost, mountains, sewage sludge, and hot springs were incubated aerobically at 55°C. Once grown in the enrichment cultures, the supernatant was spread on a 2% agar plate of medium I. After incubating overnight at 55°C, colonies were isolated by transfer to the agar plates. To screen the hydantoinase-producing thermophiles, one loopful of colony grown on agar plates was inoculated into 5 mL of medium I and cultivated aerobically at 55°C for 8 h. The cells were harvested by centrifugation at 6000*g* for 15 min. The pellet was washed twice with 0.1 *M* potassium phosphate buffer (pH 8.0) and used as whole-cell enzyme for the determination of hydantoinase activity.

Assay of Hydantoinase Activity

The reaction mixture (1.0 mL) containing 200 m*M* hydantoin, 1 m*M* $MnCl_2$, 0.1 *M* Tris-HCl buffer (pH 8.0), and intact cells (0.1–0.2 mg dry wt) or purified enzyme (1.2 μg) was incubated for 30 min at 55°C. The reaction was stopped by the addition of 0.5 mL of 12% trichloroacetic acid (TCA), and precipitated proteins were removed by centrifugation. The amount of hydantoic acid formed was determined using the colorimetric method of Takahashi et al. *(3)*. One unit of hydantoinase activity was defined as the amount of enzyme required to produce 1 μmol of hydantoic acid from hydantoin per minute under the specified condition.

Determination of Stereospecificity

Chiral TLC was used for determination of the stereospecificity of hydantoinase. *N*-Carbamoyl-amino acid, produced by whole-cell or purified enzyme, was decarbamoylated to the corresponding amino acid with equimolar nitric acid in 3.0 *M* HCl. The reaction solution was loaded on a chiral TLC plate and the TLC was run in a developing solvent of methanol:water:acetonitrile (50:50:200, v/v) for 30 min. The spots were visualized by spraying 0.2% ninhydrin in ethanol and then heating for 20 min at 105°C. The relative mobility of spots was compared with those of the D- or L-amino acid.

Preparation of an Immunoaffinity Column

D-Hydantoinase of *B. stearothermophilus* SD-1 purified in our previous work *(9)* was used as an antigen to prepare the polyclonal antibody for the ligand of affinity chromatography. The purified D-hydantoinase (150 μg) was emulsified with an equal volume of complete Freund's adjuvant and injected intradermally into rabbit. After 2 wk, the rabbit was reinjected intradermally with the enzyme (62 μg) emulsified in complete Freund's adjuvant. A booster dose of the enzyme (25 μg) was injected again intravenously after 2 wk. The polyclonal antibody was purified from serum of the rabbit using affinity chromatography packed with Protein G (Pharmacia). The polyclonal antibody was immobilized on CNBr-activated Sepharose 4B (Pharmacia) by the procedure recommended by the supplier. The immunoaffinity matrix (approx 5 mg of antibody/mL of sepharose) was packed in a column (1 × 10 cm) and used for the purification of the D-hydantoinase.

Preparation of Cell-Free Extract

Cells were cultivated in a 3-L fermentor containing 1 L of the culture medium II. The agitation and aeration rate were maintained at 400 rpm and 2 vvm, respectively. After 6 h of cultivation, the cells were harvested by centrifugation at 6000*g* for 15 min and washed twice with 0.9% saline solution. The cells (0.7 g dry cell weight) were resuspended in 50 m*M* Tris-HCl buffer (pH 8.0) containing 1 m*M* $MnCl_2$, and then disrupted by passing them twice through a French press (American Instrument, Maryland) under 1200 psi. Cell debris was removed by centrifugation at 28,000*g* for 30 min, and the supernatant was treated with 0.5 m*M* phenylmethylsulfonyl fluoride to limit proteolysis.

Immunoaffinity Chromatography

The cell-free extract was applied to a column of immunoaffinity matrix equilibrated with 50 m*M* Tris-HCl buffer (pH 8.0) containing 0.2 m*M* $MnCl_2$ and 0.15 *M* NaCl at a flow rate of 6 mL/h. The enzyme was eluted with 0.1 *M* sodium hydrogen carbonate buffer (pH 10.5) containing 2 *M* NaCl. Fractions (2.5 mL each) were collected and assayed for activity. The active

fractions were pooled and dialyzed against 10 m*M* Tris-HCl buffer (pH 8.0) containing 1 m*M* $MnCl_2$. The purified enzyme solution (1.1 mg/mL) was stored at –20°C until use.

Gel Electrophoresis and Staining

Analytical sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in slab gels was performed according to the method of Laemmli *(12)*. The stacking and separating gels were 5 and 9% acrylamide, respectively (crosslinking 2.8%). The standard proteins used were aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa). Electrophoresis was performed at a constant voltage of 150 V. Gels were stained with Coomassie brilliant blue R-250.

Determination of Molecular Mass

The molecular mass of the enzyme was determined by analytical gel filtration chromatography using a Superose-12 column (Sigma, St. Louis, MO) equilibrated with 20 m*M* Tris-HCl buffer (pH 7.2) containing 150 m*M* NaCl and 1 m*M* $MnCl_2$. The standard proteins used were bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), and carbonic anhydrase (29 kDa). The K_{av} values for the purified hydantoinase and standard proteins were calibrated and correlated with molecular mass on a semilogarithmic plot. The K_{av} was determined as $(V_e - V_o)/(V_t - V_o)$, where V_e is the elution volume, V_t is the total column volume, and V_o is the void volume.

Determination of Isoelectric Point

Isoelectric point (pI) of the purified enzyme was determined according to the procedure suggested by Laas et al. *(13)*. The slab gel for native isoelectric focusing consisted of 6% acrylamide (crosslinking 2.8%), 2.4% ampholyte with a pH range of 4.0–6.5, and 10% glycerol. The gel was run at 1500 V for 2 h at 24°C. Proteins were fixed with 10% TCA solution for 10 min, and the gel was soaked in 1% TCA solution for 3 h to remove the remaining ampholyte. Coomassie brilliant blue R-250 was used for the staining of proteins.

Determination of Metal Dependency

To investigate the metal dependency of the D-hydantoinase from *B. thermocatenulatus* GH-2, the purified enzyme was treated with 5 m*M* EDTA followed by dialysis. Various metal ions at each concentration of 1 m*M* were added to the EDTA-treated enzyme. The activity of enzyme was measured under standard assay conditions.

Determination of Kinetic Parameters

The kinetic constants of D-hydantoinase were determined by measuring the initial rate. The concentrations of HPH and hydantoin were in the

range of 0.5–20 and 70–500 m*M*, respectively. The range of HPH is limited to 25 m*M* because of its low solubility in buffer at pH 8.0 *(14)*. Enzyme solution was added to the reaction mixture and incubated for 20 min at 55°C. The reaction was stopped by the addition of 12% TCA, and the concentration of *N*-carbamoyl-amino acid formed was determined by colorometric method or HPLC. The kinetic parameters were calculated by double-reciprocal plot.

Determination of Enzyme Stability

To investigate thermostability, the enzyme was incubated for 30 min at various temperatures (30–80°C) in 0.1 *M* Tris-HCl buffer (pH 8.0) containing 1 m*M* $MnCl_2$, and residual activity was measured under standard assay conditions. For determination of pH stability of the enzyme, enzyme solution was incubated at 50°C for 30 min using different buffers, and residual activity was measured. The buffers used were Bis-Tris (pH 5.0–7.0), Tris-HCl (pH 7.0–8.5), and glycine-NaOH (pH 8.5–10.0).

Analysis

The concentrations of DL-hydroxyphenylhydantoin, *N*-carbamoyl-hydroxyphenyl glycine and D-*p*-hydroxyphenylglycine were determined using HPLC (Shimadzu, Kyoto, Japan). The column used was CLC-ODS (4.6 × 250 mm, Shimadzu). Ten percent (v/v) acetonitrile solution (pH 3.0) was used as a mobile phase, and the flow rate was 1.0 mL/min. The eluted product was monitored at 214 nm. *N*-Carbamoylglycine was assayed by using the spectrophotometric method *(3)*. Biomass concentration was estimated by measuring the absorbance at 610 nm. The concentration of protein was determined by the method of Bradford *(15)*, using bovine serum albumin as a standard.

Results and Discussion

Isolation and Identification of D-Hydantoinase-Producing Thermophile

Approximately 1500 colonies were grown on the agar plates at 55°C, and 150 colonies were shown to exhibit the hydantoinase activity. These colonies were tested in duplicate with respect to substrate specificity, stereospecificity, and thermostability. Among the isolates tested, one showed both high activity toward DL-5-*p*-hydroxyphenylhydantoin and thermostability. Biochemical and physiological characteristics were investigated for the identification of an isolated strain. This microorganism was a Gram-positive, rod shape with a size of 2–4 μm, and was spore forming. The sequence of 16S rRNA was determined as described by Lane *(16)* and aligned with reported ones. As a result, the isolated thermophile was taxonomically identified as *B. thermocatenulatus* GH-2 according to *Bergey's Manual of Systematic Bacteriology (17)*.

Production of Hydantoinase from B. thermocatenulatus *GH-2*

Culture conditions for the production of hydantoinase from *B. thermocatenulatus* GH-2 were optimized in terms of enzyme activity. First, the effect of various carbon sources on the production of hydantoinase was investigated, and glucose (0.5%, w/v) was selected as a sole carbon source because enzyme production was highest on glucose. As a nitrogen source, nutrient broth, meat extract, corn steep liquor, yeast extract, and tryptic soybean broth were tested; meat extract was found to be the most effective. The concentration of meat extract was fixed at 0.8% (w/v). Earlier investigations revealed that microbial hydantoinases are inducible *(18–20)*, and various potential inducers were tested with respect to enzyme activity. Among them, hydantoin and uracil were found to be the most effective. In this work, 0.5% (w/v) hydantoin was added to the culture medium as an inducer. It was reported that hydantoinase requires metal ions for the activity *(3,14)*, and several metal ions were tested. Enzyme catalytic activity was affected by the presence of Mn^{2+}, Co^{2+}, and Fe^{2+}, and these metal ions were added to the culture medium at a concentration of 0.1 m*M*.

Purification of Hydantoinase from B. thermocatenulatus *GH-2*

It was shown that hydantoinases from various microbial sources possess a high homology in nucleotide sequence *(21)*. Among the thermophilic bacteria that were primarily screened to express hydantoinase activity, several strains exhibited affinity for polyclonal antibody against the D-hydantoinase of *B. stearothermophilus* SD-1 purified in our previous work *(10)*. Thus, we attempted to purify the hydantoinase of *B. thermocatenulatus* GH-2 by using an immunoaffinity chromatography prepared with polyclonal antibody against the D-hydantoinase of *B. stearothermophilus* SD-1. First, an immunodiffusion test using the Ouchterlony plate method was conducted to determine whether the polyclonal antibody showed specific immunoaffinity against the hydantoinase of *B. thermocatenulatus* GH-2. As shown in Fig. 1, continuous precipitin bands were formed, indicating specific binding of hydantoinase from *B. thermocatenulatus* GH-2 to the polyclonal antibody. Based on this observation, immunoaffinity chromatography was employed in the purification of the hydantoinase from *B. thermocatenulatus* GH-2.

Table 1 summarizes the results of the purification of hydantoinase. The purification yield was 36.4%, and specific activity of the enzyme was estimated to be 461 U/mg of protein under standard assay conditions. Figure 2 shows the analytical SDS-PAGE of the sample from each step of the purification. The purified hydantoinase was electrophoretically homogeneous, and relative molecular mass of the subunit was about 56 kDa. The homogeneity of the purified enzyme was examined by different methods. The electrophoresis under denaturing and native conditions resulted in a single protein band after staining.

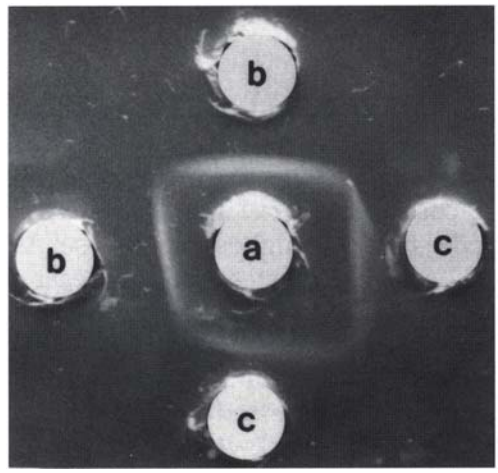

Fig. 1. Immunodiffusion test by using Ouchterony plate method: (a) antiserum from rabbit; (b) crude extract from *B. thermocatenulatus* GH-2; and (c) purified D-hydantoinase from *B. stearothermophilus* SD-1.

Table 1
Summary of the Purification of D-Hydantoinase from *B. thermocatenulatus* GH-2

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Purification fold	Activity yield (%)
Crude extract	100.00	1420	14.2	1.0	100.0
Immunoaffinity chromatography	1.12	517	461.6	32.5	36.4

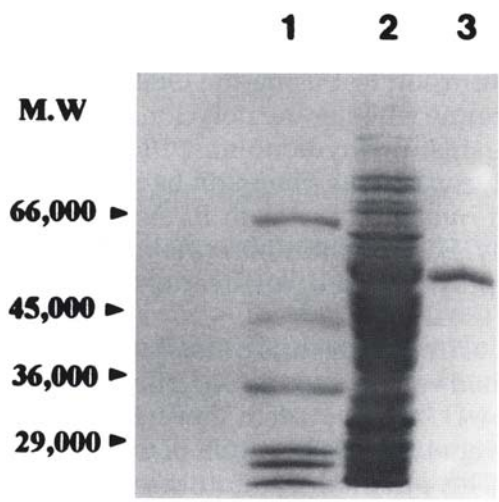

Fig. 2. SDS-PAGE of the D-hydantoinase at each purification step. Lane 1, standard marker proteins; lane 2, cell-free extract; lane 3, D-hydantoinase purified by immunoaffinity chromatography.

Several hydantoinases have been purified by using conventional chromatographic procedures *(3,14)* or by preparative gel electrophoresis *(9)*, and in these cases, the activity recovery was low. But, immunoaffinity chromatography using polyclonal antibody seems to be quite simple and powerful in the purification of hydantoinase.

Stereospecificity

Stereospecificity of the hydantoinase from *B. thermocatenulatus* GH-2 was examined, and the enzyme was strictly D-specific as previously isolated enzyme from *B. stearothermophilus* SD-1 (data not shown).

Molecular Mass and Quaternary Structure

The molecular mass of the purified D-hydantoinase was determined by gel filtration chromatography using a Superose-12 column, and its value was estimated to be about 230 kDa. From the molecular mass of the subunit, the D-hydantoinase was assumed to be composed of four identical subunits. Meanwhile, the D-hydantoinase from *B. stearothermophilus* SD-1 was found to be a dimeric protein *(9)*. Interestingly, the quaternary structures of the D-hydantoinases from the similar thermophilic Bacilli are quite different. The D-hydantoinases from *Pseudomonas (22)* and *Agrobacterium (14)* were reported to be tetramers.

Isoelectric Point

The pI of D-hydantoinase was measured by electrofocusing on 6% polyacrylamide gel. The isoelectric pH was determined as 4.3 by comparing the band position of the enzyme with those of standard proteins (data not shown). This value was similar to that of the enzyme from previously isolated *B. stearothermophilus* SD-1 *(10)*. Runser and Meyer *(14)* reported that the pI of the D-hydantoinase of *Agrobacterium* sp. is about 6.5, indicating that the enzyme is a slightly acidic protein.

Effect of Metal Ions

It was generally known that most D-hydantoinases require metal ions for the catalytic activity *(3,14,22)*. The effect of metal ions on the activity recovery was examined (Fig. 3). The enzyme catalytic activity was decreased to 5% of the original catalytic activity on treatment with EDTA, implying the requirement of metal ions for the activity. Manganese ion was found to be the most effective among the various metal ions tested. The activity of the EDTA-treated enzyme was almost fully recovered by the addition of Mn^{2+} above 1 m*M*.

Substrate Specificity

The activity of the purified D-hydantoinase was determined for various hydantoin derivatives (Table 2). D-Hydantoinase of *B. thermocatenulatus* GH-2 revealed a high affinity toward the substrate with an aromatic group

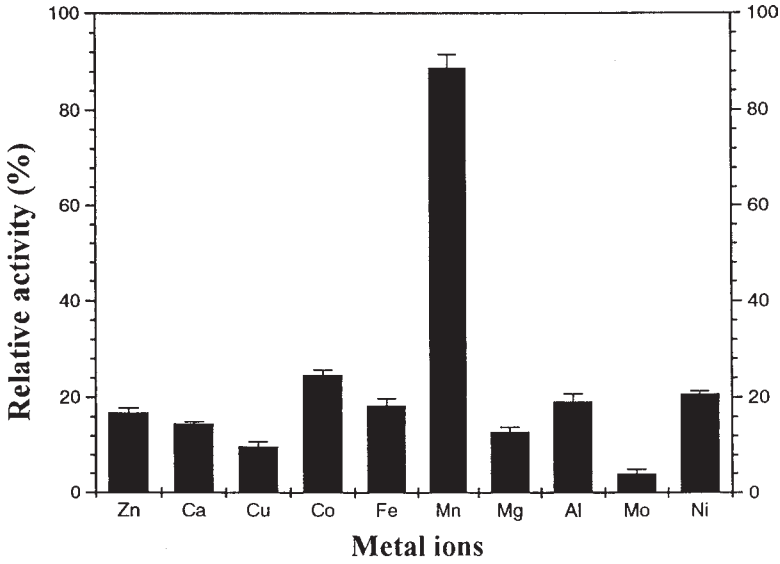

Fig. 3. Effect of metal ions on the activity of purified D-hydantoinase. The concentration of each metal ion was 1 m*M*, and the enzyme activity before dialysis was taken as 100%.

Table 2
Substrate Specificity of Purified D-Hydantoinase
from *B. thermocatenulatus* GH-2[a]

Substrate	Specific activity (U/mg enzyme)	Relative activity (%)
Hydantoin	101.5 ± 2.1	110.7
Hydroxyphenylhydantoin	91.6 ± 2.1	100.0
Phenylhydantoin	236.9 ± 5.9	258.5
Isopropylhydantoin	10.9 ± 0.1	11.9
Dihydrouracil	27.5 ± 0.6	30.0
Dihydrothymine	ND	ND
Uracil	ND	ND
2-Thiouracil	ND	ND
2,4-Dithiopyrimidine	ND	ND

[a]Each substrate at a concentration of 50 m*M* was preheated at 55°C for 20 min, and the reaction was initiated by adding the purified enzyme. The reaction was interrupted by the addition of TCA after 60 min, and *N*-carbamoyl-D-amino acids were determined by HPLC or colorimetric method. Values represent the average and standard deviation in triplicates. ND, not detected.

at the 5' position, showing a quite different feature compared to that from *B. stearothermophilus* SD-1. The K_m values for HPH of the D-hydantoinases from *B. thermocatenulatus* GH-2 and *B. stearothermophilus* SD-1 were determined to be 30.7 ± 0.7 and 47.2 ± 1.2 m*M*, respectively. The K_{cat}/K_m values for HPH and hydantoin of D-hydantoinase from *B. thermocatenulatus* GH-2

were about 6.6×10^7 and 3.5×10^7 M/h, respectively. On the other hand, D-hydantoinase from *B. stearothermophilus* SD-1 gave K_{cat}/K_m values of 4.6×10^6 and 5.9×10^6 M^{-1} h^{-1} for HPH and hydantoin, respectively. Consequently, the relative reaction rates, $v_{HPH}/v_{hydantoin}$, of the D-hydantoinases of *B. thermocatenulatus* GH-2 and *B. stearothermophilus* SD-1 were approx 1.9 and 0.7, respectively, when the concentrations of HPH and hydantoin were the same. These results clearly indicated that the D-hydantoinase from *B. thermocatenulatus* GH-2 possesses a higher affinity for the hydantoin derivative with an aromatic group at the 5' position than the previously isolated one.

As mentioned previously, D-hydantoinase possessing high affinity toward hydantoin derivatives with an aromatic group at the 5' position is desirable for practical application because these hydantoins are starting substrates for the synthesis of D-amino acids with the highest commercial demand. The D-hydantoinases from mesophiles such as *Pseudomonas* and *Agrobacterium* were reported to exhibit high affinity toward 5'-substituted hydantoins with a nonpolar or hydrophobic group *(3,14)*, and these enzymes were of tetramer. From these observations, it is likely that the substrate specificity of the hydantoinase might be linked with the oligomeric structure of the enzyme. Further studies regarding the factors affecting the substrate specificity of the enzyme will be of great interest.

Effect of Temperature and pH

The activity and stability of D-hydantoinase were determined at various temperatures (Fig. 4). Optimal temperature for the activity was about 65°C. The enzyme was stable up to 75°C, and the half-life of the enzyme was estimated to be 45 min at 80°C, showing a slightly higher thermostability than that of *B. stearothermophilus* SD-1. Thermostability of the enzyme is considered as one of the most important factors in the practical application of enzyme. Furthermore, in case of a rarely soluble substrate, thermostable enzyme has an additional advantage because enhanced production rate is expected owing to high solubility of substrate at an elevated temperature. The maximum activity was observed at pH 7.5, and the enzyme activity remained stable at a pH between 6.0 and 9.5 (data not shown).

Conclusion

D-Hydantoinase from *B. thermocatenulatus* GH-2 is highly thermostable compared with those reported so far. In addition, D-hydantoinase of *B. thermocatenulatus* GH-2 shows a higher affinity toward the substrate with the aromatic group at the 5' position than that of *B. stearothermophilus* SD-1. When we considered the thermostability and catalytic property of D-hydantoinase from a newly isolated *B. thermocatenulatus* GH-2, we found that has this enzyme significant potential in improving the enzymatic process for the production of commercially important D-amino acids from DL-5-substituted hydantoins.

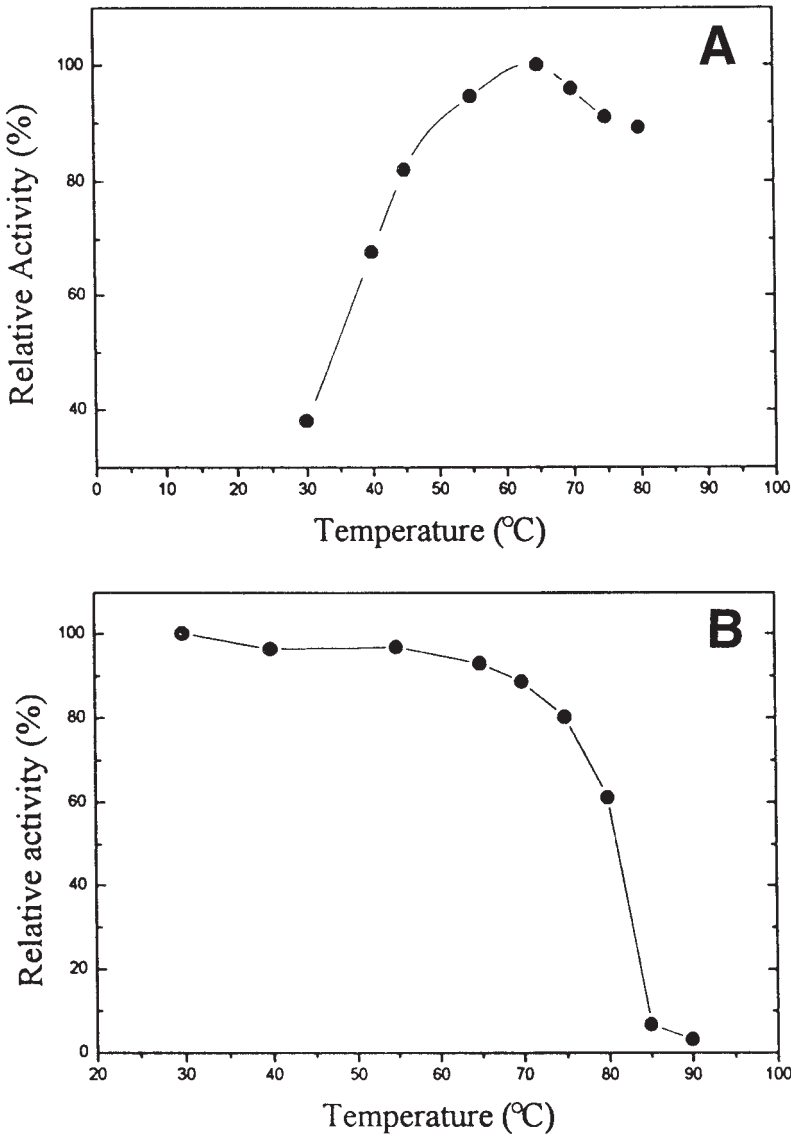

Fig. 4. Effect of temperature on the activity **(A)** and stability **(B)** of D-hydantoinase.

References

1. Yamada, H., Shimizu, S., Shimada, H., Yani, Y., Takahashi, S., and Ohashi, T. (1980), *Biochimie* **62,** 395–399.
2. Takahashi, S., Ohashi, T., Kii, Y., Kumagai, H., and Yamada, H. (1979), *J. Ferment. Technol.* **57,** 328–332.
3. Takahashi, S., Kii, Y., Kumagai, H., and Yamada, H. (1978), *J. Ferment. Technol.* **56(5),** 492–498.
4. Yamada, H., Takahashi, S., Kii, Y., and Kumagai, H. (1978), *J. Ferment. Technol.* **56(5),** 484–491.
5. Hyun, H. H. and Zeikus, J. G. (1985), *J. Bacteriol.* **164,** 1146–1152.

6. Adrian, R. P., Hugh, W. M., and Roy, M. D. (1986), *Enzyme Microb. Technol.* **8,** 668–672.
7. Jacob, E., Henco, K., Marcinowski, S., and Schenk, G. (1987), German patent DE3535987Al, BASF Aktiengesellschaft, Germany.
8. Mukohara, Y., Ishikawa, T., Watabe, K., and Nakamura, H. (1994), *Biosci. Biotech. Biochem.* **58(9),** 1621–1626.
9. Lee, S.-G., Lee, D.-C., Hong, S.-P., Sung, M.-H., and Kim, H.-S. (1995), *Appl. Microbiol. Biotechnol.* **43,** 270–276.
10. Lee, S.-G., Lee, D.-C., and Kim, H.-S. (1997), *Appl. Biochem. Biotech.* **62,** 251–266.
11. Suzuki, T., Igarash, K., Hase, K., and Tuzimura, K. (1973), *Agric. Biol. Chem.* **37,** 411–416.
12. Laemmli, U. K. (1970), *Nature* **227,** 680–685.
13. Laas, T., Olsson, I., and Soderberg, L. (1980), *Anal. Biochem.* **101,** 449–461.
14. Runser, S. M. and Meyer, P. C. (1993), *Eur. J. Biochem.* **213,** 1315–1324.
15. Bradford, M. M. (1976), *Anal. Chem.* **72,** 248–254.
16. Lane, D. J. (1991), in Stackebrandt, E. and Goodfellow, M., eds., Nucleic Acid Techniques in Bacterial Systematics, John Wiley & Sons, New York.
17. Kreig, N. R. and Holt, J. G. (1984), *Bergey's Manual of Systematic Bacteriology*, vol. 1 Williams & Wilkins, Baltimore, MD.
18. Syldatk, C., Cotoras, D., Dombach, G., Gross, C., Kallwass, H., and Wagner, F. (1987), *Biotech. Lett.* **9(1),** 25–30.
19. Ogawa, J., Kaimura, T., Yamada, H., and Shimizu, S. (1994), *FEMS Microbiol. Lett.* **122,** 55–60.
20. Nishida, Y., Nakamichi, K., Nabe, K., and Tosa, T. (1987), *Enzyme Microb. Technol.* **9,** 721–725.
21. LaPointe, G., Viau, S., Leblanc, D., Robert, N., and Morin, A. (1994), *Appl. Environ. Microbiol.* **60(3),** 888–895.
22. Morin, A., Hummel, W., Schutte, H., and Kula, M.-R. (1986), *Biotechnol. Appl. Biochem.* **8,** 564–574.