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Purification and Characterization of Thermostable D-Hydantoinase from Thermophilic *Bacillus stearothermophilus* SD-1

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ABSTRACT

A thermostable D-hydantoinase of thermophilic *Bacillus stearothermophilus* SD-1 was purified to homogeneity using an immuno-affinity chromatography. The affinity chromatography that employed polyclonal antibody immobilized on Sepharose 4B was simple to operate and gave a purification yield of 60% of enzyme activity. Molecular mass of the enzyme was determined to be about 133.9 kDa by gel filtration chromatography and the molecular mass of the subunit was 54 kDa on SDS-PAGE. Mass spectrometric analyses were also performed for the determination of the molecular mass of the native enzyme and its subunit. The apparent molecular masses were 51.1 and 102.1 kDa for the subunit and native enzyme, respectively. Based on the molecular masses determined by these two methods, it is suggested that the D-hydantoinase exists as a dimeric conformation in the cell. Isoelectric pH of the enzyme was observed to be 4.47. It was found that the enzyme requires one manganese ion per molecule of enzyme for the activity. The optimal pH and temperature for the catalytic activity were about 8.0 and 65°C, respectively. The half-life of the enzyme was estimated to be 30 min at 80°C, confirming that the enzyme purified is one of the most thermostable D-hydantoinase reported so far. Kinetic constants of the enzyme for different substrates were also determined.

Index Entries: Thermostability; D-hydantoinase; *Bacillus stearothermophilus*; immuno-affinity chromatography.

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INTRODUCTION

Dihydropyrimidinase (EC 3.5.2.2) has been found from various mammalian livers and catalyzes the hydrolysis of dihydropyrimidine and 5-monosubstituted hydantoins with enantioselectivity. The enzyme was purified from liver of bovine, calf, and rat and characterized regarding the molecular properties and the mechanism of enzyme action *(1–4)*. D-Hydantoinase, which is known to be a microbial counterpart of dihydropyrimidinase, has been found to be distributed in various microorganisms *(5)*. D-Hydantoinase of bacterial strain has been employed for the synthesis of optically active D-amino acids from DL-5-mono-substituted hydantoin derivatives *(6,7)*. Currently, optically active D-amino acids including D-*p*-hydroxyphenylglycine are widely used in the pharmaceutical field as intermediates for the synthesis of semisynthetic antibiotics, peptide hormones, pyrethroids, and pesticides.

During the past decades, much effort has been directed toward the screening of D-hydantoinase-producing microorganisms, and the D-hydantoinases from *Pseudomonas striata (8)*, *Pseudomonas fluorescence (9)*, and *Agrobacterium* sp. *(10)* have been purified to homogeneity and characterized.

From the practical standpoint, the operational stability of the enzyme is regarded as one of the main problems because the short half-life of the enzyme is often the cost-limiting factor in the enzymatic processes. There have been a few studies regarding the stability of D-hydantoinase so far. Morin et al. *(11)* reported that the D-hydantoinases of *Pseudomonas* strains were stable up to 60°C. The D-hydantoinase of *Agrobacterium* sp. was shown to be relatively stable as compared with those from *Pseudomonas* strains. After heat treatment at 70°C for 30 min, about 20% of the activity of the enzyme remained *(12)*.

As an effort to produce a thermostable enzyme with a greater potential in industrial applications, much attention has been paid to thermophilic microorganisms. Jacob et al. *(13)* first reported the cloning of genes encoding the D-hydantoinase from two thermophiles, one strain gram-negative (CBS 30380) and the other gram-positive (Lu1220). However, detailed studies regarding the identification of microorganisms and thermostability of the enzyme have not been conducted. Recently, a gene encoding thermostable D-hydantoinase of *Bascillus stearothermophilus* was cloned in *E. coli*, and biochemical properties of expressed enzyme were investigated *(14)*. Thermostability of the enzyme was shown to be dependent on the pH, and half-life at 80°C was about 25 min at pH 8.1. The maximal activity of the enzyme was observed at pH 8.8.

We have been also focusing on the screening of thermostable D-hydantoinase-producing thermophiles, and have isolated a thermophilic microorganism which produces a thermostable D-hydantoinase *(15,16)*.

The thermophilic microorganism was classified as *Bacillus stearothermophilus* SD-1 through the physiologcal and biochemical studies. In this paper, the thermostable D-hydantoinase from the thermophilic *Bacillus stearothermophilus* SD-1 is purified to homogeneity by using immunoaffinity chromatography and characteristics of the enzyme are investigated. Stability and substrate specificity of the enzyme are compared with those from different microbial sources.

MATERIALS AND METHODS

Materials

Hydantoin, *N*-carbamoylglycine, glycine, D- or L-hydroxyphenylglycine, D- or L-valine, *N*-carbamoyl-D-valine, dihydrouracil, *p*-dimethylaminobenzaldehyde, *p*-hydroxybenzaldehyde, benzaldehyde, and isobutylaldehyde were purchased from Sigma (St. Louis, MO). Water and acetonitrile as an eluent of HPLC were obtained from Budick and Jackson Lab (Muskegor, MI). All other chemicals were of analytical grade. DL-5-Hydroxyphenylhydantoin, DL-5-phenylhydantoin, and DL-5-isopropylhydantoin were synthesized with corresponding aldehyde *(17)*.

Cultivation of Microorganism

The bacterial strain used was *B. stearothermophilus* SD-1 isolated from soil samples as described before *(15)*. The composition of medium for the cultivation was 10 g of yeast extract, 2 g of meat extract, 2 g of glucose, 2 g of KH_2PO_4, 3 g of K_2HPO_4, 0.1 g of $MgSO_47H_2O$, 20 mg of $MnCl_24H_2O$ per L of distilled water, and initial pH of medium was adjusted to 7.2. *B. stearothermophilus* SD-1 was cultivated in a 5-L fermentor (Korea Fermentor, Incheon, Korea) containing 3 L of culture broth at 55°C and pH was maintained at 7.2. The agitation speed and aeration rate were maintained at 500 rpm and 0.5 v/v/m, respectively.

Enzyme Assay

The reaction mixture composed of 67 m*M* hydantoin, 1 m*M* manganese. Tris-HCl buffer, 0.1*M*, (pH 8.0) was preincubated at 55°C and then the D-hydantoinase was added into the reaction mixture. After 30 min of incubation, the reaction was stopped by the addition of 12% trichloroacetic acid, and the precipitates were removed by centrifugation. The amount of *N*-carbamoylglycine formed in the reaction mixture was determined by the colorimetric method *(8)*. One unit of hydantoinase activity was defined as the amount of enzyme required to produce one μmol of *N*-carbamoylglycine from hydantoin per min under the specified condition.

Gel Electrophoresis

Analytical sodiumdodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted according to the method of Laemmli *(18)*. The stacking gel and resolving gel contained 5 and 9% acrylamide, respectively (crosslinking 2.8%). Protein bands of the gels were fixed in 20% trichloroacetic acid and stained with silver nitrate solution.

Preparation of an Immunoaffinity Column

We used the purified D-hydantoinase as an antigen to prepare polyclonal antibody for use as the ligand in affinity chromatography. The purified D-hydantoinase (150 μg) was emulsified with equal volume of complete Freund's adjuvants (Sigma) and injected intradermally into rabbit. After 2 wk, the rabbit was reinjected id with the enzyme (62 μg) emulsified in incomplete Freund's adjuvants (Sigma). Booster dose of the enzyme (25 μg) was injected iv after 2 wk. The polyclonal antibody was purified from serum of the rabbit by using an affinity chromatography packed with Protein G (Pharmacia, Uppsala, Sweden) and was immobilized on CNBr-activated Sepharose 4B (Pharmacia) by the procedure recommended by the supplier. The immunoaffinity matrix (about 7 mL) was packed in a column (1 cm × 10 cm) and used for the purification of the D-hydantoinase.

Western Blotting

Immunoblotting was conducted by the standard procedure established by Towbin et al. *(19)*. After electrophoresis, proteins were transferred onto a nitrocellulose membrane using a electroblotting kit (Bio-Rad, Hercules, CA). The membrane was preincubated for 3 h at 30°C in TBS-Tween 20 buffer (pH 7.4) containing 5% skim milk to block the nonspecific binding of proteins. TBS-Tween 20 buffer was composed of 3 g/L of Tris, 8 g/L of NaCl, 0.2 g/L of KCl and 0.05% (v/v) of Tween 20. Skim milk was washed out with TBS-Tween 20 buffer and membrane was incubated in TBS-Tween 20 buffer containing 0.1% (v/v) of antiserum for 3 h. Unbound antibodies were washed three times with TBS-Tween 20 buffer and the membrane was incubated in the same buffer containing 0.1% (v/v) alkaline phosphatase-conjugated antirabbit IgG-antibodies (Sigma) for 3 h. Unbound secondary antibodies were carefully washed and the membrane was incubated with the substrate solution A and B (Sigma) at room temperature. The reaction bands were visualized within 5 min.

Determination of Molecular Mass by Gel-Filtration Chromatography

Analytical gel filtration chromatography was performed by using Superose 12 (Pharmacia) after the column was equilibrated with a buffer containing 20 m*M* Tris(pH7.2), 0.15*M* NaCl, and 1 m*M* $MnCl_2$. The coef-

ficients K_{av} for marker proteins and purified D-hydantoinase were determined and correlated with molecular mass on a semilogarithmic plot. The K_{av} is defined as $(V_e - V_o) / (V_i - V_o)$, where V_t is the volume required to elute small molecules which diffuse freely through the matrix, V_o the void volume, and V_e the elution volume. In this experiment, V_t and V_o were predetermined to be 20.9 and 7.68 mL by testing the elution of glycyltyrosine and blue dextran through the column, respectively.

Determination of Molecular Mass by Mass Spectrometer

A Kratos Kompact (Kratos Analytical, Manchester, UK) linear time-of-flight mass spectrometer with a nitrogen laser (337 nm, 3-ns pulse) was used for laser desorption. All experiments were performed using dihydroxybenzene acid as a matrix at a concentration of 10 μg/μL. The protein solutions were dialyzed against 1 m*M* $MnCl_2$ and 20 m*M* Tris-HCl buffer (pH 8.0) for 2 d and 0.5 μL of the sample solution was mixed with 2 μL of a matrix solution on a stainless-steel target and dried at room temperature. Each spectrum was accumulated by averaging 100 laser shots.

Determination of Isoelectric Point

Isoelectric point of the purified enzyme was determined according to the procedure suggested by Laas et al. *(20)*. Polyacrylamide gel for isoelectric focusing consisted of 6% acrylamide (crosslinking 2.8%), 2.4% ampholyte with a pH range of 4–6.5, and 10% glycerol. Protein standards for isoelectric focusing were purchased from Pharmacia. After prerunning a gel at 200 V for 1 h, 5 μg of the D-hydantoinase was loaded with protein standards on the isoelectric focusing gel. Gel was run at 1500 V for 1.5 h at 24°C. Proteins were fixed with 10% trichloroacetic acid solution for 10 min and the gel was soaked in 1% trichloroacetic acid solution for 3 h to remove ampholite. Coomassie brilliant blue G250 was used for the staining of proteins.

Determination of Manganese Content

The concentration of manganese ions was measured using a sequential plasma spectrometer (Model KPS-1000IV, Shimadzu, Kyoto, Japan). Enzyme solution was dialyzed against 0.1 m*M* Tris-HCl buffer (pH 7.8) containing different concentrations of manganese ions and 0.1*M* potassium chloride at 4°C for 2 d. Concentration of manganese ions in enzyme solution $[Mn^{2+}]_E$ and in dialysis buffer $[Mn^{2+}]_B$ were determined by measuring the emission intensity of each solution at 258 nm. Manganese content of the D-hydantoinase was calculated by $\{[Mn^{2+}]_B - [Mn^{2+}]_B\}/\{[E]/M\}$, where $[E]$ is a protein concentration of the enzyme solution and M is the molecular mass of the D-hydantoinase.

Amino Acid Composition

The purified enzyme (5 μg) was hydrolyzed at 110°C for 24 h by using a Waters Pico Tag workstation (Millipore, Bedford, MA), and the hydrolysates were analyzed with a Pico Tag amino acid analyzer (Millipore). The amounts of cysteine and cystine were determined as cysteic acid after oxidation with performic acid and tryptophan was measured after being treated with methansulfonic acid containing tryptamine HCl.

Determination of Kinetic Parameters

Kinetic constants of the D-hydantoinase were determined by measuring the initial reaction rates at standard conditions for enzyme assay described elsewhere in this paper except changing the concentrations of substrates. The concentrations of dihydrouracil used were ranged from 1 to 10 m*M*, and hydantoins were from 125 to 450 m*M*. In the cases of DL-5-phenylhydantoin and DL-5-*p*-hydroxyphenylhydantoin, substrate concentrations were controlled to be maintained below 50 m*M* because they are insoluble at concentrations higher than 50 m*M*. In order to investigate inhibition of the enzyme by reaction product, the initial reaction rates were measured in the presence of 50 m*M* *N*-carbamoylglycine and hydantoin ranged from 125 to 450 m*M*. The kinetic parameters were calculated from the double-reciprocal plot of initial reaction rates versus substrate concentrations.

Analysis

The concentrations of DL-*p*-hydroxyphenylhydantoin, *N*-carbamoyl-*p*-hydroxyphenylglycine, and D-*p*-hydroxyphenylglycine were determined by using HPLC (Shimadzu). The column used was CLC-ODS (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. Eluent was detected at 214 nm. The protein concentration was determined by the method of Bradford *(21)* using bovine serum albumin as a standard.

RESULTS

Purification of the D-Hydantoinase

The conventional chromatographic methods were used to purify the D-hydantoinase from *B. stearothermophilus* SD-1, but the purification yield was so low and the procedures were too tedious. Thus, we purified the enzyme by using an immunoaffinity chromatography as described in Materials and Methods.

Cell-free extract of *B. stearothermophilus* SD-1 was applied to the immunoaffinity column at the flow rate of 6 mL/h. The enzyme was eluted with 0.05*M* carbonate buffer (pH 10.5) containing 2*M* NaCl, and eluent

Table 1
Purification of the D-Hydantoinase by Immuno-Affinity Chromatography

Step	Total proteins (mg)	Total activity (units)	Specific activity (units/mg)	Purification fold	Activity yield (%)
Cell-free extract	890	2,200	2.47	1	100
Immuno-affinity chromatography	8.9	1,320	148.6	60.2	60

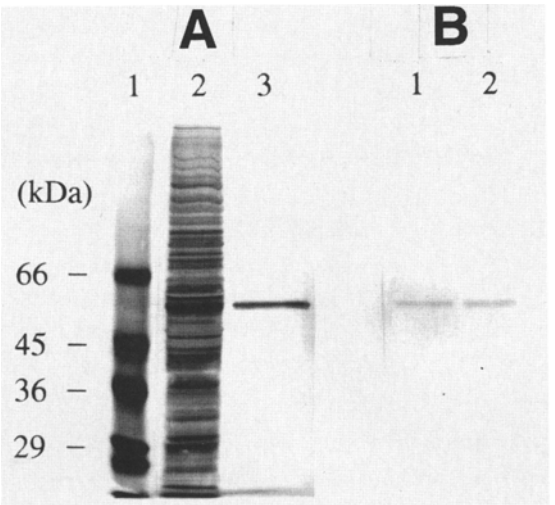

Fig. 1. Purification of D-hydantoinase from *B. stearothermophilus* SD-1. **(A)** SDS-PAGE analysis. Lanes: 1, standard proteins; 2, crude extract; 3, D-hydantoinase purified by immuno-affinity chromatography. **(B)** Western blotting of the enzyme. Lanes: 1, crude extract; 2, D-hydantoinase purified by immuno-affinity chromatography.

fractions were pooled and dialyzed against 10 m*M* Tris-HCl buffer (pH 8.0) containing 1 m*M* $MnCl_2$.

The results of the purification of the D-hydantoinase from *B. stearothermophilus* SD-1 using immunoaffinity chromatography are summarized in Table 1. The purification yield of the D-hydantoinase activity was about 60% in terms of enzyme activity, and specific activity of enzyme was about 148 U/mg protein. The analytical SDS-PAGE of the samples from each step of the purification is shown in Fig. 1A. The purified D-hydantoinase was electrophoretically homogeneous, and the apparent molecular mass of the subunit was estimated as 54 kDa from the relative migration to the molecular size standards.

Immunoaffinity of purified D-hydantoinase for antibody was confirmed by Western immunoblotting analysis as shown in Fig. 1B. The homogeneity of the purified D-hydantoinase was investigated by different methods. The electrophoresis under both denaturing and native condi-

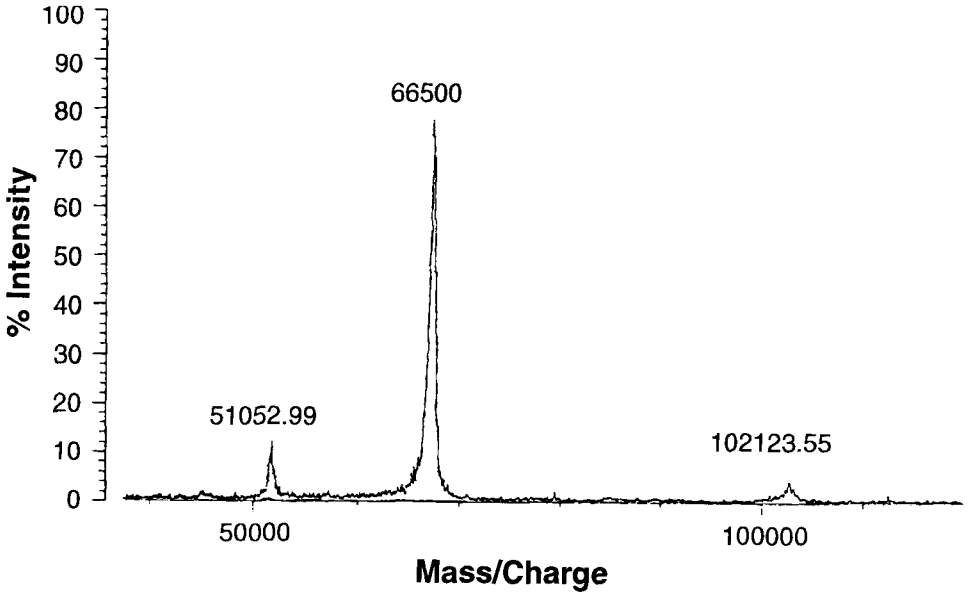

Fig. 2. MALDI-TOF spectrum of D-hydantoinase. Time-to-mass conversion was achieved by internal calibration using bovine serum albumin (66.5 kDa) as a standard protein.

tions resulted in one protein band after Coomassie brilliant blue or silver staining. Furthermore, one homogeneous peak was observed in the gel filtration chromatography (data not shown).

Determination of Molecular Mass

The molecular mass of the D-hydantoinase was determined by two independent methods. First, the molecular mass of the native enzyme was determined by gel filtration chromatography using a Superose 12 column, and its value was estimated to be about 133.9 kDa. Because the molecular mass of the native enzyme determined by gel filtration chromatography was 2.48 fold of that of subunit, it seemed to be rather difficult to determine whether the enzyme is composed of two subunits or not. It is widely accepted that gel filtration chromatography provides only an approximation of the molecular mass due to a non-ideal diffusion of the protein through the porous gel and unwanted protein-matrix interaction.

Therefore, we attempted the determination of molecular mass by using mass spectrometer. Mass spectrometer is increasingly used for the determination of molecular mass of proteins (*22*). Use of mass spectrometer seems to lead to determination of an accurate molecular mass of protein because proteins are analyzed by absolute mass/charge ratio. When the purified D-hydantoinase was analyzed by using a MALDI-TOF mass spectrometer at the standard conditions as described in Materials and Methods, two peaks corresponding to 51 and 102 kDa were observed (Fig. 2).

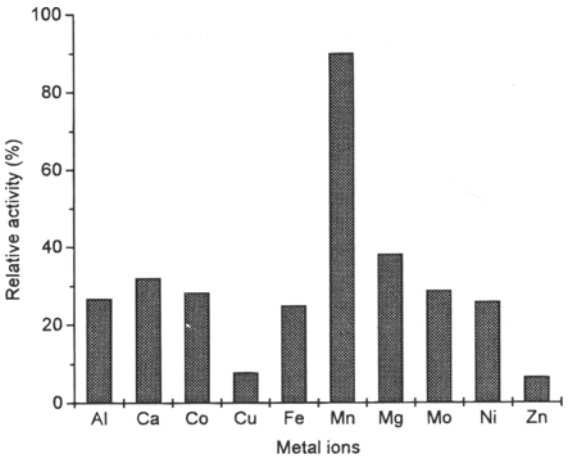

Fig. 3. Effects of metal ions on the enzyme activity. The concentration of each metal ion was 1 m*M*. The enzyme activity before dialysis was taken as 100%.

Although the second peak was different in height from sample to sample, the absolute mass/charge value was always maintained as two times that of the first peak during the repetitive runs. By comparing the mass/charge values of the two peaks with the molecular masses determined by either SDS-PAGE analysis or gel filtration chromatography, we suppose that the first peak corresponds to the molecular mass of the subunit of native enzyme and the second one to the native enzyme itself, respectively.

Isoelectric Point

The isoelectric point pI of the D-hydantoinase was measured by electrofocusing on polyacrylamide gel. The isoelectric pH was determined as 4.47 by comparing the band position of the enzyme with those of standard-size markers (data not shown).

Effects of Metal Ions on the Enzyme Activity

The purified D-hydantoinase was stringently dialyzed against metal-free buffer solution, and effects of various metal ions on the enzyme activity were investigated. The remaining enzyme activity after dialysis was about 30%. As shown in Fig. 3, manganese ions were found to be most effective, and enzyme activity was almost fully restored by addition of manganese ions. Zinc and copper ions inhibited the enzyme activity.

Manganese Ion Content

In order to determine the manganese content in the D-hydantoinase, purified enzyme was dialyzed against 0.1*M* Tris-HCl buffer (pH 7.8) containing different concentrations of manganese ions at 4°C, and then manganese content in the enzyme was determined using a sequential plasma

Table 2
Manganase Content of the D-Hydantoinase

Manganese [a] (μM)	*Manganese* [b] *(μM)*	*Protein (μM)*	*Manganese*[c] / *Protein*
0.94	3.34	4.7	0.51
2.03	4.79	3.4	0.81
29.0	34.0	5.0	1.00

[a] Concentration of manganese ions in equilibrium-dialysis buffer.
[b] Concentration of manganese ions in protein solution.
[c] (Manganese[b])—(Manganese[a])

Table 3
Composition of Amino Acids of the D-Hydantoinase

Amino acid	*No. of residues per subunit*
Asx	46.29 (46)
Thr	34.14 (34)
Ser	15.64 (16)
Glx	55.68 (56)
Pro	19.13 (19)
Gly	50.41 (50)
Ala	38.04 (38)
Cys	9.22 (9)
Val	31.22 (31)
Met	6.59 (7)
Ile	22.00 (22)
Leu	40.39 (40)
Tyr	13.06 (13)
Phe	21.25 (21)
His	9.28 (9)
Lys	37.12 (37)
Arg	11.80 (12)
Trp	6.30 (6)

spectrometer (Japan). As shown in Table 2, the manganese content of the enzyme was increased with increasing concentration of manganese ions in the dialysis buffer, and the enzyme was saturated with manganese ions when the concentrations of manganese ions in the buffer was 0.029 m*M*. When molecular mass of 102.1 kDa determined by mass spectrometer was applied, the manganese content of the D-hydantoinase was estimated to be one ion per enzyme.

Composition of Amino Acids

Experimentally determined amino acid composition of the D-hydantoinase is presented in Table 3. The composition of amino acids was calculated on the basis of molecular mass of subunit (51,050 Da) determined by

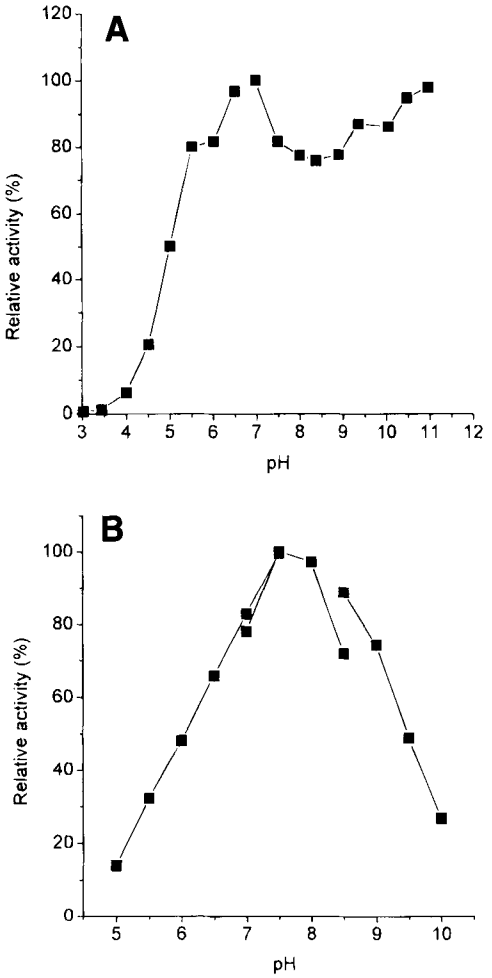

Fig. 4. Effect of pH on **(A)** the stability and **(B)** the activity of D-hydantoinase. Buffers used were: Bis-Tris buffer for pH 5–7.5, Tris-HCl buffer for pH 7–8.5, and glycine-NaOH buffer for pH 8.5–10.

mass spectrometer. Although the enzyme contained all the common amino acids, relatively high content of neutral amino acids such as glycine and alanine was observed. Hydrophobic amino acids like valine, isoleucine, and leucine were also found to exist in high content.

Effects of pH and Temperature

Purified enzyme was incubated for 12 h in various 20 m*M* buffers with different pH values at 30°C, and the residual enzyme activity was determined. As shown in Fig. 4A, the enzyme remained stable at pH values higher than 5.5.

Dependence of the D-hydantoinase activity on pH variation was also examined. Change in the activity with pH variation was quite steep and the maximum activity was observed at pH 8.0 (Fig. 4B).

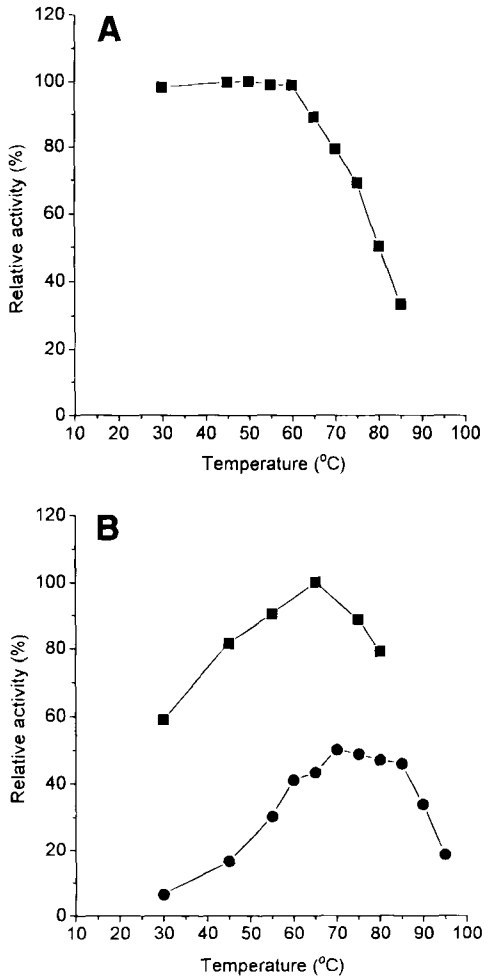

Fig. 5. Effect of temperature on **(A)** the thermostability and **(B)** the activity of D-hydantoinase. Symbols in **(B)** indicate the enzyme activity for different substrates: (■) hydantoin; (●) *p*-hydroxyphenylhydantoin.

In order to investigate the thermostability, the D-hydantoinase was incubated for 30 min at different temperatures, and the residual activity was measured. The D-hydantoinase was stable up to 60°C, and the half-life of the enzyme was estimated as long as 30 min at 80°C (Fig. 5A). The purified enzyme showed slightly higher thermostability than the one determined with cell-free extract, and it seems possible that thermally labile proteins contained in the cell-free extract may precipitate with D-hydantoinase together at elevated temperature, resulting in decreased activity of D-hydantoinase. The D-hydantoinase activity was assayed at different temperatures to evaluate the effect of temperature on the enzyme activity for substrates with different solubility. As can be seen in Fig. 5b, maximum activity for hydantoin was obtained at about 65°C, while the activity of

Table 4
Kinetic Constants of the D-Hydantoinase for Different Substrates[a]

Substrate	V_{max} (mM/h)	K_m (mM)	V_{max} / K_m (1/h)	k_{cat} (sec^{-1})	K_i (mM)
Dihydrouracil	11.2	1.1	10.2	51	n.d.
Hydantoin	121	340	0.36	553	232
Phenylhydantoin	35.0	32.1	1.1	160	n.d.
p-Hydroxyphenylhydantoin	13.4	47.2	0.28	61	n.d.

[a] n.d. means "not determined."

D-hydantoinase was maximum at 70°C for DL-*p*-hydroxyphenylhydantoin, which is slightly soluble in water.

Kinetic Parameters

Dihydrouracil, hydantoin, DL-phenylhydantoin, and DL-*p*-hydroxyphenylhydantoin were used as substrates to investigate the kinetic properties of the D-hydantoinase. The kinetic constants are summarized in Table 4. The values of K_m of the D-hydantoinase from *B. stearothermophilus* SD-1 were found to be much larger than those of D-hydantoinase from other microbial sources *(10)*. Michaelis constant K_m for dihydrouracil was about 1.1 m*M*, which implies that the D-hydantoinase has a preferential affinity for dihydrouracil. In the case of hydantoin derivatives, substrate with aromatic substituent showed a relatively higher affinity for the enzyme than hydantoin. The k_{cat} of the enzyme toward hydantoin was calculated to be about 553/s from the maximun reaction rate by assuming molecular mass of the enzyme as 102 kDa, which is contradictory to the result that the K_m value for hydantoin is largest among the substrates tested. *N*-Carbamoylglycine was found to inhibit the hydantoin-hydrolyzing activity of the enzyme in a competitive pattern.

DISCUSSION

To date, D-hydantoinase from several bacterial strains has been purified to homogeneity by typical chromatographic procedures or by preparative gel electrophoresis *(8–10)*. Purification of D-hydantoinase from thermophilic *B. stearothermophilus* SD-1 using typical chromatographies was not effective, leading to low purification yield. Meanwhile, immuno-affinity chromatography using polyclonal antibody against D-hydantoinase resulted in higher recovery yield, confirming that immunoaffinity chromatography is very simple and powerful tool in the purification of proteins.

The D-hydantoinase of *B. stearothermophilus* SD-1 was quite stable even at 60°C, and the half-life of the enzyme was about 30 min at 80°C. Recently, Mukohara et al. *(14)* reported a thermostable hydantoinase of *Bacillus stearothermophilus*. The thermostability of this enzyme was depen-

dent on the pH, and half-life at 80°C was estimated to be about 25 min at pH 8.1, showing a comparable stability to D-hydantoinase studied in this work. The hydantoinase isolated by Mukohara et al. was determined to be a homotetramer having a molecular mass of 200 kDa. The D-hydantoinase isolated in this work was found to be a homodimer with a molecular mass of about 100 kDa, which indicates that two hydantoinase are quite different each other even though these enzymes were isolated from the microorganism with the same genus and species. The D-hydantoinase of *Agrobacterium* sp. *(12)* was found to exhibit the highest thermostability among the D-hydantoinases from mesophiles reported, and residual activity of the enzyme was estimated to be about 20% after heated at 70°C for 30 min. It has been generally accepted that thermophilic microorganisms produce more thermostable enzymes than mesophiles, but the detailed mechanisms by which the enzymes are stabilized has not been well established. It is presumed that thermostable enzymes contain large number of hydrophobic amino acids such as leucine, isoleucine, and valine, suggesting that hydrophobic interactions between those amino acids could play an important role in the stabilization of protein structure against denaturants. However, no significant difference in the amino acid composition of D-hydantoinase between *B. stearothermophilus* SD-1 and *Agrobacterium* sp. or bovine liver D-hydantoinase was observed. Comparison of three-dimensional structure of D-hydantoinase of thermophilic *B. stearothermophilus* SD-1 with those of mesophiles is expected to give an important insight into the factors affecting the stability of protein.

Studies concerning the molecular mass, oligomeric structure and sedimentation coefficient of the hydantoinases from *Pseudomonas striata (8)*, *Agrobacterium* sp. *(10)*, and *Bacillus stearothermophilus (14)* revealed that these hydantoinases are large oligomeric protein possessing a low diffusion coefficient and a nonspherical shape. Most of the hydantoinases were reported to be a tetramer of identical subunits. On the other hands, native D-hydantoinase of *B. stearothermophilus* SD-1 was found to be composed of identical two subunits. Molecular mass of native D-hydantoinase of *B. stearothermophilus* SD-1 determined by gel filtration chromatography was relatively larger than that determined by mass spectrometer. Gel filtration chromatography is generally known to provide only an approximation of the molecular mass due to non-ideal diffusion of the protein through the porous gel and unwanted protein-matrix interaction. Meanwhile, mass spectrometer was employed for the accurate determination of molecular mass of the protein, and revealed a molecular mass of about 100 kDa.

Earlier investigations showed that D-hydantoinase requires metal ions for the activity *(23)*, and D-hydantoinase of *B. stearothermophilus* SD-1 was also appeared to require manganese ions for the activity. Because binding of manganese ions to the D-hydantoinase was not so tight and the dissociation of metal ions was significant during dialysis, precise determination of manganese content of the enzyme by simple dialysis procedure was diffi-

cult. In the case of zinc ions, which is known to tightly bind with bovine liver dihydropyrimidinase, simple dialysis method was attempted to estimate the metal ion content *(1)*. In order to determine the manganese content of the D-hydantoinase, equilibrium dialysis of the enzyme against buffer solution containing different concentrations of manganese ions was performed. As a result, D-hydantoinase was found to contain one manganese ion per enzyme molecule, which is quite different from bovine liver dihydropyrimidinase that possesses four zinc ions per enzyme molecule. From the manganese content in the D-hydantoinase of *B. stearothermophilus* SD-1, the role of manganese ion in the enzyme molecule seems to be linked with the assembly of two identical subunits into oligomeric structure as well as catalytic activity. In case of zinc-metallo-enzymes, zinc was suggested to contribute to the structure and/or catalytic activity of the enzyme *(24)*.

The optimal pH for the enzyme activity was 8.0, and this is similar to other D-hydantoinases from mesophiles. The temperature giving the maximum enzyme activity was varied depending on the substrate tested. For hydantoin, which is highly soluble in water, maximum activity was observed at 65°C. In the case of using *p*-hydroxyphenylhydantoin with a low solubility, maximum activity was shifted to around 70°C. One significant advantage of employing a thermostable enzyme is that enhanced production rates are expected due to increased solubility of the substrate at elevated temperatures.

The kinetic constants of the enzyme suggested that the catalytic properties of the D-hydantoinase from *B. stearothermophilus* SD-1 are different from those of other microbial sources. The K_m values of the hydantoinases from mesophiles have been reported to be about several micromolar for various hydantoin derivatives and dihydropyrimidines. From the kinetic constants, the preferred substrate of D-hydantoinase from *B. stearothermophilus* SD-1 seems to be dihydrouracil. In the case of hydantoin derivatives, substrate with a hydrophobic substituent was shown to be a good substrate for the enzyme. The D-hydantoinase from mesophiles such as *Pseudomonas*, *Agrobacterium*, and *Arthrobacter* also exhibited higher affinity toward hydantoin derivatives with non-polar hydrophobic groups *(23)*. But, close relationship between the affinity and the structure of the substrate was not found in the D-hydantoinase of *B. stearothermophilus* SD-1. It is thought that differences in the catalytic properties of the enzyme between thermophiles and mesophiles are closely linked with the structure of the enzyme. Detailed information concerning the three dimensional structure of the thermostable enzyme is expected to elucidate the catalytic properties of the enzyme further.

REFERENCES

1. Brooks, K. P., Jones, E. A., Kim, B. D., and Sander, E. G. (1983), *Archiv. Biochem. Biophys.* **226**, 469–483.
2. Jahnke, K., Podschun, B., Schnackerz, K. D., Kautz, J., and Cook, P. F. (1993), *Biochemistry* **32**, 5160–5166.

3. Kautz, J. and Schnackerz, K. D. (1989), *Eur. J. Biochem.* **181**, 431–435.
4. Kikugawa, M., Kaneko, M., Fujimoto-Sakada, S., Maeda, M., Kawasaki, K., Takagi, T., and Tamaki, N. (1994), *Eur. J. Biochem.* **219**, 393–399.
5. Yamada, H., Takahashi, S., Kii, Y., and Kumagai, H. (1978), *J. Ferment. Technol.* **56**, 484–491.
6. Takahashi, S., Ohashi, T., Kii, Y., Kumagai, H. and Yamada, H. (1979), *J. Ferment. Technol.* **57**, 328–332.
7. Yamada, H., Shimizu, S., Shimada, H., Tani, Y., Takahashi, S., and Ohashi, T. (1980), *Biochimie* **62**, 395–399.
8. Takahashi, S., Kii, Y., Kumagai, H., and Yamada, H. (1978), *J. Ferment. Technol.* **56**, 492–498.
9. Morin, A., Hummel, W., Schütte, H., and Kula, M. R. (1986), *Biotechnol. Appl. Biochem.* **8**, 564–574.
10. Runser, S. M. and Meyer, P. C. (1993), *Eur. J. Biochem.* **213**, 1315–1324.
11. Morin, A., Leblanc, D., Paleczek, A., Hummel, W., and Kula, M. R. (1990), *J. Biotechnol.* **16**, 37–48.
12. Runser, S. M. and Ohleyer, E. (1990), *Biotechnol. Lett.* **12**, 259–264.
13. Jacob, E., Henco, K., Marcinowski, S., and Schenk, G. (23.4.87) *DE* 3535987 A1, *EP* 0219034 A2.
14. Mukohara, Y., Ishikawa, T., Watabe, K., and Nakamura, H. (1994), *Biosci. Biotech. Biochem.* **58**, 1621–1626.
15. Lee, S. G., Lee, D. C., Sung, M. H., and Kim, H. S. (1994), *Biotechnol. Lett.* **16**, 461–466.
16. Lee, S. G., Lee, D. C., Hong, S. P., Sung, M. H., and Kim, H. S. (1995), *Appl. Microbiol. Biotechnol.* **43**, 270–276.
17. Suzuki, T., Igarashi, K., Hase, K., and Tuzimura, K. (1973), *Agri. Biol. Chem.* **37**, 411–416.
18. Laemmli, U. K. (1970), *Nature* **227**, 680–685.
19. Towbin, H., Staehelin, T. and Gordon, J. (1979), *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354.
20. Laas, T., Olsson, I., and Soderberg, L. (1980), *Anal. Biochem.* **101**, 449–461.
21. Bradford, M. M. (1976), *Anal. Biochem.* **72**, 248–254.
22. Yip, T. T. and Hutchens, T. W. (1992), *FEBS Lett.* **308**, 149–153.
23. Syldatk, C., Laufer, A., Muller, R., and Hoke, H. (1990) in *Advances in Biochemical Engineering/Biotechnology*, vol. 4, Fiechter, A., ed., Springer-Verlag, Berlin, Heidelberg, pp. 29.
24. Coleman, J. E. (1992), *Ann. Rev. Biochem.* **61**, 897–946.