

# Thermostable hydantoinase from a hyperthermophilic archaeon, *Methanococcus jannaschii*

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## Abstract

A hyperthermophilic hydantoinase from *Methanococcus jannaschii* with an optimum growth at 85°C was cloned and expressed in *E. coli*. The recombinant hydantoinase was purified by affinity and anion-exchange chromatography and determined to be homotetrameric protein by gel filtration chromatography. The best substrate for the hydantoinase was D,L-5-hydroxyhydantoin, which has the specific activity of 183.4 U/mg. The optimum pH and temperature for the hydantoinase activity was 8.0 and 80°C, respectively. The half-life of the hydantoinase was measured to be 100 min at 90°C in the buffer containing 500 mM KCl. Manganese ions were the most effective for the hydantoinase activity. Stereospecificity was determined to be L-specific for the 5-hydroxymethylhydantoin and 5-methylhydantoin by chiral TLC. The activity yields as well as the operational stabilities of the thermostable *M. jannaschii* hydantoinase could be significantly improved by immobilization method. © 2002 Elsevier Science Inc. All rights reserved.

**Keywords:** Hydantoinase; Hyperthermophile; *Methanococcus jannaschii*; Stereospecificity; Thermostability

## 1. Introduction

Hydantoin-hydrolyzing enzymes (hydantoinases and hydantoin amidohydrolases) belong to the cyclic amidases (EC group 3.5.2), which include dihydropyrimidinase, dihydroorotase, and allantoinase [1]. Even though the substrates on which the various cyclic amidases act are slightly different, the enzymes probably have a similar action on the cyclic amide bonds because the basic aspects of their catalytic mechanisms are believed to be similar [2]. A wide range of 5-monosubstituted hydantoins is hydrolyzed stereospecifically into corresponding D-, L-N-carbamoyl amino acids by the hydantoinases [3,4]. Hydantoinases are used for industrial production of optically pure amino acids from racemic D,L-5-monosubstituted hydantoin derivatives in combination with highly stereoselective N-carbamoylases and hydantoin racemase [5]. With growing interest in industrial production of optically pure D-amino acids for semi-synthetic antibiotics, many microbial hydantoinases

with different substrate specificity and stereospecificity have been isolated and characterized [6].

Many studies reported thermostable D-hydantoinases from thermophiles [7,8,9]. A thermostable hydantoinase from a mesophilic *Bacillus sp.* AR9 has a half life of 80 min at 70°C and loses only 33% of its activity in 4 h at 60°C [10]. Kim et al. reported that a gene encoding thermostable D-hydantoinase of *Bacillus stearothermophilus*, which has the half-life of 25 min at 80°C [11].

Hyperthermophilic microorganism provided thermostable enzymes for application in industrial processes. *M. jannaschii* is a methanogen, which grows at between 51 and 94°C, with an optimum growth at 85°C, and its entire genome has been completely sequenced [12].

In this study a hyperthermophilic hydantoinase from *M. jannaschii* was cloned and its catalytic activity and thermostability were investigated.

## 2. Materials and Methods

### 2.1. Chemicals

Hydantoin, hydantoic acid, allantoin, 5,5-diphenylhydantoin, uracil, dihydrouracil, dihydrothymine, dihydro-

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orotic acid, 5-oxo-proline, barbituric acid, creatinine, lysine-lactam, and *p*-dimethylaminobenzaldehyde were purchased from Sigma. 1-Methylhydantoin and 2-thiohydantoin were obtained from Aldrich. 5-Methylhydantoin, 5-benzylhydantoin, 5-hydroxymethylhydantoin, 5-phenylhydantoin, 5-hydroxyhydantoin, 5-( $\delta$ -hydroxybutyl)hydantoin, 5-( $\delta$ -bromobutyl) hydantoin, and 5-ethyl-3-methyl-5-phenylhydantoin were purchased from Toronto Research Chemicals Inc. (Canada). D-hydantoinase from Aduki bean (*Vigna angularis*), purchased from Sigma, was used as a control for enzyme reactions. Chiral TLC plates were also purchased from Sigma. All other reagents were of analytical grade. Restriction endonuclease and T4 DNA ligase were obtained from Promega (USA). *Taq* DNA polymerase was a product of Takara (Japan). Primers for polymerase chain reaction (PCR) were synthesized by BioSynthesis, Inc. (USA). All the instruments and columns for FPLC were supplied by Amersham Pharmacia Biotech.

## 2.2. Microorganism

The hyperthermophile *M. jannaschii* (DSM#2661) was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Germany). *M. jannaschii* was cultivated under conditions as reported by Jones et al. [13]. Genomic DNA was prepared using a Qiagen Genomic DNA Midi Kit (Qiagen GmbH, Hilden, Germany) with some modification. To improve the efficiency of cell lysis, the purification steps were modified. Five grams of cells were suspended in 20 ml of 50 mM Tris-Cl buffer, pH 8.0, containing 10 mM of EDTA and centrifuged at 10,000 $\times$  g for 20 min. Cells were resuspended in 20 ml of the same buffer containing 0.2% Triton X-100 and proteinase K (final conc. 1 mg/ml) and incubated at 65°C for 10 min. The cell extract was prepared by French pressure cell at 1,000 p.s.i. and centrifugation at 26,800 $\times$  g for 30 min.

## 2.3. Cloning of hydantoinase gene from *M. jannaschii*

Based on complete genome sequences of *M. jannaschii* [12], a hydantoinase gene (ORF *Mj0963*) of *M. jannaschii* was identified by a NCBI BLAST search (<http://www.ncbi.nlm.nih.gov/BLAST/>) [14]. *Mj0963* gene was amplified by 25 cycles of PCR using PCR machine (Perkin Elmer, GeneAmp PCR system 2400). The PCR was performed in 100  $\mu$ l reaction mixtures containing 600 ng of genomic DNA as a template, 200  $\mu$ M dNTPs, 2 units of *Taq* polymerase, 10  $\mu$ l of 10 $\times$  reaction buffer and 1 pmol of each primer. The primers for the PCR of *Mj0963* gene used was as follows: *Mj0963*-N: 5'-GCCTCACGTTTCAGGATCCGATAAAATTACAGTTGAG-3', *Mj0963*-C: 5'-GCTAGGCAATGTCGACTTACAAACCTCTTAACCTTTTT-3', (where the underlining indicates the restriction site). The PCR products were purified with QIAquick PCR purification kit (Qiagen GmbH). The PCR products encoding *Mj0963* was digested with *Bam*HI and *Sal*I. The digested fragment was cloned in

a modified thioredoxin (*Trx*) fusion vector [15] (gift from Dr. Yeon Gyu Yu, who works at Structural Biology Research Center, Korea Institute of Science and Technology). *Trx* gene of *E. coli* was inserted into the *Nde*I - *Bam*HI site of the pET28a expression vector (Novagen) and the thrombin cleavage site was produced by site-directed mutagenesis at the C-terminal end (*Bam*HI site) from *Trx* gene. The residues of site-directed mutagenesis were as follows;

Leu-val-arg-arg-gly-ser-ala  $\rightarrow$  leu-val-pro-arg-gly-ser-ala.

CTGGTTCGGCGTGGATCCGCA

$\rightarrow$  CTGGTTCGGCGTGGATCCGCA

The modified *Trx* fusion vector was designed to contain a His-Tag sequence at the N-terminal end of *Trx* gene. The *Mj0963* gene was cloned in the downstream from the *Trx* gene of the modified vector, resulting in expression of the *Trx*-fusion protein. *Mj0963* was inserted into the *Bam*HI - *Sal*I site. The DNA sequence of the gene in the expression vector was confirmed by using an ABI 373 DNA automated sequencer. The recombinant plasmid was transformed into *E. coli* strain BL21 (DE3) for protein expression [16].

## 2.4. Protein expression and purification

*E. coli* cells harboring the hydantoinase gene were grown at 37°C in Luria-Bertani broth containing 35  $\mu$ g/mL of kanamycin. The expression of hydantoinase protein was induced by addition of 0.5 mM isopropyl-D-thiogalactopyranoside (IPTG) to cells at OD<sub>600</sub> $\geq$ 0.8. After cultivation for 4 h, cells were harvested by centrifugation at 4,000 rpm for 15 min and the cell pellet was resuspended in Tris-Cl buffer (50 mM Tris-Cl, pH 8.0, 200 mM NaCl). The resuspended cells were disrupted by ultrasonication and the lysate was centrifuged at 16,000 rpm for 30 min. The supernatant was loaded onto a Ni-NTA affinity column (Qiagen GmbH). His-tagged proteins were eluted in a gradient from 150 to 200 mM imidazole. The eluted protein was dialyzed against 50 mM Tris-Cl (pH 8.0), 20 mM NaCl, 2 mM EDTA, and 1 mM DTT (buffer A). The thioredoxin protein was removed by thrombin digestion. Thrombin (100 U) was added to 1 mg of fusion protein in buffer A and the reaction was carried out for 6 to 12 h at room temperature.

The cleaved protein solution was applied to a Q-Sepharose anion-exchange FPLC column pre-equilibrated with buffer A. The column was subjected to a linear gradient of 0.02 to 1 M NaCl in Tris-Cl buffer, pH 8.0. Fractions containing hydantoinase were concentrated by ultrafiltration using an Amicon YM30 membrane (Amicon Co.). The concentrated sample was purified by gel filtration chromatography using a Superdex 200 FPLC column pre-equilibrated with buffer A. The eluted protein was concentrated to 1 mg/mL and stored at -80°C. The protein concentration was determined according to the Bradford method [17] using bovine serum albumin as a standard.

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Mj0963      MDK - - - ITVEV IKSSTSY IAEEMGI I LRNTAYSPN I KDRLDFS 40
Pshvd      MSK I H T D L K K I D P I T V Q V L G S L E N V A V E M G H K L A R M S Y S S I I R E S E D F G 50

Mj0963      C A I L S S N G E L I A Q A E H - I P V H L G S M A I G V K N T V D Y L K K E S I E I E K D D V I I 89
Pshvd      C A L V D V R G Q Q L C E S S H S T P L Q S G P I P G Y I K G I R E I M E D R N D T F N Q G D V I M 100

Mj0963      V N D P Y I A G T H L N D I T L L K P I F Y N D E I I G Y V A N K A H H V D V G G Y A P G S I S - S 138
Pshvd      H N S P Y H G A S H G P D V G F C I P V F Y K D E L I G F S V T T A H H L D I G S S T P G S C G I V 150

Mj0963      N V K E L Y H E G L I I P P S K L V I N G K L N K E L L N L I T S N V R V P K S T I G D L K A Q I A 188
Pshvd      D A V D A Y A E G L Q F K A I K V Y D Q G V K N R Y V W D I L K D N I R A P K L V V G D M E A Q I A 200

Mj0963      S L N I G V E R I L K L I E K Y G D R E V T E A W N K S L D Y S E E Y L K S K I R D - - I N C I C E 236
Pshvd      A A R I G A Q R Y I E I I E K Y G L D T V Q A A S E E L M N Y S E K M M R D A I K K L P - D G E Y T 249

Mj0963      A V - - - D - Y L E Y K - - - D K L I N I N M K I E I K N G K I K V D F T G T H R Q L - D A P L N A 278
Pshvd      A E G F L D G Y L D S D D P A K K D L R I N V T V K V D G S D L T V D L T G T S P Q V T D K P I N M 299

Mj0963      V Y G V T V A - S T S F A L K A - V I D P - - - D L P M N H G I F R V L N I I A P E E T I V N P K 322
Pshvd      P L L G T V D I A I Y L T L R S I L L D S T V Y G N F P Q N S G L I R P I K I V A P K G T L C N P I 349

Mj0963      K P A P V S V G N V E T S Q R I V D V I F K A L Y H E F P D R V P A A S N G S M N N V I I G G - - - 369
Pshvd      F P A P - T I A R F N S G N A V A D T L M K A L A Q V V P H Q V - S A G V G N L Q V V A F S G Q S N 397

Mj0963      - R G - - W A F Y E T I G G G F G G R N G K D G V D G V H A N M T N T L N T P I E V I E N E Y P I M 416
Pshvd      E N - - Y W V Y M D I M E G S Y G G R Y G K D G M D A V D T L Y A N T R N N P I E D I E S H Y P L R 445

Mj0963      I L E Y S L R E D S G G A G K Y R G G L G I R R V Y K M L S D C M - L S I I A D R I K I S P W G V N 465
Pshvd      V N R Y E L R D N D S A P G K W R G G I G S I R E V S F L A D G - S F S V E A D G H K Y A P W G F D 494

Mj0963      N G Y S G A C G E H Y V I K - D G K K - I P L S G K D - T L Y L S C G D I V E I N T P G G G G Y G S 512
Pshvd      D G Q D G Y V G S L S I R D N E T N E L V Q L P S K L P N R H A Q S G S T I Q L V G P C G G G Y G N 544

Mj0963      P Y E R D I N L I L E D V K D E K I S I K S A Y R D Y K V K I I K K D D D F V V D M E E T K K L R 561
Pshvd      P L E R E P E K V L S D Y L D G F I T K E K A L V E Y G V T I - - - T D S E E I D Y E K T N E L R 590
    
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Fig. 1. Amino acid sequence and alignment of *Mj0963* with *Pseudomonas* sp. NS671 hydantoinase. The conserved residues are boxed. The black filled boxes represent conserved histidine residues. Alignment was performed with the SEQSEE program. *Mj0963*, *Mj0963* from *Methanococcus jannaschii*; *Pshyd*, hydantoinase from *Pseudomonas* sp. NS671.

### 2.5. Assay of enzyme activity

An activity assay of hydantoinase was performed following the colorimetric method described by Takahashi et al. [18]. The reaction mixture contained of 100 mM Tris-Cl buffer, pH 8.0, 200 mM NaCl, 5 mM MnCl<sub>2</sub>, 20 mM hydantoin and 50 µg of purified protein in a total volume of 0.4 ml. The reaction was stopped by addition of 100 µl of 12% trichloroacetic acid. The amount of product formed was determined by addition of 100 µl of *p*-dimethylamino-benzaldehyde (10% w/v in 6 N HCl). The absorbance of the solution was then determined at 420 nm using a spectrophotometer. One unit of enzyme activity was defined as one µmol of product formed per minute under the assay conditions.

### 2.6. Determination of stereospecificity

The stereospecificity of hydantoinase was analyzed by chiral thin-layer chromatography (TLC) [19]. The substrates for determining stereospecificity were 5-hydroxymethylhydantoin and 5-methylhydantoin. The N-carbamoylamino acid produced from the 5-hydroxymethyl hydantoin and 5-methylhydantoin by hydantoinase was decarbamoylated to the corresponding amino acid using equimolar nitric acid in 3 N hydrochloric acid. The sample was dehydrated in a vacuum oven and dried sample was

extracted using absolute ethanol. The sample solution was loaded on a chiral TLC plate. The plate was run in a developing solution of acetone/methanol/water (10: 2: 2 by volume). Spots were visualized by spraying 2% ninhydrin in ethanol, then heating for 10 min at 105°C. The relative mobility of spots was compared with spots of the D-, L-serine and D-, L-alanine.

### 2.7. Determination of thermostability

The enzyme was incubated at 90°C in Tris-Cl buffer, pH 8.0 with respective salts. Aliquots were withdrawn at periodic intervals and kept on ice. The residual activity of aliquots was assayed after all samples had been collected at various times. The relative activity of the enzymes was defined as the residual activity of the enzyme compared to the activity of the enzyme, which was not heated.

## 3. Results

### 3.1. Cloning and purification of hydantoinase

We identified the ORF *Mj0963* from *M. jannaschii* as a presumed hydantoinase showing sequence homology to hydantoinases of other organisms by the BLAST program at NCBI [14]. Using the SEQSEE program [20], the amino

acid sequence of *Mj0963* aligns with that of hydantoinase from *Pseudomonas* sp. NS671 [21] (Fig. 1). From the analysis of sequence alignment, several regions are highly conserved and these proteins have the conserved histidines. The amino acid sequence of *Mj0963* also exhibited 20–50% identity with those of some hydantoinases from *Aeropyrum pernix* [22], *Aquifex aeolicus* [23], and *Helicobacter pylori* [24].

In order to obtain the soluble protein encoded by cloned hydantoinase gene, *Mj0963*, we used the various expression vector systems. We first cloned the gene into the pET3a expression vector (Novagen). The recombinant plasmid was introduced into *E. coli* BL21 (DE3) for protein expression, which was induced by addition of IPTG to the cells harboring the recombinant plasmid. Unfortunately, this protein was insoluble (data not shown). The expressed proteins using the pET15b and pET22b vectors (Novagen) were also insoluble. Consequently, we attempted protein expression using the pET28a-*Trx* fusion vector system (see the description under methods). The *Trx* gene of *E. coli*, which encodes the thioredoxin protein, was inserted into the polycloning site of the pET28a expression vector, resulting in expression of a *Trx*-fusion protein. The hydantoinase gene was inserted downstream from the *Trx* gene of the vector and introduced into *E. coli* BL21 (DE3) cells. When the cell lysate was fractionated and analyzed by electrophoresis on 10% SDS-polyacrylamide gel, 50% of the fusion protein was recovered in the soluble fraction. The fusion protein was purified with the Ni-NTA affinity column, while the other proteins flowed through. Binding of the fusion protein to the Ni-NTA agarose resin through the His-Tag was specific enough to allow single-step purification because of a His-Tag sequence at the N-terminus of the *Trx* gene. After cleavage of the fusion protein with thrombin, the resultant expressed hydantoinase has additional two amino acids (Gly and Ser) at their N-terminus. The hydantoinase was purified with anion-exchange and gel filtration chromatography. The protein was purified with > 95% homogeneity. The purified hydantoinase showed single band of molecular weight 62,000 on SDS-PAGE (Fig. 2). The molecular mass of the native protein was 240 kDa, which is determined by gel filtration chromatography using a Superdex 200 FPLC column and marker proteins (carbonic anhydrase; 29 kDa, bovine serum albumin; 66 kDa, yeast alcohol dehydrogenase; 150 kDa, sweet potato-amylase; 200 kDa, horse spleen apoferritin; 443 kDa, and bovine thyroglobulin; 669 kDa). It could be suggested that this hydantoinase is tetramer consisting of four identical subunits. The final yield of the hydantoinase after purification ranged up to 1 mg/liter per culture.

### 3.2. Substrate specificity and stereospecificity

Among the different kinds of substrates, D,L-5-substituted hydantoin were the preferred substrate for the hydantoinase from *M. jannaschii* (Table 1). The best substrate for the hydantoinase was D,L-5-hydroxyhydantoin. The aromatic substrate, D,L-5-phenylhydantoin and D,L-5-benzylhydantoin, showed 10–20% of relative activities lower than that of D,L-5-hydroxyhydantoin. However, 5,5-diphenylhydantoin and 5-ethyl-3-methyl-5-phenylhydantoin (mephentoin) were not hydrolyzed by this hydantoinase (Table 1). Structurally analogous pyrimidines (uracil, dihydrouracil and dihydrothymine) examined were not digested by this hydantoinase. Besides the substrates in Table 1, barbituric acid, dihydroorotic acid, 5-oxo-proline (pyroglutamic acid), creatinine, and lysine-lactam, which are the substrates for cyclic amidases, were examined as substrates for hydantoinase, but they were not digested by this hydantoinase (data not shown).

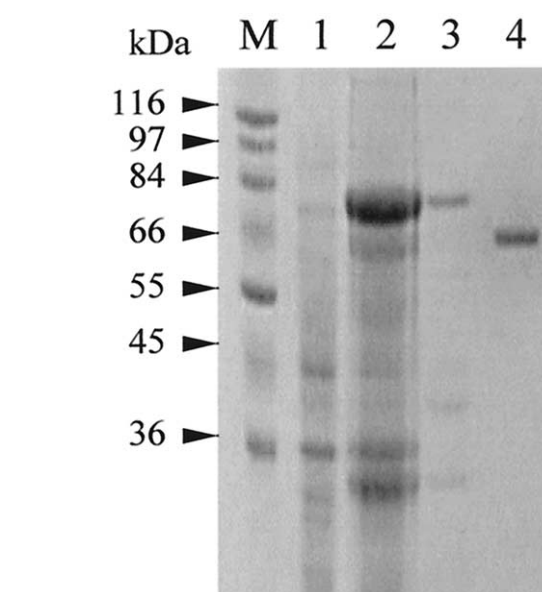


Fig. 2. Purification of *Mj0963* showing SDS-PAGE gel stained with Coomassie brilliant blue. Lane M, protein molecular markers; lane 1, control (crude extract of cells harboring plasmid without insert); lane 2, crude extract of cells harboring plasmid inserted *Mj0963*; lane 3, sample eluted from Ni-NTA column; lane 4, purified sample from gel filtration after thrombin treatment.

Hydantoinase was incubated with 5-hydroxymethylhydantoin and 5-methylhydantoin separately in 100 mM Tris-Cl buffer, pH 8.0 in order to determine the stereospecificity of this protein. After chemical decarbamylation, amino acid produced was analyzed with chiral TLC plate as described in materials and methods. The product of hydantoinase has the  $R_f$  value of 0.77 for the 5-hydroxymethylhydantoin and 0.73 for the 5-methylhydantoin on TLC, which is consistent with  $R_f$  values of 0.77 of L-serine and 0.74 of L-alanine, whereas  $R_f$  values of D-serine and D-alanine were 0.74 and 0.7, respectively. Therefore, this hydantoinase was confirmed to be L-specific. There was absolutely no D-amino acid detected, indicating an highly stereospecific enzyme.

Table 1  
Substrate specific activity and relative activity of hydantoinase

Substrates	Specific activity (units/mg)	Relative activity (%)
Hydantoin	43.6	23.8
5-Methylhydantoin	41.8	22.8
5-Benzylhydantoin	30.6	16.7
5-Hydroxymethylhydantoin	113.5	61.9
5-Phenylhydantoin	19.2	10.5
5-Hydroxyhydantoin	183.4	100
5-Hydroxybutylhydantoin	8.8	4.8
5-Bromobutylhydantoin	6.2	3.4
Mephentoin	ND	ND
5,5-Diphenylhydantoin	ND	ND
1-Methylhydantoin	4.9	2.7
Allantoin	3.5	1.9
Uracil	ND	ND
Dihydrouracil	ND	ND
Dihydrothymine	ND	ND

\* ND : not detected

### 3.3. Effects of pH and temperature

Hydantoinase activity was analyzed in the pH range of 6.0–10.5 with D,L-5-hydroxyhydantoin as a substrate. The optimum pH of this protein was approx. 8.0 (Fig. 3A). The optimum pH of hydantoinase of *Pseudomonas striata* was found to be around 8.0–8.3 for hydantoin, 8.3–8.5 for DL-5-(2-methylthioethyl)hydantoin and 8.8–9.0 for dihydrouracil, which is different from the reported value of pH 9.5 for that of mesophilic *Bacillus* sp. AR9 [18,25]. More than 43% of the maximum activity was retained in a pH 6.5 buffer. At pH 9.5, the activity was 47% of the maximum (Fig. 3A). The optimum pH for hydantoinases from *Bacillus* sp. AR9 [19], *Agrobacterium tumefaciens* [26], and *Pseudomonas fluorescens* DSM84 [27] have been reported to be pH 8.5–10. Dependence of hydantoinase activity on temperature was also investigated with D,L-5-hydroxyhydantoin as a substrate. The optimum temperature was approx. 80°C (Fig. 3B). However, the hydantoinase from *M. jannaschii* exhibit catalytic activity over a broad temperature range, retaining more than 37% of the activity from 40 to 80°C at pH 8.0 (Fig. 3B).

### 3.4. Metal ion dependence of hydantoinase

The effects of various metal ions on enzyme activity were investigated using D,L-5-hydroxyhydantoin as a substrate (Fig. 3C). The metal contents of the purified hydantoinase were determined with an Inductively Coupled Plasma-Mass Spectrometer (ICP-MS, Perkin-Elmer Elan 5000). After dialysis against 50 mM Tris-Cl (pH 8.0) containing 2 mM EDTA during the purification process, metals associated with the hydantoinase was less than 1 µg/mol of

enzyme that were negligible. Enzyme was incubated with various metal ions of 5 mM concentration. The enzyme exhibited significantly higher hydantoinase activity in the presence of some divalent metal ions (Fig. 3C). Manganese ions were the most effective for the activity of the hydantoinase. Magnesium, nickel, and zinc ions also enhanced enzyme activity. This finding suggests that this hydantoinase is metalloenzyme. Several hydantoinases are metal dependent, e.g. for the hydantoinase from *Arthrobacter aureus* DSM 3745, zinc plays an essential role not only for the catalytic activity but also for the stabilization of the active quaternary structure of the hydantoinase [28]

### 3.5. Thermostability of hydantoinase

Thermostability was investigated by incubation of hydantoinase from *M. jannaschii* at 90°C in the buffer containing respective salts, KCl, K-Glutamate, and NaCl. Aliquots were taken at periodic intervals and the residual activity of each aliquot was measured with D,L-5-hydroxyhydantoin as a substrate by standard methods described in the materials and methods section. As shown in Fig. 4, the hydantoinase is relatively stable against heat treatment in the buffer containing a high concentration of salts. The half-life of the hydantoinase was estimated to be approx. 100 min at 90°C in the buffer containing 500 mM KCl. When the optimum salt concentration for the thermostability of hydantoinase was examined between 0.02 and 1 M of KCl, the thermostability of hydantoinase was the highest near 500 mM KCl (data not shown).

## 4. Discussion

We cloned a hyperthermophilic hydantoinase from *Methanococcus jannaschii*. The hydantoinase expressed in *E. coli* was purified and characterized. The amino acid sequence of the Mj0903 was aligned with the hydantoinase from *Pseudomonas* sp. and found to have the conserved histidines (Fig. 1). The role of four strictly conserved histidines has been investigated in mammalian dihydroorotase [29]. The conserved histidines possibly play an essential role in metal binding and participate in catalysis. Site-directed mutagenesis was performed with the D-hydantoinase gene of *B. stearrowthermophilus* to confirm the functional significance of the conserved histidine residues [2]. The four histidines were replaced with Asn, resulting in an almost complete loss of enzyme activity.

The optimum temperature of hydantoinase of *M. jannaschii* was approx. 80°C, retaining more than 37% of their activity at 40°C (Fig. 3B). Recently, thermostable D-hydantoinases from several *Bacillus* sp. have been isolated [19, 30]. They exhibited maximum activities at approx. 65°C, which was reduced to 20–30% of the maximum at 80°C. Studies on the effects of metal ions on enzyme activity indicate that *M. jannaschii* hydantoinase is metalloenzyme

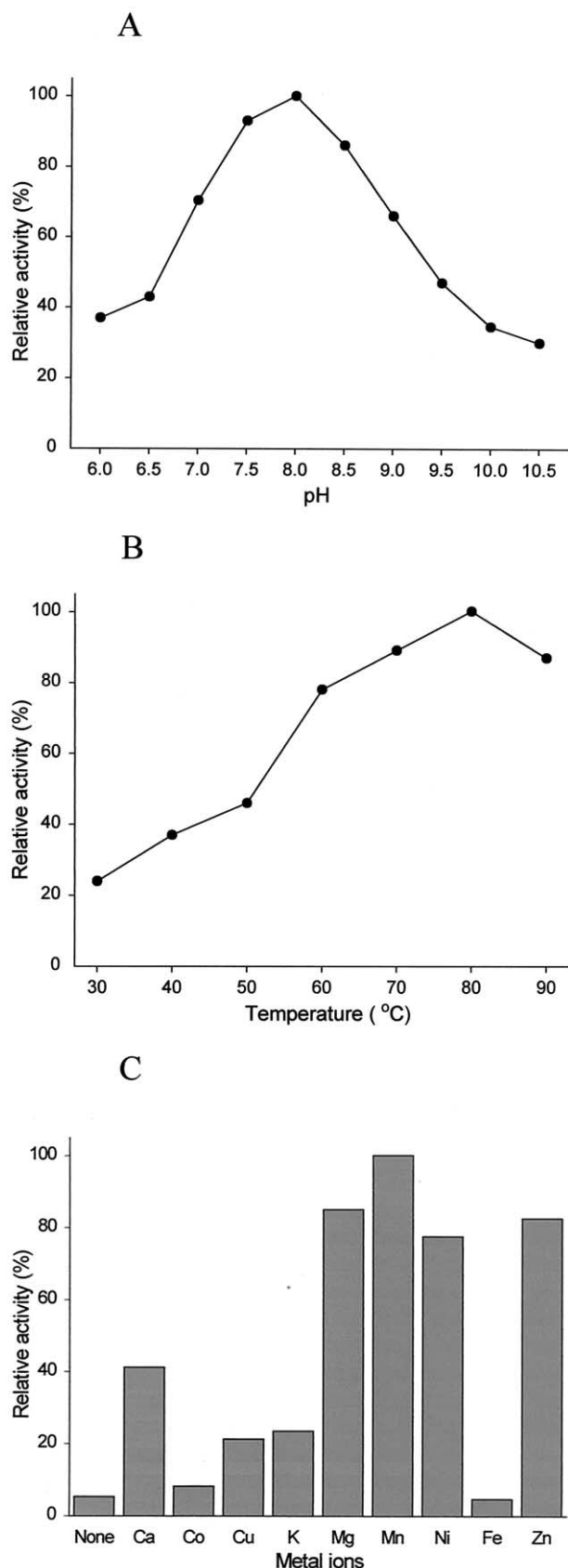


Fig. 3. The effects of pH, temperature and metal ions on hydantoinase activity. (A) The activity assays of pH effect were performed in various buffers of 100 mM. The following buffers were used; MES buffer for pH 6.0–6.5, HEPPES buffer for pH 7.0–7.5, Tris-Cl buffer for pH 8.0–8.5, CHES buffer for pH 9.0–9.5, and CAPS buffer for pH 10.0–10.5. (B) Assays of temperature effect were performed at various temperatures in 100 mM Tris-Cl buffer, pH 8.0. (C) The effects of metal ions were assayed in 100 mM Tris-Cl buffer, pH 8.0. The concentration of each metal ion was 5 mM.

(Fig. 3C). The specific activity of purified hydantoinase from *Agrobacterium* sp. was activated by addition of either 2 mM  $\text{Ni}^{2+}$  ions (up to 2-fold) or  $\text{Mg}^{2+}$  ions (1.5-fold) [31], whereas the D-hydantoinase of *Pseudomonas fluorescens* was activated by  $\text{Fe}^{2+}$  and  $\text{Mn}^{2+}$  ions [27]. The hydantoinase activity of *Bacillus circulans* was stimulated by  $\text{Co}^{2+}$ ,  $\text{Ni}^{2+}$ , and  $\text{Mn}^{2+}$  ions, but the hydantoinase from *Agrobacterium tumefaciens* was not affected by metal ion [26].

The preferred substrate for *M. jannaschii* hydantoinase was D,L-5-hydroxyhydantoin (Table 1). The hydantoinase has the relative activity of 10–20% for the aromatic substrates compared to that for the D,L-5-hydroxyhydantoin. It has been reported that unsubstituted hydantoin is the best substrate for the hydantoinases from mesophilic *Bacillus* sp. AR9 [19] and thermophilic *Bacillus stearothermophilus* SD1 [30]. The hydantoinase from *Bacillus* sp. AR9 had a relative activity of 40% and 19% with D,L-phenylhydantoin and D,L-p-hydroxyphenylhydantoin, respectively [19].

Hydantoinases were classified as D-, L- and non-stereospecific depending on their stereospecificity [32] *M. jannaschii* hydantoinase was confirmed to be L-specific with 5-hydroxymethylhydantoin and 5-methylhydantoin. L- and non-stereospecific hydantoinases have been exploited for the production of optically pure L-amino acids, but their functional roles in microorganisms have not been elucidated. Yamashiro et al. described the mechanism of stereospecific production of L-amino acids from the corresponding 5-substituted hydantoin by *Bacillus brivis* AJ-12299 [25], consisting of the following two successive reactions. The first step was the ring-opening hydrolysis to N-carbamoyl amino acid catalyzed by an ATP dependent L-5-substituted hydantoinase, which was stereospecific and the N-carbamoyl amino acid produced was exclusively the L-form. In the second step, N-carbamoyl-L-amino acid was hydrolyzed to L-amino acid by an N-carbamoyl-L-amino acid hydrolase, which was also an L-specific enzyme.

Under the buffer condition containing 500 mM of KCl, the half-life of *M. jannaschii* hydantoinase was estimated to be approx. 100 min at 90°C (Fig. 4). The thermostable D-hydantoinase from *Bacillus* sp. AR9 exhibited a half-life of 80 min at 70°C [19], whereas the half-life of thermostable D-hydantoinase from the thermophile *Bacillus stearothermophilus* SD1 was 30 min at 80°C [33]. The purified hydantoinase of *M. jannaschii* was suggested to be a tetramer according to the result of the gel filtration chromatography. It has been reported that the native hydantoinases from *Bacillus circulans* [34], *Agrobacterium* sp. [35],

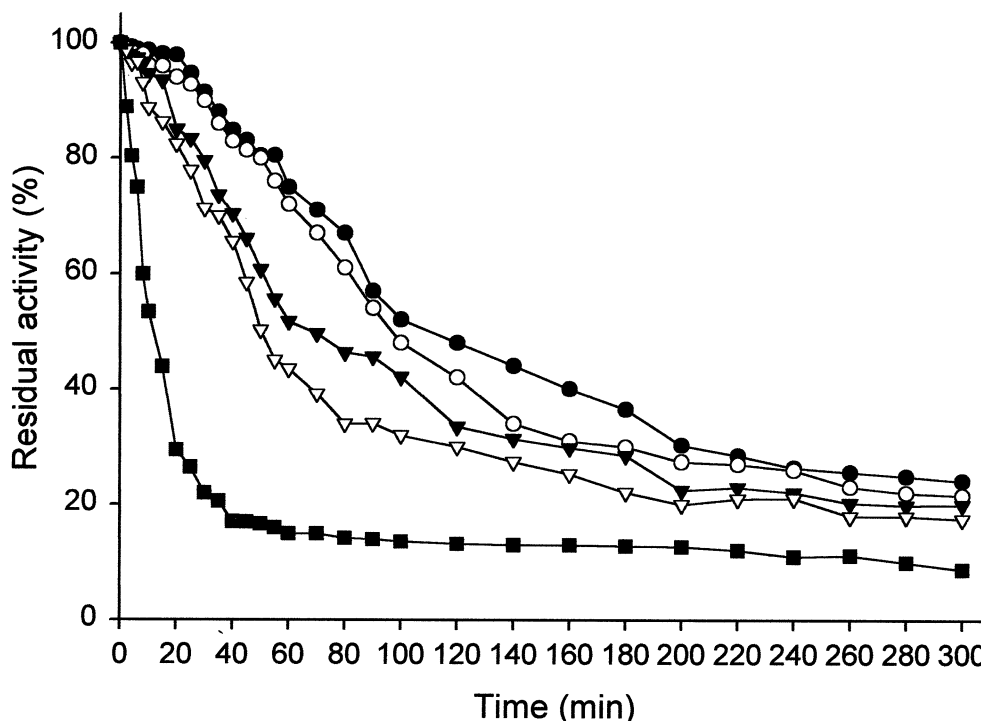


Fig. 4. Thermostability of hydantoinase. The protein was incubated at 90°C in buffer containing each salt. Aliquots were withdrawn at periodic intervals and kept on ice. Residual activity was assayed after all samples had been collected. The relative residual activity was expressed as a percentage compared to the activity of unheated protein. Closed circles, 500 mM KCl; open circles, 500 mM K-Glutamate; closed triangles, 500 mM NaCl; open triangles, 20 mM NaCl; closed squares, thermostability of D-hydantoinase from *Vigna angularis* (Aduki bean) as a control.

and *Bacillus stearothermophilus* NS1122A [36] are tetramers. For the hydantoinase of *Bacillus stearothermophilus* NS1122A, oligomeric structure is required for activity and suggested to contribute to thermostability [36]. The indolepyruvate ferredoxin oxidoreductase (IOR) from hyperthermophilic archaeon *Pyrococcus kodakaraensis* needs high temperature environments for the hetero-oligomerization of IOR subunits [37]. The thermostability of a protein arises from the several forces, including hydrogen bonds, ion pairs, or hydrophobic bonds [38]. Formylmethanofuran: tetrahydromethanopterin formyltransferase (Ftr) protein from *Methanopyrus kandleri* is dependent on the presence of lyotropic salts for activity and thermostability [39]. Ftr is inactive and unstable as a monomer at low concentrations of  $K_2HPO_4$ , but it assembles as an active and thermostable tetramer in the presence of 1 M  $K_2HPO_4$ . As shown in Fig. 4, the *M. jannaschii* hydantoinase was thermally stable in buffer containing high concentration of salts. Oligomerization of hyperthermophilic hydantoinase, which is dependent on heat and salt concentration as stated above, may contribute to its stability. Its thermostability and strict stereospecificity indicate a significant potential for commercial production of amino acids. A great variety of amino acids and derivatives are highly desirable molecules for synthesis of peptides and peptido-mimetics.

The activity yield as well as the operational stabilities of the free enzymes could be significantly improved by immobilization [40]. For the hydantoinase and the L-N-car-

bamoylase from *Arthrobacter aurescens*, the use of the recombinant proteins resulted in enhanced specific activities especially when using a hydrophilic support for immobilization such as Sepharose. If the stability of the *M. jannaschii* hydantoinase can be enhanced, e.g. by immobilization as shown for the hydantoinase from *Arthrobacter aurescens*, the hyperthermophilic hydantoinase will become an important tool in biotransformation of simple precursors to D- and L- amino acids.

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