Antonie van Leeuwenhoek (2005) 88: 189–197
DOI 10.1007/s10482-005-5270-3

Phylogenetic analysis and biochemical characterization of a thermostable dihydropyrimidinase from alkaliphilic *Bacillus* sp. TS-23

Long-Liu Lin[1], Wen-Hwei Hsu[2], Wei-Yi Hsu[2], Shu-Chen Kan[2] and Hui-Yu Hu[3,*]
*[1]Department of Applied Chemistry, National Chiayi University, Chiayi 60083, Taiwan; [2]Institute of Molecular Biology, National Chung Hsing University, Taichung 402, Taiwan; [3]Department of Food and Nutrition, Hungkuang University, Shalu, Taichung 433, Taiwan; *Author for correspondence (e-mail: huiyu @sunrise.hk.edu.tw; fax: +886-4-2631-9176)*

Received 22 February 2005; accepted in revised form 11 April 2005

Key words: Bacillus sp. TS-23, Dihydropyrimidinase, Gene cloning, Phylogeny, Overexpression

Abstract

Two degenerate primers established from the alignment of highly conserved amino acid sequences of bacterial dihydropyrimidinases (DHPs) were used to amplify a 330-bp gene fragment from the genomic DNA of *Bacillus* sp. TS-23 and the amplified DNA was successfully used as a probe to clone a *dhp* gene from the strain. The open reading frame of the gene consisted of 1422 bp and was deduced to contain 472 amino acids with a molecular mass of 52 kDa. The deduced amino acid sequence exhibited greater than 45% identity with that of prokaryotic D-hydantoinases and eukaryotic DHPs. Phylogenetic analysis showed that *Bacillus* sp. TS-23 DHP is grouped together with *Bacillus stearothermophilus* D-hydantoinase and related to dihydroorotases and allantoinases from various organisms. His_6-tagged DHP was over-expressed in *Escherichia coli* and purified by immobilized metal affinity chromatography to a specific activity of 3.46 U mg^{-1} protein. The optimal pH and temperature for the purified enzyme were 8.0 and 60 °C, respectively. The half-life of His_6-tagged DHP was 25 days at 50 °C. The enzyme activity was stimulated by Co^{2+} and Mn^{2+} ions. His_6-tagged DHP was most active toward dihydrouracil followed by hydantoin derivatives. The catalytic efficiencies (k_{cat}/K_m) of the enzyme for dihydrouracil and hydantoin were 2.58 and 0.61 s^{-1} mM^{-1}, respectively.

Introduction

Dihydropyrimidinase (5,6-dihydropyrimidine amidohydrolase, DHP; EC 3.5.2.2), the second enzyme in the breakdown of the pyrimidine bases, catalyzes the degradation of dihydrouracil and dihydrothymine to β-ureidopropionic acid and β-ureidoisobutyric acid, respectively. DHPs are described to have broad substrate specificity and can hydrolyze 5-monosubstituted hydantoins (Syldatk et al. 1990). Yamada et al. (1978) first reported the D-selective cleavage of 5′-monosubstituted hydantoins in microorganisms. They postulated the identity of microbial D-hydantoinases (D-HYDs) with DHPs and proved this hypothesis for the enzyme from *Pseudomonas striata* (Takahashi et al. 1978). Several publications have described D-selective HYDs from *Pseudomonas fluorescens* DSM84 (Morin et al. 1986), *Agrobacterium* sp. IP-I 671 (Runser and Ohleyer 1990), and several *Bacillus* spp. (Lee et al. 1997; Luksa et al. 1997; Sharma and Vohra 1997; Park et al. 1998). Although HYD is frequently used as synonym for prokaryotic DHP, an HYD from *Agrobacterium* species was proved to have no DHP activity (Runser and Meyer 1993). In bioconversion, DHPs are used in cooperation with carbamoylases for the enantioselective production

of non-proteinogenic amino acids from racemic hydantoins (Oliver et al. 1981; Lo et al. 2003). The products serve as the important building blocks for a variety of biologically active pharmaceuticals like semisynthetic β-lactam antibiotics, peptide hormones, angiotensin-converting enzyme inhibitors and pesticides (Syldatk et al. 1990).

Recently, the macromolecular structure of homotetrameric *Thermus* sp. DHP has been determined by X-ray crystallography (Abendroth et al. 2002a). The core of each monomer consists of a triose phosphate isomerase (TIM) barrel domain, which is flanked by a β-sheet domain and some additional helices. The crystal structures of D-HYDs from both *Bacillus stearothermophilus* (Cheon et al. 2002) and *Burkholderia pickettii* (Xu et al. 2003), and L-HYD from *Arthobacter aurescens* (Abendroth et al. 2002b) have also been solved. Additionally, structures of several other amidohydrolases including *Klebsiella aerogenes* urease α-subunit (Jabri et al. 1996), *Escherichia coli* dihydroorotase (DHO) (Thoden et al. 2001), and *Pseudomonas diminuta* phosphotriesterase (Vanhooke et al. 1996) were determined in the past 10 years. Although the amino acid sequences of these enzymes share low similarity, their tertiary structures adopt the same TIM barrel fold. Therefore, a close evolutionary relationship between DHPs and other amidohydrolases was proposed by Holm and Sander (1997) and by May et al. (1998).

We isolated an alkaliphilic and thermoaerobic bacterium, *Bacillus* sp. TS-23, from a hot spring of Taiwan (Lin et al. 1994). The amylolytic enzymes produced by this bacterium have been intensively studied (Lin et al. 1996, 1997; Lo et al. 2001, 2002; Chang et al. 2003). In this investigation, we demonstrate the cloning and biochemical characterization of a DHP enzyme (bsDHP) from this strain. These results will expand our knowledge of *Bacillus* sp. TS-23 D-specific hydantoin amidohydrolase and serves as a solid foundation for subsequent studies on the molecular structure of this enzyme.

Materials and methods

Bacterial strains, plasmids and growth conditions

The bacteria used included *Bacillus* sp. TS-23 (Lin et al. 1994), *Escherichia coli* XL1-Blue MRF' (Stratagene, CA, USA), and *E. coli* Novablue (Novagen, Inc., WI, USA). Vectors used were ZAP Express vector (Stratagene) and pQE-30 (Qiagen, Inc., CA, USA). The medium and growth conditions for *Bacillus* sp. TS-23 were described previously (Lin et al. 1994). *E. coli* cultures were grown in Luria–Bertani (LB) medium supplemented with 100 μg ampicillin ml^{-1}.

DNA methods

Standard DNA manipulation techniques were performed according to Sambrook and Russel (2001). Chromosomal DNA of *Bacillus* sp. TS-23 was prepared as described by Doi et al. (1983). Plasmid DNA was isolated from *E. coli* strains by a Viogene plasmid miniprep kit (Viogene, Inc., Taipei, Taiwan) according to manufacturer's protocol. The primers used were synthesized under the instructions of Oligo analysis software (National Biosciences, MI, USA) with an Applied Biosystems DNA synthesizer model 380A. DNA sequence was determined by Quality Systems (Taipei, Taiwan). Amino acid sequences were analyzed with the program BLAST-X (Altschul et al. 1990) from the National Center for Biotechnology Information (National Library of Medicine, National Institute of Health, MD, USA) and alignment from the ExPASy molecular biology server (Swiss Institute of Bioinformatics)

Preparation of a DNA probe and cloning of the dhp gene

Two degenerate primers, 5′-GCSGCVTTYG-GNGGNACNAC-3′ and 5′-TCNCCRTTY-TCNGCRTGNAC-3′, were designed for PCR preparation of a 330-bp *dhp* gene fragment. PCR amplification was carried out with *Pfu* DNA polymerase in a RoboCycler Temperature Cyclers (Stratagene). PCR amplification was initiated at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 2 min, annealing at 44–66 °C for 2 min, and primer extension at 72 °C for 1 min. Labeling of the amplified fragment with a Megaprime DNA-labeling system (Amersham Biosciences, NJ, USA) and [α–^{32}P]dCTP was performed in accordance with manufacturer's instructions.

A genomic library of *Bacillus* sp. TS-23 was constructed in *Bam*HI/CIAP-treated ZAP Express vector and maintained in *E. coli* XL1-Blue MRF' cells. Cosmid clones containing DNA predicted to encode *dhp* gene were identified by Southern hybridization of the amplified library with the [$\alpha-^{32}$P] dCTP-labeled probe. Hybridizations were performed at 65 °C and membranes were washed under high-stringency conditions. One clone, designated *E. coli* (pBK-CMV5), was selected and subjected to DNA sequencing. Sequences were assembled with Lasergene sequence analysis software (DNASTAR, Inc., WI, USA). Open reading frame (ORF) was identified by similarity to known *dhp* and *hyd* genes.

DHP expression and purification

For high-level gene expression, a DNA fragment encoding the entire DHP was amplified from pBK-CMV5 by PCR with the primers Dhpf, (5′-AGATCTATGAAAAAGCTGATAAAA-3′) and Dhpr (5′-AAGCTTTTAAATCGTTAATTTC-3′) containing artificial *Bgl*II or *Hin*dIII restriction sites. The PCR product was cloned as a *Bgl*II–*Hin*dIII fragment (1.4 kb) into the corresponding sites of pQE-30. After the selected clones had been verified by restriction enzyme analysis, one expression plasmid in which the *dhp* gene is transcribed by a T5 promoter was obtained and designated pQE-DHP.

Escherichia coli Novablue cells containing pQE-DHP were grown in 50 ml of LB medium supplemented with 100 μg ampicillin ml^{-1} at 37 °C for overnight. This preculture (5 ml) was used to inoculate 500 ml of the above-mentioned medium to an optical density of 0.8 at 600 nm. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 1 mM, and the cultivation was continued with shaking at 28 °C for 6 h. The cells were pelleted by centrifugation at 12,000 × *g* for 10 min at 4 °C, washed twice with 50 mM Tris–HCl buffer (pH 8.0), resuspended in 3 ml of binding buffer (5 mM imidazole, 0.5 M NaCl, and 20 mM Tris–HCl; pH 7.9), and disrupted by sonication. The extracts were clarified by centrifugation at 12,000 × *g* for 10 min at 4 °C and the resulting materials were mixed with Ni^{2+}–nitrilotriacetate resin (Ni^{2+}–NTA; Qiagen) pre-equilibrated with the binding buffer. The His_6-tagged DHP was eluted from the Ni^{2+}–NTA resin with a buffer containing 0.5 M imidazole, 0.5 M NaCl, and 20 mM Tris–HCl (pH 7.9).

Protein analyses

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 4% polyacrylamide stacking and 10% polyacrylamide separating gels was performed on a Bio-Rad Mini-Protein III gel apparatus (Bio-Rad Laboratories, MA, USA). Before electrophoresis, the cell-free extracts and purified enzyme were mixed with 2× SDS-sample buffer, heated at 100 °C for 5 min, and centrifuged at 12,000 × *g* for 10 min. Appropriate amounts of the samples were subjected to SDS-PAGE. Protein bands were stained with 0.5% Coomassie Brilliant Blue dissolved in 50% methanol–10% acetic acid, and destained in a 30% methanol–10% acetic acid solution. The protein size markers were myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and trypsin inhibitor (21 kDa). Protein concentration was measured with Bio-Rad Protein Assay kit using bovine serum albumin as the standard.

Enzyme assay

DHP activity was assayed by the method described by Takahashi et al. (1978) with some modifications. Briefly, the reaction mixture (1 ml) contained 10 mM D,L-*p*-hydroxyphenylhydantoin (D,L-*p*-HPH), 50 mM Tris–HCl buffer (pH 8.0) and an appropriate amount of the purified DHP. The mixture was incubated at 50 °C for 10 min and the reaction was stopped by the addition of 200 μl of 12% TCA (w/v). After centrifugation at 12,000 × *g* for 5 min, the resulting material (200 μl) was withdrawn and mixed with 80 μl of *p*-dimethylaminobenzaldehyde (Sigma–Aldrich D-2004, MO, USA). The absorbance at 450 nm was then measured. One unit of DHP activity is defined as the amount of the enzyme that produces 1 μmol of *N*-carbamyol-D-hydroxyphenylglycine per minute under the assay conditions.

Biochemical studies

The effect of pH on DHP activity was performed at 50 °C in 50 mM Tris–maleate buffer (pH 4 – 7), 50 mM Tris – HCl buffer (pH 7 – 9), and 50 mM glycine–NaOH buffer (pH 8.5 – 10). To determine the effect of temperature on DHP activity, assays were performed in Tris – HCl buffer (pH 8.0) at temperatures between 30 and 90 °C. Thermostability of the enzyme was performed by incubating 1.5 ml of the purified DHP (4 U ml^{-1}) in 50 mM Tris – HCl buffer (pH 8.0) and 500 μM $CoCl_2$ for designated times. The residual activity was measured under standard conditions.

A steady-state kinetics study of the purified enzyme was performed at 50 °C in 50 mM Tris – HCl buffer (pH 8.0) and 500 μM $CoCl_2$ with dihydrouracil or hydantoin at concentrations ranging from 1 to 10 mM. The K_m and k_{cat} values were determined by fitting the initial rates as a function of substrate concentration to the Michaelis – Menten equation.

Nucleotide sequence accession number

The sequence of the *Bacillus* sp. strain TS-23 *dhp* gene is available in GenBank under Accession No. DQ 001902.

Results and discussion

Cloning and sequencing of dhp gene from Bacillus *sp. TS-23*

A comparison of the amino acid sequence of DHPs from *B. stearothermophilus* NS1122A (Swiss-Prot Q45515), *B. pickettii* (Swiss-Prot Q8VTT5), *Pseudomonas putide* (Swiss-Prot Q59699), *Agrobacterium tumefaciens* (Swiss-Prot Q44184), and *Streptomyces coelicolor* (Swiss-Prot O69809) identified four conserved motifs. Based on the AAAFGG and VHAENG motifs, two degenerate primers were synthesized and used for PCR amplification of the corresponding fragment. The amino acid sequence deduced from the 330-bp DNA fragment showed high similarity with that of microbial HYDs and the [$\alpha-^{32}$P]dCTP-labeled PCR product could hybridize with *Bam*HI- or *Hin*dIII-restricted *Bacillus* sp. TS-23 genomic DNA (data not shown). Ten positive clones were obtained from the λ-ZAP genomic library by plaque hybridization, in which one clone displayed hydantoinase activity on LB plate containing 1% hydantoin and 0.005% phenol red (Kim et al. 1997a). This clone was mapped with restriction enzymes and subjected to DNA sequencing. The nucleotide sequence of the 3.8-kb insert contained a complete ORF encoding 472 amino acids with a calculated molecular mass of approximately 52 kDa. The DHP-encoding sequence starts with an ATG codon preceded by 9 bp of spacing by a potential ribosome-binding site (5′-AGGAGGA). A putative promoter region (TTAAAA-N19-TAGGAT) is located 164 bp upstream of the ATG start codon. The translational stop codon TAG is followed by a stem–loop structure. Additionally, an incomplete ORF upstream of the *dhp* gene exhibited 40, 42, and 76% identities, respectively, with part of the amino acid sequence of pyrimidine dehydrogenases from *Oryza sativa* (GenBank BAD15901), *Salmonella typhimurium* LT2 (GenBank NP_461132), and *Brevibacillus agri* (Hsu and Kao 2003) and greater than 50% identity with the amino acid sequence of dihydroorotate dehydrogenases from a variety of microorganisms. The 1.3-kb downstream sequence can be transcribed into an incomplete ORF, which shows more than 30% identity in the aligned region with transcription regulators of microbial origin.

Amino acid sequence comparison and phylogenetic analysis

BLAST searching of the GenBank database with the translated ORF resulted in 40 – 87% identities with DHP and HYD sequences from a variety of organisms. The closest match was the D-HYD sequence of *B. stearothermophilus* SD1 (87% amino acid identity; 90% similarity). Multiple sequence alignment between *Bacillus* sp. TS-23 DHP, *B. stearothermophilus* SD1 D-HYD, *Thermus* sp. DHP, *B. pickettii* D-HYD, and *A. aurescens* L-HYD revealed several significantly conserved regions (Figure 1). As reported by Abendroth et al. (2002a), each subunit of the tetrameric *Thermus* sp. DHP consists of an elliptically distorted $(\alpha/\beta)_8$-barrel domain, which hosts the active site, and a β-sheet domain. In the active site, the two zinc ions are bridged by the carbamate group of a

carboxylated lysine residue and these two ions play a role to activate a water molecule for nucleophilic attack on the amide bond of the substrate. Interestingly, the molecular structures of *B. stearothermophilus* D-HYD, *B. pickettii* D-HYD, and *A. aurescens* L-HYD exhibit very little change in the geometry of the active site (Abendroth et al. 2002b; Cheon et al. 2002; Xu et al. 2003). Allantoinases (ALLs) and DHOs are known to catalyze the ring-opening reaction on the cyclic amide bond, like HYDs, and it has been suggested that all belong to the amidohydrolase family (Lapointe et al. 1994). Previously, Zimmermann et al. (1995) have investigated the role of the conserved histidine residues in catalysis and metal binding in mammalian DHO. It was suggested that the active-site zinc ion is coordinated by three histidine residues and that the fourth histidine residue participates in catalysis. In an analysis of deletion mutants of the *hyd* gene from *B. stearothermophilus* SD1, enzyme activity was lost on the removal of His-58 and His-60, but the deletion mutant retaining these two residues exhibited the enzyme activity (Kim et al. 1997b). The aligned data showed that the amino acid residues essential for the metal-binding and the catalytic sites of these enzymes are conserved in *Bacillus* sp. TS-23 DHP (Figure 1).

A phylogenetic analysis showed that *Bacillus* sp. TS-23 DHP is closely related to bacterial D-HYDs and animal DHP-related proteins (data not shown). The *A. aurescens* L-HYD has some unusual properties in regard to the stereospecific hydrolysis of hydantoins (Ogawa and Shimizu 1997). *A. aurescens* is one of the few microorganisms that produce L-HYD, which converts 5-monosubstituted hydantoins into L-amino acids in catalysis (Ogawa and Shimizu 1997), while most other microorganisms produce D-enantiomers. When *A. aurescens* L-HYD is used for the production of methionine, it behaves like normal D-HYD and produces more D-enantiomer than L-enantiomer (Ogawa and Shimizu 1997; May et al. 1998). Surprisingly, a single mutation inverted enantioselectivity to more than 99% of L-enantiomer (May et al. 1998). According to phylogenetic analysis, *A. aurescens* L-HYD is not closely related to the other D-HYDs but the

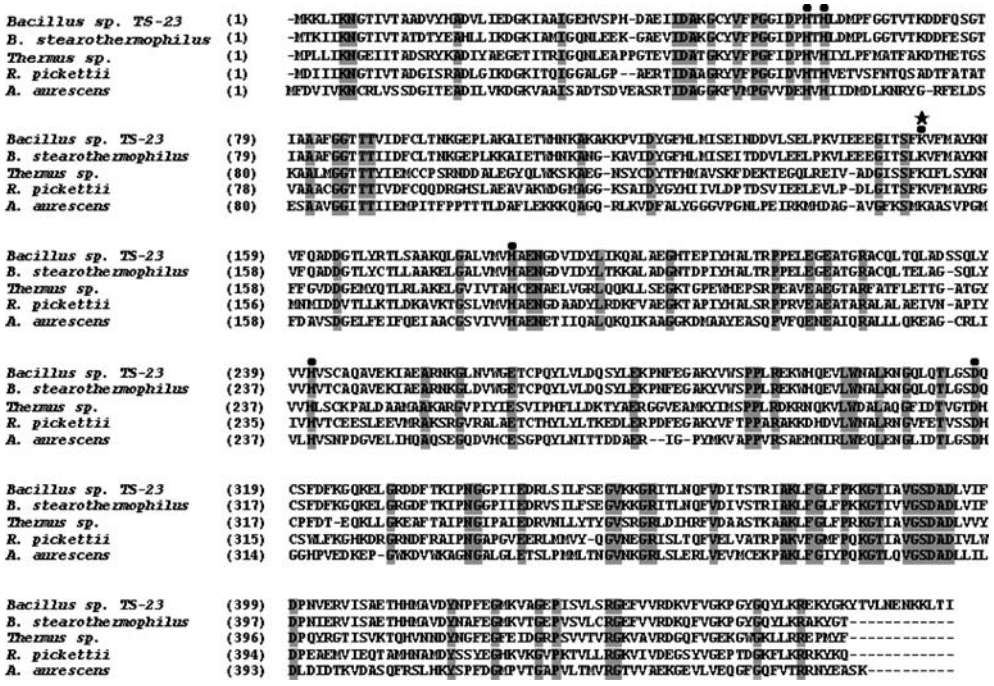

Figure 1. Alignment of the deduced amino acid with microbial DHP and HYDs. The conserved residues are shaded. Gaps are represented by dashes. The active-site residues are marked by closed circles. The carboxylated lysine residue in the amino acid sequences is represented by a star.

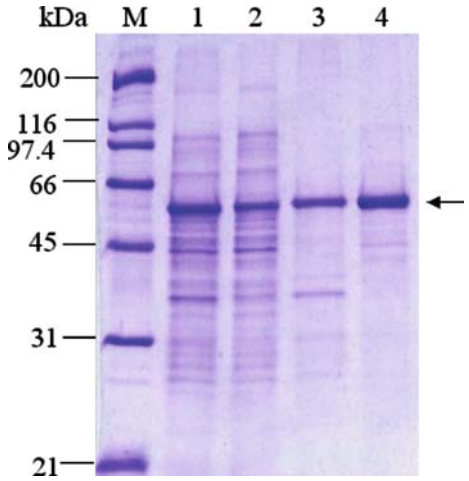

Figure 2. SDS-PAGE analysis of crude cell extract from *E. coli* Novablue (pQE-DHP) and the purified His_6-tagged bsDHP. Lane M, molecular mass marker; lane 1, crude cell extract of the transformed *E. coli*; lane 2, supernatant of crude cell extract; lane 3, pellet of the crude cell extract; lane 4, purified enzyme from the crude cell extract. Arrow indicates the position of His_6-tagged bsDHP.

catalytic ligands of microbial D-HYDs are present in the enzyme (Abendroth et al. 2002b), implying that there is a conserved hydrolytic mechanism. The relative locations of D,L-HYDs, DHPs, ALLs, and DHOs suggested that these enzymes have a common ancestor.

Expression and purification of His_6-tagged bsDHP

For high-level expression of His_6-tagged bsDHP, the PCR-amplified DNA fragment encoding the entire bsDHP was subcloned into the expression vector pQE-30. The expressed protein has 10 additional amino acids, namely MRGSHHHHHH, at its N-terminus. SDS-PAGE analysis of total cell proteins produced by transformed *E. coli* NovaBlue is shown in Figure 2. *E. coli* NovaBlue (pQE-DHP) produced a predominant protein band with an apparent molecular mass of approximately 52 kDa, comparing well with the calculated mass of the affinity-tagged translational product of the *dhp* gene. The recombinant bsDHP in the cell-free extract had a specific activity of 0.11 U mg^{-1} protein. To investigate its biochemical properties, the His_6-tagged enzyme was purified by a Ni^{2+}–NTA resin (Figure 2). The purification resulted in a yield of 15% of the DHP activity and 31-fold increase in specific activity.

Biochemical characterization

The pH and temperature optima for the hydrolysis of D,L-*p*-HPH were determined. The enzyme was active over the pH range 7–9 with maximal activity at pH 8.0 (Figure 3a). The enzyme displayed DHP activity over a temperature range of 50–80 °C and had optimal activity at 60 °C (Figure 3b). The His_6-tagged bsDHP was stable within 10 days at 50 °C with approximately 50% of the enzymatic activity retained after 25 days incubation. The activity and stability of His_6-tagged bsDHP under alkaline conditions and at high temperatures allow rapid bioconversion of aromatic substituted hydantoins. Since the solubilities of aromatic substituted hydantoins in aqueous solution are very low, the bioconversion of these compounds must be performed at high temperature to obtain better productivity. Moreover, spontaneous racemization of aromatic substituted hydantoins by ketoenol tautomerism can occur easily in weak alkaline solution, in which theoretical quantitative yields of enantiopure amino acids may be obtained (Faber 2000).

It has been known that the enzyme activity of the cyclic amidohydrolase family is partly or completely affected by the presence of their cofactors (Ogawa and Shimizu 1997; Syldatk et al. 1999). To determine the specificity with respect to the metal requirement, the enzyme solution (specific activity, 3.46 U mg^{-1} protein; 250 μg ml^{-1}) was first dialyzed with the chelating agent EDTA against metal-free buffer (50 mM Tris–HCl, pH 8.0). Enzyme activity was then determined in the presence of different metal ions under standard assay conditions. The results indicated that bsDHP is a metal-dependent enzyme. Though the enzymatic activity of bsDHP can be stimulated by various metal ions, the most effective metal was Co^{2+} (Table 1). This observation is consistent with that of *B. stearothermophilus* NS1122A and *B. pickettii* D-HYDs (Mukohara et al. 1994; Xu et al. 2003). The substitution of zinc with cobalt (Brooks et al. 1983; Huang and Yang 2002) and cadmium (Jahnke et al. 1993) has been carried out in DHPs. Interestingly, the activity of metal-reconstituted pig liver DHP is closely related to the

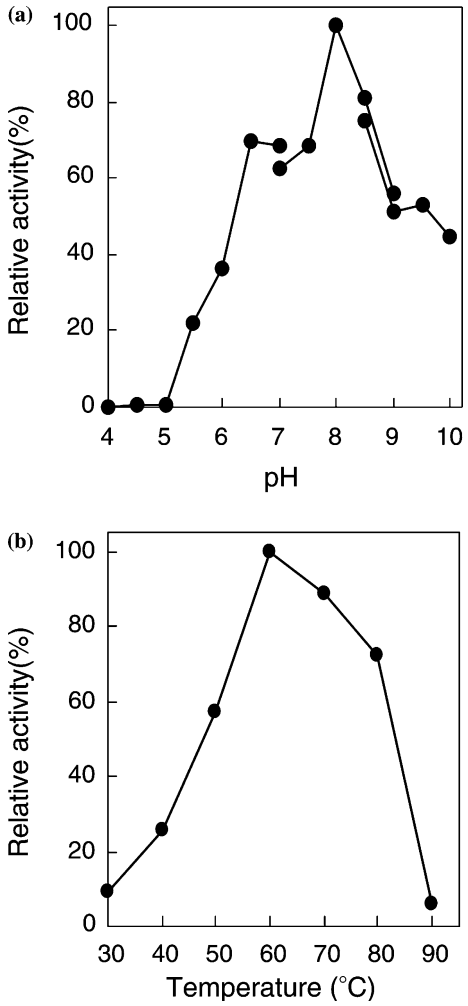

Figure 3. Effect of pH (a) and temperature (b) on the activity of the purified His_6-tagged bsDHP. Enzyme activities were measured at various pH values and temperatures under standard assay conditions.

Table 1. Metal dependency of *Bacillus* sp. TS-23 DHP.

Metal ions	Size of metal ion (pm)	Specific activity (U mg^{-1})
None	–	0.52 ± 0.04
Co^{2+}	72	3.60 ± 0.39
Mn^{2+}	80	2.38 ± 0.18
Zn^{2+}	69	0.73 ± 0.12
Cu^{2+}	71	0.68 ± 0.09
Mg^{2+}	71	0.54 ± 0.04
Ni^{2+}	69	0.49 ± 0.02
Ca^{2+}	114	0.46 ± 0.02
Fe^{2+}	74	0.36 ± 0.01

The EDTA-dialyzed enzyme was incubated with different metal ions at a concentration of 500 μM at 50 °C for 10 min. Enzyme activity was measured under standard assay conditions in the absence of 500 μM $CoCl_2$.

Table 2. Substrate specificity of *Bacillus* sp. TS-23 DHP.

Substrate	Specific activity (U mg^{-1})
Dihydrouracil	3.56 ± 0.25
D,L-*p*-Hydroxyphenylhydantoin	1.69 ± 0.17
D,L-5-[2-(Methylthio)-ethyl]hydantoin	0.62 ± 0.09
Hydantoin	0.17 ± 0.04
Allantoin	0.03 ± 0.01
Dihydroorotate	0.02 ± 0.01
1-*n*-Butylhydantoin	–[a]
1-Methylhydantoin	–

[a]–, not detected.

size of metal ions and the metal ion size similar to zinc ion has a profoundly stimulatory effect (Huang and Yang 2002); however, the stimulation of bsDHP by metal ions was independent of their sizes. More study is needed to elucidate the inconsistence between pig liver DHP and bsDHP.

The hydrolytic activity of the EDTA-dialyzed/Co^{2+}-substituted enzyme against several potential substrates was determined. Putative natural substrates like allantoin or dihydroorotate were not accepted by the DHP (Table 2). The enzyme had a highest activity toward dihydrouracil, which is described to be the substrate for DHPs, while the D-HYD from *B. stearothermophilus* SD1 showed a considerable activity for both dihydrouracil and allantoin (Kim and Kim 1998). To our knowledge, DHP and HYD with DHO activity have not yet been reported. Similarly, no DHO and ALL have been shown to exhibit activity toward dihydropyrimidines and structurally related hydantoins. As shown in Table 2, bsDHP also exhibited significant activity against D,L-*p*-hydroxyphenylhydantoin and D,L-5-[2-(methylthio)-ethyl] hydantoin. These results indicated that the substrate-binding site of bsDHP allows only a limited number of structural modifications for a potential substrate and this enzyme is highly specialized for the degradation of pyrimidines.

The affinity for dihydrouracil (K_m), turnover rate (k_{cat}), and catalytic efficiency (k_{cat}/K_m) of the purified bsDHP were estimated to be 2.4 mM, 6.2 s^{-1}, and 2.58 s^{-1} mM^{-1}, respectively, while that for hydantoin were 5 mM, 3.04 s^{-1}, and 0.61 s^{-1} mM^{-1}, respectively. Kinetic constants of *B. stearothermophilus* SD1 D-HYD for hydantoin were 98 mM and 440 s^{-1} (Cheon et al. 2004). From the point of view of structure and phylogenetics, as well as substrate

specificity, bsDHP and *B. stearothermophilus* SD1 D-HYD represent a very uniform group, but the kinetic properties differ significantly from one enzyme to the other.

Conclusion

These results show that stereoselectivity cannot be the only basis for the classification of the cyclic amidohydrolase family, which includes DHP, HYD, ALL, and DHO. This superfamily is part of the $(\beta/\alpha)_8$-barrel group of enzymes with conserved metal binding and catalytic residues at the end of β-sheets (Holm and Sander 1997; Thoden et al. 2001; Abendroth et al. 2002a, b; Cheon et al. 2002). It is believed that extant proteins have evolved from a limited number of ancestral proteins (Chothia 1992). The driving force of protein evolution is a random mutagenesis of the amino acid sequence. Natural selection modulates the rate of protein divergence by imposing constraints on changes in the amino acid sequence. Resolution of the three-dimensional structure of related enzymes is expected to provide a more definitive and evolutionary relationship among this enzyme family.

Acknowledgments

This work was supported by a research grant (92AS-4.2.1-FD-Z4) from the Council of Agriculture of the Republic of China.

References

Abendroth J., Niefind K. and Schomburg D. 2002a. X-ray structure of a dihydropyrimidinase from *Thermus* sp. at 1.3 Å resolution. J. Mol. Biol. 320: 143–156.

Abendroth J., Niefind K., May O., Siemann M., Syldatk C. and Schomburg D. 2002b. The structure of L-hydantoinase from *Arthobacter aurescens* leads to an understanding of dihydropyrimidinase substrate and enantio specificity. Biochemistry 41: 8589–8597.

Altschul S.F., Gish W., Miller W., Meyers E.W. and Lipman D.J. 1990. Basic local alignment search tool. J. Mol. Biol. 215: 403–410.

Brooks K.P., Jones E.A., Kim B.D. and Sander E.B. 1983. Bovine liver dihydropyrimidine amidohydrolase: purification, properties and characterization as a zinc metalloenzyme. Arch. Biochem. Biophys. 226: 469–483.

Chang C.T., Lo H.F., Chi M.C., Yao C.Y., Hsu W.H. and Lin L.L. 2003. Identification of essential histidine residues in a recombinant α-amylase of thermophilic and alkaliphilic *Bacillus* sp. strain TS-23. Extremophiles 7: 505–509.

Cheon Y.H., Kim H.S., Han K.H., Abendroth J., Niefind K., Schomburg D., Wang J. and Kim Y. 2002. Crystal structure of D-hydantoinase from *Bacillus stearothermophilus*: insight into the stereochemistry of enantioselectivity. Biochemistry 41: 9410–9417.

Cheon Y.H., Park H.S., Kim J.H., Kim Y.S. and Kim H.S. 2004. Manipulation of the active site loops of D-hydantoinase, a (β/α)8-barrel protein, for modulation of the substrate specificity. Biochemistry 43: 7413–7420.

Chothia C. 1992. Proteins: one thousand families for the molecular biologist. Nature (London) 357: 543–544.

Doi R.H., Rodriguez R.L. and Trait R.C. 1983. Recombinant DNA Techniques: An Introduction. Addison-Wesley, Reading, MA, pp. 162–164.

Faber K. 2002. Biotransformations in Organic Chemistry. Springer-Verlag, Berlin, pp. 60–62.

Holm L. and Sander C. 1997. An evolutionary treasure: unification of a broad set of amidohydrolases related to urea. Proteins 28: 72–82.

Hsu W.H. and Kao C.H. 2003. A gene cluster involved in pyrimidine reductive catabolism from *Brevibacillus agri* NCHU 1002. Biochem. Biophys. Res. Commun. 303: 848–854.

Huang C.Y. and Yang Y.S. 2002. The role of metal on imide hydrolysis: metal content and pH profiles of metal ion-replaced mammalian imidase. Biochem. Biophys. Res. Commun. 297: 1027–1032.

Jabri J. and Karplus P.A. 1996. Structures of the *Klebsiella aerogenes* urease apoenzyme and two active-site mutants. Biochemistry 35: 10616–10626.

Jahnke K., Podschun B., Schnackerz K.D., Kautz J. and Cook P.F. 1993. Acid–base catalytic mechanism of dihydropyrimidinase from pH studies. Biochemistry 32: 5160–5166.

Kim G.J. and Kim H.S. 1998. Identification of the structural similarity in the functionally related amidohydrolases acting on the cyclic amide ring. Biochem. J. 330: 295–302.

Kim G.J., Lee S.G., Park J.H. and Kim H.S. 1997a. Direct detection of the hydantoinase activity on solid agar plates and electrophoretic acrylamide gels. Biotechnol. Tech. 11: 511–513.

Kim G.J., Park J.H., Lee D.C., Ro H.S. and Kim H.S. 1997b. Primary structure, sequence analysis, and expression of the thermostable D-hydantoinase from *Bacillus stearothermophilus* SD1. Mol. Gen. Genet. 255: 152–156.

Lapointe G., Viau S., Leblanc D., Robert N. and Morin A. 1994. Cloning, sequencing, and expression in *Escherichia coli* of the D-hydantoinase gene from *Pseudomonas putida* and distribution of homologous genes in other microorganisms. Appl. Environ. Microbiol. 60: 888–895.

Lee S.G., Lee D.C. and Kim H.S. 1997. Purification and characterization of thermostable D-hydantoinase from thermophilic *Bacillus stearothermophilus* SD-1. Appl. Biochem. Biotechnol. 62: 251–266.

Lin L.L., Tsau M.R. and Chu W.S. 1994. General characteristics of thermostable pullulanases and amylases from an alkaliphilic *Bacillus* sp. TS-23. Appl. Microbiol. Biotechnol. 42: 51–56.

Lin L.L., Tsau M.R. and Chu W.S. 1996. Purification and properties of a 140 kDa amylopullulanase from thermophilic and alkaliphilic *Bacillus* sp. strain TS-23. Biotechnol. Appl. Biochem. 24: 101–107.

Lin L.L., Hsu W.H. and Chu W.S. 1997. A gene encoding for an α-amylase from thermophilic *Bacillus* sp. strain TS-23 and its expression in *Escherichia coli*. J. Appl. Microbiol. 82: 325–334.

Lo H.F., Lin L.L., Li C.C., Hsu W.H. and Chang C.T. 2001. The N-terminal signal sequence and the last 98 amino acids are not essential for the secretion of *Bacillus* sp. TS-23 α-amylase in *Escherichia coli*. Curr. Microbiol. 43: 170–178.

Lo H.F., Lin L.L., Chiang W.Y., Chi M.C., Hsu W.H. and Chang C.T. 2002. Deletion analysis of the C-terminal region of the α-amylase of *Bacillus* sp. strain TS-23. Arch. Microbiol. 178: 115–123.

Lo H.H., Kao C.H., Lee D.S., Yang T.K. and Hsu W.H. 2003. Enantioselective synthesis of (*S*)-2-amino-4-phenylbutanoic acid by the hydantoinase method. Chirality 15: 699–702.

Luksa V., Starkuviene V., Starkuviene B. and Dagys R. 1997. Purification and characterization of the D-hydantoinase from *Bacillus circulans*. Appl. Biochem. Biotechnol. 62: 219–231.

May O., Habenicht A., Mattes R., Syldatk C. and Siemann M. 1998. Molecular evolution of hydantoinases. Biol. Chem. 379: 743–747.

Morin A., Hummel W., Schutte H. and Kula M.R. 1986. Characterization of hydantoinase from *Pseudomonas fluorescens* strain DSM 84. Biotechnol. Appl. Biochem. 8: 564–574.

Mukohara Y., Ishikawa T., Watabe K. and Nakamura H. 1994. A thermostable hydantoinase of *Bacillus stearothermophilus* NS1122A: cloning, sequencing, and high expression of the enzyme gene, and some properties of the expressed enzyme. Biosci. Biotechnol. Biochem. 58: 1621–1626.

Ogawa J. and Shimizu S. 1997. Diversity and versatility of microbial hydantoin-transforming enzymes. J. Mol. Catal. B: Enzym. 2: 163–176.

Oliver R., Fascetti E., Angelini L. and Degen L. 1981. Microbial transformation of racemic hydantoins to D-amino acids. Biotechnol. Bioeng. 23: 2173–2183.

Park J.H., Kim G.J., Lee S.G. and Kim H.S. 1998. Biochemical properties of thermostable D-hydantoinase from *Bacillus thermocatenulatus* GH-2. Ann. N. Y. Acad. Sci. 864: 337–340.

Runser S.M. and Ohleyer E. 1990. Properties of the hydantoinase from *Agrobacterium* sp. IP I-671. Biotechnol. Lett. 12: 259–264.

Runser S.M. and Meyer P.C. 1993. Purification and biochemical characterization of the hydantoin hydrolyzing enzyme from *Agrobacterium* species: a hydantoinase with no 5,6-dihydropyrimidine amidohydrolase activity. Eur. J. Biochem. 213: 1315–1324.

Sambrook J. and Russel D.W. 2001. Molecular Cloning: A Laboratory Manual, 3rd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA.

Sharma R. and Vohra R.M. 1997. A thermostable D-hydantoinase isolated from a mesophilic *Bacillus* sp. AR9. Biochem. Biophys. Res. Commun. 234: 485–488.

Syldatk C., Laüfer A., Müller R. and Höke H. 1990. Production of optically pure D- and L-α-amino acids by bioconversion of D,L-5-monosubstituted hydantoin derivatives. Adv. Biochem. Eng. Biotechnol. 41: 29–75.

Syldatk C., May O., Altenbuchner J., Mattes R. and Siemann M. 1999. Microbial hydantoinases – industrial enzymes from the origin of life? Appl. Microbiol. Biotechnol. 51: 293–309.

Takahashi S., Kii Y., Kumagai H. and Yamada H. 1978. Purification, crystallization and properties of hydantoinase from *Pseudomonas striata*. J. Ferment. Technol. 56: 492–498.

Thoden J.B., Philips G.N.J., Neal T.M., Raushel F.M. and Holden H.M. 2001. Molecular structure of dihydroorotase: a paradigm for catalysis through the use of a binuclear metal center. Biochemistry 40: 6989–6997.

Vanhooke J.L., Benning M.M., Raushel F.M. and Holdens H.M. 1996. Three-dimensional structure of the zinc-containing phosphotriesterase with the bound substrate analog diethyl 4-methylbenzylphosphonate. Biochemistry 35: 6020–6025.

Xu Z., Liu Y., Yang Y., Jiang W., Arnold E. and Ding J. 2003. Crystal structure of D-hydantoinase from *Burkholderia pickettii* at a resolution of 2.7 angstroms: insights into the molecular basis of enzyme thermostability. J. Bacteriol. 185: 4038–4049.

Yamada H., Takahashi S., Kii Y. and Kumagai H. 1978. Distribution of hydantoin hydrolyzing activity in microorganisms. J. Ferment. Technol. 56: 484–491.

Zimmermann M.K., Kemling N.M. and Evans D.R. 1995. Function of conserved histidine residues in mammalian dihydroorotase. Biochemistry 34: 7038–7046.