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Characterization of Thermostable Hydantoinases Cloned from *Geobacillus stearothermophilus*

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Keywords: *hydantoinase, thermophiles, Geobacillus stearothermophilus***,** *optical pure amino acids.*

Introduction. Among cyclic amidohydrolases those displaying the hydantoinase activity are employed in a two-step biocatalytical production of optically pure D- and L-amino acids from hydantoin racemates. Hydantoinases stereospecifically cleave the amide ring of D,L-5-monosubstituted hydantoins to form the corresponding *N*-carbamoyl amino acids, which can be further converted to free D- or L-amino acids by stereospecific carbamoylases [(*N*-carbamoylamidohydrolase; EC 3.5.1.6) [1]. Enzymes of pyrimidine metabolism are considered to exhibit the hydantoinase activity [2]. Both non-stereospecific and D- or L-stereospecific hydantoinases have been detected in many soil bacteria [3, 4, 5]. In *Arthrobacter aurescens* DSM 3745 the stereospecificity of hydantoinase has been shown to be substrate dependent [6]. The hydantoinase gene has been located on an indigenous 172-kb plasmid in the *Pseudomonas* sp. NS671 strain [7] that allowed postulating an extrachromosomal origin for this gene in bacteria.

Hydantoinases of thermophilic bacteria are of particular interest for biocatalysis. ATP-independent hydantoinases have been detected in *G. stearothermophilus* NS1122A and SD1 strains [8, 9] and the corresponding genes have been cloned and characterized [10, 11]. Though amino acid sequences of *G. stearothermophilus* hydantoinases have been found to be very similar, but the *G. stearothermophilus* SD1 enzyme exhibits strict D-stereospecificity whereas the *G. stearothermophilus* NS1122A enzyme is non-stereospecific.

The discrepancy in substrate specificity exhibited by similar cyclic amidohydrolases as well as the scarcity of data on genes involved in hydantoin metabolism emphasizes the necessity to obtain further insights into their structure-function relationships. In this study we have cloned and characterized

two thermostable hydantoinases of *G. stearothermophilus*. The genetic and enzymatic analyses indicate that the hydantoinases are involved in pyrimidine metabolism.

Methods. **Genomic bank construction and hydantoinase gene cloning**. DNA of *G. stearothermophilus* ATCC 31783 was partially digested with *Sau3A* endonuclease and 2-5 kb fraction was ligated with *Bam*HI digested λZAP phagemid vector (Stratagene). The ligated DNA was packaged into λ phage particles and the *E. coli* XL1 Blue MRF' strain was infected with these particles. The phage plaques were transferred onto nylon filtres and hybridized with a 371-bp hydantoinase- specific DNA probe of *G. stearothermophilus* ATCC 31783. The hydantoinase-specific DNA was labeled by random-primer incorporation of digoxigenin labeled 11-dUTP (DIG DNA labeling kit, Boehringer Mannheim). Coding DNA of ATCC 31783 and ATCC 31195 hydantoinase gene was amplified by PCR, with simultaneous creation of *Nco*I and *EcoR*I flanking sites and inserted into pET2ld(+) vector. The cloned hydantoinase genes were overexpressed in *E.coli* BL2l(DE3) cells.

Hydantoinase activity. The *G. stearothermophilus* and *E. coli* cells were harvested by centrifugation, washed in Tris-HCl buffer 0.1 M, pH 8.0 and sonicated (10 min by pulsing 10 s at 19 kHz) in the same buffer in presence of 2 mM $MnS0_4$ at 4°C. Hydantoinase activity was checked over *G. stearothermophilus* cells growing at 56°C in the presence of 50-100 mM substrate, 500 µl intact cells, $MnSO_4$ 10 mM and Tris-HCl buffer 25 mM, pH 8.8. Characterization of the recombinant enzymes was performed on a partially purified extract obtained after heat treatment (15 min at 60°C) and desalting on a PD-10 column (Pharmacia) as described previously [12] and the *N*-carbamoyl derivatives produced were quantified as described [13].

Pulsed-Field Gel Electrophoresis (PFGE). *G. stearothermophilus* cells grown at 56°C in LB-broth were harvested in the exponential phase (OD_{600} 0.8), pelleted by centrifugation for 10 min at 5000 g, washed in Tris 10 mM, Na_2EDTA 1 mM, pH 8.0 and then resuspended in Tris 10 mM, Na_2EDTA 100 mM, pH 7.5 at a final titer 10^8 cells/ml. An aliquot of a suspension was immediately mixed with an equal volume of 1.5% low melting agarose and the embedded cells were then treated with lysozyme and pronase as described by Daniel [14]. Agarose blocks were treated by 30 U of *Sma*I, *Not*I or *Sfi*I restriction endonucleases (New England Biolabs) for 3 h, respectively at 25°C, 37°C and 50°C. Electrophoresis was carried out in a 1% agarose gel on a TAFE apparatus (Beckman Geneline I) with running buffer composed of Tris 10 mM, acetate 4.35 mM, free acid EDTA 0.5 mM, pH 8.2 at l0°C at 140 mA (constant) and with pulse times of 20 s for 16 h.

Results and discussion. **Cloning and analysis of the hydantoinase gene.** Bacterial strains of *G. stearothermophilus* were tested on their capacity to convert hydantoin to *N*-carbamoyl glycine. High hydantoinase activity was detected in *G. stearothermophilus* ATCC 3l783 strain. Therefore, a gene library of this strain was constructed using lZap vector. Hydantoinase gene carrying clones were detected among 25.000 tested phage plaques; all positive clones acquired from 1-kb to 3-kb DNA fragments. Next, a 1539-bp DNA fragment

carrying the hydantoinase gene from another strain, ATCC 31195 was also amplified by PCR using a pair of degenerated oligonucleotides.
The cloned DNA fragments from two strains were sequenced and 93.9% identity was found between their hydantoinase genes. Alignment of cloned *G. stearothermophilus* and two other hydantoinase genes with revealed the highest relatedness between the ATCC 31783 and SD1 enzymes (Fig. 1).

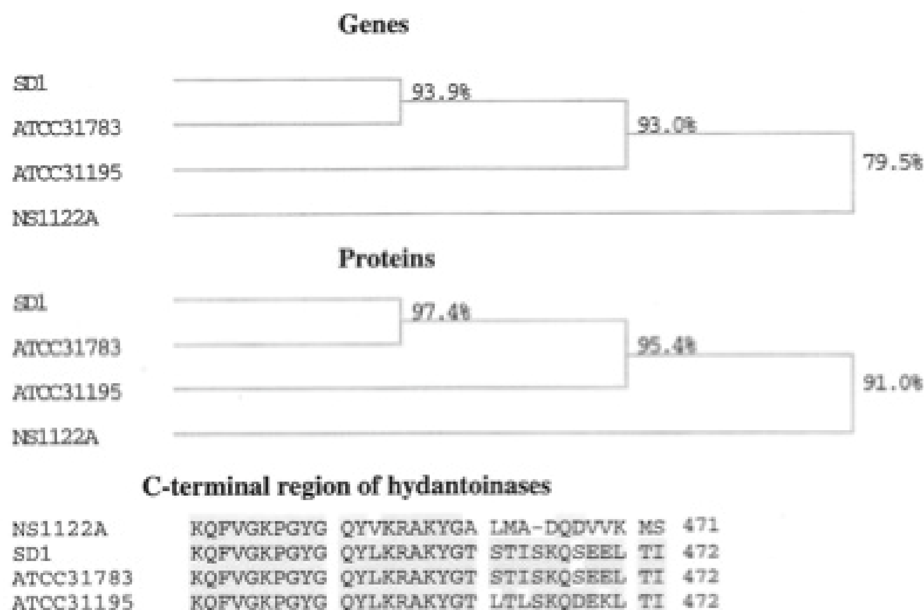

Fig. 1. A relatedness of *G. stearothermophilus* hydantoinases established by nucleotide and amino acid sequence comparisons. Only C-terminal portions of proteins are shown.

With the exception of the C-terminal region, the amino acid sequences of G. stearothermophilus hydantoinases were found to be highly similar. The G. stearothermophilus hydantoinases share high similarity with dihydropyrimidinases and to a lesser extent with dihydroorotases, allantooinases and ureases (data not shown).

Overexpression and substrate specificity of hydantoinases. The cloned hydantoinase genes were overexpressed in *E. coli* cells. The recombinant hydantoinases retained their activity towards hydantoin within pH 8-10 with the highest activity at pH 9.0 and at 60°C. The activity of the enzymes was ATP-independent. Enzymes lost their activity after dialysis against EDTA; however, addition of Mn^{2+} to the reaction mixture restored the activity to almost its initial level. The thermostable enzymes hydrolysed D-5-(2-methylthioethyl) hydantoin but not L-5-(2-methylthioethyl) hydantoin (Table 1). The specific activity of recombinant enzymes was found to be rather high in the pellets obtained after centrifugation of *E. coli* cell extracts (1.4 and 4.9 $U \cdot mg^{-1}$ protein, respectively for ATCC 31195 and ATCC 31783 enzymes) in comparison with the supernatant fraction (3.0 and 4.7 $U \cdot mg^{-1}$ protein, respectively for *G. stearothermophilus* ATCC 31195 and ATCC 31783 encoded enzymes). This

could be related with low solubility of recombinant enzymes under the conditions used.

Table 1

Affinity and activity of partially purified recombinant hydantoinases towards different substrates

Bacteria	Substrate	Km (mM)	Vmax ($U \cdot mg^{-1}$ protein)
G. stearothermophilus ATCC 31195	Dihydrouracil Dihydrothymine Dihydroorotate D-5-(2-methylthioethyl)hydantoin	1.5 0.3 - 4.0	71 6.2 N.D. 3.4
G. stearothermophilus ATCC 31783	Dihydrouracil Dihydrothymine Dihydroorotate D-5-(2-methylthioethyl)hydantoin	0.8 0.7 - 2.5	30 6.1 N.D. 5.9

N.D. - non detectable

The partially purified hydantoinases were also tested for their ability to cleave the amide ring of pyrimidine metabolism intermediates (see Table 1). No activity was detected towards dihydroorotate. Both enzymes exhibited higher affinity towards dihydrouracil and dihydrothymine, than towards D-5-(2-metltylthioethyl)hydantoin. Thus, the catalytic properties of recombinant hydantoinases indicated their affiliation to dihydropyrimidinase family of enzymes.

Location of the hydantoinase gene. PFGE analysis revealed the presence of several high molecular weight plasmid DNAs in *G. stearothermophilus* ATCC 31783 and ATCC 31195 strains (Fig. 2). Both undigested total and plasmid DNAS as well as *Sma*I-, *Not*I- or *Sfi*I-generated DNA fragments were probed to hydantoinase-specific DNAs. Analysis of the hybridized DNA fragments clearly showed that the hydantoinase gene is located on the chromosome of both *G. stearothermophilus* strains.

The gene of hydantoinase has been found to be clustered with L-*N*-carbamoylase and hydantoin racemase genes in the *Pseudomonas sp*. NS671 strain [7, 15] and in *Microbacterium liquefaciens* AJ 3912 [16]. Recently, we have cloned large 12.7-kb region of *G. stearothermophilus* ATCC 31783 genome. Partial sequencing of this region shows that the hydantoinase gene is preceded by a sequence sharing high similarity with eukaryotic dihydropyrimidine dehydrogenase. This observation along with the data presented here indicates that hydantoinase is involved in pyrimidine degradation *via* reductive pathway in a thermophile bacterium *G. stearothermophilus*. Noteworthy, that because of the biotechnological importance of *Geobacillus* species, five genome-sequencing projects are running at the moment in different

laboratories (http://www.ncbi.nlm.nih.gov/genome), which will provide valuable information on metabolic pathways in these bacteria.

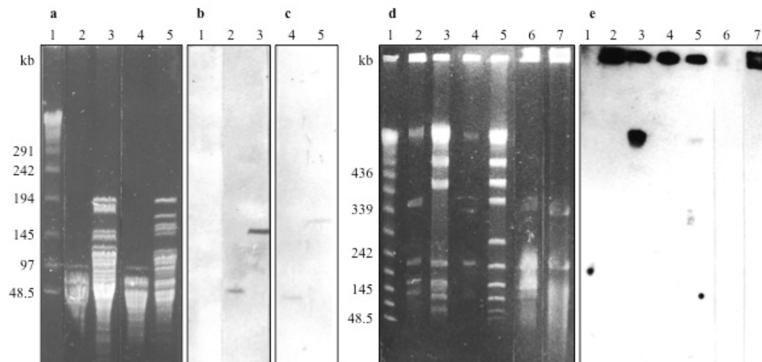

Fig. 2. PFGE of *G. stearothermophilus* ATCC 31195 and ATCC 31783 total and plasmid DNAs (a and d) and hybridization with two hydantoinase-specific DNA probes: a 371-bp DNA fragment of ATCC 31783 (c and e) and a complete 1.4-kb hydantoinase sequence of ATCC 31195 (b). Lanes 2, 3 (a, b), and 5 (d, e) - total DNA of ATCC 31195 digested with *Sma*I (2a, 2b), *Not*I (3a, 3b) and *Sfi*I (5d, 5e); Lanes 4, 5 (a, c) and 3 (d, e) - total DNA of ATCC 31783 digested with *Sma*I (4a, 4c), *Not*I (5a, 5c) and *Sfi*I (3d, 3e). Lanes 2 (d, e) - total DNA of ATCC 31783. Lanes 6 (d, e) –plasmids of ATCC 31783. Lanes 4 (d, e) - total DNA of ATCC 31195. Lanes 7 (d, e) – plasmids of ATCC 31195. Lanes 1 (a, b, d, e), DNA concatemers of the phage λ genome (New England Biolabs) indicated in kb beside the gel.

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Characterization of Thermostable Hydantoinases Cloned from *Geobacillus stearothermophilus*

Recombinant hydantoinases are thermostable metallo-dependent enzymes, which exhibit D-stereospecificity and show high affinity for dihydrouracil and dihydrothimine and no for dihydroorotic acid. The protein sequences are closer to eukaryotic dihydropyrimidinase than to bacterial dihydroorotase and other cyclic amidohydrolases. Analysis total and large plasmid DNAs separated by pulsed-field gel electrophoresis

and hybridized with specific molecular probes revealed that the hydantoinase gene is located on chromosome in both strains. The data presented show that hydantoinase is involved in pyrimidine reductive degradation pathway in *G. stearothermophilus.*

Պ. Վեյգել, Ֆ. Մարկ, Հ. Ա. Ազանյանց,
ՀՀ ԳԱԱ արտասահմանյան անդամ Վ. Ա. Սաքանյան

Geobacillus stearothermophilus-ից կլոնավորված ջերմակայուն հիդանտոինազի բնութագրումը

Ցույց է տրվել, որ ռեկոմբինանտ հիդանտոինազները ջերմակայուն, մետաղ-կախյալ, D-ստերեոսպեցիֆիկ ֆերմենտներ են և ունեն բարձր խնամակցություն դիհիդրոուրացիլի և դիհիդրոթիմինի նկատմամբ և չեն փոխազդում դիհիդրոօրո-տաթթվի հետ: Սպիտակուցների առաջնային հաջորդականություններն ավելի մոտ են էուկարիոտների դիհիդրոպիրիմիդինազին, քան բակտերիալ դիհիդրոօրոտազին և այլ ցիկլիկ ամիդոհիդրոլազների հաջորդականություններին: Քրոմոսոմային և խոշոր պլազմիդային ԴՆԹ-երի ուսումնասիրումը, որոնք բաժանվել են “Pulsed-Field Gel Electrophoresis” մեթոդով և հիբրիդացվել սպեցիֆիկ մոլեկուլային նմուշներին, ցույց է տվել հիդանտոինազ գենի քրոմոսոմային բույթը: Ներկայացված տվյալները ցույց են տալիս, որ հիդանտոինազը ընդգրկված է *G. stearothermophilus*-ի պիրիմիդինների ճեղքման ուղղում:

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иностранный член НАН РА В. А. Саканян

Характеристика термостабильной гидантоиназы, клонированной из _Geobacillus stearothermophilus_

Показано, что рекомбинантные гидантоиназы являются термостабильными, металлозависимыми и D-стереоспецифичными ферментами. Первичная последовательность белков более схожа с дигидропиримидиназами эукариотов, чем с бактериальными дигидрооротазами и другими циклическими амидогидролазами. Анализ хромосомной и больших плазмидных ДНК (обнаруженных в этих штаммах), обработанных эндонуклеазами рестрикции, разделенных методом “Pulsed-Field Gel Electrophoresis” и гибридизированных со специфичными молекулярными пробами, указал на хромосомальную природу гена гидантоиназы. Показано, что гидантоиназа участвует в редуктивном пути деградации пиримидинов в *G. stearothermophilus*.

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