Biochemical Properties of Thermostable D-Hydantoinase from *Bacillus thermocatenulatus* GH-2

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INTRODUCTION

D-Hydantoinase is currently employed as a biocatalyst for the production of optically pure D-amino acids, which are intermediates for the synthesis of semisynthetic antibiotics, peptide hormone, pyrethroids, and pesticides. In the process developed by the Yamada group,[1,2] DL-5-substituted hydantoin is asymmetrically hydrolyzed to the *N*-carbamoyl-D-amino acid by D-specific hydantoinase, and this product is further chemically or biologically converted to the corresponding D-amino acid. The operational stability of the enzyme is considered as one of the most important factors because the short half-life of the enzyme often limits the development of the enzymatic process. As an effective way of producing thermostable enzymes with great biotechnological potential, isolation of an enzyme from thermophiles has attracted much attention.

We have been focusing on the screening of thermostable D-hydantoinase-producing thermophiles[3] and have attempted to isolate a thermostable D-hydantoinase with high affinity toward hydantoins with an aromatic group at the 5′-position. A thermostable D-hydantoinase was isolated from *Bacillus thermocatenulatus* GH-2 and purified to homogeneity by using immunoaffinity chromatography. Biochemical characteristics of the enzyme were investigated.

PURIFICATION OF HYDANTOINASE FROM *BACILLUS THERMOCATENULATUS* GH-2

We attempted to purify the hydantoinase of *B. thermocatenulatus* GH-2 by using immunoaffinity chromatography prepared with polyclonal antibody. The results of the purification of hydantoinase are summarized in TABLE 1. The purification yield was 36.4%, and the specific activity of the enzyme was estimated to be 461 U/mg-protein under standard assay conditions. The analytical SDS/PAGE of the sample from each step of the purification is shown in FIGURE 1. The purified hydantoinase

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

Purification Step	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg-protein)	Purification (fold)	Activity Yield (%)
Crude extract	100	1420	14.2	1	100
Immunoaffinity chromatography	1.12	517	461.6	32.5	36.4

was electrophoretically homogeneous, and the relative molecular mass of the subunit was about 56 kDa.

MOLECULAR MASS AND QUATERNARY STRUCTURE

The molecular mass of the purified D-hydantoinase was determined by gel filtration chromatography using a Superose-12 column, and its value was estimated to be about 230 kDa. From the molecular mass of the subunit, the D-hydantoinase was assumed to be composed of four identical subunits.

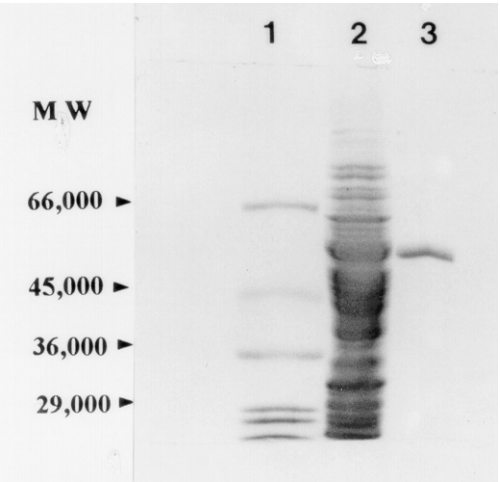

FIGURE 1. SDS/polyacrylamide gel electrophoresis of the D-hydantoinase at each purification step. Lanes: 1, standard marker proteins; 2, cell-free extract; 3, D-hydantoinase purified by immunoaffinity chromatography.

STEREOSPECIFICITY

The relative mobility of *p*-hydroxyphenylglycine and valine produced by the purified hydantoinase on chiral TLC was compared, and the hydantoinase of *B. thermocatenulatus* GH-2 was found to be strictly D-specific as previously isolated enzyme from *B. stearothermophilus* SD-1 (data not shown).

ISOELECTRIC POINT

The isoelectric point p*I* of the D-hydantoinase was measured by electrofocusing on 6% polyacrylamide gel. The isoelectric pH was determined as 4.3 by comparing the band position of the enzyme with those of standard proteins. Runser and Meyer[4] reported that the isoelectric point of the D-hydantoinase of *Agrobacterium thermocatenulatus* is about 6.5, indicating that the enzyme is a slightly acidic protein.

EFFECT OF METAL IONS

It was generally known that most D-hydantoinases require metal ions for their activity.[3,5] The enzyme activity was decreased to 5% of the original activity upon treatment with EDTA, and the activity of the EDTA-treated enzyme was almost fully recovered by the addition of Mn^{2+} above 1 mM.

SUBSTRATE SPECIFICITY

The relative reaction rates, $V_{HPH}/V_{hydantoin}$, of the D-hydantoinases from *B. thermocatenulatus* GH-2 and *B. stearothermophilus* SD-1 were approximately 1.9 and 0.7, respectively, when the concentrations of HPH and hydantoin were the same. These results clearly indicate that the D-hydantoinase from *B. thermocatenulatus* GH-2 possesses higher affinity for hydantoin derivatives with an aromatic group at the 5′-position than the previously isolated one.[3] As mentioned earlier, D-hydantoinase having high affinity toward hydantoin derivatives with an aromatic group at the 5′-position is desirable for practical application because these hydantoins are the starting substrates for the synthesis of D-amino acids with the highest commercial demand. The D-hydantoinases from mesophiles such as *Pseudomonas*,[5] *Agrobacterium*, and *Arthrobacter* were reported to show high affinity toward 5-substituted hydantoins with nonpolar or hydrophobic groups, and these enzymes were tetramers. From these observations, it is likely that the substrate specificity of the hydantoinase might be linked with the oligomeric structure of the enzyme. Further studies regarding the factors affecting the substrate specificity of the enzyme will be of great interest.

EFFECT OF pH AND TEMPERATURE

The maximum activity was observed at pH 7.5 and the enzyme activity remained stable at pH between 6.0 and 9.5.

TABLE 2. Summary of the Properties of D-Hydantoinase from *B. thermocatenulatus* GH-2

Optimal temperature	65 °C
Optimal pH	7.5
Molecular mass (subunit)	56 kDa
Oligomeric structure	homotetramer
p*I*	4.3
K_m (against hydroxyphenylglycine)	30.67 mM
Stoke's radius	53.4 Å
Metal requirement	Mn^{2+}

Optimal temperature for the activity was about 65 °C. The enzyme was stable up to 75 °C, and the half-life of the enzyme was estimated to be 45 min at 80 °C.

CONCLUSIONS

The biochemical properties of the purified D-hydantoinase from *Bacillus thermocatenulatus* GH-2 are summarized in TABLE 2. Thermostability of the enzyme is considered as one of the most important factors in the practical application of the enzyme. Furthermore, in the case of rarely soluble substrate, thermostable enzyme has an additional advantage because an enhanced production rate is expected due to the high solubility of substrate at elevated temperature. When considering the catalytic property and thermostability of the D-hydantoinase from a newly isolated *Bacillus thermocatenulatus* GH-2, this enzyme is thought to be of significant potential in improving the enzymatic process for the production of commercially important D-amino acids from DL-5-substituted hydantoins.

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