Purification and Characterization of Hydantoin Racemase from Microbacterium liquefaciens AJ 3912

Shun’ichi SUZUKI,† Norimasa ONISHI, and Kenzo YOKOZEKI

AminoScience Laboratories, Ajinomoto Co., Inc., Kawasaki-ku, Kawasaki, Kanagawa 210-8681, Japan

Received September 29, 2004; Accepted November 30, 2004

A hydantoin racemase that catalyzed the racemization of 5-benzyl-hydantoin was detected in a cell-free extract of Microbacterium liquefaciens AJ 3912, a bacterial strain known to produce L-amino acids from their corresponding DL-5-substituted-hydantoins. This hydantoin racemase was purified 658-fold to electrophoretic homogeneity by serial chromatography. The N-terminal amino acid sequence of the enzyme showed homology with known hydantoin racemases from other microorganisms. The apparent molecular mass of the purified enzyme was 27 kDa on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and 117 kDa on gel-filtration in the purification conditions, indicating a homotetrameric structure. The purified enzyme exhibited optimal activity at pH 8.2 and 55 °C, and showed a chiral preference for L-5-benzyl- rather than D-5-benzyl-hydantoin.

Key words: hydantoin racemase; hydantoin; amino acid production; Microbacterium liquefaciens

Both naturally occurring and unnatural amino acids have been identified as valuable intermediates in the production of various antibiotics, pesticides, sweeteners, and pharmaceuticals.1,2) One of the most useful methods of producing optically pure amino acids is the stereoselective hydrolysis of DL-5-monosubstituted hydantoins.3) The advantage of this method lies in the stereoselectivities and broad substrate specificities of hydantoin hydrolyzing enzymes, i.e., hydantoinases and N-carbamoyl α-amino acids amido hydrolases (N-carbamoylases). Using these enzymes, various kinds of optically pure D- or L-amino acids can be obtained from the corresponding DL-5-substituted hydantoins.4) In the stereoselective hydantoin hydrolysis method, hydantoin compounds, which can be chemically synthesized in many cases,5) are used as the initial substrates. These are first hydrolyzed by hydantoinases to form N-carbamoyl-α-amino acids, then converted to the corresponding D- or L-amino acids by N-carbamoylases. The stereoselectivities of the hydantoinases and/or N-carbamoylases lead to the optical purities of the produced amino acids; however, the racemization of hydantoin is a rate limiting step, which results in unacceptable levels of residual substrates. In order to avoid this disadvantage, it is necessary to improve the effectiveness of hydantoin racemization during the process. The chemical racemization of some 5-substituted hydantoins proceeds optimally under alkaline conditions and at high reaction temperatures;6) however, the rate of racemization also depends greatly on the structure of the substituent in position 5 and is usually slower than that of hydantoin hydrolysis. Relatively rapid spontaneous racemization has been observed only with D,L-phenyl- and D,L-5-p-hydroxyphenyl-hydantoin.7) With other hydantoin compounds, many hours were required to achieve complete racemization.6)

Hydantoin racemases (HRases) are the enzymes responsible for racemizing hydantoins, and their inclusion in the hydrolysis process speeds the production of optically pure amino acids from racemic hydantoins. HRase proteins have been reported to occur in various microorganisms.7–11) In addition, their genetic organization and recombinant gene expression have been studied, and the effectiveness of HRase proteins in the production of optically pure amino acids from D,L-hydantoin compounds has been demonstrated.12–17)

In this study, we focused on a bacterial strain, Microbacterium liquefaciens AJ 3912, as a potent producer of a new HRase. This microorganism was originally known as Flavobacterium sp. AJ 3912 in our previous series of studies on L-amino acid production, and was observed to possess a D,L-hydantoinase and a L-stereoselective N-carbamoylase.18,19) This strain was able to produce L-Phe from DL-5-benzyl-hydantoin (DL-BH) at yields of more than 50%, suggesting that racemization of DL-BH occurred under the reaction conditions. At the time, we discussed the possibility of spontaneous racemization of 5-benzyl-hydantoin, since this has been reported to be more readily racemized under alkaline conditions than other 5-substituted hydantoin compounds.20) However, the rate of racemi-

† To whom correspondence should be addressed. Fax: +81-44-244-6581; E-mail: shunichi.suzuki@ajinomoto.com

Abbreviations: AMS, ammonium sulfate; BH, 5-benzyl-hydantoin; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; HRase, hydantoin racemase; IAA, isoionic acid; MHR, hydantoin racemase from Microbacterium liquefaciens AJ 3912; N-carbamoylase, N-carbamoyl α-amino acids amido hydrolase; NEM, N-ethylmaleimide; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.
zation occurring in the presence of the microorganism appeared to be faster than the spontaneous racemization rate under the same reaction conditions, suggesting the existence of a hydantoin-racemizing enzyme in this strain. Here we describe the subsequent purification and characterization of the HRase from *M. liquefaciens* AJ 3912 (MHR).

**Materials and Methods**

**Bacterial strain and culture conditions.** A bacterial strain, *M. liquefaciens* AJ 3912, was used as the HRase producer. The culture medium and conditions were as described in our previous publication.20

**Chemicals.** D- and L-BH were synthesized chemically from D- or L-Phe.21 Other chemicals were commercial products of the highest grade available.

**Purification of MHR.** Sixty-six grams of wet *M. liquefaciens* AJ 3912 cells, obtained from 3.7 liters of culture broth, was used as the starting material. The cells were washed with 0.1 M potassium phosphate buffer (pH 7.0), suspended in 130 ml of the same buffer, and disrupted by agitation with glass beads (0.1 mm diameter) for 3 min. The liquor was separated from the glass beads and treated with deoxyribonuclease I (added to a final concentration of 5 μg/ml) at room temperature for 20 min. The supernatant obtained by centrifugation at 100,000g for 60 min was used as the crude extract.

The protein in the crude extract was precipitated with a 70% saturated solution of ammonium sulfate (AMS; pH 7.0), separated from the supernatant and dissolved in a small amount of 20 mM potassium phosphate buffer (pH 7.0). The protein solution was dialyzed against 20 mM potassium phosphate buffer (pH 7.0) containing 1.2 M AMS and 0.5 mM CoCl₂, then applied to a Phenyl Sepharose HP 26/10 column (Amersham Bioscience, Piscataway, NJ) pre-equilibrated with the same buffer. The proteins adsorbed on the column were eluted by gradually decreasing the concentration of AMS in the buffer. The fractions exhibiting HRase activity were collected, concentrated, and dialyzed against 20 mM potassium phosphate buffer (pH 7.0). The dialyze was applied to a Phenyl Sepharose HP 16/10 column (Amersham Bioscience) pre-equilibrated with the same buffer, and the adsorbed proteins were eluted by adding NaCl to a concentration of 0.5 M. The fractions containing HRase activity were collected, concentrated, and applied to a Superdex 200 pg 16/60 column (Amersham Bioscience) pre-equilibrated with the same buffer. The resulting HRase-containing fractions were purified further by three passages through a Phenyl Superose 5/5 column (Amersham). The buffer used for the first and third passages was 20 mM potassium phosphate buffer (pH 7.0) containing 1 M AMS, 1 mM L-BH; the concentration of AMS was then gradually decreased to elute the proteins. The same technique was used for the second passage, but the buffer did not contain L-BH.

The fractions obtained from the third passage through the Phenyl Superose 5/5 column were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and the fractions containing electrophoretically pure MHR were used as the enzyme source for the characterization studies.

**Enzyme assay.** HRase activity was determined by measuring the rate of racemization of 5-benzyl-hydantoin. Standard conditions were defined as a reaction mixture consisting of 6.3 mM D-BH, 50 mM potassium phosphate buffer (pH 8.0), 5 mM dithiothreitol (DTT), and the enzyme solution which was incubated at 37°C for 30 min. Under these conditions, spontaneous racemization of the substrate appeared to be negligible. The reaction was terminated by adding CuSO₄ and phosphoric acid to final concentrations of 1 mM and 10 mM respectively. The rate of racemization was then determined by high-performance liquid chromatography (HPLC). HPLC analysis was carried out using CHIRALPAK WH resin (Dichem Chemical Industry, Osaka, Japan): column length, 25 cm; column temperature, 50°C; mobile phase, 1 mM CuSO₄ and 5% (v/v) methanol; flow rate, 1.5 ml/min; detection, ultraviolet detector (210 nm). One unit of HRase activity was defined as the formation of 1 μmol l-BH from d-BH in 1 min. The activity was calculated from the initial velocity of the reaction, in which formation of racemized isomer did not exceed 20% of the initial amount of the substrate.

To determine the optimal conditions for the reaction, the same assays were carried out at pH 8.0 (for the optimal reaction temperature) and at 37°C (for the optimal pH). The stability of MHR was determined as the residual activity of the standard reaction mixture (see above) measured after incubation for 30 min at various pHs or temperatures. The buffers used were 0.1 M sodium acetate (pH 3.1, 3.9, 4.9, and 6.1), potassium phosphate (pH 6.4, 7.2, and 8.0) and sodium carbonate (pH 8.2, 9.1, 10.2, and 10.9).

The sensitivity of the enzyme to potent inhibitors was determined as the residual activity under conditions after pre-incubation with each reagent for 30 min at pH 8.0 on ice. The inhibitors used were N-ethylmaleimide (NEM), iodoacetic acid (IAA), CuSO₄, EDTA, and methanol.

The methods used for the assays of hydantoinase and N-carbamoylase were as described in our previous publication.20 However, in this study, one unit of activities of these enzymes was defined as the hydrolysis of 1 μmol substrate per min.

**Analysis.** Protein concentrations were measured by the Bradford method with bovine serum albumin as a standard.

SDS–PAGE was performed under reduced conditions using 15–25% gradient gel (Daichi Pure Chemicals, Tokyo, Japan). Proteins were visualized by staining with
Coomassie brilliant blue R-250 or silver staining (Silvest Stain Kit, Nacalai Tesque, Kyoto, Japan). For analysis of the N-terminal amino acid sequence of MHR, the purified MHR was blotted from the SDS–PAGE gel onto a polyvinylidene difluoride membrane (Trans-Blot, Bio-Rad Laboratories, Hercules, CA) and sequenced using a protein sequencer (Model 476A, Applied Biosystems, Foster City, CA).

**Results**

**Purification of MHR**

Hydantoin-racemizing activity was detected in some of the fractions eluted from the Phenyl Sepharose column during the purification process (Fig. 1). Furthermore, HRase, hydantoinase, and N-carbamoylase activities were detected in different fractions, indicating that enzymatic racemization of BH occurred independently of hydrolysis of the substrate, and hence that *M. liquefaciens* AJ 3912 contained a HRase.

Although the specific activity of the HRase was increased by chromatography on three different kinds of resin, the purity of the resulting proteins as assessed by SDS–PAGE remained low (data not shown). Extra chromatographic steps using a hydrophobic interaction column (Phenyl Superose) were therefore introduced in an attempt to purify the MHR to electrophoretic homogeneity. In these steps, buffers with and without t-BH were used. In the preliminary tests of these chromatographic steps, the elution position of the HRase activity was affected by the addition of t-BH to the buffer, although the overall elution pattern of the total proteins was almost the same (Fig. 2A and B). Similar

---

**Fig. 1.** Purification of HRase on Phenyl Sepharose HR 26/10.
A protein sample obtained by ammonium sulfate precipitation of a cell free extract of *M. liquefaciens* AJ 3912 was loaded onto a Phenyl Sepharose HR 26/10 column pre-equilibrated with 20 mM potassium phosphate buffer (pH 7.0) containing 1.2 M ammonium sulfate and 0.5 mM CoCl₂. The proteins were eluted by gradually decreasing the ammonium sulfate concentration from 1.2 M to 0. The eluted proteins and ammonium sulfate concentration were measured by monitoring the absorbance at 280 nm (solid line) and the electrical conductivity (dotted line) respectively. The HRase (○), N-carbamoylase (●), and hydantoinase (△) activities in the eluted fractions were assayed.

**Fig. 2.** Purification of HRase on Phenyl Superose with and without t-BH.
Chromatographic purification of HRase on Phenyl Superose using buffer without (A) or with (B) t-BH. An aliquot of the HRase protein solution after partial purification by ammonium sulfate precipitation and serial chromatography on Phenyl Sepharose, Q-Sepharose, and Superdex 200 pg columns was dialyzed against 20 mM potassium phosphate buffer (pH 7.0) containing 1 M ammonium sulfate and 0.5 mM CoCl₂. The proteins were eluted by gradually decreasing the ammonium sulfate concentration from 1 M to 0. The eluted proteins and ammonium sulfate concentration were measured by monitoring the absorbance at 280 nm (solid line) and the electrical conductivity (dotted line) respectively. The HRase (○), N-carbamoylase (●), and hydantoinase (△) activities in the eluted fractions were assayed.
results were observed on SDS–PAGE, in that almost all of the proteins were eluted in roughly the same position except for a few bands, the elution positions of which were influenced by the presence of L-BH (Fig. 2C and D). Among these proteins, a protein with a molecular mass of approximately 27 kDa on SDS–PAGE exhibited an elution pattern consistent with that of HRase activity, and was perhaps the candidate MHR protein. Thus, the partially purified MHR solution was purified to electrophoretic homogeneity by sequential chromatography including a Phenyl Superose step.

As predicted in the preliminary purification test, the purified MHR had a molecular mass of 27 kDa on SDS–PAGE (Fig. 3). However, on gel-filtration chromatography (data not shown), MHR was eluted at a position corresponding to an estimated molecular mass of 117 kDa, suggesting that MHR may have a homotetrameric structure.

After all the purification steps, the specific activity of the purified protein had increased to 79 U/mg, a 658-fold increase compared with the crude extract (Table 1).

**Enzymatic characteristics of the purified MHR**

The purified enzyme was able to act on both D- and L-BH to cause complete racemization (Fig. 4). When either D- or L-BH was used as the substrate, the final molecular ratio between the isomers was 1:1, but the initial rate of racemization was slightly different. While the initial specific activity against D-BH was calculated at 79 U/mg, that against L-BH was 20% higher, at 100 U/mg.

The optimal reaction conditions and stability against pH and temperature changes were then investigated. The optimal pH was 8.2, with the stable range lying between pH 6 and 9. The optimal temperature was 55 °C, and the HRase remained stable at temperatures below 37 °C.

Next, the inhibitory effects of various reagents on HRase activity were investigated. HRase activity was completely inhibited by reagents that react with the thiol group, i.e., NEM, IAA, and CuSO₄, suggesting the existence of cysteine residues in the catalytic center of the HRase. EDTA had no clear inhibitory effect on the HRase activity, indicating that the enzyme does not require divalent ions to exert its activity. The activity was not inhibited by methanol at concentrations of up to 10% (v/v).

These characteristics of MHR are summarized in Table 2.

**Comparison of the N-terminal amino acid sequence of MHR with those of other HRases**

The 30 amino acid residues in the N-terminal region of the purified MHR were determined, and compared with those of other HRases. The comparison revealed a high degree of sequence identity with other HRases, indicating a common evolutionary origin.

---

**Table 1. Summary of Purification of HRase**

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Purification (x fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysate</td>
<td>1963</td>
<td>236</td>
<td>0.12</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>AMS</td>
<td>1812</td>
<td>236</td>
<td>0.13</td>
<td>1.1</td>
<td>100</td>
</tr>
<tr>
<td>Phenyl</td>
<td>220</td>
<td>121</td>
<td>0.55</td>
<td>4.6</td>
<td>51</td>
</tr>
<tr>
<td>Superose</td>
<td>34</td>
<td>44</td>
<td>1.3</td>
<td>11</td>
<td>19</td>
</tr>
<tr>
<td>Phenyl</td>
<td>8.6</td>
<td>34</td>
<td>3.9</td>
<td>33</td>
<td>14</td>
</tr>
<tr>
<td>Phenyl</td>
<td>0.91</td>
<td>25</td>
<td>28</td>
<td>233</td>
<td>11</td>
</tr>
<tr>
<td>Superose</td>
<td>0.25</td>
<td>17</td>
<td>67</td>
<td>558</td>
<td>7.1</td>
</tr>
<tr>
<td>Phenyl</td>
<td>0.09</td>
<td>7</td>
<td>79</td>
<td>658</td>
<td>3.0</td>
</tr>
</tbody>
</table>

---

**Fig. 3.** SDS–PAGE of the Purified HRase.  
Lane 1, Molecular weight markers; lane 2, purified HRase.

**Fig. 4.** Time Courses of the Racemization of D- and L-BH by the Purified HRase.  
D- (○) or L-BH (●) (initial concentration 6.3 mM) was added to 50 mM potassium phosphate buffer (pH 8.0) containing 5 mM DTT and 0.3 μg/ml of the purified HRase. The reaction mixture was incubated at 37 °C. Aliquots were removed at suitable time-points and the degree of racemization was assayed by HPLC. Spontaneous racemization (incubation without HRase) of D- (△) and L-BH (▲) was also measured under the same conditions. Values are expressed as means with standard deviations for triplicate experiments.
of MHR, M. liquefaciens sp. NS761, and P. meliloti DSM 3747, were determined and aligned with the sequences of other HRase proteins reported in gel-filtration studies. 

**TABLE 2. Enzymatic Characteristics of HRase**

The enzymatic characteristics of purified HRase were determined. The specific activities were measured under standard conditions, as described in “Materials and Methods”. In the inhibitory profile, the concentration of each reagent is the final concentration in the assay of residual activity. The residual activity is expressed as a percentage of the activity without any reagents added.

<table>
<thead>
<tr>
<th>Specific activity</th>
<th>79 U/mg (with t-BH)</th>
<th>100 U/mg (with t-BH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular mass</td>
<td>27 kDa (SDS-PAGE)</td>
<td>117 kDa (Gel-filtration)</td>
</tr>
</tbody>
</table>

**Optimal pH**
- pH 8.2

**Optimal temperature**
- 55°C

**Stability range for pH**
- pH 6–9

**Stability range for temperature**
- <−37°C

**Inhibitory profile**

<table>
<thead>
<tr>
<th>Reagents added</th>
<th>Concentration</th>
<th>Residual Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>100</td>
</tr>
<tr>
<td>NEM</td>
<td>5 mM</td>
<td>0</td>
</tr>
<tr>
<td>IAA</td>
<td>10 mM</td>
<td>0</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>2.5 mM</td>
<td>0</td>
</tr>
<tr>
<td>EDTA</td>
<td>10 mM</td>
<td>86</td>
</tr>
<tr>
<td>methanol</td>
<td>10% (v/v)</td>
<td>99</td>
</tr>
</tbody>
</table>

**Discussion**

This study confirmed the existence of HRase activity in a cell-free extract of *M. liquefaciens* AJ 3912 (Fig. 1). As a result, this strain is now known to possess three hydantoin-metabolizing enzymes, i.e., a HRase, a D,L-non-stereoselective hydantoinase, and a L-stereoselective N-carbamoylase. The production of the hydantoinase and the N-carbamoylase enzymes, which is probably sensitive to hydantoin compounds. Such gene clusters have already been reported for the hydantoin-metabolizing enzymes of other microorganisms, including *Pseudomonas* sp. NS761, *A. aurescens* DSM 3747, and *Agrobacterium* sp. IP I-671.

When considering the mechanism by which L-Phe is produced from DL-BH by *M. liquefaciens* AJ 3912 in our previous paper, we discussed only the contribution of chemical racemization. However, the rate at which BH was racemized in the presence of the microorganism was found to be faster than the spontaneous racemization rate under the same reaction conditions (data not shown). The activity of the newly identified MHR in this strain explains the above observations. In our initial attempts to confirm the existence of an HRase in *M. liquefaciens* AJ 3912, the crude extract was first purified using the same anion-exchange resin that we used to partially purify the hydantoinase and N-carbamoylase enzymes. Unfortunately, this led to the detection of only very low HRase activity levels. Furthermore, these occurred at almost the same elution position as the hydantoinase activity, which was strong enough to deprive the HRase of its substrate (data not shown). Perhaps this prevented us from discovering MHR in our previous studies.

Once it was identified, MHR was purified to electrophoretic homogeneity (Fig. 3). There have been several studies concerning the purification of HRase proteins, but all of them used recombinant microorganisms as the enzyme producers. The only study in which a HRase was isolated from its authentic host involved *Pseudomonas* sp. NS761, and did not achieve homogeneous purification. Therefore, to the best of our knowledge, this is the first report of the homogeneous purification of an HRase from an authentic producer. The purification of MHR was greatly assisted by the finding that its elution position on hydrophobic-interaction chromatography (Phenyl Superose) moved in specific response to the addition of L-BH to the chromatography buffer (Fig. 2). This might be explained by a specific interaction between the MHR molecule and L-BH, which might bind more tightly to the phenyl group in the resin than MHR alone. As another possibility, some changes might occur in the assembly of subunits in response to the addition of the substrate. This possibility ought to be confirmed in further studies.

The apparent molecular mass of 27 kDa on SDS-PAGE and the N-terminal amino acid sequence of MHR both suggest structural similarity with previously reported HRase proteins from other microorganisms. The molecular masses deduced from the reported sequences of other HRase proteins are almost the same, 25–27 kDa. Although only a limited portion of the amino acid sequence of MHR has been determined so far, it exhibits some homology with those of the HRase proteins from other microorganisms. Judging from the alignment scores calculated during this study, MHR shows the greatest similarity to the HRase from *A. aurescens*. However, the native molecular masses of HRase proteins reported in gel-filtration studies lie...
between 100 and 190 kDa, corresponding to tetrameric (AtHyuA and AtHyuA2 from *A. tumefaciens*, and SmeHyuA from *S. meliloti*), hexameric (PsHyuE from *Pseudomonas* sp.), and hexameric/heptamer/octameric (AaHyuA from *A. aurescens*) structures.\(^7\)\(^10\) In consideration of these features, the suggested tetrameric structure of MHR might indicate similarity to the HRase proteins from *A. tumefaciens* or *S. meliloti*.

The other enzymatic properties of MHR, i.e., its optimal reaction conditions and stability, are similar to those previously reported for HRase proteins, but further investigation might discover some intrinsic characteristics of MHR. From the industrial viewpoint, substrate specificity is one of the most interesting aspects, although D- and L-BH are the only known substrates of this racemase as yet, mainly due to the low yield of the purified enzyme. In this study, a chiral preference for L-(100 U/mg) rather than D-BH (79 U/mg) was observed. A similar preference has also been observed for the HRase proteins from *Pseudomonas* sp., *A. aurescens*, and *S. meliloti*.*\(^7\)\(^8\)\(^10\)* Further investigation of the substrate specificity of MHR is an important topic for future research, for which gene cloning and high-level expression of this enzyme might be required.

**Acknowledgments**

We thank Dr. T. Oonuki, and Dr. K. Izawa of our laboratories for encouragement and useful suggestions. We are also grateful to Dr. N. Fukuchi and Dr. M. Oota of our laboratories for technical assistance with N-terminal amino acid sequencing.

**References**


