Microbial Conversion of DL-5-Substituted Hydantoins to the Corresponding L-Amino Acids by *Pseudomonas* sp. Strain NS671

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Received January 7, 1993

A bacterial strain, NS671, which converts DL-5-(2-methylthioethyl)hydantoin stereospecifically to L-methionine, was isolated from soil and was classified into the genus *Pseudomonas*. With growing cells of *Pseudomonas* sp. strain NS671, DL-5-(2-methylthioethyl)hydantoin was effectively converted to L-methionine. Under adequate conditions, 34 g of L-methionine per liter was produced with a molar yield of 93% from DL-5-(2-methylthioethyl)hydantoin added successively. In addition to L-methionine, other amino acids such as L-valine, L-leucine, L-isoleucine, and L-phenylalanine were also produced from the corresponding 5-substituted hydantoins, but these L-amino acids produced were partially consumed by strain NS671. The hydantoinase, by which 5-substituted hydantoin rings are opened, was ATP-dependent. The N-carbamyl-amino acid amidohydrolase was found to be strictly L-specific, and its activity was inhibited by high concentration of ATP.

5-Substituted hydantoins are classical precursors for DL-amino acids and can be easily synthesized from aldehydes at a low cost by the Bucherer–Bergs reaction. For the production of L-amino acids from the corresponding 5-substituted hydantoins, some bacterial strains belonging to the genera *Flavobacterium*, *Arthrobacter*, *Pseudomonas*, and *Bacillus* have been reported. Among these genera, only the genus *Bacillus* could convert DL-5-(2-methylthioethyl)hydantoin (DL-Met-Hyd) stereospecifically to L-methionine. The microbial conversion of 5-substituted hydantoins to L-amino acids consists of the following three successive steps: (1) spontaneous or enzymatic racemization of 5-substituted hydantoins; (2) a ring-opening hydrolysis of 5-substituted hydantoins to produce N-carbamyl-amino acids with an enzyme called hydantoinase; (3) an L-isomer-specific hydrolysis of N-carbamyl-L-amino acids to produce L-amino acids.

We found a bacterial strain, NS671, that produces L-methionine effectively from DL-Met-Hyd. Strain NS671 harbored a plasmid containing all the genes for the three enzymes (hydantoinase, racemase, and hydantoinase, and N-carbamyl-L-amino acid amidohydrolase) concerned with the conversion of DL-5-substituted hydantoins to the corresponding L-amino acids.

This paper deals with the taxonomic studies of strain NS671, the conditions for effective production of L-methionine by strain NS671, and several properties of the enzymes concerned with the L-amino acid production.

Materials and Methods

Microorganism. The bacterial strain NS671 isolated from soil was used.

Media. Medium I contained 5 g of glucose, 1 g of DL-Met-Hyd, 0.5 g of KH₂PO₄, 1 g of Na₂HPO₄, 0.1 g of MgSO₄·7H₂O, 0.01 g of FeSO₄·7H₂O, and 0.01 g of MnSO₄·4·H₂O per liter of distilled water. The pH was adjusted to 7.0 with NaOH. Medium II contained 15 g of tryptone, 5 g of soya peptone, and 5 g of NaCl per liter of distilled water. Medium III contained 5 g of glucose, 1 g of DL-Met-Hyd, 0.25 g of KH₂PO₄, 0.5 g of Na₂HPO₄, 0.1 g of MgSO₄·7H₂O, 0.01 g of FeSO₄·7H₂O, and 0.01 g of MnSO₄·4·H₂O per liter of distilled water. The pH was adjusted to 7.2 with NaOH. Medium IV contained 5 g of sucrose in place of glucose in medium III.

Chemicals. 5-Substituted hydantoins were prepared from the corresponding aldehydes and amino acids. N-Carbamyl-methionine was prepared from methionine. Other chemicals were the best available commercial products.

Screening methods. For the isolation of microorganisms which hydrolyze DL-Met-Hyd to L-methionine, adequate amounts of soil suspension were spread on medium I agar plates containing 1 g of DL-Met-Hyd per liter instead of the DL-form and the plates were kept for 2–3 days at 30°C. Resultant colonies that used L-Met-Hyd as the sole source of nitrogen were inoculated into 1 ml of the same liquid medium containing L-Met-Hyd in test tubes. After aerobic cultivation at 30°C until growth was detected visually, L-methionine produced in the culture was detected by thin-layer chromatography (TLC).

Identification of strain NS671. An isolated microorganism, strain NS671, which produces L-methionine from DL-Met-Hyd, was taxonomically identified by the criteria given in Bergey's Manual of Systematic Bacteriology.

Preparation of crude extracts. The cells of strain NS671 grown in medium III at 30°C for 24 h were harvested by centrifugation, suspended in 0.1 M phosphate buffer (pH 7.5), and then disrupted with a sonic oscillator. The cell debris was removed by centrifugation and the supernatant was used as crude extract.

Analytical methods. Methionine produced and Met-Hyd remaining in the culture were separated by TLC in the solvent system of butanol-acetic acid-water (9:1:1, by volume), and they were detected by a method using potassium iodoplatinate as the color-producing reagent. Amino acids, N-carbamyl-amino acids, and 5-substituted hydantoins were measured by high performance liquid chromatography (HPLC) using a TSKgel ODS-80Tm column (4.6 mm i.d. x 150 mm, Tosoh). The mobile phase of 5 mm NH₄H₂PO₄·H₂O buffer (pH 2.8) was used at a flow rate of 1 ml/min. The configurations of methionine and other amino acids produced were identified by ligand-exchange HPLC by using a Chiralpak WH column (4.6 mm i.d. x 250 mm, Daicel Chemical Industries) or ligand-exchange TLC by using a Chiralplate (Machery-Nagel), or by specific optical rotation measurement using a digital polarimeter (DIP-360, Japan

Abbreviations: Met-Hyd, 5-(2-methylthioethyl)hydantoin; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography.
Spectroscopic Co., Ltd.). ATP and ADP were measured by HPLC using a Wako pak WA-NH2-10H column (4.0 mm i.d. x 250 mm, Wako Pure Chemical Industries). The mobile phase of 0.5 M KH2PO4-H3PO4 buffer (pH 4.0) was used at a flow rate of 0.7 ml/min. Total sugar was measured by the phenol-sulfuric acid method.149

Results

Screening of microorganisms producing L-methionine from L-Met-Hyd

Through screening for microorganisms that can hydrolyze L-Met-Hyd to L-methionine, several bacterial strains were isolated from about 400 soil samples. Among them, strain NS671 showed strong abilities to consume L-Met-Hyd and to produce L-methionine with growing cells. Strain NS671 was used for further experiments.

Identification of bacterial strain NS671

The results of detailed taxonomical studies on strain NS671 are summarized in Table I. According to Bergey’s Manual of Systematic Bacteriology, strain NS671 belongs to the genus Pseudomonas, because strain NS671 was Gram-negative aerobic rods, motile (polar flagella), decomposed glucose oxidatively, and did not require growth factors, and the oxidase test was positive. Pseudomonas species are divided into four sections in Bergey’s Manual of Systematic Bacteriology (Vol. 1), and strain NS671 belongs to section II or III because it did not require growth factors and accumulated poly-β-hydroxybutyrate. In sections II and III, 15 species are already known. The taxonomic characteristics of strain NS671 were different from those of the species in sections II and III already known as to the following; oxidase test, denitrification, liquefaction of gelatin, growth at 40°C, assimilation of carbon sources, etc. From these results, strain NS671 was identified as a

Table I. Characteristics of Strain NS671

[a] Morphological characteristics
   Rods (0.8-1.0 x 1.7-2.5 μm), non-pneumonic, motile (polar flagella),
   Gram negative, non-spore forming, and non-acid fast
[b] Cultural characteristics
   (1) Nutrient agar plate: good growth, circular, convex, opaque, smooth,
       and straw color
   (2) Nutrient agar slant: good growth, filiform
   (3) Nutrient broth: moderate growth, ring, turbid and viscid
   (4) Gelatin stab: not liquefied
   (5) Litmus milk: unchanged
[c] Physiological characteristics
   (1) Reduction of nitrate: negative
   (2) Denitrification: negative
   (3) V-P test: negative
   (4) M-R test: negative
   (5) Production of indole: negative
   (6) Production of H2S: negative
   (7) Hydrolysis of starch: negative
   (8) Utilization of citrate:
       Koser’s medium, positive
       Christensen’s medium, positive
   (9) Utilization of nitrogen sources:
       ammonia, positive
       nitrate, positive
   (10) Water soluble pigments: not produced
   (11) Urease test: negative
   (12) Oxidase test: negative
   (13) Catalase test: positive
   (14) Temperature for growth: grown at 37°C but not at 40°C
   (15) pH for growth: 5-9
   (16) Oxygen requirement: aerobic
   (17) O-F test: oxidative
   (18) Acid or gas production from sugars:
       produces acid but not gas from L-arabinose, D-glucose, sucrose,
       D-mannitol (weak), and glycerol. No acid or gas from D-xylene,
       D-mannose, D-fructose, D-galactose, maltose, lactose, trehalose,
       D-sorbitol, inositol, and starch.
   (19) Oxidation of glucuronate: negative
   (20) Utilization of malonate: negative
   (21) Decarboxylase reaction (Møller’s method): negative to lysine,
       ornithine, and arginine
   (22) Hydrolysis of DNA: positive
   (23) Accumulation of poly-β-hydroxybutyrate: positive
   (24) Organic growth factor: not required
   (25) Assimilation of carbon sources: assimilates L-arabinose, D-xylene,
       D-glucose, D-fructose, sucrose, trehalose, D-ribose, L-rhamnose,
       ethanol, β-alanine, L-arginine, butyrate, DL-lactate, m-hydroxy-
       benzole, DL-β-hydroxybutyrate, propylene glycol, and 2,3-
       butylene glycol. Does not assimilate adonitol, erythritol, sorbitol,
       L-valine, D-tartarate, meso-tartarate, p-hydroxybenzoate, gly-
       colate, malonate, levulinate, citraconate, mesaconate, tryptia-
       mine, or betaine.

Fig. 1. Course of L-Methionine Production from DL-Met-Hyd by
Pseudomonas sp. Strain NS671.

Cells of Pseudomonas sp. strain NS671 subcultured on a medium II agar plate was inoculated into 50 ml of medium I (A) or medium I containing 10 g of DL-Met-Hyd per liter (B) in a 300-ml flask. The cultivation was done at 30°C on a rotary shaker. Bacterial growth was measured by the optical density at 660 nm. Symbols: O, L-methionine; △, N-carbamyl-methionine; ●, DL-Met-Hyd; ●, growth.
Pseudomonas sp.

Conversion of DL-Met-Hyd to L-methionine by growing cells of Pseudomonas sp. strain NS671

L-Met-Hyd was effectively hydrolyzed to L-methionine with growing cells of strain NS671. Figure 1 (A) shows the course of the production of L-methionine from 1 g of DL-Met-Hyd per liter by growing cells of strain NS671. The amount of L-methionine produced by strain NS671 coincided stoichiometrically with that of DL-Met-Hyd consumed, and N-carbamyl-methionine, an intermediate of the reaction, was hardly detected in 36 h of cultivation at 30°C. It suggests that D-Met-Hyd was easily racemized in this reaction system and that L-methionine produced was hardly metabolized by strain NS671 in spite of the growth of this bacterium. Therefore, DL-Met-Hyd seems to be used as both a substrate and a main nitrogen source for this cultivation.

As shown in Fig. 1(B), in the presence of 10 g DL-Met-Hyd per liter, DL-Met-Hyd was not completely consumed and N-carbamyl-methionine was formed. Bacterial growth was delayed, and the growth was completely repressed in the presence of 30 g DL-Met-Hyd per liter (data not shown).

For the effective production of L-methionine, it is necessary to maintain the concentration of DL-Met-Hyd below 10 g/liter in this medium.

Typical production of L-methionine from DL-Met-Hyd by Pseudomonas sp. strain NS671

A typical production of L-methionine from DL-Met-Hyd is illustrated in Fig. 2. The seed culture was inoculated into 2.5 liters of medium IV containing 5 g of DL-Met-Hyd per liter in a 5-liter jar fermentor. After aerobic cultivation at 30°C for 15 h, 94 g of DL-Met-Hyd and 134 g of molasses were successively added to maintain the concentration of DL-Met-Hyd below 5 g/liter in the medium during further cultivation. After aerobic cultivation at 30°C for 72 h, the DL-Met-Hyd added was converted completely to L-methionine and the concentration of L-methionine produced reached 34 g/liter, with a molar yield of 93%. Little N-carbamyl-methionine was accumulated.

Isolation of L-methionine from culture broth

L-Methionine was isolated from the culture broth described above by ordinary procedures. The material isolated as colorless crystals gave the same decolorized spot by the method using potassium iodoplatinate and the same Rf value on a silica-gel thin-layer chromatogram as those of authentic L-methionine. From the liquid chromatographical analysis, its purity was found to be 98%. It gave almost the same value of specific optical rotation as that of authentic L-methionine and its optical purity was calculated to be 98% as the L-form (Table II). From these results, the reaction product was confirmed to be entirely the L-form of methionine.

Production of different L-amino acids from the corresponding 5-substituted hydantoins

The production of different L-amino acids from the corresponding 5-substituted hydantoins was examined with growing cells of Pseudomonas sp. strain NS671. As shown in Fig. 3 and Table III, in addition to L-methionine, the other L-amino acids such as L-phenylalanine, L-leucine, L-

![Fig. 2. Typical Production of L-Methionine from DL-Met-Hyd by Pseudomonas sp. Strain NS671.](image)

![Fig. 3. Production of L-Phenylalanine from DL-5-Benzylhydantoin by Pseudomonas sp. Strain NS671.](image)

<table>
<thead>
<tr>
<th>Methionine</th>
<th>Specific rotation [α]D (c 1.0, 6N-HCl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolated</td>
<td>+22.2° (98%)</td>
</tr>
<tr>
<td>Authentic</td>
<td>+22.7° (100%)</td>
</tr>
</tbody>
</table>

In parenthesis, the calculated optical purity based on authentic L-methionine.
isoleucine, and l-valine were produced, but the amounts of l-amino acids accumulated were very small compared to those of 5-substituted hydantoins consumed. This suggests that the l-amino acids produced, except l-methionine, were metabolized by strain NS671.

Properties of the enzymes concerning L-amino acid production

It is recognized that the enzymatic conversion of 5-substituted hydantoins to l-amino acids proceeds by the mechanism via the corresponding N-carbamyl-amino acids as the reaction intermediates. For measurement of the stereospecificity of L-amino acid-forming enzymes from strain NS671, L, D-Met-Hyd and N-carbamyl-L-, N-carbamyl-D-methionine were used as substrates. As shown in Fig. 4, L, D-Met-Hyd, and N-carbamyl-L-methionine were completely converted to methionine. These methionine molecules were confirmed to be the L-form by using TLC for optical resolution. On the other hand, l-methionine production from the D-form of N-carbamyl-methionine was not observed and N-carbamyl-D-methionine was recovered. This suggests that the 5-substituted hydantoin is racemized in the reaction system but the N-carbamyl-methionine is not, and that the stereospecificity of N-carbamyl-amino acid amidohydrolase from strain NS671 is strictly L-isomer-specific.

Further, by using the crude extract of strain NS671 as an enzyme source, little conversion of DL-Met-Hyd to L-methionine was observed, but when N-carbamyl-DL-methionine was used as the substrate, L-methionine was produced. By the addition of ATP to this reaction system, the production of N-carbamyl-L-methionine and L-methionine from DL-Met-Hyd was observed (Fig. 5). At high concentrations of ATP, L-Met-Hyd was converted to N-carbamyl-L-methionine, but not to L-methionine. This suggests that the hydantoinase of strain NS671 is an ATP-dependent enzyme and that the activity of the N-carbamyl-L-amino acid amidohydrolase was inhibited at high concentrations of ATP.

The stoichiometry of the hydantoinase reaction was examined by measuring the amounts of L-Met-Hyd and ATP consumed, and that of N-carbamyl-L-methionine and ADP produced. As shown in Fig. 6, the amount of L-Met-Hyd consumed was in fair agreement with that of ATP consumed, and N-carbamyl-L-methionine produced coincided with ADP produced. These results indicate that the hydrolysis of L-Met-Hyd to N-carbamyl-L-methionine requires equi-

Table III. Production of L-Amino Acids from the Corresponding 5-Substituted Hydantoins by Pseudomonas sp. Strain NS671

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product (Yield)</th>
<th>Substrate remained</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-Met-Hyd</td>
<td>l-Methionine (98%)</td>
<td>0%</td>
</tr>
<tr>
<td>DL-5-Isopropylhydantoin</td>
<td>l-Valine (trace)</td>
<td>48%</td>
</tr>
<tr>
<td>DL-5-Isobutylhydantoin</td>
<td>l-Leucine (68%)</td>
<td>6%</td>
</tr>
<tr>
<td>DL-5-sec-Butylhydantoin</td>
<td>l-Isoleucine (8%)</td>
<td>22%</td>
</tr>
<tr>
<td>DL-5-Benzhydantoin</td>
<td>l-Phenylalanine (24%)</td>
<td>6%</td>
</tr>
</tbody>
</table>

One loopful of cells of Pseudomonas sp. strain NS671 subcultured on a medium II agar plate was inoculated into 3 ml of medium III containing 5 g of DL-5-substituted hydantoin per liter instead of DL-Met-Hyd in a test tube. The cultivation was done at 30°C for 72 h. For l-phenylalanine, the cultivation was done as described in Fig. 3.

Fig. 4. Stereospecificity of L-Amino Acid-forming Enzymes in the Cells of Pseudomonas sp. Strain NS671.

One loopful of cells of Pseudomonas sp. strain NS671 subcultured on a medium II agar plate was inoculated into 3 ml of medium III containing 1 g of each substrate per liter instead of DL-Met-Hyd in a test tube. The cultivation was done at 30°C. The resultant culture media were analyzed by TLC. HYD, Met-Hyd; NCA, N-carbamyl-methionine; MET, methionine. The substrates used were the following with their corresponding lane number: (1), L-Met-Hyd; (2), D-Met-Hyd; (3), N-carbamyl-L-methionine; (4), N-carbamyl-D-methionine; (5), standards.

Fig. 5. L-Amino Acid-forming Activities in the Crude Extract of Pseudomonas sp. Strain NS671.

The crude extract of Pseudomonas sp. strain NS671 was incubated with 5 mg of DL-Met-Hyd (A) or N-carbamyl-DL-methionine (B) per ml and each concentration of ATP in 0.1 M phosphate buffer (pH 7.5). Each reaction was done at 30°C for 18 h (A) or for 2 h (B). The resultant reaction mixtures were analyzed by TLC. HYD, Met-Hyd; NCA, N-carbamyl-methionine; MET, methionine.
molecular amounts of ATP.

Discussion

A bacterial strain NS671, which converts DL-Met-Hyd effectively to L-methionine, was isolated from a soil sample. It was identified as *Pseudomonas* sp.

DL-Met-Hyd can be used as both a substrate and a nitrogen source for the cultivation of *Pseudomonas* sp. strain NS671 and was stoichiometrically hydrolyzed to L-methionine.

The growth of strain NS671 was reduced and the yield of L-methionine produced was lower in the presence of high concentrations (over 10 g/liter) of DL-Met-Hyd. These inhibitory effects could be eliminated by stepwise feeding of the substrate. Thus, the inhibitory effects on L-methionine production by strain NS671 were brought about by substrate inhibition and not by product inhibition, that is, the same case as for L-tryptophan production by *Flavobacterium* sp. 21

Under the best conditions so far found, the concentration of L-methionine produced from DL-Met-Hyd reached 34 g/liter with a molar yield of 93%. Strain NS671 can produce various aliphatic and aromatic L-amino acids from the corresponding DL-5-substituted hydantoins but L-amino acids produced were partially consumed except for L-methionine.

The hydantoinase of strain NS671 required the coupled hydrolysis of ATP for the ring-opening hydrolysis of 5-substituted hydantoin. Yamashiro et al. 11 reported an ATP-dependent L-specific hydantoinase from *Bacillus brevis*. However, the hydantoinase of *Pseudomonas* sp. strain NS671 is not a stereospecific enzyme because it was indicated that *Escherichia coli* transformed with a plasmid containing the genes encoding the hydantoinase of strain NS671 was capable of hydrolyzing D- and L-5-substituted hydantoin. 13 Therefore, the hydantoinase of strain NS671 was different from the L-specific enzyme of *B. brevis*. Kim et al. 22 reported a N-methylhydantoin amidohydrolase which hydrolyzes N-methylhydantoin to N-carbamyl-sarcosine from *Pseudomonas putida*. This enzyme also required the coupled hydrolysis of ATP for cleavage of N-methylhydantoin. The hydantoinase of strain NS671 is different from the enzyme of *Pseudomonas putida* because it did not hydrolyze N-methylhydantoin (data not shown).

With growing cells of strain NS671, DL-Met-Hyd is effectively hydrolyzed to L-methionine. This effective conversion during the cultivation of strain NS671 might be caused by the effective supply of ATP for the hydrolysis of this 5-substituted hydantoin.

The N-carbamyl-amino acid amidohydrolase is strictly L-isomer-specific and its activity is inhibited by ATP at high concentrations (over 10 mM). The hydantoinase requires ATP and the N-carbamyl-amino acid amidohydrolase is inhibited by ATP in the successive reactions catalyzed by these two enzymes. These findings are interesting in studying a function of ATP.

Acknowledgment. The authors wish to thank Dr. T. Kojima, Dr. H. Kimura, and Mr. T. Kojima for helpful advice and technical contributions.

References

1) E. Ware, *Chemical Reviews*, 46, 403–470 (1950).