

5-SUBSTITUTED HYDANTOIN RACEMASE, DNA CODING FOR THE SAME,
RECOMBINANT DNA, TRANSFORMED CELLS, AND
PROCESS FOR PRODUCING OPTICALLY ACTIVE AMINO ACIDS

5 FIELD OF THE INVENTION

The present invention relates to a novel 5-substituted hydantoin racemase efficiently catalyzing racemization reaction with a higher working optimum temperature for racemization reaction, DNA coding for the same, recombinant
10 DNA, transformed cells and a process for producing optically active amino acids.

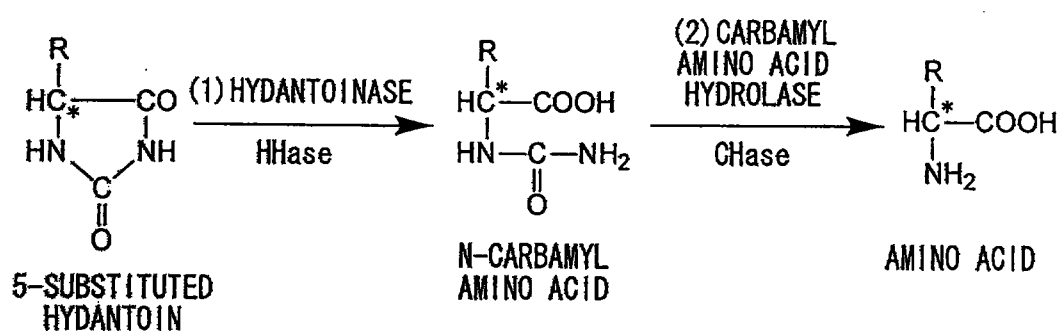
BACKGROUND OF THE INVENTION

The 5-substituted hydantoin racemase (hereinafter
15 abbreviated to HRase) is an enzyme acting on an optically active 5-substituted hydantoin compound, that is, a D- or L-5-substituted hydantoin compound, to catalyze the racemization reaction of the compound.

The 5-substituted hydantoin compound undergoes
20 hydrolysis reaction with enzymes (1) and (2) to form an amino acid as shown in the following reaction scheme (I).

(1) An enzyme (hydantoinase) acting on the
5-substituted hydantoin compound and hydrolyzing the
compound thereby catalyzing the reaction of forming
25 N-carbamyl amino acid.

(2) An enzyme (N-carbamyl amino acid hydrolase) acting on the formed N-carbamyl amino acid and hydrolyzing the compound thereby catalyzing the reaction of forming an optically active amino acid.



5

. . . (1)

For producing the optically active amino acid from the 5-substituted hydantoin compound, at least one of (1) hydantoinase and (2) N-carbamyl amino acid hydrolase used should be an optically selective enzyme, and a method of using a microbial enzyme system and a method of combining a bacterial enzyme system with a chemical reaction system have been known heretofore. The process for producing the optically amino acid from the 5-substituted hydantoin compound is important for production of pharmaceutical preparations, products in chemical industry, food additives etc.

For optico-selective hydrolysis of the total amount of the DL-5-substituted hydantoin compound to convert it into the optically active amino acid by a microbial enzyme

DK200 (Japanese Patent Application Laid-Open (JP-A) No. 62-122591), and 45 °C for the enzyme derived from *Pseudomonas* sp. NS671.

Generally, as the working optimum temperature in the reaction of an enzyme is increased, the industrial utility value of the enzyme is also increased. That is, if the reaction temperature can be increased, the reaction rate can be also increased, which results not only in efficient progress of the desired reaction but also in a reduction in the risk of contamination of the reaction solution with microorganisms during the reaction, thus bringing about the advantage of easy process control including quality control.

As described above, HRase having a higher optimum temperature for the reaction is useful from the viewpoint of industrial applicability. However, the upper limit of the working optimum temperatures of the previously reported HRases is 50 °C, and an HRase having a higher working optimum temperature has been desired to achieve efficient progress of the racemization reaction and to reduce the risk of contamination of the reaction solution with microorganisms.

SUMMARY OF THE INVENTION

It is an object of the present invention to isolate a novel HRase having a higher reaction optimum temperature than conventional and to provide a process for producing

an optically active amino acid by use of the enzyme.

As a result of extensive study in view of the problem described above, the present inventors found that a novel HRase having a desired higher reaction optimum temperature
5 is present in a microorganism of the genus *Microbacterium*, thus arriving at completion of the present invention.

The 5-substituted hydantoin racemase according to one aspect of the present invention catalyzes racemization reaction of 5-substituted hydantoin. In this case, the
10 working optimum pH is in the range of pH 7 to 9, and the working optimum temperature is in the range of 50 to 60 °C.

The protein according to another aspect of the present invention has 5-substituted hydantoin racemase activity. Moreover, the protein has amino acid sequence (a) or (b),
15 where (a) is the amino acid sequence set forth in SEQ ID NO:2 in the Sequence Listing, and (b) is an amino acid sequence wherein in the amino acid sequence set forth in SEQ ID NO:2 in the Sequence Listing, one or more amino acid residues have been replaced, deleted, inserted, added or inverted.

20 The 5-substituted hydantoin racemase fraction according to still another aspect of the present invention is derived from a microorganism of the genus *Microbacterium*. Moreover, the 5-substituted hydantoin racemase fraction is obtained by culturing a microorganism of the genus
25 *Microbacterium* and disrupting or lyzing the resulting

microorganism followed by purification treatment thereof.

The method of racemizing an optically active 5-substituted hydantoin compound according to still another aspect of the present invention comprises allowing the
5 above-described 5-substituted hydantoin racemase or the 5-substituted hydantoin racemase fraction to act on an optically active 5-substituted hydantoin compound.

The process for producing N-carbamyl amino acid according to still another aspect of the present invention
10 comprises allowing the above-described 5-substituted hydantoin racemase or the 5-substituted hydantoin racemase fraction and an enzyme for optico-selectively hydrolyzing 5-substituted hydantoin or a material containing the enzyme to act on 5-substituted hydantoin.

15 The process for producing an optically active amino acid according to still another aspect of the present invention comprises allowing the above-described 5-substituted hydantoin racemase or the 5-substituted hydantoin racemase fraction, an enzyme for hydrolyzing
20 5-substituted hydantoin or a material containing the enzyme and an enzyme for optico-selectively hydrolyzing N-carbamyl amino acid or a material containing the enzyme to act on 5-substituted hydantoin.

DNA according to still another aspect of the present
25 invention is coding for the above-described 5-substituted

hydantoin racemase.

DNA according to still another aspect of the present invention is coding for a protein having a 5-substituted hydantoin racemase activity. Moreover, the DNA has a
5 nucleotide sequence (a) or (b), where (a) is the nucleotide sequence set forth in SEQ ID NO:1 in the Sequence Listing, and (b) is a nucleotide sequence hybridizing under stringent conditions with the nucleotide sequence set forth in SEQ ID NO:1 in the Sequence Listing.

10 DNA according to still another aspect of the present invention is coding for a protein having a 5-substituted hydantoin racemase activity. Moreover, the DNA has a amino acid sequence (c) or (d), where (c) is the amino acid sequence set forth in SEQ ID NO:2 in the Sequence Listing, and (d)
15 is an amino acid sequence wherein in the amino acid sequence set forth in SEQ ID NO:2 in the Sequence Listing, one or more amino acid residues have been replaced, deleted, inserted, added or inverted.

Recombinant DNA according to still another aspect of
20 the present invention is obtained by linking the above-described DNA to a vector DNA.

Cells according to still another aspect of the present invention are transformed with the above-described recombinant DNA.

25 The process for producing a protein having a

5-substituted hydantoin racemase activity according to still another aspect of the present invention comprises culturing the above-described cells in a medium and accumulating a protein having a 5-substituted hydantoin
5 racemase activity in the medium and/or the cells.

Other objects and features of this invention will become apparent from the following description with reference to the accompanying drawings.

10 BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a drawing showing a group of structural genes coding for hydantoin racemase, hydantoinase and N-carbamyl-L-amino acid hydrolase.

Fig. 2 is a flow chart showing a process for producing
15 the hydantoin racemase of the present invention.

Fig. 3 is a drawing showing the optimum pH curve of the hydantoin racemase of the present invention.

Fig. 4 is a drawing showing the pH stability curve of the hydantoin racemase of the present invention.

20 Fig. 5 is a drawing showing the optimum temperature curve of the hydantoin racemase of the present invention.

Fig. 6 is a drawing showing the temperature stability curve of the hydantoin racemase of the present invention.

Fig. 7 is a drawing showing the time course of
25 racemization of BH by the hydantoin racemase of the present

invention. Where, ○: Substrate D-BH (HRase added), △: Substrate D-BH (HRase not added), ●: Substrate L-BH (HRase added), ▲: Substrate L-BH (HRase not added).

Fig. 8 is a drawing showing the time course of formation of D-Phe. Where, □: Substrate D-BH (HRase not added), ○: Substrate DL-BH (HRase not added), △: Substrate L-BH (HRase not added), ■: Substrate DL-BH (HRase added), ▲: Substrate L-BH (HRase added).

10 DESCRIPTION OF THE PREFERRED EMBODIMENTS

Hereinafter, the present invention is described in detail by reference to the accompanying drawings in the order of:

[I] HRase, and

15 [II] Process for producing an optically active amino acid by use of HRase.

[I] HRase

Among microorganisms of the genus *Flavobacterium*, a bacterial strain hydrolyzing DL-5-indolyl methyl hydantoin optico-selectively to form its corresponding L-tryptophan is reported. When this microbial strain forming L-tryptophan, that is, *Flavobacterium* sp. AJ3912 (FERM-P3133), is used in the reaction of forming L-tryptophan from DL-5-indolyl methyl hydantoin as the substrate, the
25 molar yield of L-tryptophan formed reaches 80 % or more (Agric.

Biol. Chem., 51, 363 (1987)).

Since spontaneous racemization of optically active indolyl methyl hydantoin rarely occurs under neutral conditions where the enzyme reaction is carried out, the present inventors estimated that a novel HRase is present in the microbial strain. On the basis of this estimation, the present inventors purified and isolated a HRase from the cultured microorganism of the microbial strain to reveal the presence of a novel HRase, and found that this enzyme is a novel HRase having a desired high optimum reaction temperature.

Flavobacterium sp. AJ3912 (FERM-P3133) was initially deposited as Flavobacterium sp. AJ3912 (FERM-P3133) on June 27, 1975 with the National Institute of Bioscience and Human-Technology, the Agency of Industrial Science of Technology, the Ministry of International Trade and Industry, Japan, but it was revealed as a result of re-identification that this microorganism is classified into Microbacterium liquefaciens. At present, this microorganism is deposited as Microbacterium liquefaciens AJ3912 (FERM-P3133) with the National Institute of Bioscience and Human-Technology, the Agency of Industrial Science of Technology, the Ministry of International Trade and Industry.

The physiological properties of Microbacterium liquefaciens AJ3912 (FERM-P3133) were examined in light of



a microbial classification book, Berjey's Manual of Determinative Bacteriology, Vol. 1 (9th edition, 1994, William & Wilkins Publishing Company), and the test results are shown in Table 1.

5 [Table 1] Results of re-identification of *Microbacterium liquefaciens* AJ3912

	Gram stainability	positive
	Motility	none
	Nitrate reduction	-
10	Pyrimidinase	-
	Pyridonyl allyl amidase	-
	Alkali phosphatase	+
	β -Glucuronidase	-
	β -Galactosidase	+
15	α -Glucosidase	+
	N-Acetyl- β -glucosaminidase	+
	Aesculin (glucosidase)	+
	Urease	-
	Gelatin liquefaction	+
20	Fermentability of hydrocarbons	
	glucose	-
	ribose	-
	xylose	-
	mannitol	-
25	maltose	-

	lactose	-
	white sugar	-
	glycogen	-
	Anaerobic growth	-
5	Casein hydrolyzability	+

Further, the present inventors purified HRase derived from *Microbacterium liquefaciens* AJ3912, and the amino acid sequence of the HRase was determined. Furthermore, an about 30-bp DNA molecule deduced from the amino acid sequence of the HRase was synthesized, and by using this DNA molecule as a probe, a full-length DNA coding for the HRase derived from the microorganism of the genus *Microbacterium* could be successfully isolated from a chromosomal gene library of the microorganism of the genus *Microbacterium*.

Further, the present inventors estimated that a nucleotide sequence downstream from the HRase gene, which was simultaneously obtained in isolation of the HRase gene derived from the microorganism of the genus *Microbacterium*, is a part of a hydantoinase (HHase) gene, and the DNA fragment was amplified by the PCR method and utilized as a probe, whereby the HHase gene could be successfully isolated and obtained from the gene library of the bacterial strain. The present inventors estimated that a nucleotide sequence downstream from the HHase gene, which was simultaneously obtained in isolation of the HHase gene, is a part of an

N-carbamyl amino acid hydrolase (CHase) gene, and the DNA fragment was amplified by the PCR method and utilized as a probe, whereby the full-length CHase gene could be successfully isolated and obtained.

5 That is, as shown in Fig. 1, the HRase gene of the present invention is considered to form an operon together with the HHase gene and CHase gene. In Fig. 1, (1) is an EcoRI/PstI fragment, (2) is a KpnI/SacI fragment, and (3) is a BglII fragment.

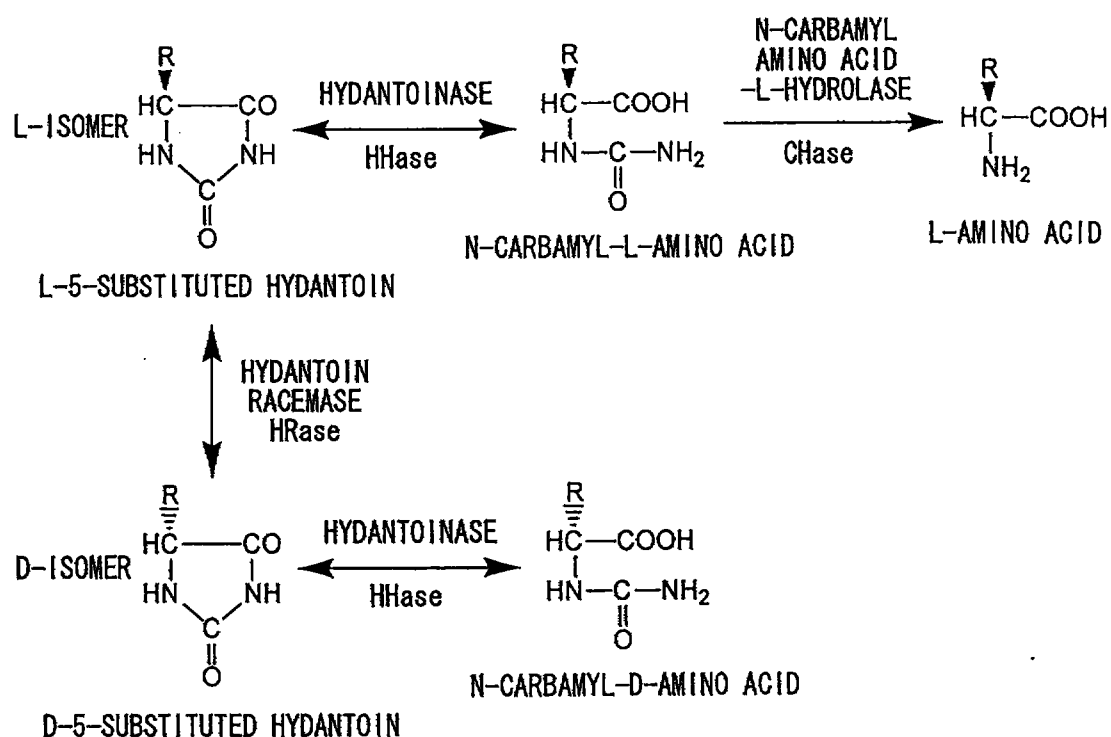
10 In the accompanying sequence listing of the present invention, the DNA coding for the HRase of the present invention is shown in SEQ ID NO:1, the DNA coding for the HHase is shown in SEQ ID NO:3, and the DNA coding for the CHase is shown in SEQ ID NO:5, as determined by the method
15 described above. Further, DNA coding for a group of structural genes including the HRase gene, HHase gene and CHase gene is set forth in SEQ ID NO:7. In the nucleotide sequence set forth in SEQ ID NO:7, the nucleotide residues 1 to 708 code for the HRase of the present invention, the
20 nucleotide residues 729 to 2105 code for the HHase, and the nucleotide residues 2105 to 3340 code for the CHase.

 These DNAs were those isolated from the chromosomal DNA in *Microbacterium liquefaciens* AJ3912 and coding for those proteins related to production of L-amino acids.

25 Further, the amino acid sequence of the HRase encoded

by the nucleotide sequence in SEQ ID NO:1 in the Sequence Listing is set forth in SEQ ID NO:2 in the Sequence Listing, the amino acid sequence of the HHase encoded by the nucleotide sequence in SEQ ID NO:3 in the Sequence Listing is set forth
5 in SEQ ID NO:4 in the Sequence Listing, and the amino acid sequence of the CHase encoded by the nucleotide sequence in SEQ ID NO:5 in the Sequence Listing is set forth in SEQ ID NO:6 in the Sequence Listing.

As shown in the reaction scheme below, the HRase set
10 forth in SEQ ID NO:2 in the Sequence Listing, the HHase in SEQ ID NO:4 in the Sequence Listing and the CHase in SEQ ID NO:6 in the Sequence Listing catalyze the reaction of forming an optically active amino acid represented by L-tyrosine from 5-substituted hydantoin represented by
15 5-(4-hydroxybenzyl) hydantoin as shown in the reaction scheme (II).



. . . (11)

For the novel HRase of the present invention, (1) DNA coding for HRase, (2) properties of HRase, and (3) process for producing HRase are now described in this order.

5 (1) DNA coding for HRase

As described above, the present HRase gene having the nucleotide sequence of SEQ ID NO:1 in the Sequence Listing was isolated from the chromosomal DNA of *Microbacterium liquefaciens* AJ3912. The nucleotide sequence of SEQ ID NO:1

10 in the Sequence Listing shows 48 % homology in the amino acid sequence and 49 % homology in the nucleotide sequence to a known HRase derived from a microorganism of the genus *Pseudomonas* (J. Bacteriol. 174, 962 (1992)).

The method of acquiring DNA coding for the HRase from

the microorganism of the genus *Microbacterium* is described.

First, the amino acid sequence of the purified HRase is determined. The amino acid sequence can be determined by use of the Edman method (Edman, P., *Acta Chem. Scand.* 4, 227 (1950)). Alternatively, the amino acid sequence can
5 be determined by use of a sequencer manufactured by Applied Biosystems. By determining the amino acid sequence of 30 residues from the N-terminus in the HRase of the present invention derived from the microorganism of the genus
10 *Microbacterium*, the sequence shown in SEQ ID NO:8 in the Sequence Listing was revealed.

On the basis of the revealed amino acid sequence, the nucleotide sequence of DNA coding for the same can be deduced. To deduce the nucleotide sequence of DNA, universal codon
15 is adopted.

On the basis of the deduced nucleotide sequence, an about 30-bp DNA molecule is synthesized. The method of synthesizing the DNA molecule is disclosed in *Tetrahedron Letters*, 22, 1859 (1981)). Alternatively, the DNA molecule
20 can be synthesized by use of a synthesizer manufactured by Applied Biosystems. The DNA molecule can be used as a probe so that full-length DNA coding for the HRase derived from the microorganism of the genus *Microbacterium* is isolated from a chromosomal gene library of the microorganism of the
25 genus *Microbacterium*. Alternatively, the DNA molecule can

be used as a primer by which DNA coding for the HRase derived from the microorganism of the genus *Microbacterium* is amplified by the PCR method. However, the DNA amplified by the PCR method does not contain the full-length DNA coding for the HRase derived from the microorganism of the genus *Microbacterium*, and thus the DNA amplified by the PCR method is used as a probe by which the full-length DNA coding for the HRase derived from the microorganism of the genus *Microbacterium* is isolated from a chromosomal gene library of the microorganism of the genus *Microbacterium*.

The operation in the PCR method is described by White, T. J. et al., in *Trends Genet.* 5, 185 (1989), etc. The method of preparing chromosomal DNA and the method of isolating the desired DNA molecule from a gene library by use of the above DNA molecule as a probe are described in *Molecular Cloning*, 2nd edition, Cold Spring Harbor Press (1989), etc.

The method of determining the nucleotide sequence of the isolated DNA coding for the HRase derived from the microorganism of the genus *Microbacterium* is described in *A Practical Guide to Molecular Cloning*, John Wiley & Sons, Inc. (1985). Alternatively, the nucleotide sequence can be determined by use of a DNA sequencer manufactured by Applied Biosystems. The DNA coding for the HRase derived from the microorganism of the genus *Microbacterium* is shown in SEQ ID NO:1 in the Sequence Listing.

The DNA coding for the HRase derived from the microorganism of the genus Microbacterium is not limited to the DNA set forth in SEQ ID NO:1 in the Sequence Listing. This is because the nucleotide sequence must be varied
5 depending on the species and strain of each microorganism belonging to the genus Microbacterium.

As a matter of course, the DNA of the present invention encompasses not only the isolated DNA coding for the HRase but also HRase-coding DNA which was prepared by artificially
10 mutating the HRase-coding DNA isolated from the chromosomal DNA in the microorganism of the genus Microbacterium. As a method used frequently for artificial mutation, there is the site-specific mutagenesis method described in Methods in Enzymol., 154 (1987).

15 The DNA of the present invention also encompasses DNA having a nucleotide sequence hybridizing under stringent conditions with the nucleotide sequence set forth in SEQ ID NO:1 in the Sequence Listing and coding for a protein having a 5-substituted hydantoin racemase activity. As
20 used herein, the "stringent conditions" refer to those conditions under which a specific hybrid is formed whereas an unspecific hybrid is not formed. These conditions are hardly expressed numerically, but by way of example, mention is made of those conditions under which DNA molecules having
25 higher homology, for example 50 % or more, more preferably

80% or more and most preferably 90% or more homology hybridize with each other while DNA molecules having lower homology do not hybridize with each other, or to those conditions under which hybridization occurs under usual washing conditions in Southern hybridization, that is, at 60 °C, a salt concentration of 1xSSC and 0.1 % SDS, preferably at 60 °C, 0.1xSSC and 0.1 % SDS and more preferably at 65 °C, 0.1xSSC and 0.1 % SDS. Further, the "5-substituted hydantoin racemase activity" may be any activity of racemizing a 5-substituted hydantoin compound. However, the nucleotide sequence hybridizing under stringent conditions with the nucleotide sequence set forth in SEQ ID NO:1 in the Sequence Listing preferably maintain about at least half of the enzyme activity of a protein having the amino acid sequence set forth in SEQ ID NO:2 in the Sequence Listing under the conditions of 50 °C and pH 8.

The DNA of the present invention also encompasses DNA coding for substantially the same protein as the HRase encoded by the DNA set forth in SEQ ID NO:1 in the Sequence Listing. That is, the DNA of the present invention encompasses DNA coding for a protein having a 5-substituted hydantoin racemase activity and having the following amino acid sequence:

(a) the amino acid sequence set forth in SEQ ID NO:2 in the Sequence Listing, or

(b) an amino acid sequence wherein in the amino acid sequence set forth in SEQ ID NO:2 in the Sequence Listing, one or more amino acid residues have been replaced, deleted, inserted, added or inverted. Here, the number of "one or
5 more amino acid residues" is in such a range that the stereostructure of the proteins of amino acid residues or the 5-substituted hydantoin racemase activity is not significantly deteriorated; specifically, the number of such amino acids is 2 to 50, preferably 2 to 30 and more
10 preferably 2 to 10. Further, the "5-substituted hydantoin racemase activity" means any activity of racemizing a 5-substituted compound as described above. However, the amino acid sequence (b) wherein in the amino acid sequence set forth in SEQ ID NO:2 in the Sequence Listing, one or
15 more amino acid residues have been replaced, deleted, inserted, added or inverted, preferably has about at least half of the enzyme activity of a protein having the amino acid sequence set forth in SEQ ID NO:2 in the Sequence Listing under the conditions of 50 °C and pH 8.

20 (2) Properties of HRase

Hereinafter, the properties of the purified HRase derived from the microorganism of the genus *Microbacterium* are described.

As revealed by isolation and analysis of the
25 above-described gene, the HRase of the present invention

has the amino acid sequence set forth in SEQ ID NO:2 in the Sequence Listing. However, the present invention also encompasses a protein having a 5-substituted hydantoin racemase activity and having an amino acid sequence wherein
5 in the amino acid sequence set forth in SEQ ID NO:2 in the Sequence Listing, one or more amino acid residues have been replaced, deleted, inserted, added or inversed.

That is, the HRase of the present invention is a protein having a 5-substituted hydantoin racemase activity and
10 having the following amino acid sequence (a) or (b):

(a) the amino acid sequence set forth in SEQ ID NO:2 in the Sequence Listing, and

(b) an amino acid sequence wherein in the amino acid sequence set forth in SEQ ID NO:2 in the Sequence Listing,
15 one or more amino acid residues have been replaced, deleted, inserted, added or inverted.

The terms "one or more amino acid residues" and "5-substituted hydantoin racemase activity" have the same meanings as defined in item (1) DNA coding for HRase.

20 The HRase of the present invention catalyzes the racemization reaction of a 5-substituted hydantoin compound.

Measurement of the HRase activity of the HRase of the present invention can be carried out by measuring a change
25 in the optical rotation as the degree of racemization of

the optically active D- or L-5-substituted hydantoin compound as the substrate or by high performance liquid chromatography (HPLC) using an optical resolution column.

Specifically, a reaction solution containing 120 mg/dl L- or D-benzyl hydantoin (BH), 50 mM dibasic potassium phosphate buffer (KPB) (pH 8.0), 5 mM dithiothreitol (DTT) and an HRase enzyme solution was incubated at 37 °C for 30 minutes, and the reaction was stopped by adding a 9-fold excess volume of 1.1 mM CuSO₄ and 11.1 mM H₃PO₄ solution. The precipitates were removed by centrifugation at 20,000 g×10 minutes, and the amount of racemized BH was quantitatively determined by HPLC to estimate the racemization activity. The enzyme activity causing racemization of 1 μmol BH per minute under these conditions was defined as 1 U of the enzyme activity.

The quantitative analysis of optically active BH by analysis in HPLC was conducted by use of Daicel Chemical CHIRALPAK WH 0.46 cmφ×25 cm. The analysis conditions are as follows:

Mobile phase: 5 % (v/v) methanol, 1 mM CuSO₄

Column temperature: 50 °C

Flow rate: 1.5 ml/min.

Detection: UV₂₁₀

Under these conditions, D-BH (retention time: 4.2 minutes) and L-BH (retention time: 5.3 minutes) were eluted.

Then, the enzymatic chemical properties of the HRase of the present invention measured by the above analysis method are described below.

The working optimum temperature of the HRase of the present invention lies in a higher temperature range than that of the previously reported HRases, and thus the present enzyme is characterized by the ability to catalyze racemization reaction efficiently. That is, the HRase of the present invention having the amino acid sequence (a) or (b) above is characterized in that its working optimum temperature, though varying at a certain degree due to a difference in the amino acid sequence, is 50 °C or more. The working optimum temperature is preferably 52 °C or more, more preferably 55 °C or more. The upper limit of the working optimum temperature is not particularly limited, but in consideration of the thermostability of HRase, the upper limit is preferably 60 °C or less. The "working optimum temperature" in the present specification means the temperature at which the maximum activity is exhibited under the condition of pH 8.

Optimum pH: in the vicinity of about 7 to about 9.

To determine the range of the working optimum pH, the reaction was carried out for 30 minutes under the condition of 40 °C.

pH stability: in the vicinity of about 6 to about 9.

The pH stability after treatment at each pH for 30 minutes under the condition of 0 °C was examined.

Temperature stability: stable at about 40 °C or less.

The temperature stability after heating treatment for 5 30 minutes under the condition of pH 8.0 was examined.

Influence of inhibitors: Strongly inhibited by NEM (N-ethyl maleimide), copper ion, or IAA (monoiodoacetic acid). The HRase is hardly inhibited by EDTA (ethylenediaminetetraacetic acid). The HRase of the 10 invention is activated by adding DTT (dithiothreitol).
Molecular weight: a) about 107,000 (gel filtration), b) about 27,000 (SDS-PAGE).

It is estimated that the HRase of the present invention has a tetramer structure of subunits each having a molecular 15 weight of 27,000.

(3) Process for producing HRase and a protein having a 5-substituted hydantoin racemase activity

Now, a process for producing the HRase of the invention and a protein having a 5-substituted hydantoin racemase 20 activity is described. The process for producing the HRase of the invention and a protein having a 5-substituted hydantoin racemase activity includes two methods, that is, (i) a process which comprises culturing a microorganism to form and accumulate HRase, and (ii) a process which comprises 25 preparing a transformant forming HRase or a protein having

a 5-substituted hydantoin racemase activity by recombinant DNA technology and culturing the transformant to form and accumulate the protein.

(i) Process of formation and accumulation by culturing a
5 microorganism

In the process of formation and accumulation by culturing a microorganism, the microorganism as a source of providing HRase includes microorganisms belonging to the genus *Microbacterium*. Preferable microorganisms include
10 *Microbacterium liquefaciens* AJ3912 (FERM-P3133).

The form of culture of microorganisms belonging to the genus *Microbacterium* may be either liquid culture or solid culture, but an industrially advantageous method is a submerged aeration shake culture method. Nutrient
15 sources in the liquid medium can make use of carbon sources, nitrogen sources, inorganic salts and other trace nutrient sources, which are usually used in culture of microorganisms. Any nutrient sources can be used insofar as they can be utilized by the bacterial strain used.

20 As the aeration conditions, aerobic conditions are used. The culture temperature may be in any range in which the microorganism grows and produces HRase. Accordingly, there are no strict conditions, but the culture temperature is usually 10 to 50 °C, preferably 30 to 40 °C. The culture
25 time is varied depending on other culture conditions. For

example, the microorganism may be cultured until HRase is produced most, and the culture time is usually 5 hours to 7 days, preferably 10 hours to 3 days or thereabout.

After culture, the microorganism is collected by
5 centrifugation (e.g., 10,000×g, 10 minutes). Because the enzyme is present in the microorganism, this microorganism is disrupted or lyzed, whereby the enzyme is solubilized. Disruption of the microorganism can make use of methods such as disruption by sonication, disruption by a French press,
10 disruption by glass beads etc., or when the microorganism is to be lyzed, a method of treatment with egg white lysozyme or a peptidase or a method of suitable combination thereof is used.

To purify the HRase derived from a microorganism of
15 the genus *Microbacterium*, the solution containing the solubilized enzyme is purified as the starting material, and if undisrupted or non-lyzed microbial residues are present, it is advantageous to centrifuge the solubilized solution again to remove the precipitated residues.

20 For purification of the enzyme, any conventional methods used in conventional enzyme purification, such as salting-out with ammonium sulfate, gel filtration, ion-exchange chromatography, hydrophobic chromatography etc. can be used. As a result, a fraction containing HRase
25 of higher specific activity can be obtained.

(ii) Process by recombinant DNA technology

Now, a process for producing HRase or a protein having a 5-substituted hydantoin racemase activity (hereinafter referred to collectively as HRase in (ii) Production by recombinant DNA technology) by recombinant DNA technology is described. A large number of examples where useful proteins such as enzymes, physiologically active substances etc. by utilizing recombinant DNA technology are known, and by use of recombinant DNA technology, useful proteins present in a very small amount in nature can be produced in a large amount.

Fig. 2 is a flow chart of the process for producing the HRase of the present invention.

First, the DNA coding for the HRase of the present invention is prepared (step S1).

Then, the prepared DNA is ligated to a vector DNA to prepare a recombinant DNA (step S2), and cells are transformed with the recombinant DNA, to prepare a transformant (step S3). Subsequently, the transformant is cultured in a medium to form and accumulate HRase in the medium and/or the cells (step S4).

Thereafter, the enzyme is recovered and purified in step S5, whereby the purified HRase is produced.

Further, the purified HRase produced in step S5 or the medium containing the HRase accumulated therein in step

S4 is used in synthesis of amino acid, whereby the desired optically active amino acid can be produced in a large amount (step S6).

The DNA ligated to the vector DNA may be any DNA capable
5 of expressing the HRase of the present invention.

As the HRase gene ligated to the vector DNA, it is possible to use the above-described DNA, for example:

(a) DNA having the nucleotide sequence set forth in SEQ ID NO:1 in the Sequence Listing,

10 (b) DNA having a nucleotide sequence hybridizing under stringent conditions with the nucleotide sequence set forth in SEQ ID NO:1 in the Sequence Listing,

(c) DNA coding for the amino acid sequence set forth in SEQ ID NO:2 in the Sequence Listing, and

15 (d) DNA coding for an amino acid sequence wherein in the amino acid sequence set forth in SEQ ID NO:2 in the Sequence Listing, one or more amino acid residues have been replaced, deleted, inserted, added or inverted.

When the protein is produced in a large amount by
20 recombinant DNA technology, the protein preferably aggregates in the transformant producing the protein, to form a protein inclusion body. The advantage of this method of expression and production lies in protection of the desired protein against digestion by proteases present in
25 the microorganism as well as in easy purification of the

desired protein by disrupting and centrifugation of the microorganism.

The protein inclusion body thus obtained is solubilized by a protein denaturant, and after it was
5 subjected to the procedure of activity restoration mainly by removing the denaturant, the inclusion body is converted into a correctly folded, physiologically active protein. For example, there are many examples such as restoration of the activity of human interleukin-2 (Japanese Patent
10 Application Laid-Open (JP-A) No. 61-257931).

To obtain the active protein from the protein inclusion body, a series of procedures such as solubilization and activity restoration etc. are necessary, and thus the procedures are more complex than in direct production of
15 the active protein. However, if a protein exerting an influence on growth of the microorganism is produced in a large amount in the microorganism, the influence can be suppressed by accumulating the protein as an inert inclusion body in the microorganism.

20 The method of producing a large amount of the desired protein as an inclusion body includes not only a method of expressing the desired protein solely under the control of a strong promoter but also a method of expressing a fusion protein with a protein known to be expressed in a large amount.

25 It is also effective that a sequence recognized by

a restriction protease is arranged in a suitable position in order to cut the desired protein off after expression thereof as a fusion protein.

The host cells to be transformed for producing a large
5 amount of the protein by recombinant DNA technology can make use of microbial cells, Actinomyces cells, yeast cells, fungal cells, plant cells, animal cells etc., but in general Escherichia coli is preferably used. This is because there are a large number of techniques for a large production of
10 proteins by use of Escherichia coli. Hereinafter, a process for producing HRase by use of transformed Escherichia coli is described.

As the promoter expressing DNA coding for HRase, a promoter ordinarily used in production of a heterogeneous
15 protein in E. coli can be used, and mention can be made of e.g. strong promoters such as T7 promoter, trp promoter, lac promoter, tac promoter, PL promoter etc.

To produce HRase as a fusion protein inclusion body, a gene coding for another protein, preferably a hydrophilic
20 peptide, is ligated to a region upstream or downstream of the HRase gene to produce a fusion-protein gene. The gene coding for another protein may be any gene which increases the amount of a fusion protein accumulated and raises the solubility of the fusion protein after denaturation and
25 restoration, and for example, T7 gene 10, β -galactosidase

gene, dehydrofolate reductase gene, interferon γ gene, interleukin-2 gene, prochymosin gene etc. can be cited as candidates.

For ligating these genes to the gene coding for HRase,
5 their codon open reading frames shall be in accordance with one another. They may be ligated at suitable restriction enzyme sites, or synthetic DNA of suitable sequence may be utilized.

To increase the amount of the fusion protein produced,
10 a terminator that is a transcription termination sequence is preferably ligated to a downstream region of the fusion-proteingene. The terminator includes T7 terminator, fd phage terminator, T4 terminator, a terminator for tetracycline resistance gene, a terminator for E. coli trp
15 A gene, etc.

The vector for introducing the gene coding for HRase or a fusion protein of HRase with another protein into E. coli is preferably the so-called multicopy vector, and includes plasmids having an origin of replication derived
20 from Col E1, for example, pUC series plasmids, pBR322 series plasmids or derivatives thereof. The "derivatives" are those obtained by modifying plasmids by replacement, deletion, insertion, addition or inversion of some nucleotides. The modification referred to here includes
25 modification by natural mutation or by mutation treatment

with a mutagen or UV irradiation.

To select the transformant, the vector preferably has a marker such as ampicillin resistance gene etc. As such plasmids, expression vectors having a strong promoter are
5 commercially available (pUC series (Takara Shuzo Co., Ltd.), pPROK series (Clontech), pKK233-2 (Clontech) etc.).

A DNA fragment having a promoter, a gene coding for HRase or a fusion protein of HRase with another protein and a terminator ligated in this order is then ligated to a vector
10 DNA to give a recombinant DNA.

The recombinant DNA is used to transform E. coli, and by culturing the E. coli, HRase or a fusion protein of HRase with another protein is expressed and produced.

Although the host to be transformed can be any strain
15 ordinarily used for expressing a heterogeneous gene, Escherichia coli JM109, particularly JM109 (DE3) is preferable. The method for conducting transformation and the method for selecting the transformant are described in Molecular Cloning, 2nd edition, Cold Spring Harbor Press
20 (1989), etc.

When HRase is expressed as a fusion protein, HRase may be cleaved off with a restriction protease such as blood coagulation factor Xa or kallikrein whose recognition sequence is a sequence not present in HRase.

25 The production medium used may be a medium such as

M9-casamino acid medium, LB medium etc. which is usually used for culturing E. coli. Further, culture conditions and production-inducing conditions are selected suitably depending on a marker and a promoter in the vector used,
5 the type of host microorganism, etc.

To recover HRase or a fusion protein of HRase and another protein, there are the following methods. If HRase or its fusion protein has been solubilized in the microorganism, the microorganism is recovered, disrupted
10 or lyzed and the resulting crude enzyme solution can be used. As necessary, HRase or its fusion protein can be used in a pure form after purification by techniques such as conventional precipitation, filtration and column chromatography. In this case, a method of purification by
15 use of an antibody against HRase or its fusion protein can also be used.

When the protein inclusion body is formed, it is solubilized with a denaturant. The inclusion body may be solubilized together with microbial proteins, but in
20 consideration of the subsequent purification procedures, it is preferable that the inclusion body is removed and solubilized. The inclusion body can be recovered from the microorganism by a method known in the art. For example, the microorganism is disrupted and centrifuged etc. to
25 recover the inclusion body. The denaturant for

solubilizing the protein inclusion body includes guanidine hydrochloride (e.g., 6 M, pH 5 to 8) and urea (e.g. 8 M).

By removing the denaturant by dialysis, HRase is regenerated as a protein having activity. The dialysis
5 solution used in dialysis may make use of Tris-HCl buffer or phosphate buffer, and the concentration is 20 mM to 0.5 M, and the pH is 5 to 8.

The concentration of the protein in the regeneration step is preferably limited to about 500 µg/ml or less. To
10 prevent self-crosslinkage of the regenerated HRase, the dialysis temperature is preferably 5 °C or less. Further, the method of removing the denaturant includes a dilution method and an ultrafiltration method besides the dialysis method, and using any method, restoration of the activity
15 can be expected.

If the DNA set forth in SEQ ID NO:1 in the Sequence Listing is used as DNA coding for HRase, HRase having the amino acid sequence set forth in SEQ ID NO:2 is produced.

[II] Process for producing an optically active amino acid
20 by HRase

Now, the process for producing an optically active amino acid from a 5-substituted hydantoin compound by use of the HRase of the present invention is described.

The HRase of the present invention used in the reaction
25 of producing an optically active amino acid includes a

protein having a hydantoin racemase activity, which has the amino acid sequence (a) or (b):

(a) the amino acid sequence set forth in SEQ ID NO:1 in the Sequence Listing, and

5 (b) an amino acid sequence wherein the amino acid sequence in SEQ ID NO:1 in the Sequence Listing, one or more amino acid residues have been replaced, deleted, inserted, added or inversed.

As the HRase described above, it is possible to use
10 (1) HRase obtained by culturing a microorganism of the genus Microbacterium or (2) HRase obtained by preparing an HRase-producing transformant by recombinant DNA technology from a microorganism of the genus Microbacterium and then culturing the transformant.

15 When a microorganism of the genus Microbacterium or cells transformed by the recombinant DNA are used to produce HRase, a substrate may be added directly to a culture thereof during culturing, and either the microorganism separated from the culture or the washed microorganism can be used.

20 Further, a microbial treated material prepared by disrupting or lyzing the microorganism may be used as it is, or HRase may be recovered from the microbial treated material and used as a crude enzyme solution, and the enzyme may be further purified before use. That is, the enzyme and materials
25 containing the enzyme as a fraction having HRase activity

can be used. The "materials containing the enzyme" may be any materials containing the enzyme, and include e.g. a culture, a cultured microorganism, a washed microorganism, a microbial treated material obtained by disrupting or lysing the microorganism, a crude enzyme solution, a purified enzyme, etc.

In the process for producing an optically active amino acid according to the present invention, it is necessary to use the HRase of the present invention and two other enzymes:

(1) hydantoinase (HHase) acting on a 5-substituted hydantoin compound and hydrolyzing the compound thereby catalyzing the reaction of forming N-carbamyl amino acid, and

(2) N-carbamyl amino acid hydrolase (CHase) acting on the formed N-carbamyl amino acid and hydrolyzing the compound thereby catalyzing the reaction of forming an optically active amino acid.

In the above, if the optical selectivity of HHase hydrolyzing a 5-substituted hydantoin compound is high, either optically active N-carbamyl-L-amino acid or N-carbamyl-D-amino acid can be formed in higher yield (50 % or more molar yield by the action of HRase) by allowing the HHase having high optical selectivity and the HRase of the present invention to act on the 5-substituted hydantoin.

In this case, CHase or a material containing the enzyme may be subsequently used to produce the optically active amino acid, or the optically active amino acid can be produced in higher yield while maintaining optical activity by
5 chemical hydrolysis treatment with nitrite (a method by combination of a microbial enzyme system and a chemical reaction system).

HHase for optico-selectively hydrolyzing a 5-substituted hydantoin compound can be obtained in the
10 following manner. For example, the presence of a thermostable D-HHase enzyme in microorganisms of the genus *Bacillus* as microorganisms having D-HHase forming N-carbamyl-D-amino acid is known; for example, HHase or an HHase-containing fraction may be prepared from *Bacillus*
15 *stearothermophilus* ATCC 31195 etc. (Appl. Microbiol. Biotechnol., 43, 270 (1995)). ATCC 31195 can be obtained from American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, United States of America. Further, the presence of L-HHase acting specifically on
20 L-hydantoin compounds is known in e.g. *Bacillus* sp. AJ12299 (Japanese Patent Application Laid-Open (JP-A) No. 63-24894). *Bacillus* sp. AJ12299 is a microorganism deposited on July 5, 1986 under FERM-P8837 with the National Institute of Bioscience and Human-Technology, the Agency of Industrial
25 Science of Technology, the Ministry of International Trade

and Industry.

Even if D- has no optico-selective hydrolysis activity, the formed amino acid will be a D- or L-optically active isomer insofar as CHase has optical selectivity. In this case, it is estimated that when L-amino acid is formed by CHase through selective decomposition of N-carbamyl-L-amino acid that is N-carbamyl amino acid as the unreacted enantiomer, N-carbamyl-D-amino acid will remain in the reaction solution, while D-amino acid is formed, N-carbamyl-L-amino acid will remain. In this case, however, HHase will slightly catalyze the reverse reaction of dehydrating and condensing N-carbamyl amino acid as the remaining unreacted enantiomer, to form the 5-substituted hydantoin compound again, and therefore the optically active amino acid can be produced in high yield (at least 50 % molar yield by the action of HRase) by the three enzymes, that is, HRase, HHase and highly optically selective CHase, or a material containing the three enzymes.

The presence of HHase having no optical selectivity in e.g. *Arthrobacter aurescens*, besides *Microbacterium liquefaciens* AJ3912 shown in the present invention, is known (J. Biotechnol. 61, 1 (1998)).

The presence of CHase selectively hydrolyzing a D-isomer of N-carbamyl amino acid in e.g. *Pseudomonas* sp. AJ 11220 is known (Japanese Patent Application Publication



(JP-B) No. 56-003034). As a result of our re-classification, *Pseudomonas* sp. AJ11220 is classified as *Agrobacterium* sp.. *Agrobacterium* sp. AJ11220 is a micororganism deposited on December 20, 1977 under FERM-P4347 with the National Institute of Bioscience and Human-Technology, the Agency of Industrial Science of Technology, the Ministry of International Trade and Industry. Further, the presence of CHase selectively hydrolyzing an L-isomer of N-carbamyl amino acid in e.g. *Flavobacterium* sp. AJ3912 (Japanese Patent Application Publication (JP-B) No. 56-008749) and *Bacillus* sp. AJ12299 is known. As described above, *Flavobacterium* sp. AJ3912, which is now classified into *Microbacterium liquefaciens* AJ3912 (FERM-P3133), is a microorganism deposited on June 27, 1975 under FERM-P3133 with the National Institute of Bioscience and Human-Technology, the Agency of Industrial Science of Technology, the Ministry of International Trade and Industry. Further, *Bacillus* sp. AJ12299 is a microorganism deposited on July 5, 1986 under FERM-P8837 with the National Institute of Bioscience and Human-Technology, the Agency of Industrial Science of Technology, the Ministry of International Trade and Industry.

The substrate of HRase or a fraction containing HRase can make any 5-substituted hydantoin compound which can be racemized with the substrate specificity of the enzyme. Examples of the 5-substituted hydantoin compound include 5-indolylmethylhydantoin, 5-(p-hydroxybenzyl) hydantoin,



5-isobutylhydantoin, 5-(3'-pyridyl)-methylhydantoin etc.

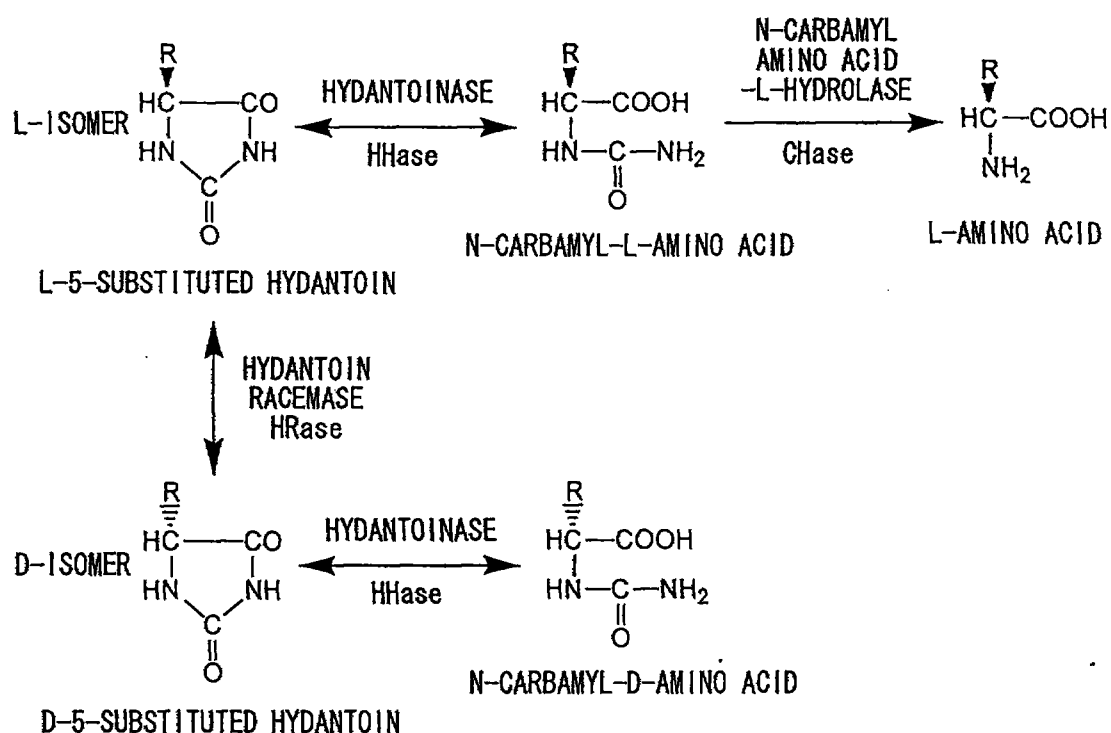
A preferable combination of HRase, HHase and CHase includes a combination of:

HRase having the amino acid sequence set forth in SEQ
5 ID NO:2 in the Sequence Listing,

HHase having the amino acid sequence set forth in SEQ
ID NO:4 in the Sequence Listing, and

CHase having the amino acid sequence set forth in SEQ
ID NO:6 in the Sequence Listing. Any of these enzymes are
10 enzyme derived from *Microbacterium liquefaciens* AJ3912.

When HRase having the amino acid sequence set forth
in SEQ ID NO:2 in the Sequence Listing, HHase having the
amino acid sequence in SEQ ID NO:4 in the Sequence Listing
and CHase having the amino acid sequence in SEQ ID NO:6 in
15 the Sequence Listing are used in combination, mixed proteins
consisting of HRase, HHase and CHase, obtained by culturing
cells transformed with a recombinant DNA comprising a vector
ligated to a group of structural genes set forth in SEQ ID
NO:7 coding for all the proteins involved in producing
20 L-amino acid can also be used. If the mixed proteins are
used, the hydantoin racemase contained in the mixed proteins
catalyses racemization of the 5-substituted hydantoin
compound as shown in the reaction scheme (II) below, so that
from the DL-5-substituted hydantoin compound, L-amino acid
25 can be produced in theoretically 100 % molar yield.



... (11)

The above mixed proteins can also be used to produce N-carbamyl amino acid. For example, N-carbamyl amino acid can be produced by adding e.g. an inhibitor of the

5 N-carbamyl-L-amino acid hydrolase to terminate the hydrolysis reaction at the stage of N-carbamyl amino acid.

When the culture liquid of the microorganism of the genus *Microbacterium* or of cells transformed with the recombinant DNA, or the separated microorganism, the washed

10 microorganism, the microbial treated material, or the crude or purified enzyme obtained from the microbial treated material is used for progress of the amino acid-forming reaction, a reaction solution containing a 5-substituted hydantoin compound and the culture liquid, the separated

microorganism, the washed microorganism, the microbial treated material or the crude or purified enzyme is adjusted to a suitable temperature of 25 to 40 °C and left or stirred for 8 hours to 5 days while the pH is kept at 5 to 9.

5 Further, when the amino acid-forming reaction is allowed to proceed while the microorganism of the genus *Microbacterium* or cells transformed with the recombinant DNA are cultured in a water-soluble medium, the water-soluble medium contains a 5-substituted hydantoin compound and
10 nutrients such as a carbon source, a nitrogen source and inorganic ions necessary for growth of the transformed cells. Further, desirable results are often obtained by adding organic trace nutrients such as vitamins, amino acids etc. The 5-substituted hydantoin compound may be added in divided
15 portions. Culturing is conducted preferably for 8 hours to 5 days under aerobic conditions while maintaining a suitable range of pH 5 to 9 and a temperature of 25 to 40 °C.

 The formed amino acid can be separated and purified
20 by techniques known in the art. For example, mention is made of a method of adsorbing the basic amino acid by contacting with an ion-exchange resin, then eluting it followed by crystallization thereof, a method of discoloring the amino acid by filtration with activated carbon and then
25 crystallizing it, etc.

Hereinafter, the present invention is described in more detail by the way of examples. The present invention is not limited to the description of the Examples.

Example 1: Production and purification of HRase

5 *Microbacterium liquefaciens* AJ3912 (FERM-P3133) was cultured to give the microorganism having a sufficient HRase activity.

First, the microorganism was refreshed at 30 °C for 24 hours in CM2G agar medium (0.5 g/dl D-glucose, 1 g/dl yeast extract, 1 g/dl peptone, 0.5 g/dl NaCl, 2 g/dl agar, 10 pH 7.0), followed by seed culture in CM2G liquid medium at 30 °C for 24 hours.

Thereafter, 1 ml seed culture liquid was inoculated into a 500-ml Sakaguchi flask containing 50 ml main culture 15 medium and cultured under shaking at 30 °C for 18 hours. The main culture medium was prepared by autoclaving a medium of 0.5 g/dl D-glucose, 0.5 g/dl (NH₄)₂SO₄, 1 g/dl yeast extract, 1 g/dl peptone, 0.1 g/dl KH₂PO₄, 0.3 g/dl K₂HPO₄, 0.01 g/dl MgSO₄·7H₂O, 0.35 g/dl DL-5-indolyl methyl hydantoin (IMH), 20 pH 7.0, at 120 °C for 20 minutes, and then adding 1 mg/dl each of FeSO₄·7H₂O, MnSO₄·4-5H₂O, and CaCl₂·2H₂O thereto and leaving the medium at 4 °C to precipitate IMH sufficiently.

After culture, the microorganism was collected by centrifugation, washed with 0.1 M KPB (pH 7.0) to give the 25 washed microorganism.



The microorganism thus obtained was used as a material from which HRase was to be purified.

1. Disruption of the microorganism

66 g of the washed microorganism (corresponding to 3.7 L culture liquid) was used as the starting material, and this microorganism was suspended in 130 ml of 0.1 M KPB (pH 7.0) and disrupted with 0.1 mm ϕ glass beads for 3 minutes (30 seconds $\times$ 6 times at 90-second intervals) with a bead beater. The solution was recovered and treated with DNase I at a final concentration of 5 μ g/ml at room temperature for 20 minutes. Thereafter, the undisrupted cells were removed by centrifugation at 13,000 g $\times$ 10 minutes, and the membrane fraction was further removed by ultracentrifugation at 100,000 g $\times$ 60 minutes, and the supernatant was used as a cell-free extract.

2. Ammonium sulfate fractionation

59 g ammonium sulfate was added at a final concentration of 70 % saturation to 125 ml of the cell-free extract, and the extract was adjusted to pH 7.0 with KOH and stirred at 5 $^{\circ}$ C for 60 minutes. The precipitates were recovered by centrifugation at 13,000 g $\times$ 15 minutes, dissolved in a small amount of 20 mM KPB (pH 7.0) and dialyzed against 1.2 M (NH₄)₂SO₄, 20 mM KPB, 0.5 mM CoCl₂, pH 7.0 (Buffer A). After dialysis, the dialyzate was centrifuged at 13,000 g $\times$ 30 minutes, and the resulting supernatant, 40 ml, was used in the following

purification.

3. Hydrophobic chromatography

The resulting enzyme solution was subjected to a hydrophobic chromatography column Phenyl Superose HP 26/10 (Pharmacia) previously equilibrated with Buffer A. The non-adsorbed protein was eluted with Buffer A, and then the adsorbed protein was eluted with a linear gradient (-1.2 M/12 CV) of from 1.2 to 0 M ammonium sulfate. When the HRase activity of each eluted fraction was measured, the activity was recognized in elution positions of from about 500 mM to 100 mM ammonium sulfate. Fractions with the HRase activity were recovered, concentrated through a membrane and dialyzed against 20 mM KPB (pH 7.0).

4. Anion-exchange chromatography

The resulting enzyme solution was applied onto an anion-exchange chromatography column Q-Sepharose HP 16/10 (Pharmacia) previously equilibrated with 20 mM KPB (pH 7.0). After the non-adsorbed protein was eluted with 20 mM KPB (pH 7.0), the adsorbed protein was eluted with a linear gradient (0.5 M/12 CV) of from 0 to 0.5 M NaCl. When the HRase activity of each eluted fraction was measured, the activity was recognized in elution positions of from about 300 mM to 400 mM NaCl. Fractions with the HRase activity were recovered and concentrated through a membrane.

5. Gel filtration

The resulting enzyme solution was applied onto Superdex 200 pg 16/60 (Pharmacia) previously equilibrated with 20 mM KPB (pH 7.0), and the sample was developed with the same buffer. When the HRase activity of each eluted
5 fraction was measured, the activity was recognized at the position of an estimated molecular weight of about 107,000. Fractions with the HRase activity were recovered, concentrated through a membrane and dialyzed against 1.0 M $(\text{NH}_4)_2\text{SO}_4$, 20 mM KPB, 1 mM L-benzyl hydantoin (BH), pH 7.0
10. (Buffer B).

6. Hydrophobic chromatography

The resulting enzyme solution was applied onto Phenyl Superose 5/5 (Pharmacia) previously equilibrated with Buffer B. The non-adsorbed protein was eluted with Buffer
15 B, and then the adsorbed protein was eluted with a linear gradient (-1.2 M/17 CV) of from 1.2 to 0 M ammonium sulfate. The HRase activity of each eluted fraction was measured, and fractions with the HRase activity were recovered and concentrated through a membrane.

20 Thereafter, this purification step using Phenyl Superose was performed once in an L-BH-free buffer system and then once in an L-BH-containing buffer system.

The enzyme solution obtained in the above procedures was used as purified HRase solution.

25 The increase in specific activity brought about by

the above purification was measured. By measuring the HRase activity of both the cell-free extract after disruption of the microorganism and the active fraction obtained after purification, it was found that the specific activity per
5 unit weight of the protein was increased 658-fold. In the activity measurement method described later, the specific activity of the purified HRase was estimated to be 79 U/mg.

The resulting active fraction was subjected in a usual manner to SDS-polyacrylamide gel electrophoresis and
10 stained with Coomassie Brilliant Blue, and as a result it was confirmed that the HRase was purified to a single band, and its molecular weight was estimated to be 27,000. In consideration of the result of gel filtration, it was estimated that this HRase has a tetramer structure of
15 subunits each having a molecular weight of 27,000.

7. Determination of an amino acid sequence in the vicinity of the N-terminus

A sequence in the vicinity of the N-terminus of the HRase purified in the manner described above was determined
20 in the following manner.

That is, about 10 µg protein out of the purified HRase fraction was subjected to polyacrylamide gel electrophoresis in the presence of SDS, and then the desired enzyme was transferred from the electrophoresed gel onto
25 a polyvinylidene fluoride (PVDF (Bio-Rad, Trans-Blot))

membrane by use of a Milliblot (Millipore) in a semi-dry system ("Tanpakushitsu Kozo Kaiseki" (Structural Analysis of Protein) written by Hisashi Hirano, Tokyo Kagaku Dojin). Subsequently, the desired enzyme on the PVDF membrane was
5 subjected to a protein sequencer (model 476A manufactured by ABI), to analyze its N-terminal amino acid sequence.

The amino acid sequence of 30 residues from the N-terminus was determined. The determined amino acid sequence in the vicinity of the N-terminus of HRase is set
10 forth in SEQ ID NO:8 in the Sequence Listing.

Example 2: Effect of pH on HRase

The change (optimum pH) of the enzyme activity by reaction pH was determined in the following manner.

0.3 µg/ml purified HRase was used and reacted at 37
15 °C for 30 minutes in the presence of 0.1 M sodium acetate buffer (pH 3.1, 3.9, 4.9, 6.1), KPB (pH 6.4, 7.2, 8.0), or sodium carbonate buffer (pH 8.2, 9.1, 10.2, 10.9), to determine the reaction optimum pH of the HRase.

The measurement results were shown as relative enzyme
20 activity against the actually measured pH of each reaction solution. For convenience sake, the highest activity was given 100. The measurement results are shown in Fig. 3.

The optimum pH of the HRase of the present invention is in the pH range of about 7 to about 9, strictly about
25 pH 8.0 to 9.0 (see Fig. 3).

Example 3: pH stability of HRase

3.0 $\mu\text{g/ml}$ purified HRase was left at 0 °C for 30 minutes in the presence of 0.1 M buffer at each pH, and then adjusted to pH 8.0, and the enzyme was subjected to reaction at pH 8.0, at 37 °C for 30 minutes, and the residual activity was measured to determine the pH stability of the HRase.

The measurement results were shown as relative enzyme activity against pretreatment at each pH. For convenience sake, the highest activity was given 100. The measurement results are shown in Fig. 4.

It was found that the HRase of the present invention is stable in the range of about pH 6 to 9 (see Fig. 4).

Example 4: Effect of temperature on HRase

0.3 $\mu\text{g/ml}$ purified HRase was used and reacted at pH 8.0 for 30 minutes at 0, 21, 30, 40, 50, 60, 70 and 80 °C respectively, to determine the working optimum temperature of the HRase.

The measurement results were shown as relative enzyme activity against the actually measured temperature of each reaction solution. For convenience sake, the highest activity was given 100. The measurement results are shown in Fig. 5.

It was found that the optimum temperature of the HRase of the present invention is in the range of 50 to 60 °C (see Fig. 5). This optimum temperature in reaction is the highest

among known HRases.

Example 5: Temperature stability of HRase

3.0 µg/ml purified HRase was left at pH 8.0 for 30 minutes at 0, 21, 30, 40, 50, 60, 70 and 80 °C, and then
5 the enzyme was subjected to reaction at pH 8.0, at 37 °C for 30 minutes, and the residual activity was measured to determine the temperature stability of the HRase.

The measurement results were shown as relative enzyme activity against pretreatment at each temperature. For
10 convenience sake, the highest activity was given 100. The measurement results are shown in Fig. 6.

It was found that the HRase of the present invention is stable in the range of 40 °C or less (see Fig. 6).

Example 6: Effect of various reagents such as enzyme 15 inhibitors on HRase activity

1.5 µg/ml purified HRase was pre-incubated at 0 °C for 30 minutes at pH 8.0 with 25 mM DTT, 25 mM N-ethylmaleimide (NEM), 12.5 mM CuSO₄, 50 mM monoiodoacetic acid (IAA), 50 mM EDTA or 50 % (v/v) methanol. As the enzyme source, each
20 of these mixtures was reacted at pH 8.0, at 30 °C for 30 minutes to measure the residual activity, and the susceptibility of the HRase to each inhibitor was determined. The concentration of each of the enzyme and the inhibitor during the reaction was about 1/5 relative to the
25 concentration during the pre-incubation.

The residual activity was shown as relative activity to the activity (100 %) in the absence of the reagent (see Table 2). The HRase of the present invention was activated by DTT (not shown in the table) and significantly inhibited by a cysteine residue-modifying reagent such as NEM, iodoacetic acid or Cu^{2+} , suggesting the contribution of a cysteine residue to expression of the activity. Because no significant inhibitory effect was observed when EDTA was added, it was considered that no divalent ion is necessary for expression of the HRase activity. Further, pre-incubation with 50 % (v/v) methanol did not influence the HRase activity (Table 2).

[Table 2]

Reagent	Concentration	Residual Activity (%)
None	-	100
NEM	5 mM	0
CuSO_4	2.5 mM	0
IAA	10 mM	0
EDTA	10 mM	86
Methanol	10 % (v/v)	99

Example 7: Racemization of BH by purified HRase

A reaction solution containing 0.3 mg/ml purified HRase, 0.12 g/dl D- or L-BH, 50 mM KPB (pH 8.0) and 5 mM DTT was incubated at 37 °C and sampled with time to quantify the amount of BH by HPLC. As the control, the same experiment was performed in an experimental group not containing the enzyme, to quantify the spontaneous racemization of BH. The results are shown in Fig. 7.

The HRase of the present invention recognized both D-BH and L-BH as substrates, and whichever substrate was used as the starting substrate, the reaction proceeded until the D, L-isomer ratio reached 1 : 1. However, the initial
5 reaction rate of the racemization reaction was higher when L-BH was used as the starting substrate, and the specific activity of the purified HRase as calculated from the initial reaction rate was estimated to be 100 U/mg for formation of D-BH from L-BH and 79 U/mg for formation of L-BH from
10 D-BH (see Fig. 7).

On the other hand, even in the control group where HRase was not added, spontaneous racemization of BH was observed, but the rate of this racemization was significantly low as compared with the rate of racemization catalyzed by
15 HRase (see Fig. 7).

Example 8: Production of D-Phe from DL-BH or L-BH by a combination of *Agrobacterium* sp. AJ11220 and HRase

Pseudomonas sp. AJ11220 is a microorganism deposited on December 20, 1977 under FERM-P4347 with the National
20 Institute of Bioscience and Human-Technology, the Agency of Industrial Science of Technology, the Ministry of International Trade and Industry. The microbial strain is known to hydrolyze a D-5-substituted hydantoin compound into its corresponding D-amino acid (*Agric. Biol. Chem.*, 51, 721
25 (1987)). However, AJ11220 does not have any hydantoin



racemase so that unless the hydantoin compound spontaneously racemizes, the L-5-substituted hydantoin compound not forming D-amino acid will remain, and therefore, the molar yield of D-amino acid formed from the DL-5-substituted hydantoin compounds that can be prepared inexpensively by chemical synthesis is 50 % at the maximum. Accordingly, the production of D-Phe by a combination of the AJ11220 microorganism and the purified HRase was examined using the substrate BH that could hardly spontaneously racemize.

10. 1. Culturing of AJ 11220 strain

The microorganism was refreshed at 30 °C for 24 hours in GM2G agar medium (0.5 g/dl D-glucose, 1 g/dl yeast extract, 1 g/dl peptone, 0.5 g/dl NaCl, 2 g/dl agar, pH 7.0), followed by seed culture in GM2G liquid medium at 30 °C for 24 hours.

15 The main-culture medium used was prepared by autoclaving a medium consisting of 0.5 g/dl D-glucose, 0.5 g/dl $(\text{NH}_4)_2\text{SO}_4$, 1 g/dl yeast extract, 0.1 g/dl KH_2PO_4 , 0.3 g/dl K_2HPO_4 , 0.01 g/dl $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, pH 7.0, at 120 °C for 20 minutes and then adding 1 mg/dl each of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 20 $\text{MnSO}_4 \cdot 4-5\text{H}_2\text{O}$ and 2 g/dl CaCO_3 thereto. 50 ml of this main-culture medium was introduced into a 500-ml Sakaguchi flask, and 1 ml of the seed culture liquid was inoculated thereinto and subjected to shake culture at 30 °C for 22 hours. For inducing the enzyme, D-glucose and DL-BH were 25 added at final concentrations of 2 g/dl and 0.2 g/dl

respectively in 14 hours after the main culture was initiated, and DL-BH was added at a final concentration of 0.2 g/dl in 16 and 18 hours after the main culture was initiated.

After the culture was finished, the microorganism was
5 collected by centrifugation (10,000×g, 4 °C, 10 minutes) and washed with 0.1 M KPB (pH 7.0), to give the washed microorganism.

2. Production of D-Phe from DL-BH or L-BH by a combination of AJ 11220 and HRase

10 0.5 g/dl (26 mM) DL-BH or L-BH, together with 0.84 µg/ml purified HRase, 0.1 M KPB (pH 8.0), 0.5 mM CoCl₂ and 2 g/dl wet AJ11220 microorganism, was subjected to stationary reaction at 30 °C. Oxygen was previously removed from the reaction solution by replacing it by nitrogen. The reaction
15 solution was sampled with time and analyzed by HPLC to quantify the formed D-Phe.

The time course of D-Phe formation is shown in Fig. 8. In the control where D-BH was used as the substrate in the absence of HRase, almost every substrate was converted
20 into D-Phe in the reaction for 30 hours (amount of D-Phe produced, 25.4 mM and molar yield, 98 % in □ in Fig. 8). On the other hand, the amount of D-Phe formed from DL-BH was 12.9 mM at the maximum (reaction, 22 hours and molar yield, 49 % in ○ in Fig. 8), while the amount of D-Phe formed
25 from L-BH was limited to 1.5 mM (reaction, 30 hours and molar

yield, 6 % in Δ in Fig. 8). The amount of D-Phe formed from DL-BH as the substrate was about half of the amount of D-Phe formed from D-BH as the substrate, and D-Phe was hardly formed from L-BH as the substrate, thus indicating that the
5 rate-determining reaction of forming D-Phe from BH in this reaction system is the reaction of racemizing L-BH into D-BH.

When HRase was added, on the other hand, it was observed that in the reaction for 30 hours, 24.6 mM D-Phe (molar yield, 94 %) ($\blacksquare$ in Fig. 8) was formed from DL-BH, and 24.6 mM D-Phe
10 (molar yield, 94 %) ($\blacktriangle$ in Fig. 8) was formed from L-BH. These values were almost equivalent to those achieved by using D-BH as the substrate, suggesting that the rate-determining reaction of racemizing L-BH into D-BH was almost completely resolved by adding HRase.

15 From the foregoing, when the DL- or L-5-substituted hydantoin compound is allowed to act in the D-isomer-selective hydrolysis reaction system to form its corresponding D-amino acid, in the case of 5-substituted hydantoin compound used as the substrate having no or less
20 spontaneous racemization ability, the L-5-substituted hydantoin compound not undergoing hydrolysis will remain unreacted in the reaction solution; however, by combining the reaction system with HRase, the whole of the substrate can be efficiently hydrolyzed to form its corresponding
25 D-amino acid in higher yield.

As a matter of course, when the 5-substituted hydantoin compound is allowed to act similarly in the L-isomer-selective hydrolysis reaction system to form its corresponding L-amino acid, the whole of the substrate can be efficiently hydrolyzed by combining the reaction system with HRase, whereby its corresponding L-amino acid can be formed in higher yield.

Example 9: Racemization of various optically active 5-substituted hydantoin compounds other than BH

The ability of the present HRase to catalyze racemization of optically active 5-substituted hydantoin compounds other than BH was examined. The formation of D-amino acid from L-5-substituted hydantoin by a combination of *Pseudomonas* sp. AJ 11220 and HRase was confirmed in the same manner as in Example 8.

1 g/dl 5-substituted hydantoin of various kinds, together with 1.3 µg/ml purified HRase, 0.1 M KPB (pH 8.0), 0.5 mM CoCl₂ and 2 g/dl wet AJ 11220, was subjected to stationary reaction at 37 °C for 22 hours. Each hydantoin consisted of D- and L-isomers as the substrate, and it was confirmed that D-amino acid was formed from the D-isomer substrate in the absence of HRase, and also that D-amino acid was not formed from the L-isomer substrate in the absence of HRase, and then it was judged that when D-amino acid was formed from the L-isomer substrate in the presence of HRase,

the substrate was recognized. The formation of D-amino acid was qualitatively estimated by development with optical resolution TLC (MERCK, HPTLC CHIR) with methanol : H₂O : acetonitrile = 1 : 1 : 4.

5 As a result, it was recognized that HRase racemized L-5-indolyl methyl hydantoin, L-5-(p-hydroxybenzyl) hydantoin and L-5-isobutyl hydantoin to form their corresponding D-tryptophan, D-tyrosine and D-leucine respectively.

10 Example 10: Isolation of HRase gene

Hereinafter, isolation of HRase gene and expression of HRase in *E. coli* are described where *Microbacterium liquefaciens* AJ3912 (FERM-P3133) was used as the bacterial strain. Isolation of the gene and expression of HRase were
15 conducted using *E. coli* JM109 as the host and pUC18 as the vector.

1. Preparation of PCR primers based on the determined amino acid sequence

On the basis of the N-terminal amino acid sequence
20 (SEQ ID NO:8 in the Sequence Listing) of HRase of *Microbacterium liquefaciens* AJ3912 (FERM-P3133), mix primers shown SEQ ID NOS:9 and 10 respectively were prepared.

2. Acquisition of the microorganism

Microbacterium liquefaciens AJ3912 (FERM-P3133) was
25 refreshed by culturing it at 30 °C for 24 hours in CM2G agar

medium (0.5 g/dl glucose, 1.0 g/dl yeast extract, 1.0 g/dl peptone, 0.5 g/dl NaCl, 2 g/dl agar, pH 7.0). The microorganism was inoculated via one loop of platinum into a 500-ml Sakaguchi flask containing 50 ml CM2G liquid medium, and then cultured under shaking at 30 °C for 16 hours under aerobic conditions.

3. Acquisition of the chromosomal DNA from the microorganism

50 ml of the culture liquid was centrifuged (12,000xg, 4 °C, 15 minutes), whereby the microorganism was collected. This microorganism was suspended in 10 ml of 50 : 20 TE (50 mM Tris-HCl, pH 8.0, 20 mM EDTA), washed and centrifuged, whereby the microorganism was recovered. This microorganism was suspended again in 10 ml of 50 : 20 TE. 0.5 ml of 20 mg/ml lysozyme solution and 1 ml of 10 % SDS solution were added to the above suspension, followed by incubation at 55 °C for 20 minutes. After incubation, a 1-fold volume of 10:1 TE-saturated phenol was added thereto to remove proteins. A 1-fold volume of 2-propanol was added to the separated aqueous layer, to precipitate and recover DNA. After the precipitated DNA was dissolved in 0.5 ml of 50 : 20 TE, 5 µl of 10 mg/ml RNase and 5 µl of 10 mg/ml Proteinase K were added thereto and the mixture was reacted at 55 °C for 2 hours. After reaction, a 1-fold volume of 10 : 1 TE-saturated phenol was added thereto to remove proteins. Further, a 1-fold volume of 24 : 1

chloroform/isoamyl alcohol was added thereto and stirred, and the aqueous layer was recovered. This procedure was repeated further twice, and 3 M sodium acetate solution (pH 5.2) was added at a final concentration of 0.4 M to the
5 resulting aqueous layer, followed by adding a 2-fold volume of ethanol thereto. The DNA occurring as precipitates was recovered, washed with 70 % ethanol, dried and dissolved in 1 ml of 10 : 1 TE.

4. Acquisition of a DNA fragment containing a part of the 10 HRase gene by the cassette PCR method

For isolation and amplification of the DNA molecule containing the gene (fhr) coding for HRase by the cassette PCR method, TaKaRa LA PCR in vitro Cloning Kit (Takara Shuzo Co., Ltd) was used. Unless otherwise noted, the experiment
15 was carried out according to instructions of the kit. In the cassette PCR method, when primer 1 (1st PCR) and primer 2 (2nd PCR) were used as the primers, an about 0.4-kb band (fragment 1) was amplified with HindIII cassette. By determining the nucleotide sequence of this fragment,
20 fragment 1 was confirmed to be a part of fhr.

5. Cloning of the HRase gene from a gene library

Then, Southern hybridization with fragment 1 as a probe was carried out to obtain the full-length fhr.

The concentration of the DNA fragment serving as a
25 probe was adjusted to about 50 ng/ μ l, and 16 μ l of this DNA

solution was labeled as the probe by incubating it at 37 °C for 24 hours with DIG High Prime (Boehringer Mannheim) according to its protocol.

1 µg chromosomal DNA was completely digested with a combination of various restriction enzymes, subjected to electrophoresis on 0.8 % agarose gel, and blotted onto a nylon membrane (Boehringer Mannheim, Nylon membranes positively charged). Subsequently, Southern hybridization was conducted according to the conventional method. Hybridization was carried out using DIG Easy Hyb (Boehringer Mannheim), and after pre-hybridization at 50 °C for 30 minutes, the probe was added and hybridized at 50 °C for 18 hours. The sample was detected using a DIG Nucleotide Detection Kit (Boehringer Mannheim).

As a result, a band was detected at a position of about 2.9 kb in the product cleaved with EcoRI/PstI.

This 2.9-kb fragment was recovered and ligated to pUC18, to prepare a library (120 strains) with E. coli JM109. Subsequently, colony hybridization was conducted according to the conventional method. The colonies were transferred onto a nylon membrane filter (Boehringer Mannheim, Nylon membranes for colony and plaque hybridization), followed by alkali denaturation, neutralization and immobilization. Hybridization was conducted using DIG Easy Hyb. The filter was immersed in a buffer and subjected to pre-hybridization

at 42 °C for 30 minutes. Thereafter, the above-described labeled probe was added thereto and hybridized at 42 °C for 18 hours. After washing with SSC buffer, 1 positive clone strain was selected by using a DIG Nucleotide Detection Kit.

5 6. Nucleotide sequence of the HRase gene derived from *Microbacterium liquefaciens* AJ3912 (FERM-P3133)

The plasmid maintained by the selected transformant was prepared by a method described in Molecular Cloning, 2nd edition, Cold Spring Harbor Press (1989), and a
10 nucleotide sequence near to the sequence hybridized with the probe was determined. An open reading frame (ORF) coding for a protein containing the N-terminal amino acid sequence of 30 residues in HRase was present therein and confirmed to be the gene *fhr* coding for HRase. The nucleotide sequence
15 of the full-length HRase gene is shown in SEQ ID NO:1 in the Sequence Listing. The resulting ORF showed 48% homology to known HRases (J. Bacteriol., 174, 962 (1992)) derived from microorganisms of the genus *Pseudomonas*.

7. Expression of HRase gene in *E. coli*

20 To express *fhr* in *E. coli*, plasmid pUCFHR was constructed by ligating *fhr* to a downstream region of a lac promoter in pUC18. PCR was performed where the chromosomal DNA from *Microbacterium liquefaciens* AJ3912 (FERM-P3133) was used as the template and the oligonucleotides shown in
25 Table 2 were used as the primers, and the amplified fragment

was treated with EcoRI and BamHI and ligated to an EcoRI/BamHI-cleaved product of pUC18, and then transformed into E. coli JM109. From ampicillin resistant strains, a strain having the desired plasmid was selected, and the constructed plasmid was designated expression plasmid pUCFHR.

[Table 3]

Primer	Sequence	
5'-side	GCCGAATTCGCGACTGGCAACACGAAGG EcoR I	SEQ ID NO:11
3'-side	CGGGGATCCTTCTCGTTAGAGGTACTGC BamH I	SEQ ID NO:12

The HRase E. coli transformant harboring pUCFHR was subjected to seed culture at 37 °C for 16 hours in LB medium containing 0.1 mg/ml ampicillin. 1 ml of this pre-culture liquid was seeded in a 500-ml Sakaguchi flask containing 50 ml LB medium, and then cultured at 37 °C. In 2.5 hours after culture was initiated, isopropyl-1-thio- β -D-galactopyranoside (IPTG) was added thereto at a final concentration of 1 mM, and culture was further continued for 4 hours.

After the culture was finished, the microorganism was collected, washed, suspended in 5 ml of 50 mM KPB (pH 8.0) and disrupted with 0.1 mm ϕ glass beads for 3 minutes (30 secondsx6 times at 90-second intervals) with a beads beater. The solution was recovered and centrifuged at 20,000 gx10 minutes, and the supernatant was used as the cell-free

extract.

8. Measurement of HRase activity

After the culture, the cell-free extract was prepared as an enzyme source and measured for its HRase activity.

5 For measurement of the HRase activity, a reaction solution containing 120 mg/dl L-BH, 50 mM KPB (pH 8.0), 5 mM DTT, 5 mM EDTA, 150 mM NaCl and the enzyme solution was incubated at 37 °C for 30 minutes, and the reaction was terminated by adding a 9-fold volume of 1.1 mM CuSO₄ and
10 11.1 mM H₃PO₄. By centrifugation at 20,000 g×10 minutes, precipitates were removed, and the amount of BH racemized was quantified by HPLC to determine the HRase activity. The enzyme activity causing racemization of 1 μmol BH per minute under these conditions was defined as 1 U of the enzyme
15 activity.

The HPLC conditions used in analysis are as follows:

Column: Daicel Chemical CHIRALPAK WH 0.46 cmφ×25 cm

Mobile phase: 5 % (v/v) methanol, 1 mM CuSO₄

Column temperature: 50 °C

20 Flow rate: 1.5 ml/min.

Detection: UV₂₁₀

As a result, the HRase activity was detected in only the E. coli having pUC18FHR introduced therein, and it was confirmed that the cloned fhr gene was expressed in E. coli
25 (Table 4).

[Table 4]

Expression of HRase by E. coli having pUC18FHR introduced therein

	Addition of IPTG	Protein concentration (mg/ml) in the cell-free extract	HRase activity (U/mg)
pUC18FHR	+	1.9	0.15
pUC18FHR	-	3.4	0.06
pUC18	+	2.2	Not detected
pUC18	-	3.7	Not detected

According to the present invention, HRase not known so far can be obtained from a microorganism of the genus Microbacterium. As compared with HRases known in the art, this HRase is characterized by a higher optimum reaction temperature, so the reaction temperature can be increased, resulting in an increase in the reaction rate and efficient progress of the desired reaction. Further, the risk of contamination of the reaction solution with microorganisms during the reaction can be reduced and thus there is the advantage of easy process control including quality control. Further, by using the hydantoin racemase of the present invention in combination with a system of hydrolyzing 5-substituted hydantoin compounds optico-selectively, optically active amino acids useful for production pharmaceutical preparations, products in chemical industry, food additives can be formed in higher yield.

20

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195

200

205

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190

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15

195

200

205

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210

215

220

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225

230

235

240

Ser Asn Pro Gly Gly Val Glu Leu Ile His Lys Ala Gln Ser Glu Gly

245

250

255

25

Gln Asp Val His Cys Glu Ser Gly Pro Gln Tyr Leu Asn Leu Thr Met

260

265

270

Asp Asp Ala Glu Lys Val Gly Pro Tyr Met Lys Ile Ala Pro Pro Val

5

275

280

285

Arg Ser Ala Glu Leu Asn Ala Val Leu Trp Glu Gln Leu Glu Lys Gly

290

295

300

10 Tyr Ile Asp Thr Leu Gly Ser Asp His Gly Gly His Pro Val Glu Asn

305

310

315

320

Lys Glu Gly Gly Trp Asp Asp Ile Trp Thr Ala Ser Asn Gly Ala Leu

325

330

335

15

Gly Leu Glu Thr Ser Leu Pro Met Met Leu Thr Asn Gly Val Asn Lys

340

345

350

Gly Arg Val Ser Leu Glu Arg Leu Val Glu Val Met Cys Glu Asn Pro

20

355

360

365

Ala Lys Leu Phe Gly Ile Tyr Pro Gln Lys Gly Thr Leu Gln Val Gly

370

375

380

25 Ser Asp Ala Asp Leu Leu Ile Leu Asp Leu Glu Ile Glu Asp Arg Lys

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405 410 415

5

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15

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Thr Leu Ser Arg Phe Ser Val Glu Gly Pro Gly Val Thr Arg Leu Thr

20 25 30

10

tac act ccg gag cac gcc gcc gcg cga gag gtg atc gtc gcc gcc atg 144

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10

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25

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 His Ile Glu Gln Gly Pro Ile Leu Glu Gln Glu Lys Val Glu Ile Gly
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385

390

395

400

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1239

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5

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<213> Microbacterium liquefaciens

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15

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5

10

15

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20

25

30

20 Tyr Thr Pro Glu His Ala Ala Ala Arg Glu Val Ile Val Ala Ala Met

35

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25

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15																
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	Ala Leu Val Pro Ala Ala Leu Met Val Arg Glu Ile Asn Arg Phe Val		
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	Asp Leu Arg Ser Pro His Glu Glu Ser Ile Arg Leu Leu Val Asp Arg		
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340

345

350

5 Arg Asp Ile Thr Ser Gly Ala Gly His Asp Ser Met Phe Ile Ala Gln

355

360

365

Ile Thr Asp Val Gly Met Val Phe Val Pro Ser Arg Ala Gly Arg Ser

370

375

380

10

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390

395

400

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Although the invention has been described with respect
 to a specific embodiment for a complete and clear disclosure,
 25 the appended claims are not to be thus limited but are to

be construed as embodying all modifications and alternative constructions that may occur to one skilled in the art which fairly fall within the basic teaching herein set forth.

WHAT IS CLAIMED IS:

1. 5-Substituted hydantoin racemase catalyzing racemization reaction of 5-substituted hydantoin, wherein the working optimum pH being greater than or equal to 7 and less than or equal to 9, and the working optimum temperature being greater than 50 and less than or equal to 60 °C.
2. A protein having a 5-substituted hydantoin racemase activity, the protein having amino acid sequence (a) or (b), where (a) is the amino acid sequence set forth in SEQ ID NO:2 in the Sequence Listing, and (b) is an amino acid sequence wherein in the amino acid sequence set forth in SEQ ID NO:2 in the Sequence Listing, one or more amino acid residues have been replaced, deleted, inserted, added or inverted.
3. A 5-substituted hydantoin racemase fraction derived from a microorganism of the genus Microbacterium, the 5-substituted hydantoin racemase fraction is obtained by culturing a microorganism of the genus Microbacterium and disrupting or lyzing the resulting microorganism followed by purification treatment thereof.
4. A method of racemizing an optically active 5-substituted hydantoin compound, which comprises allowing a 5-substituted hydantoin racemase to act on an optically



active 5-substituted hydantoin compound,

wherein the 5-substituted hydantoin racemase catalyzing racemization reaction of 5-substituted hydantoin, and the working optimum pH being greater than
5 or equal to 7 and less than or equal to 9, and the working optimum temperature being greater than 50 and less than or equal to 60 °C.

5. A method of racemizing an optically active
10 5-substituted hydantoin compound, which comprises allowing a protein having a 5-substituted hydantoin racemase activity to act on an optically active 5-substituted hydantoin compound,

wherein the protein having amino acid sequence (a)
15 or (b), where (a) is the amino acid sequence set forth in SEQ ID NO:2 in the Sequence Listing, and (b) is an amino acid sequence wherein in the amino acid sequence set forth in SEQ ID NO:2 in the Sequence Listing, one or more amino acid residues have been replaced, deleted, inserted, added
20 or inverted.

6. A method of racemizing an optically active 5-substituted hydantoin compound, which comprises allowing a 5-substituted hydantoin racemase fraction derived from
25 a microorganism of the genus *Microbacterium* to act on an



optically active 5-substituted hydantoin compound,

wherein the 5-substituted hydantoin racemase fraction is obtained by culturing a microorganism of the genus *Microbacterium* and disrupting or lyzing the resulting microorganism followed by purification treatment thereof.

7. A process for producing N-carbamyl amino acid, which comprises allowing a 5-substituted hydantoin racemase and an enzyme for optico-selectively hydrolyzing 5-substituted hydantoin or a material containing the enzyme to act on 5-substituted hydantoin,

wherein the 5-substituted hydantoin racemase catalyzing racemization reaction of 5-substituted hydantoin, and the working optimum pH being greater than or equal to 7 and less than or equal to 9, and the working optimum temperature being greater than 50 and less than or equal to 60 °C.

8. A process for producing N-carbamyl amino acid, which comprises allowing a protein having a 5-substituted hydantoin racemase activity and an enzyme for optico-selectively hydrolyzing 5-substituted hydantoin or a material containing the enzyme to act on 5-substituted hydantoin,

wherein the protein having amino acid sequence (a)



or (b), where (a) is the amino acid sequence set forth in SEQ ID NO:2 in the Sequence Listing, and (b) is an amino acid sequence wherein in the amino acid sequence set forth in SEQ ID NO:2 in the Sequence Listing, one or more amino acid residues have been replaced, deleted, inserted, added or inverted.

9. A process for producing N-carbamyl amino acid, which comprises allowing a 5-substituted hydantoin racemase fraction derived from a microorganism of the genus Microbacterium and an enzyme for optico-selectively hydrolyzing 5-substituted hydantoin or a material containing the enzyme to act on 5-substituted hydantoin, wherein the 5-substituted hydantoin racemase fraction is obtained by culturing a microorganism of the genus Microbacterium and disrupting or lysing the resulting microorganism followed by purification treatment thereof.

10. A process for producing an optically active amino acid, which comprises allowing a 5-substituted hydantoin racemase, an enzyme for hydrolyzing 5-substituted hydantoin or a material containing the enzyme and an enzyme for optico-selectively hydrolyzing N-carbamyl amino acid or a material containing the enzyme to act on 5-substituted hydantoin,



wherein the 5-substituted hydantoin racemase catalyzing racemization reaction of 5-substituted hydantoin, and the working optimum pH being greater than or equal to 7 and less than or equal to 9, and the working optimum temperature being greater than 50 and less than or equal to 60 °C.

11. A process for producing an optically active amino acid, which comprises allowing a protein having a 5-substituted hydantoin racemase activity, an enzyme for hydrolyzing 5-substituted hydantoin or a material containing the enzyme and an enzyme for optico-selectively hydrolyzing N-carbamyl amino acid or a material containing the enzyme to act on 5-substituted hydantoin,

15 wherein the protein having amino acid sequence (a) or (b), where (a) is the amino acid sequence set forth in SEQ ID NO:2 in the Sequence Listing, and (b) is an amino acid sequence wherein in the amino acid sequence set forth in SEQ ID NO:2 in the Sequence Listing, one or more amino acid residues have been replaced, deleted, inserted, added or inverted.

12. A process for producing an optically active amino acid, which comprises allowing a 5-substituted hydantoin racemase fraction derived from a microorganism of the genus



Microbacterium, an enzyme for hydrolyzing 5-substituted hydantoin or a material containing the enzyme and an enzyme for optico-selectively hydrolyzing N-carbamyl amino acid or a material containing the enzyme to act on 5-substituted
5 hydantoin,

wherein the 5-substituted hydantoin racemase fraction is obtained by culturing a microorganism of the genus Microbacterium and disrupting or lyzing the resulting microorganism followed by purification treatment thereof.

10

13. DNA coding for a 5-substituted hydantoin racemase,

wherein the 5-substituted hydantoin racemase catalyzing racemization reaction of 5-substituted hydantoin, and the working optimum pH being greater than
15 or equal to 7 and less than or equal to 9, and the working optimum temperature being greater than 50 and less than or equal to 60 °C.

14. DNA coding for a protein having a 5-substituted
20 hydantoin racemase activity, the protein having amino acid sequence (a) or (b), where (a) is the nucleotide sequence set forth in SEQ ID NO:1 in the Sequence Listing, and (b) is a nucleotide sequence hybridizing under stringent conditions with the nucleotide sequence set forth in SEQ
25 ID NO:1 in the Sequence Listing.



15. DNA coding for a protein having a 5-substituted hydantoin racemase activity, the protein having amino acid sequence (c) or (d), where (c) is the amino acid sequence set forth in SEQ ID NO:2 in the Sequence Listing, and (d) is an amino acid sequence wherein in the amino acid sequence set forth in SEQ ID NO:2 in the Sequence Listing, one or more amino acid residues have been replaced, deleted, inserted, added or inverted.

10 16. A recombinant DNA obtained by linking a DNA coding for a 5-substituted hydantoin racemase to a vector DNA, wherein the 5-substituted hydantoin racemase catalyzing racemization reaction of 5-substituted hydantoin, and the working optimum pH being greater than or equal to 7 and less than or equal to 9, and the working optimum temperature being greater than 50 and less than or equal to 60 °C.

17. The recombinant DNA according to claim 16, wherein the vector DNA is a pUC series plasmid, a pBR322 series plasmid, or a plasmid derived from derivatives thereof.

18. A recombinant DNA obtained by linking a DNA coding for a protein having a 5-substituted hydantoin racemase activity to a vector DNA,



wherein the protein having amino acid sequence (a) or (b), where (a) is the nucleotide sequence set forth in SEQ ID NO:1 in the Sequence Listing, and (b) is a nucleotide sequence hybridizing under stringent conditions with the nucleotide sequence set forth in SEQ ID NO:1 in the Sequence Listing.

19. The recombinant DNA according to claim 18, wherein the vector DNA is a pUC series plasmid, a pBR322 series plasmid, or a plasmid derived from derivatives thereof.

20. A recombinant DNA obtained by linking a DNA coding for a protein having a 5-substituted hydantoin racemase activity to a vector DNA,

15 wherein the protein having amino acid sequence (c) or (d), where (c) is the amino acid sequence set forth in SEQ ID NO:2 in the Sequence Listing, and (d) is an amino acid sequence wherein in the amino acid sequence set forth in SEQ ID NO:2 in the Sequence Listing, one or more amino acid residues have been replaced, deleted, inserted, added or inverted.

21. The recombinant DNA according to claim 20, wherein the vector DNA is a pUC series plasmid, a pBR322 series plasmid, or a plasmid derived from derivatives thereof.



22. Cells transformed with a recombinant DNA, the recombinant DNA being obtained by linking a DNA coding for a 5-substituted hydantoin racemase to a vector DNA,

wherein the 5-substituted hydantoin racemase
5 catalyzing racemization reaction of 5-substituted hydantoin, and the working optimum pH being greater than or equal to 7 and less than or equal to 9, and the working optimum temperature being greater than 50 and less than or equal to 60 °C.

10

23. The cells according to claim 22 being derived from *Escherichia coli*.

24. Cells transformed with a recombinant DNA, the
15 recombinant DNA being obtained by linking a DNA coding for a protein having a 5-substituted hydantoin racemase activity to a vector DNA,

wherein the protein having amino acid sequence (a) or (b), where (a) is the nucleotide sequence set forth in
20 SEQ ID NO:1 in the Sequence Listing, and (b) is a nucleotide sequence hybridizing under stringent conditions with the nucleotide sequence set forth in SEQ ID NO:1 in the Sequence Listing.

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25. The cells according to claim 23 being derived from *Escherichia coli*.

26. Cells transformed with a recombinant DNA, the
5 recombinant DNA being obtained by linking a DNA coding for a protein having a 5-substituted hydantoin racemase activity to a vector DNA,

wherein the protein having amino acid sequence (c) or (d), where (c) is the amino acid sequence set forth in
10 SEQ ID NO:2 in the Sequence Listing, and (d) is an amino acid sequence wherein in the amino acid sequence set forth in SEQ ID NO:2 in the Sequence Listing, one or more amino acid residues have been replaced, deleted, inserted, added or inverted.

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27. The cells according to claim 26 being derived from *Escherichia coli*.

28. A process for producing a protein having a
20 5-substituted hydantoin racemase activity, which comprises culturing cells in a medium and accumulating a protein having a 5-substituted hydantoin racemase activity in the medium and/or the cells,

wherein the cells being transformed with a recombinant
25 DNA, the recombinant DNA being obtained by linking a DNA



coding for a 5-substituted hydantoin racemase to a vector DNA,

wherein the 5-substituted hydantoin racemase catalyzing racemization reaction of 5-substituted hydantoin, and the working optimum pH being greater than or equal to 7 and less than or equal to 9, and the working optimum temperature being greater than 50 and less than or equal to 60 °C.

29. A process for producing a protein having a 5-substituted hydantoin racemase activity, which comprises culturing cells in a medium and accumulating a protein having a 5-substituted hydantoin racemase activity in the medium and/or the cells,

wherein the cells being transformed with a recombinant DNA, the recombinant DNA being obtained by linking a DNA coding for a protein having a 5-substituted hydantoin racemase activity to a vector DNA,

wherein the protein having amino acid sequence (a) or (b), where (a) is the nucleotide sequence set forth in SEQ ID NO:1 in the Sequence Listing, and (b) is a nucleotide sequence hybridizing under stringent conditions with the nucleotide sequence set forth in SEQ ID NO:1 in the Sequence Listing.

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30. A process for producing a protein having a
5-substituted hydantoin racemase activity, which comprises
culturing cells in a medium and accumulating a protein having
a 5-substituted hydantoin racemase activity in the medium
5 and/or the cells,

wherein the cells being transformed with a recombinant
DNA, the recombinant DNA being obtained by linking a DNA
coding for a protein having a 5-substituted hydantoin
racemase activity to a vector DNA,

10 wherein the protein having amino acid sequence (c)
or (d), where (c) is the amino acid sequence set forth in
SEQ ID NO:2 in the Sequence Listing, and (d) is an amino
acid sequence wherein in the amino acid sequence set forth
in SEQ ID NO:2 in the Sequence Listing, one or more amino
15 acid residues have been replaced, deleted, inserted, added
or inverted.

