

PROCESS FOR PRODUCING D-N-CARBAMOYL- α -AMINO ACIDS

Field of the Invention

The present invention relates to a process for producing D-N-carbamoyl- α -amino acids. More specifically, it relates to a process for producing D-N-carbamoyl- α -amino acids utilizing a novel transformant having a gene for an enzyme which converts 5-substituted hydantoins into corresponding D-N-carbamoyl- α -amino acids by asymmetric cleavage hydrolysis (hereinafter referred to as hydantoinase).

Backgrounds of the Invention

Optically-active D-N-carbamoyl- α -amino acids can be converted into corresponding D- α -amino acids and optically-active D- α -amino acids are important compounds as intermediate compounds for drugs. In particular, examples of industrially useful compounds include D-phenylglycine and D-(p-hydroxyphenyl)glycine as intermediate compounds for the production of semisynthetic penicillins and semisynthetic cephalosporins.

For the production of D-N-carbamoyl- α -amino acids, processes utilizing microbial enzymatic reactions have been known to convert 5-substituted hydantoins into corresponding D-N-carbamoyl- α -amino acids by asymmetric cleavage hydrolysis and such processes are described in JP-A 53-91189, JP-A 53-44690, JP-A 53-69884, JP-A 53-133688 and the like.



obtain from thermophilic microorganisms and it is inserted into mesophilic microorganisms to produce transformant microorganisms having hydantoinase activity. Furthermore, in WO94/00577, a hydantoinase gene fragment is isolated from a microorganism belonging to the genus Agrobacterium and introduced into Escherichia coli or a microorganism of the genus Agrobacterium to express the activity.

Objects of the Invention

In the above-described processes utilizing microbial enzymatic reactions, there are such drawbacks that yields of the enzyme by microorganisms known to be its source is insufficient and, further, culture mediums are expensive.

In addition, with regard to the transformant microorganisms disclosed in JP-A 62-87089, the improvement of the enzymatic activity is only several times in comparison with sporulating thermophilic microorganisms having hydantoinase activity and yields of the enzyme is yet insufficient.

Furthermore, there is no example in the prior art that a 5-substituted hydantoin is subjected to asymmetrical cleavage hydrolysis by using the above-described transformant microorganism to produce and accumulate the corresponding D-N-carbamoyl- α -amino acid.

The present invention is to solve these problems and is directed to the creation of a microorganism having very

high enzyme productivity and to the production of D-N-carbamoyl- α -amino acids efficiently by using the enzyme source thus obtained.

Summary of the Invention

5 JP-A 62-87089 and W094/00577 disclose the method for obtaining hydantoinase producer microorganisms by using recombinant DNA techniques. However, the source microorganisms for supplying hydantoinase genes are completely different from microorganisms used for obtaining hydantoinase genes in
10 the present invention. In addition, the nucleotide sequences and amino acid sequences of the hydantoinase genes are considerably different from those of the present invention.

The present invention is to provide a process for producing a D-N-carbamoyl- α -amino acid which comprises
15 reacting a 5-substituted hydantoin in an aqueous medium with hydantoinase produced by a transformant obtained by transforming a host microorganism such as a microorganism belonging to the genus Escherichia, Pseudomonas, Flavobacterium, Bacillus, Serratia, Agrobacterium, Corynebacterium or
20 Brevibacterium with a recombinant DNA of a DNA fragment containing a gene for hydantoinase derived from Bacillus sp. KNK245 (FERM BP-4863), Agrobacterium sp. KNK712 (FERM BP-1900) or Pseudomonas sp. KNK003A (FERM BP-3181) and a vector DNA.

A transformant having very high hydantoinase
25 productivity such as that obtained in the present invention

has not been known heretofore in the prior art and the extremely efficient and economical production of D-N-carbamoyl- α -amino acids become possible by utilizing the reaction of the enzyme of the transformant.

5 Hereinafter, the present invention will be described in detail with reference to the accompanying drawings.

Brief Explanation of the Drawings

Fig. 1 is a restriction enzyme map of plasmid pAH1043.

10 Fig. 2 is a restriction enzyme map of plasmid pTH102.

Fig. 3 is a restriction enzyme map of plasmid pTH103.

15 Fig. 4 is a restriction enzyme map of plasmid pTH104.

Fig. 5 is a restriction enzyme map of plasmid pPHD301.

Fig. 6 is a restriction enzyme map of plasmid pTHB301.

Detailed Explanation of the Invention

A DNA fragment containing the gene to be used in the present invention can be obtained from Bacillus sp. KNK245, Agrobacterium sp. KNK712 or Pseudomonas sp. KNK003A. Agrobacterium sp. KNK712 and Pseudomonas sp. KNK003A have been deposited at National Institute of Bioscience and Human-Technology, Agency of Industrial Science & Technology (NIBH), Ministry of International Trade & Industry by the present applicant under accession numbers of FERM BP-1900 and FERM BP-3181, respectively and are known strains which described in detail in WO92/10579. Bacillus sp. KNK245 is a strain newly found by the present inventors and has been deposited at NIBH as of November 2, 1994 under the accession number of FERM BP-4863.

Microbiological characteristics of Bacillus sp. KNK245 are as follows.

Bacillus sp. KNK245

Form	rods, filamentous
Size	0.5-0.8 x 3-10 (µm)
Gram stain	+
Spores	+
Motility	-
Growth temperature	37-60°C
Growth pH	5.0-8.9
Growth in 5% NaCl	-

	Growth in 7% NaCl	-
	Growth in 0.02% azide	-
	Growth in 0.001% lysozyme	-
	Nitrate reduction	-
5	Starch hydrolysis	+
	Casein decomposition	+
	Tyrosine decomposition	-
	Catalase	-
	Oxidase	+
10	Indole production	-
	Production of acids from carbohydrates	
	Mannose	(+)
	Xylose	-
15	Arabinose	+
	Rhamnose	-
	Glucose	+
	Fructose	+
	Raffinose	-
20	Sucrose	+
	Maltose	+
	Lactose	-

For obtaining a hydantoinase gene from these strains, normally, the following procedure is carried out.

25 Firstly, genomic DNA is extracted from a microbial cell and then the DNA is partially hydrolyzed with a suitable

restriction enzyme, for example, Sau3AI or the like. The resulting fragments are ligated to vector DNA to obtain recombinant DNA molecules having various genomic DNA fragments as a gene library (see JP-A 58-126789 and U.S. Patent No. 4,237,224).

Then, a recombinant containing the desired gene is selected from this gene library. For example, the selection can be carried out by detection of an expressed protein using its enzymatic activity as an indication, or the selection can be carried out by analyzing the N-terminal sequence of a protein, synthesizing a corresponding DNA probe and detecting the presence of the desired gene by colony hybridization or the like. In addition, the desired gene can be obtained by colony hybridization or polymerase chain reaction (PCR) method using DNA primers which are synthesized suitable portions of a known hydantoinase base sequence. Once the desired gene is obtained, its nucleotide sequence is analyzed. In addition, a hydantoinase producer microorganism and a recombinant containing this enzyme gene are cultivated. The resultant enzyme is purified and the molecular weight of the resultant protein is determined. At the same time, the amino acid sequence about its N-terminal region is determined with a gas phase protein sequencer or the like.

Then, by comparison of these DNA nucleotide sequences and N-terminal amino acid sequences, the initiation

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5 site for translation of the nucleotide sequence portion
encoding a hydantoinase protein into the protein is determined
and, by considering the relation to the molecular weight of
the protein, it is confirmed that the enzyme protein is
10 encoded in the gene portion extending from this site to the
termination codon, thereby verifying the desired gene
(Molecular Cloning, A Laboratory Manual, 2nd Ed., Cold Spring
Harbor Laboratory Press, Chapters 4 and 13). Thus, DNA
fragments of SEQ ID Nos. 1, 2 and 3 were obtained from
15 Bacillus sp. KNK245, Agrobacterium sp. KNK712 and Pseudomonas
sp. KNK003A. As is well known, the gene encoding the enzyme
thus obtained and/or DNA fragment containing it are equivalent
to DNA fragments having another nucleotide sequence which
encodes an amino acid sequence corresponding to the above gene
15 and/or DNA fragment because one amino acid usually corresponds
to a plurality of base codons.

As the vector to be used in the present invention,
plasmids, phages or derivatives thereof can be used, which are
derived from microorganisms and can be autonomously grown in
20 cells of bacteria belonging to the genus Escherichia,
Pseudomonas, Flavobacterium, Bacillus, Serratia,
Corynebacterium or Brevibacterium.

For example, host-vector systems described in
"Guidelines on Recombinant DNA Experiments, -Commentation, Q
25 and A-" (the Life Science Section of the Research Development

Office in the Science and Technology Agency, Ed., revised September 16, 1987), pages 25 to 27 and 36 to 38 can be used. In addition, recombinant DNA having a translation initiation site linked to a suitable position of a vector which has been
5 modified to have a strong structural promoter can be used for the purpose of increasing the amount of enzyme to be produced. Both ends of these fragments are cleaved with a restriction enzyme to produce a recombinant with an appropriate vector. A hydantoinase producer recombinant microorganism can be
10 produced by directly transforming a microorganism belonging to the genus Escherichia, Pseudomonas, Flavobacterium, Bacillus, Serratia, Agrobacterium, Corynebacterium or Brevibacterium.

A hydantoinase producer recombinant microorganism
15 can also be obtained without direct transformation of a microorganism of the above-described genus; that is, the desired gene is once cloned in a host vector system using other microorganisms such as Escherichia coli, after which recombinant DNA with an appropriate vector is produced.

20 The description of the following documents can be widely applied to the production of recombinants: the specification of U.S. Patent No. 4,237,244 to S. N. Cohen et al.; "Idenshi-sousa Jikken-hou (Experimental Method of Gene Manipulation)" [Ed., Yasuyuki TAKAGI, Kohdan-sha Scientific
25 (1980)]; Method in Enzymology, 68, Recombinant DNA [Ed., Ray

Mv, Academic Press (1979)], the specification of JP-A 58-126789 and the like.

5 The desired gene is cloned and unnecessary DNA thereof is removed. Recombinant DNA having its translation initiation site linked to a suitable position of a vector which has been modified to have a strong structural promoter can be used for transformation of the above host cell to increase the amount of the enzyme to be produced.

10 The transformant is cultivated in a conventional nutrient culture medium to express the introduced recombinant DNA. When a character derived from gene DNA or vector DNA is conferred to the recombinant DNA, an appropriate component can be added to the culture medium according to a particular character.

15 To take the transformant thus obtained as a source of enzyme supply, it can be cultivated by using the conventional culture medium. If necessary, it is also possible to carry out a treatment for enzyme induction such as an addition of hydantoin compounds, uracil, isopropyl-1-thio- β -D-galactosides (IPTG) or the like and a temperature increase.
20 Usually, the culture medium used for cultivating the transformant can be a conventional culture medium containing carbon sources, nitrogen source and inorganic ions. In many cases, desired results can be obtained by further adding
25 organic trace nutrients such as vitamins, amino acids and the

like. As the carbon sources, carbohydrates such as glucose and sucrose, organic acids such as acetic acid, alcohols and the like can be conveniently used. As the nitrogen sources, ammonia gas, ammonia water, ammonium salts and the like can be used. As the inorganic ions, phosphate ion, magnesium ion, potassium ion, iron ion, manganese ion, cobalt ion, nickel ion, copper ion, aluminum ion, molybdenum ion and the like can be used.

If cultivation is carried out under aerobic conditions for 1 to 10 days, while adjusting the pH and temperature to an appropriate range of 4 to 8 and 25 to 60°C, respectively, desired results can be obtained.

The enzymatic activity of an enzyme produced by the transformant can be exhibited in the form of, for example, a culture solution of the transformant, its microbial cells, the enzyme extracted from the microbial cells, immobilized microbial cells and the like.

As the microbial cells, any of the culture solution as it is after completion of cultivation, microbial cells separated from the culture solution, washed microbial cells and the like can be used. As the treated microbial cells, lyophilized microbial cells, acetone-dried microbial cells, microbial cells brought into contact with toluene or a detergent, lysozyme treated microbial cells, ultrasonicated microbial cells, mechanically ground microbial cells and the

like can be used. In addition, enzyme extracts having hydantoinase activity obtained from these treated microbial cells, these immobilized microbial cells, insolubilized treated microbial cells, enzyme proteins fixed on a support for immobilization (e.g., anion exchange resin) and the like can be used. For an immobilization method, for example, the specification of JP-A 63-185382 and the like can be referred to.

As the support used for immobilization, suited are phenol-formaldehyde anion exchange resins such as Duolite A568 or DS17186 (Rohm & Haas Co.: registered trade mark); and various anion exchange resins containing various amines, ammonium salts, or functional groups of the diethanolamine type, for example, polystyrene resins such as Amberlite IRA935, IRA945, IRA901 (Rohm & Haas Co.: registered trade mark), Lewatit OC1037 (Bayer A.G.: registered trade mark) and Diaion EX-05 (Mitsubishi Chemical Industries, Ltd.: registered trade mark). Other supports such as DEAE-cellulose can also be used.

Further, in order to obtain stronger and more stable adsorption of enzymes, usually, cross-linking agents are used, the preferred examples thereof being glutaraldehyde. As the enzyme to be used, not only purified enzymes, but also those with different degrees of purification such as partially purified enzymes, suspensions of disintegrated microbial cells

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and cell-free extracts can be used. The preparation of immobilized enzymes can be carried out according to a conventional method, for example, by adsorbing enzymes on a support, followed by cross-linking treatment.

5 The 5-substituted hydantoin to be used as a substrate of the enzymatic reaction of the present invention is not specifically limited and can be any compound normally used in this kind of reaction. Examples thereof include that represented by the general formula (1):

10

 The substituent R in the compound of the formula (1) can be selected in a wide range. In order to provide industrially useful products such as intermediate compounds of drugs, preferably, R is phenyl, phenyl substituted with hydroxy, alkyl, substituted alkyl, aralkyl or thienyl. In the
15 case of phenyl substituted with hydroxy, the number of hydroxy may be one or more and they can be located at any of o-, m- and p-positions, with the typical example thereof being p-hydroxyphenyl. The alkyl group is such a group of 1 to 4
20 carbon atoms that the corresponding D-N-carbamoyl- α -amino acid is D-N-carbamoyl-alanine, D-N-carbamoyl-valine, D-N-carbamoyl-

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leucine, D-N-carbamoyl-isoleucine or the like. The substituted alkyl group is such an alkyl group of 1 to 4 carbon atoms substituted with hydroxy, alkylthio, carboxyl, amino, phenyl, phenyl substituted with hydroxy, amido or the like
5 that the corresponding D-N-carbamoyl-amino acid is D-N-carbamoyl-serine, D-N-carbamoyl-threonine, D-N-carbamoyl-methionine, D-N-carbamoyl-cysteine, D-N-carbamoyl-asparagine, D-N-carbamoyl-glutamine, D-N-carbamoyl-tyrosine, D-N-carbamoyl-tryptophane, D-N-carbamoyl-aspartic acid, D-N-carbamoyl-glutamic acid, D-N-carbamoyl-histidine, D-N-carbamoyl-lysine, D-N-carbamoyl-arginine, D-N-carbamoyl-citrulline or the like. The aralkyl group is such a group having 7 to 8 carbon atoms, for example, benzyl or phenethyl, that the corresponding D-N-carbamoyl- α -amino acid is D-N-carbamoyl-phenylalanine or the like.
10
15

As the aqueous medium, those containing water, buffers or organic solvents such as ethanol can be used. Further, when necessary, nutrients required for the growth of microorganisms, antioxidants, detergents, coenzymes, hydroxylamines, metals and the like can also be added to the aqueous medium.
20

In the case where, while cultivating the microbial cells of the above microorganisms in a water-soluble medium, the microbial cells are brought into contact with a particular
25 5-substituted hydantoin, an aqueous medium containing not only

the 5-substituted hydantoin but also nutrients required for the growth of microorganisms such as carbon sources, nitrogen sources and inorganic ions is used. If organic trace nutrients such as vitamins and amino acids are further added thereto, satisfactory results can be obtained in many cases. As the carbon sources, carbohydrates such as glucose, sucrose; organic acids such as acetic acid; alcohols and the like are conveniently used. As the nitrogen sources, ammonia gas, ammonia water, ammonium salts and the like can be used. As the inorganic ions, phosphate ion, magnesium ion, potassium ion, iron ion, manganese ion, cobalt ion, nickel ion, copper ion, aluminum ion, molybdenum ion and the like can be used.

The cultivation is carried out under aerobic conditions, while adjusting the pH and temperature to an appropriate range of 4 to 8 and 25 to 60°C, respectively. If the cultivation is carried out for 1 to 10 days, 5-substituted hydantoins are converted into only D-N-carbamoyl- α -amino acids with high efficiency.

On the other hand, in the case where the culture solution of the above-microorganisms is allowed as it is to react with cultivated microbial cells, treated microbial cells, enzyme extracts, immobilized microbial cells, insolubilized microbial cells or fixed enzyme proteins in an aqueous medium containing a 5-substituted hydantoin dissolved or suspended, the reaction mixture can be allowed to stand for

some time or stirred, while maintaining at an appropriate temperature of 10 to 80°C and pH of 4 to 9.5. Thus, if 5 to 100 hours have run their course, D-specific hydrolysis reaction of the substrate by hydantoinase and chemical racemization of the substrate proceed and any of D-, DL- or L-5-substituted hydantoins are converted into the corresponding D-N-carbamoyl- α -amino acids to accumulate a large amount of the amino acids in the aqueous medium. In addition, 5-substituted hydantoins can be added in separate portions with the progress of reaction.

The produced D-N-carbamoyl- α -amino acid can be isolated and purified by a conventional separation method.

D- α -amino acids can be easily obtained by using the isolated and purified D-N-carbamoyl- α -amino acids obtained by the above-described method or the reaction mixtures obtained by the hydantoinase reaction as they are by the action and converting the D-N-carbamoyl- α -amino acids into the corresponding D- α -amino acids chemically or enzymatically with enzymes having decarbamylase activity.

The following Examples further illustrate the present invention in detail.

Example 1

Isolation of microorganism having hydantoinase activity from soil

After suspending 0.5 g of a sample of soil in 2 ml of a physiological saline, the suspension was allowed to stand and one drop of the supernatant was smeared on an isolation agar plate medium (1.0% of meat extract, 1.0% of peptone, 1.0% of yeast extract, 0.3% of sodium chloride, 2.0% of agar; pH 7.5). This was cultivated at 50°C for 2 days and the colony obtained was transferred to the same isolation plate medium to which 0.1% of uracil and 20 ppm of manganese chloride and then further cultivated at 50°C for 1 day. The microbial cells thus obtained were suspended in 100 µl of a reaction substrate solution [DL-(p-hydroxyphenyl)hydantoin 30 mM, sodium sulfite 0.1%, carbonate buffer 50 mM] and they were reacted at 50°C for 2 hours. Then, the reaction mixture was spotted on a TLC plate and developed with a developing solvent of butanol : acetic acid : water (4 : 1 : 1), followed by developing color with a 10% solution of p-dimethylamino-benzaldehyde in conc. hydrochloric acid and detecting a yellow spot to select microbial strains producing N-carbamoyl-p-hydroxyphenylglycine. Thus, among 2740 colonies, Bacillus sp. KNK245 (FERM BP-4863) having high hydantoinase activity was isolated.

Example 2

N-Terminal amino acid sequencing of hydantoinase derived from Bacillus sp. KNK245

20

1

5

10

at its N-terminal.

Example 3

25

derived from Agrobacterium sp. KNK712 (FERM Bp-1900)

It was found that a hydantoinase gene was located on the DNA fragment cloned in the plasmid pAHD101 described in WO92/10579 by a study according to the following method.

5 Escherichia coli JM109 was transformed with pAHD101 according to a conventional method, inoculated into 50 ml of L-broth (10 g/liter of peptone, 5 g/liter of yeast extract, 5 g/liter of sodium chloride; pH 7.0) containing 100 mg/liter of ampicillin and cultivated at 37°C for 16 hours. Then, 50 ml of the
10 culture broth was collected, followed by removing the supernatant, suspending the microbial cells in 50 ml of a substrate solution (0.5% of 5-(p-hydroxyphenyl)hydantoin, 0.05% of Triton X-100, 0.05 M phosphate buffer; pH 8.7) and reacting at 37°C for 3 hours in a stream of nitrogen. The reaction
15 mixture was spotted on a TLC plate, developed and subjected to color development with a solution of p-dimethylamino-benzaldehyde in hydrochloride to detect a spot of D-N-carbamoyl-(p-hydroxyphenyl)glycine in agreement with an authentic sample. In view of the above, it was confirmed that
20 the transformant of pAHD101 expresses a gene encoding a hydantoinase.

A 2.1 kbp fragment obtained by cleaving the plasmid pAHD101 with SacI and SalI to shorten the inserted fragment was ligated to pUC18 fragment cleaved by SacI and SalI to
25 obtain the plasmid pAH1043 (see Fig. 1).

In addition, the nucleotide sequencing of the hydantoinase gene was carried out by using DNA sequence kit (manufactured by Applied Biosystems). The results are shown in SEQ ID No. 1

5 Escherichia coli HB101 was transformed with the plasmid pAH1043 to obtain a transformant, Escherichia coli HB101 pAH1043 (FERM BP-4865).

Example 4

10 Preparation of recombinant DNA of genomic DNA of Bacillus sp. KNK245 and vector DNA

15 Bacillus sp. KNK245 (FERM BP-4863) was cultivated in 2 liters of a broth (10 g/liter of meat extract, 10 g/liter of peptone, 5 g/liter of yeast extract; pH 7.5) at 45°C for 24 hours and microbial cells were collected. Genomic DNA was extracted from the microbial cells obtained according to Marmur method. Ten units of BamHI was added to 1 µg of the genomic DNA and they were reacted at 37°C for 16 hours.

20 On the other hand, the plasmid pUC19 was completely cleaved with BamHI and ligated thereto the above-obtained genomic DNA fragment with T4DNA ligase to obtain a mixture of various recombinant plasmids.

Example 5

To obtain hydantoinase gene derived from Bacillus sp. KNK245

Based on the nucleotide sequences of hydantoinase genes derived from Agrobacterium sp. KNK712 (FERM BP-1900) of Example 3 and Lul220 strain disclosed in JP-A 62-87089, two kinds of primers 1 and 2 (see Table 1) having sequences of each complementary strand of the above nucleotide sequences oriented toward opposite directions from 1155 bp interrupting nucleotides were synthesized. A polymerase chain reaction (PCR) was carried out by using two primers which were synthesized by using genomic DNA derived from Bacillus sp. KNK245 prepared in Example 4 as a template to obtain about 1.2 kbp fragment. The nucleotide sequencing of this fragment was carried out by using DNA sequence kit (manufactured by Applied Biosystems). As a result, it had high complementation to a known hydantoinase gene and it was found that the PCR fragment obtained was a part of the hydantoinase gene. Then, two primers 3 and 4 (Table 1) having oppositely directed sequences of respective complementary strands of this fragment were synthesized.

Further, primer 5 (Table 1) having the sequence of the vector portion in the recombinant DNA of Example 4 was synthesized and, by using the above synthesized primers and the latter primer and using the recombinant DNA of Example 4 as a template, PCR was carried out to obtain two kinds of DNA fragments containing the front half and back half parts of the hydantoinase gene, respectively. These DNA fragments were

subjected to nucleotide sequencing. Based on nucleotide sequences expected from the N-terminal amino acid sequence of the hydantoinase protein of Example 2, the translation codon was determined. Together with the results of the above-determined nucleotide sequences, the entire nucleotide sequence of the gene encoding hydantoinase was determined.

The results are shown in SEQ ID No. 2.

Then, based on the nucleotide sequence thus obtained, primer 6 (Table 1) having a sequence about the initiation codon of the hydantoinase gene and NdeI restriction site and primer 7 (Table 1) having a sequence about PstI restriction site in the hydantoinase gene were synthesized and PCR was carried out by using the genomic DNA of Example 4 as a template to obtain a 1.2 kb fragment 1. Then, primer 8 (Table 1) having an oppositely directed sequence at the above PstI restriction site and primer 9 (Table 1) having a sequence corresponding to the downstream of the termination codon and HindIII restriction site were synthesized and PCR was carried out by using the genomic DNA of Example 4 as template to obtain a 0.25 kb fragment 2. Fragment 1 was restricted with NdeI and PstI, fragment 2 was restricted with PstI and HindIII and pUCNT which was an improved vector plasmid of pUC19 described in W094/03613 was restricted with NdeI and HindIII. The restricted fragments were ligated with T4DNA ligase to

obtain plasmid pTH102 containing the hydantoinase gene (see Fig. 2).

Table 1

Primer 1

5'-AAA(^T_C)GGAAC(^G_C)AT(^A_C)GT(^G_C)ACCGC-3'

Primer 2

5'-GCGTC(^G_C)GAACC(^G_C)ACCGCGATCGT-3'

Primer 3

5'-TAACATCCTTTTGCATCAACCACTTC-3'

Primer 4

5'-AAAAAAGGAAGAATCACGTTAAA-3'

Primer 5

5'-GTTTTCCCAGTCACGAC-3'

Primer 6

5'-TACGAGCATATGAAAAAGATCATTAAAGAACGG
AAC-3'

Primer 7

5'-GTGTGTTTCTGCAGAAATTACCCGTTC-3'

Primer 8

5'-TAATTTCTGCAGAAACACACCATAT-3'

Primer 9

5'-GCTGAAAGCTTCGAAATAAGCCATTTTCGAGC
AG-3'

Primer 10

5'-TAATTTCTGCAGAAACACACCACATGGCCGT
CG-3'

Primer 11

5'-CGAGCAAGCTTCTACTAAATGGTTAATTTCTC
AT-3'

Primer 12

5'-GGAAGCTTTCATTAAATGGTTAATTTCTCATC
TTGTT-3'

Primer 13

5'-TACGAGGGATCCTATAAATTCCAACAAGTGC
TATA-3'

Example 6

Preparation of recombinant DNA for expression of
Bacillus sp. KNK245 hydantoinase gene

According to the same manner for obtaining fragment
5 2 in Example 5, PCR was carried out to obtain a 0.25 kb
fragment 3 except that, instead of the primer having PstI
restriction site used for the production of fragment 2,
synthesized primer 10 (Table 1) wherein one nucleotide at NdeI
restriction site near PstI restriction site was substituted
10 to block cleavage with NdeI was used and instead of the primer
having the sequence about the termination codon, synthesized
primer 11 (Table 1) wherein the number of nucleotides at the
downstream of the termination codon were further reduced was
used. Then, according to the same manner for obtaining pTH102
15 in Example 5, the plasmid pTH103 was obtained from the
fragments 1 and 3 and pUCNT (see Fig. 3).

Further, according to the same manner for obtaining
pTH103, pTH104 was obtained except that, instead of the primer
11 having the downstream sequence about the termination codon,
20 primer 12 (Table 1) was used.

Escherichia coli HB101 transformed with pTH104 was
designated as Escherichia coli HB101 pTH104. This strain has
been deposited at National Institute of Bioscience and Human-
Technology as of November 2, 1994 under the accession number
25 of FERM BP-4864.

Example 7

To obtain plasmid containing gene for hydantoinase derived from Pseudomonas sp. KNK003A (FERM BP-3181)

According to the same manner as described in Example 3, it was found that hydantoinase gene was located on the plasmid pPHD301 described in WO92/10579.

Escherichia coli HB101 was transformed by pPHD301 to obtain Escherichia coli HB101pPHD301 (FERM BP-4866). The restriction enzyme map of pPHD301 is shown in Fig. 5.

The nucleotide sequencing of the hydantoinase gene was carried out according to the same manner as described in Example 3. The results are shown in SEQ No. 3.

Example 8

Conversion of 5-(p-hydroxyphenyl)hydantoin into D-N-carbamoyl-(p-hydroxyphenyl)glycine by transformant obtained

The transformant obtained in Example 6, Escherichia coli HB101pTH104 was inoculated into 50 ml of 2YT broth (16 g/liter of polypeptone, 10 g/liter of yeast extract, 5 g/liter of sodium chloride; pH 7.0) containing 100 µg/ml of ampicillin and 400 ppm manganese chloride tetrahydrate and cultivated at 37°C for 24 hours. The microbial cells were collected from 50 ml of this culture broth and suspended in 0.05 M carbonate buffer (pH 8.7) to make up the volume to 50 ml. Triton X-100 was added so that the final concentration was 0.05%. To this

was added 1.5 g of 5-(p-hydroxyphenyl)hydantoin and the mixture was allowed to react with stirring at 40°C for 3 hours in the stream of nitrogen with adjusting the pH at 8.7 by 6N sodium hydroxide. After completion of the reaction, the reaction mixture was centrifuged and the supernatant was subjected to a colorimetric determination of D-N-carbamoyl-(p-hydroxyphenyl)glycine therein by color development with 10% solution of p-diemthylaminobenzaldehyde in conc. hydrochloric acid. As a result, D-N-carbamoyl-(p-hydroxyphenyl)glycine was produced in the conversion yield of 97.5%.

The reaction mixture was adjusted to pH 5 with hydrochloric acid. After centrifugation, the supernatant was concentrated, adjusted to pH 2 with conc. hydrochloric acid and stored at 4°C. The precipitated crystals were filtered off and recrystallized from water-ethanol to obtain 970 mg of D-N-carbamoyl-(p-hydroxyphenyl)glycine, m.p. 176-177°C, $[\alpha]_D^{20} = -177.0^\circ$ (c=0.5, 5% ethanol).

Example 9

Preparation of immobilized hydantoinase

Microbial cells were collected from 1 liter of a culture broth of Escherichia coli HB101pTH104 obtained according to the same manner as described in Example 7, suspended in aqueous 1 mM manganese sulfate to make up the volume to 60 ml, adjusted to pH 8.5 and disrupted by ultrasonication. To the enzyme solution thus obtained was

added 20 g of an anion exchange resin, Duolite A-568 equilibrated to pH 8.5 and the mixture was stirred at 15°C for 20 hours to adsorb the enzyme. To this mixture was added glutaraldehyde so that the final concentration thereof was 0.1% and the mixture was stirred for 1 hour to proceed crosslinking reaction. Then, the resin was collected and washed to obtain 20 g of an immobilized hydantoinase.

Example 10

Conversion of 5-(p-hydroxyphenyl)hydantoin into D-carbamoyl-(p-hydroxyphenyl)glycine by immobilized enzyme

Five grams of the immobilized hydantoinase obtained in Example 9 was added to 100 ml of aqueous 1 mM manganese sulfate at 40°C which was bubbled with nitrogen. To this was added 3 g of 5-(p-hydroxyphenyl)hydantoin and the reaction was carried out with stirring at 40°C for 3 hours with bubbling nitrogen. After completion of the reaction, the reaction mixture was allowed to stand and sucked off to collect the reaction mixture. According to the same manner as described in Example 8, the carbamoyl amino acid product was purified to obtain 2.2 g of D-N-carbamoyl-(p-hydroxyphenyl)glycine.

Example 11

Expression of Bacillus sp. KNK245 hydantoinase gene in Bacillus subtilis

According to the same manner as described in Example 5, PCR was carried out by using the genomic DNA of Bacillus sp. KNK245 as a template and using primers 12 and 13 (Table 1) to obtain a 1.7 kb fragment. This was cleaved with BamHI and HindIII and ligated to the plasmid pHY300PLK (Takara Shuzo) which had been cleaved in a similar manner to obtain the plasmid pTHB301. Its restriction enzyme map is shown in Fig. 6.

This plasmid pTHB301 was introduced into Bacillus subtilis ISW1214 (available from Takara Shuzo), Bacillus subtilis YS-11 (IAM12019) or Bacillus subtilis 3115 (IAM12020) by electroporation (using Shimadzu Corporation GTE-10 Model, 10 KV/cm, 2 ms) and cultivated in the culture medium as shown in Example 4 to which 0.02 g/liter of manganese chloride tetrahydrate was added at 37°C for 20 hours. A cell-free extraction was prepared by collecting the microbial cells and disrupting them with ultrasonication. As a result, each hydantoinase activity was about 2.2 times higher than that obtained by cultivation of Bacillus sp. KNK245 under the same conditions.

Example 12

Expression of Bacillus sp. KNK245 hydantoinase gene in thermophile

Bacillus stearothermophilus IF012550 was transformed with the plasmid pTHB301 prepared in Example 11 by protoplast

method according to J. Bacteriol., 149, 824 (1982) and cultivated according to the same manner as described in Example 11 to prepare a cell-free extract. The hydantoinase activity was about 3.6 times higher than that of Bacillus sp. KNK245 prepared under the same conditions.

As described hereinabove, according to the present invention, a microorganism having very high hydantoinase activity can be created and, by using this as a source of enzyme, D-N-carbamoyl- α -amino acids can be produced, efficiently.

Deposit of Microorganisms

Agrobacterium sp. KNK712 has been deposited as of May 31, 1988 under the accession number of FERM BP-1900; Pseudomonas sp. KNK003A has been deposited as of December 1, 1990 under the accession number of FERM BP-3181; Bacillus sp. KNK245 has been deposited as of November 2, 1994 under the accession number of FERM BP-4863; and Escherichia coli HB101 pAH1043 has been deposited as of November 2, 1994 under the accession number of FERM BP-4866 at the following Depsoitary Authority under Budapest Treaty, respectively.

Name: National Institute of Bioscience and Human-Technology (formerly Fermentation Research Institute), Agency of Industrial Science & Technology, Ministry of International Trade & Industry

- 31 -

Address: 1-3, Higashi 1 chome Tsukuba-shi Ibaraki-ken 305,
Japan

THIS SHEET HAS BEEN
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- 32 -

Sequence Listing

SEQ ID NO: 1

SEQUENCE LENGTH: 2518

SEQUENCE TYPE: nucleic acid

5 SEQUENCE:

	10	20	30	40	50	60
	GAGCTCGGCCTCGTCGGTGAAATGCCAGCGCGGGAAGAAGGTCGTAAGCGCGAGTTCGGG					
	70	80	90	100	110	120
	GAAGACAATGAAATTCGCGCCCCGGCTCGCGGCTTTCGTCAGCATGTCGAGAAGACGAAC					
10	130	140	150	160	170	180
	GACGACCTGTTTCGCGTGTCTCCGCGCGCGGATCGGACCTTGTTGTCCCACTGCAAGTAT					
	190	200	210	220	230	240
	CATCTGACGTGTCATGAACCTCTGCTCCTTCCTAGGTGATGTCCGGCCCAGCGGCCGGGA					
	250	260	270	280	290	300
15	AAACGTTGTGAAACACATCGCCGAAAACCGGCTTTGTGACTCGCGGCGCAATGCAAATTC					
	310	320	330	340	350	360
	TACATAAAGTACGAAATATTACATGCAGCACGACAATAACAGGCGTAAAATTTTGTATCT					
	370	380	390	400	410	420

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- 33 -

GTCAACCGGAGCGGAACCGGAATTTGCGCTGCTTGACAAGCTCTCGCGCGAGCCCGCCGT
430 440 450 460 470 480
CGACTTCAACTCGTTCCGCCTCGAACGAGAGCGCTCTGCACAGAATCGTCTTCGCTAGGC
490 500 510 520 530 540
5 CGCCGTTCCGCTTGACAGCTTCAAAACACCATGCAAACCTGTATAAAGAATTACATTCCA
550 560 570 580 590 600
TATACGGAACAAACTCTTCAATCCTGAGAGCGACATCATGGACATCATTATCAAAAACGG
MetAspIleIleIleLysAsnGly
610 620 630 640 650 660
10 AACCATCGTGACCGCGGATGGCATTCTCGCGCCGATCTCGGGATCAAGGATGGCAAGAT
ThrIleValThrAlaAspGlyIleSerArgAlaAspLeuGlyIleLysAspGlyLysIle
670 680 690 700 710 720
CACCCAGATCGGCGGCGCGCTCGGCCAGCGGAGCGGACGATCGACGCGCGCCGGCCGCTA
ThrGlnIleGlyGlyAlaLeuGlyProAlaGluArgThrIleAspAlaAlaGlyArgTyr
730 740 750 760 770 780
15 CGTCTTTCCGGGCGGCATAGACGTTACACGCATGTCGAAACGGTCAGCTTCAACACGCA
ValPheProGlyGlyIleAspValHisThrHisValGluThrValSerPheAsnThrGln
790 800 810 820 830 840
GTCGGCCGACACGTTTCGCAACAGCGACGGTCGCGGCCGCTGTGGCGGAACGACAACCAT



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- 34 -

SerAlaAspThrPheAlaThrAlaThrValAlaAlaAlaCysGlyGlyThrThrThrIle

850 860 870 880 890 900

CGTCGATTTCTGTCAGCAGGATCGCGGCCACAGCCTGGCGGAACCCGTCCCCAAGTGGGA

ValAspPheCysGlnGlnAspArgGlyHisSerLeuAlaGluProValProLysTrpAsp

5 910 920 930 940 950 960

CGGTATGGCCGGCGGCAAGTCGGCGATCGATTACGGCTACCACATCATCGTGCTCGACCC

GlyMetAlaGlyGlyLysSerAlaIleAspTyrGlyTyrHisIleIleValLeuAspPro

970 980 990 1000 1010 1020

GACCGACAGCGTGATTGAGGAGCTGGAGGTGCTTCCCGATCTTGGCATTACCTCCTTCAA

10 ThrAspSerValIleGluGluLeuGluValLeuProAspLeuGlyIleThrSerPheLys

1030 1040 1050 1060 1070 1080

GGTCTTCATGGCCTATCGCGGCATGAACATGATCGACGACGTGACGCTGCTGAAGACGCT

ValPheMetAlaTyrArgGlyMetAsnMetIleAspAspValThrLeuLeuLysThrLeu

1090 1100 1110 1120 1130 1140

15 CGACAAGGCGGTCAAGACCGGATCGCTCGTCATGGTGACGCGGAAAACGGCGACGCCGC

AspLysAlaValLysThrGlySerLeuValMetValHisAlaGluAsnGlyAspAlaAla

1150 1160 1170 1180 1190 1200

CGACTATCTGCGCGACAAGTTCGTGGCCGAGGGCAAAACCGCGCCGATCTACCACGCGCT

AspTyrLeuArgAspLysPheValAlaGluGlyLysThrAlaProIleTyrHisAlaLeu

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- 35 -

	1210	1220	1230	1240	1250	1260
	CAGCCGCCCCGCCCCGGGTCGAAGCCGAGGCAACCGCGCGGGCCCTCGCCCTGGCCGAAAT					
	SerArgProProArgValGluAlaGluAlaThrAlaArgAlaLeuAlaLeuAlaGluIle					
	1270	1280	1290	1300	1310	1320
5	CGTCAACGCCCCGATCTACATAGTCCATGTGACCTGCGAGGAGTCCCTTGAGGAGGTGAT					
	ValAsnAlaProIleTyrIleValHisValThrCysGluGluSerLeuGluGluValMet					
	1330	1340	1350	1360	1370	1380
	GCGCGCAAAATCGCGAGGCGTCCGCGCTCTGGCGGAAACCTGCACGCATTACCTTTACCT					
	ArgAlaLysSerArgGlyValArgAlaLeuAlaGluThrCysThrHisTyrLeuTyrLeu					
10	1390	1400	1410	1420	1430	1440
	CACCAAGGAAGACCTGGAGCGGCCGATTTCGAAGGTGCGAAATACGTTTTACACCGCC					
	ThrLysGluAspLeuGluArgProAspPheGluGlyAlaLysTyrValPheThrProPro					
	1450	1460	1470	1480	1490	1500
	GGCCCGCGCGAAGAAAGACCATGACGTTCTCTGGAACGCACTCAGAAACGGTGTGTTTCA					
15	AlaArgAlaLysLysAspHisAspValLeuTrpAsnAlaLeuArgAsnGlyValPheGlu					
	1510	1520	1530	1540	1550	1560
	AACGGTTTCCTCCGACCATTTGCTCCTGGCTCTTCAAGGGGCACAAGGACCGGGGCCGAA					
	ThrValSerSerAspHisCysSerTrpLeuPheLysGlyHisLysAspArgGlyArgAsn					
	1570	1580	1590	1600	1610	1620

This Sheet Has Been Replaced



- 36 -

CGACTTTCGCGCCATCCCGAACGGCGCGCCGGGCGTCGAGGAACGGTTGATGATGGTCTA
AspPheArgAlaIleProAsnGlyAlaProGlyValGluGluArgLeuMetMetValTyr

1630 1640 1650 1660 1670 1680
5 TCAGGGCGTCAACGAAGGCCGGATTTCCTTACCCAGTTCGTGGAACGGTCGCCACGCG
GlnGlyValAsnGluGlyArgIleSerLeuThrGlnPheValGluLeuValAlaThrArg

1690 1700 1710 1720 1730 1740
CCCGGCCAAGGTCTTCGGAATGTTTCCGCAAAGGGGACGATCGCGGTCTGGTTCGGACGC
ProAlaLysValPheGlyMetPheProGlnLysGlyThrIleAlaValGlySerAspAla

1750 1760 1770 1780 1790 1800
10 CGACATCGTCCTTTGGGACCCCGAGGCCGAAATGGTGATCGAACAGACCGCCATGCACAA
AspIleValLeuTrpAspProGluAlaGluMetValIleGluGlnThrAlaMetHisAsn

1810 1820 1830 1840 1850 1860
CGCCATGGATTACTCCTCCTACGAGGGACACAAGGTCAAGGGCGTGCCGAAGACGGTGCT
AlaMetAspTyrSerSerTyrGluGlyHisLysValLysGlyValProLysThrValLeu

1870 1880 1890 1900 1910 1920
15 CCTGCGTGGAAGGTTATCGTCGACGAAGGTTCTATGTCGGCGAACCGACGGACGGGAA
LeuArgGlyLysValIleValAspGluGlySerTyrValGlyGluProThrAspGlyLys

1930 1940 1950 1960 1970 1980
ATTCTGAAACGTCGCAAATACAAGCAGTAAACCGGCATTCTACGAGAACGATGATGACA



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- 37 -

PheLeuLysArgArgLysTyrLysGln***

457

	1990	2000	2010	2020	2030	2040
	ATACACACTTCGACAAGTGTCTCGCGCCTGACGGCGACAGCGTTGCAAGCGGCTTCGAC					
5	2050	2060	2070	2080	2090	2100
	AGGCGTTACGGCGCACCGACATTCAATCAGATACCATCGGGCGAACGGCTGGTGTCTGGAC					
	2110	2120	2130	2140	2150	2160
	TGCATCGCGCTGATGCTGAAGCACCGTTCCGTCAGACGCTACACGGGGGAACCTCTCCCC					
	2170	2180	2190	2200	2210	2220
10	CCAGGCACGCTGGAAATGCTGATGGCGGCGGGTCAGTCGGCTGCGACATCCTCCAACATG					
	2230	2240	2250	2260	2270	2280
	CAGACCGTGTCTGGTCATCGCGGTGACGGACCCCGAGAAAAAGACCCGTCTCGCGAAGACA					
	2290	2300	2310	2320	2330	2340
	TGCGCCGGGCAGGACTTCATCGCCAACGCTCCTGTGCTCCTGTGTTTCGTGACGGATCTT					
15	2350	2360	2370	2380	2390	2400
	GCGCGCCCCGCCAGGGTCGCCTCCACGATTGGCGCCGATCTTTTCGCGTTGCCGATGATC					
	2410	2420	2430	2440	2450	2460

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- 38 -

GACACCTTTCTGGCGTCCATCAGCGATTGTTCCATCTTCGCGCAAAACCTTGTTCTCGCC

2470 2480 2490 2500 2510

GCGGAATCGCTCGGCATGGGCACCTGCTATGTCGGGAGCCTGCGCAACCGGCCTGATC

SEQ ID NO: 2

5SEQUENCE LENGTH: 2105

SEQUENCE TYPE: nucleic acid

SEQUENCE:

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GTCGACTTCAACTCGTTCCGCCTCGAACGAGAGCGCTCTGCACAGAATCGTCTTCGCTAG

10 70 80 90 100 110 120

GCCGCCGTTCCGCTTGACAGCTTCAAAACACCATGCAAACCTTGATATAAAGAATTACATTC

130 140 150 160 170 180

CATATACGGAACAACTCTTCAATCCTGAGAGCGACATCATGGACATCATTATCAAAAAC

MetAspIleIleIleLysAsn

15

190 200 210 220 230 240

GGAACCATCGTGACCGCGGATGGCATTCTCGCGCCGATCTCGGGATCAAGGATGGCAAG

GlyThrIleValThrAlaAspGlyIleSerArgAlaAspLeuGlyIleLysAspGlyLys

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- 39 -

	250	260	270	280	290	300
	ATCACCCAGATCGGCGGCGCGCTCGGCCCAGCGGAGCGGACGATCGACGCGGCCGGCCGC					
	IleThrGlnIleGlyGlyAlaLeuGlyProAlaGluArgThrIleAspAlaAlaGlyArg					
	310	320	330	340	350	360
5	TACGTCTTTCCGGGCGGCATAGACGTTACACGCATGTCGAAACGGTCAGCTTCAACACG					
	TyrValPheProGlyGlyIleAspValHisThrHisValGluThrValSerPheAsnThr					
	370	380	390	400	410	420
	CAGTCGGCCGACACGTTGCAACAGCGACGGTCGCGGCCGCCTGTGGCGGAACGACAACC					
	GlnSerAlaAspThrPheAlaThrAlaThrValAlaAlaAlaCysGlyGlyThrThrThr					
10	430	440	450	460	470	480
	ATCGTCGATTTCTGTCAGCAGGATCGCGGCCACAGCCTGGCGGAACCCGTCCCCAAGTGG					
	IleValAspPheCysGlnGlnAspArgGlyHisSerLeuAlaGluProValProLysTrp					
	490	500	510	520	530	540
	GACGGTATGGCCGGCGGCAAGTCGGCGATCGATTACGGCTACCACATCATCGTGCTCGAC					
15	AspGlyMetAlaGlyGlyLysSerAlaIleAspTyrGlyTyrHisIleIleValLeuAsp					
	550	560	570	580	590	600
	CCGACCGACAGCGTGATTGAGGAGCTGGAGGTGCTTCCCGATCTTGGCATTACCTCCTTC					
	ProThrAspSerValIleGluGluLeuGluValLeuProAspLeuGlyIleThrSerPhe					
	610	620	630	640	650	660

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- 40 -

AAGGTCTTCATGGCCTATCGCGGCATGAACATGATCGACGACCTGACGCTGCTGAAGACG
LysValPheMetAlaTyrArgGlyMetAsnMetIleAspAspValThrLeuLeuLysThr

670 680 690 700 710 720
CTCGACAAGGCGGTCAAGACCGGATCGCTCGTCATGGTGCACGCGGAAAACGGCGACGCC
5 LeuAspLysAlaValLysThrGlySerLeuValMetValHisAlaGluAsnGlyAspAla

730 740 750 760 770 780
GCCGACTATCTGCGCGACAAGTTCGTGGCCGAGGGCAAACCGCGCCGATCTACCACGCG
AlaAspTyrLeuArgAspLysPheValAlaGluGlyLysThrAlaProIleTyrHisAla

790 800 810 820 830 840
10 CTCAGCCGCCCCGCCCCGGGTCTGAAGCCGAGGCAACCGCGCGGGCCCTCGCCCTGGCCGAA
LeuSerArgProProArgValGluAlaGluAlaThrAlaArgAlaLeuAlaLeuAlaGlu

850 860 870 880 890 900
ATCGTCAACGCCCCGATCTACATAGTCCATGTGACCTGCGAGGAGTCCCTTGAGGAGGTG
IleValAsnAlaProIleTyrIleValHisValThrCysGluGluSerLeuGluGluVal

910 920 930 940 950 960
15 ATGCGCGCAAAATCGCGAGGCGTCCGCGCTCTGGCGGAAACCTGCACGCATTACCTTTAC
MetArgAlaLysSerArgGlyValArgAlaLeuAlaGluThrCysThrHisTyrLeuTyr

970 980 990 1000 1010 1020
CTCACCAAGGAAGACCTGGAGCGGCCGGATTTCTGAAGGTGCGAAATACGTTTTTCACACCG

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- 41 -

LeuThrLysGluAspLeuGluArgProAspPheGluGlyAlaLysTyrValPheThrPro

1030 1040 1050 1060 1070 1080

CCGGCCCCGCGCAAGAAAGACCATGACGTTCTCTGGAACGCACTCAGAAACGGTGTGTTC

ProAlaArgAlaLysLysAspHisAspValLeuTrpAsnAlaLeuArgAsnGlyValPhe

5 1090 1100 1110 1120 1130 1140

GAAACGGTTTCCTCCGACCATTGCTCCTGGCTCTTCAAGGGGCACAAGGACCGGGGCCGG

GluThrValSerSerAspHisCysSerTrpLeuPheLysGlyHisLysAspArgGlyArg

1150 1160 1170 1180 1190 1200

AACGACTTTCGCGCCATCCCGAACGGCGCGCCGGGCGTCGAGGAACGGTTGATGATGGTC

10 AsnAspPheArgAlaIleProAsnGlyAlaProGlyValGluGluArgLeuMetMetVal

1210 1220 1230 1240 1250 1260

TATCAGGGCGTCAACGAAGGCCGGATTTCCTTACCCAGTTCTGGAACCTGGTCGCCACG

TyrGlnGlyValAsnGluGlyArgIleSerLeuThrGlnPheValGluLeuValAlaThr

1270 1280 1290 1300 1310 1320

15 CGCCCGGCCAAGGTCTTCGGAATGTTTCGCAAAAGGGGACGATCGCGGTCTGGTTCGGAC

ArgProAlaLysValPheGlyMetPheProGlnLysGlyThrIleAlaValGlySerAsp

1330 1340 1350 1360 1370 1380

GCCGACATCGTCCTTTGGGACCCCGAGGCCGAAATGGTGATCGAACAGACCGCCATGCAC

AlaAspIleValLeuTrpAspProGluAlaGluMetValIleGluGlnThrAlaMetHis

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- 42 -

	1390	1400	1410	1420	1430	1440
	AACGCCATGGATTACTCCTCCTACGAGGGACACAAGGTCAAGGGCGTGCCGAAGACGGTG					
	AsnAlaMetAspTyrSerSerTyrGluGlyHisLysValLysGlyValProLysThrVal					
	1450	1460	1470	1480	1490	1500
5	CTCCTGCGTGGCAAGGTTATCGTCGACGAAGGTTCTATGTGCGCGAACCGACGGACGGG					
	LeuLeuArgGlyLysValIleValAspGluGlySerTyrValGlyGluProThrAspGly					
	1510	1520	1530	1540	1550	1560
	AAATTCCTGAAACGTCGCAAATACAAGCAGTAAACCGGCATTCTACGAGAACGATGATGA					
	LysPheLeuLysArgArgLysTyrLysGln***					
10	1570	1580	1590	1600	1610	1620
	CAATACACACTTCGACAAGTGTCTGTCGCGCCTGACGGCGACAGCGTTGCAAGCGGCTTCG					
	1630	1640	1650	1660	1670	1680
	ACAGGCGTTACGGCGCACCGACATTCAATCAGATACCATCGGGCGAACGGCTGGTGTCTGG					
	1690	1700	1710	1720	1730	1740
15	ACTGCATCGCGCTGATGCTGAAGCACCGTTCCGTCAGACGCTACACGGGGGAACCTCTCC					
	1750	1760	1770	1780	1790	1800
	CCCCAGGCACGCTGGAAATGCTGATGGCGGGCGGGTCAGTCGGCTGCGACATCCTCCAACA					
	1810	1820	1830	1840	1850	1860

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- 43 -

TGCAGACCGTGTCTCGGTCATCGCGGTGACGGACCCCGAGAAAAAGACCCGTCTCGCGAAGA

1870 1880 1890 1900 1910 1920

CATGCGCCGGGCAGGACTTCATCGCCAACGCTCCTGTGCTCCTGTGTTTCGTGACGGATC

1930 1940 1950 1960 1970 1980

5 TTGCGCGCCCCGCCAGGGTCGCCTCCACGATTGGCGCCGATCTTTTCGCGTTGCCGATGA

1990 2000 2010 2020 2030 2040

TCGACACCTTTCTGGCGTCCATCAGCGATTGTTCCATCTTCGCGCAAAACCTTGTTCTCG

2050 2060 2070 2080 2090 2100

CCGCGGAATCGCTCGGCATGGGCACCTGCTATGTCGGGAGCCTGCGCAACCGGCCTGATC

10

CCGGG

SEQ ID NO: 3

SEQUENCE LENGTH: 1569

SEQUENCE TYPE: nucleic acid

15SEQUENCE:

10 20 30 40 50 60

TCGGAACCTGAAAACGAAATATCCCGCCGGACGGTGGGAAGGTGAAAGGAGGAGTCTCCA

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Met

	70	80	90	100	110	120
	TGACAACAGTTATCAAGGGTGAACGATCGTCGCCGCCGATCGCAGCTATGAAGCCGATA					
	ThrThrValIleLysGlyGlyThrIleValAlaAlaAspArgSerTyrGluAlaAspIle					
5	130	140	150	160	170	180
	TCCTGATCGAAGGCGAAAAGATCGCCCAGATCGGCAGGGATCTGCAGGGCGACAAGATTG					
	LeuIleGluGlyGluLysIleAlaGlnIleGlyArgAspLeuGlnGlyAspLysIleVal					
	190	200	210	220	230	240
	TAGACGCCGAGGGTGCCTATATCATTCCCGGCGGCATCGATCCTCACACGCATCTTGAAA					
10	AspAlaGluGlyAlaTyrIleIleProGlyGlyIleAspProHisThrHisLeuGluMet					
	250	260	270	280	290	300
	TGCCCTTTATGGGCACGACCACTGCCGAGACCTGGGAGTCCGGCACTTTCGCGGCTCTCT					
	ProPheMetGlyThrThrThrAlaGluThrTrpGluSerGlyThrPheAlaAlaLeuSer					
	310	320	330	340	350	360
15	CCGGCGGCACCACGATGGTTGTGGATTTCGTCATTCCCGGGCCGGCGGGAATGCTTGCCG					
	GlyGlyThrThrMetValValAspPheValIleProGlyProAlaGlyMetLeuAlaAla					
	370	380	390	400	410	420
	CCTTCGATCAATGGCAGGAGAGGGCAGCGCGCCAGGCTTCGTCCGACTACTCGCTGCATA					
	PheAspGlnTrpGlnGluArgAlaAlaArgGlnAlaSerSerAspTyrSerLeuHisMet					

	430	440	450	460	470	480
	TGTGCGTGACCGGCTGGTCAAAGCAGATCTTCGAGGACATGGCGAAGGTGGTCGAGCGCG					
	CysValThrGlyTrpSerLysGlnIlePheGluAspMetAlaLysValValGluArgGly					
	490	500	510	520	530	540
5	GTGTCAACACCTTCAAGCATTTTCATGGCCTATAAGGGCGCGCTGATGGTGAACGACGACG					
	ValAsnThrPheLysHisPheMetAlaTyrLysGlyAlaLeuMetValAsnAspAspGlu					
	550	560	570	580	590	600
	AGATGTTGCGCTCCTTCCAGCGCTGCGCCGCCCTGGGCGCCCTCCCGCTCGTGCGATGCGG					
	MetPheAlaSerPheGlnArgCysAlaAlaLeuGlyAlaLeuProLeuValHisAlaGlu					
10	610	620	630	640	650	660
	AAAACGGCGACATCGTCGCCTCGCTGCAGCAGAAATATATGCAGGAAGGCCTCACGGGTC					
	AsnGlyAspIleValAlaSerLeuGlnGlnLysTyrMetGlnGluGlyLeuThrGlyPro					
	670	680	690	700	710	720
	CGGAGGCTCATGCCTATTCCCGCCCGCCGGAGGTCGAGGGCGAGGCCACCAACCGCGCCA					
15	GluAlaHisAlaTyrSerArgProProGluValGluGlyGluAlaThrAsnArgAlaIle					
	730	740	750	760	770	780
	TCATGATCGCCGATGCCGCAGGCGTGCCGGTCTATATCGTGCGATGTCTCCTGTGAACAGG					
	MetIleAlaAspAlaAlaGlyValProValTyrIleValHisValSerCysGluGlnAla					
	790	800	810	820	830	840

CGCACGAAGCGATCCGCCGCGCACGCCAGAAGGGAATGCGCGTCTATGGCGAGCCGCTCA
HisGluAlaIleArgArgAlaArgGlnLysGlyMetArgValTyrGlyGluProLeuIle

850 860 870 880 890 900
TCCAGCACCTGCTGCTTGACGAGTCCGAGTACAAGAGAGCCGACTGGGACGAGGCGGCTC
5 GlnHisLeuLeuLeuAspGluSerGluTyrLysArgAlaAspTrpAspGluAlaAlaArg

910 920 930 940 950 960
GGCGGGTGATGTCCGCGCCATTCCGCGATAAAAGCCATCAGGACAGCCTCTGGGCTGGGC
ArgValMerSerAlaProPheArgAspLysSerHisGlnAspSerLeuTrpAlaGlyLeu

970 980 990 1000 1010 1020
10 TCGCAGCGGGCTCGCTGCAGGTCGTGGCAACGGACCACTGCGCCTTTACCACTGAACAGA
AlaAlaGlySerLeuGlnValValAlaThrAspHisCysAlaPheThrThrGluGlnLys

1030 1040 1050 1060 1070 1080
AGCGGCTGGGACTCAACGACTTCACGAAAATCCCGAACGGCACCAGGGGGGTTAGAGGACC
ArgLeuGlyLeuAsnAspPheThrLysIleProAsnGlyThrGlyGlyLeuGluAspArg

1090 1100 1110 1120 1130 1140
15 GGTGTGCTGCTGCTCTGGACCTATGGCGTGAAGACGGGGCGGCTCACGCCTAACGAATTCG
LeuSerLeuLeuTrpThrTyrGlyValLysThrGlyArgLeuThrProAsnGluPheVal

1150 1160 1170 1180 1190 1200
TCGCCGTACCTCCACCAATATCGCACAAATCCTGAACATCTATCCGCAAAGGGCGCAA

AlaValThrSerThrAsnIleAlaGlnIleLeuAsnIleTyrProGlnLysGlyAlaIle

1210 1220 1230 1240 1250 1260

TCCTGCCCTGGGTCCGATGCCGACCTCGTCGTCTGGGATCCAGCCAAATCGAAGAAGATCA

LeuProGlySerAspAlaAspLeuValValTrpAspProAlaLysSerLysLysIleThr

5 1270 1280 1290 1300 1310 1320

CTGCTTCCGCTCAGAAATCCATCATCGACTACAATATCTTCGAGGGCTACGAGGTGACCG

AlaSerAlaGlnLysSerIleIleAspTyrAsnIlePheGluGlyTyrGluValThrGly

1330 1340 1350 1360 1370 1380

GCATGCCGCGATACACGCTCTCACGCGGCGAGGTCGTATGGGGCGAGGAAGGCAGCAACG

10 MetProArgTyrThrLeuSerArgGlyGluValValTrpGlyGluGluGlySerAsnGlu

1390 1400 1410 1420 1430 1440

AACCGCGGCCCCGGTCGCGGTAGGTTCGTCGAGCGCCGGCCCTTTGCCGCCGTCAGCCGTG

ProArgProGlyArgGlyArgPheValGluArgArgProPheAlaAlaValSerArgAla

1450 1460 1470 1480 1490 1500

15 CCCTTTCAACCTGGAAGGAGATCGTCGCTCCGCGTGCGGTGGAGCGCTCGGCAAAGCATA

LeuSerThrTrpLysGluIleValAlaProArgAlaValGluArgSerAlaLysHisMet

1510 1520 1530 1540 1550 1560

TGCCGATCGGCGTTTGAATTTTGGGAGGCAGGCTCGAACTACGGTTTGGCCTGTCTCAGG

ProIleGlyVal***

- 48 -

ACAATCCAT



What is claimed is:

1. A transformant microorganism obtained by transforming a host microorganism with a recombinant DNA of a DNA fragment containing a gene encoding an enzyme which converts 5-substituted hydantoins into corresponding D-N-carbamoyl- α -amino acids by asymmetric cleavage hydrolysis (hereinafter referred to as hydantoinase) derived from Bacillus sp. KNK245 (FERM BP-4863), Agrobacterium sp. KNK712 (FERM BP-1900) or Pseudomonas sp. KNK003A (FERM BP-3181) and a vector DNA.

2. A transformant microorganism according to claim 1, wherein the host microorganism is selected from a microorganism belonging to the genus Escherichia, Pseudomonas, Flavobacterium, Bacillus, Serratia, Agrobacterium, Corynebacterium or Brevibacterium.

3. A transformant microorganism according to claim 2, wherein the transformant microorganism is Escherichia coli HB101 pTH102, Escherichia coli HB101 pTH103, Escherichia coli HB101 pTH104 (FERM BP-4864), Escherichia coli HB101 pAH1043 (FERM BP-4865) or Escherichia coli HB101 pPHD301 (FERM BP-4866).

4. A process for producing an enzyme which converts 5-substituted hydantoins into corresponding D-N-carbamoyl- α -amino acids by asymmetric cleavage hydrolysis thereof which



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comprises using the transformant microorganism according to any one of claims 1 to 3.

5 5. A process for producing D-N-carbamoyl- α -amino acids which comprises using the enzyme obtained by a process according to claim 4.

6. A process according to claim 5, wherein the 5-substituted hydantoin is a compound represented by the general formula (1):

10 wherein R is phenyl, phenyl substituted with hydroxy, alkyl, substituted alkyl, aralkyl or thienyl.

7. A recombinant plasmid obtained by recombination of a DNA fragment encoding hydantoinase derived from Bacillus sp. KNK245 (FERM BP-4863) and a plasmid (pUCNT) and having the restriction enzyme map of any of Figs. 2 to 4.

15 8. A recombinant plasmid according to claim 7, wherein the recombinant plasmid is pTH102, pTH103 or pTH104.

20 9. A recombinant plasmid obtainable by recombination of a DNA fragment encoding hydantoinase derived from Agrobacterium sp. KNK712 (FERM BP-1900) and a plasmid pUC18 and having the restriction enzyme map of Fig. 1.

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10. A recombinant plasmid according to claim 9, wherein the recombinant plasmid is pAH1043.

11. A recombinant plasmid obtainable by recombination of a DNA fragment encoding hydantoinase derived from
5 Pseudomonas sp. KNK003A (FERM BP-3181) and having the recombinant enzyme map of Fig. 5.

12. A recombinant plasmid according to claim 11, wherein the recombinant plasmid is pPHD301.

13. A gene for a protein having an enzymatic
10 activity for conversion of 5-substituted hydantoins into corresponding D-N-carbamoyl- α -amino acids by asymmetric cleavage hydrolysis thereof, said gene encoding all or a part of the amino acid sequence of the first to 472th amino acids shown in SEQ ID No. 2.

14. A DNA fragment containing a gene for a protein
15 having an enzymatic activity for conversion of 5-substituted hydantoins into corresponding D-N-carbamoyl- α -amino acids by asymmetric cleavage hydrolysis thereof, said gene having all or a part of the nucleotide sequence of the first to 1776th
20 nucleotides shown in SEQ ID No. 2 or an equivalent thereto.

15. A gene for a protein having an enzymatic activity for conversion of 5-substituted hydantoins into corresponding D-N-carbamoyl- α -amino acids by asymmetric cleavage hydrolysis thereof, said gene encoding all or a part



of the amino acid sequence of the first to 457th amino acids shown in SEQ ID No. 1.

5 16. A DNA fragment containing a gene for a protein having an enzymatic activity for conversion of 5-substituted hydantoins into corresponding D-N-carbamoyl- α -amino acids by asymmetric cleavage hydrolysis thereof, said gene having all or a part of the nucleotide sequence of the first to 2518th nucleotides shown in SEQ ID No. 1 or an equivalent thereto.

10 17. An enzyme protein containing all or a part of the amino acid sequence of the first to 472th amino acids shown in SEQ ID No. 2 and having an enzymatic activity for conversion of 5-substituted hydantoins into corresponding D-N-carbamoyl- α -amino acids by asymmetric cleavage hydrolysis thereof.

15 18. An enzyme protein containing all or a part of the amino acid sequence of the first to 457th amino acids shown in SEQ ID No. 1 and having an enzymatic activity for conversion of 5-substituted hydantoins into corresponding D-N-carbamoyl- α -amino acids by asymmetric cleavage hydrolysis thereof.

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19. A gene for a protein having an enzymatic activity for conversion of 5-substituted hydantoins into corresponding D-N-carbamoyl- α -amino acids by asymmetric cleavage hydrolysis thereof, said gene encoding all or a part



of the amino acid sequence of the first to 485th amino acids shown in SEQ ID No. 3.

5 20. A DNA fragment containing a gene for a protein having an enzymatic activity for conversion of 5-substituted hydantoins into corresponding D-N-carbamoyl- α -amino acids by asymmetric cleavage hydrolysis thereof, said gene having all or a part of the nucleotide sequence of the first to 1569th nucleotides shown in SEQ ID No. 3 or an equivalent thereto.

10 21. An enzyme protein containing all or a part of the amino acid sequence of the first to 485th amino acids shown in SEQ ID No. 3 and having an enzymatic activity for conversion of 5-substituted hydantoins into corresponding D-N-carbamoyl- α -amino acids by asymmetric cleavage hydrolysis thereof.