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SPECIFICATION

PROCESS FOR PRODUCTION OF D- α -AMINO ACIDS

Technical field

The present invention relates to a process for the production of D- α -amino acids, and more particularly, to a process for the production of D- α -amino acids using a novel transformant having a gene which is related to an enzyme capable of converting D-N-carbamoyl- α -amino acids into the corresponding D- α -amino acids.

Background art

Optically-active D- α -amino acids are important compounds as intermediates of drugs, and particularly, D-phenylglycine, D-parahydroxyphenyl-glycine and other intermediates for the production of semisynthesized penicillin or cephalosporin antibiotics are industrially useful compounds. As a process for the production of such D- α -amino acids, there is a well-known process in which carbamoyl groups of the corresponding D-N-carbamoyl- α -amino acids are removed to give the desired D- α -amino acids. The removal of carbamoyl groups in this process is achieved by a chemical process (e.g., the specification of Japanese Patent Publication No. 58-4707) or by a process utilizing the enzymatic reaction of microorganisms (e.g., the

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specifications of Japanese Patent Publication Nos. 57-18793, 63-20520, and 1-48758).

Problems to be solved by the invention

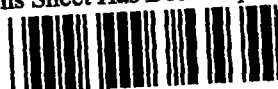
In a chemical process employed for the removal of carbamoyl groups as described above, a great amount of mineral acid such as sulfuric acid is used, and therefore, there will occur serious environmental problems regarding to the disposal thereof and the like. On the other hand, a process utilizing the enzymatic reaction of microorganisms has several drawbacks that microorganisms hitherto known as a source of enzyme supply cannot produce a sufficient amount of enzymes and that expensive hydantoin or N-carbamoylamino acid compounds are required for the production of enzymes.

Means for solving the problems

For the purpose of solving such problems, the objects of the present invention are to prepare microorganisms having high productivity of enzymes, as well as to produce D- α -amino acids with high efficiency by use of a source of enzyme supply thus obtained.

A similar technique is disclosed in Japanese Patent Laid-open Publication No. 63-24894. However, this technique relates to the production of L- α -amino acids, and there is no experimental example describing the production of D- α -amino acids.

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The present invention provides a process for the production of D- α -amino acids, characterized in that D-N-carbamoyl- α -amino acids are converted into the corresponding D- α -amino acids in an aqueous medium with the aid of an action of an enzyme produced from a transformant which is obtained by transformation of host bacterial cells selected from the microorganisms belonging to the genera Escherichia, Pseudomonas, Flavobacterium, Bacillus, Serratia, Corynebacterium, and Brevibacterium, with a recombinant DNA comprising a vector DNA and a DNA fragment containing a gene which is related to that enzyme capable of converting D-N-carbamoyl- α -amino acids by removal of their carbamoyl groups into the corresponding D- α -amino acids, after which the D- α -amino acids produced are collected.

By the way, no example has hitherto been known that a recombinant DNA comprising a vector and a gene which is related to an enzyme capable of converting D-N-carbamoyl- α -amino acids by removal of their carbamoyl groups into the corresponding D- α -amino acids is incorporated into microorganisms to achieve the expression of the gene. Such a technique was not succeeded until the present invention has been completed.

The enzymes capable of converting D-N-carbamoyl- α -amino acids by removal of their carbamoyl groups into the corresponding D- α -amino acids are, in fact, not limited to those which specifically act on the D-isomers or those which

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act on either D- or L-isomers. In particular, enzymes having a strict stereoselectivity to D-N-carbamoyl- α -amino acids may be referred to as D-N-carbamoyl- α -amino acid amidohydrolases

Examples of the DNA fragment containing a gene which can be used in the present invention are those which are derived from eucaryotes, prokaryotes, viruses, bacteriophages or plasmids, and which contain a gene related to a particular D-N-carbamoyl- α -amino acid amidohydrolase. As the gene derived from prokaryotes, preferred are those which are derived from bacteria belonging to the genus, for example, Pseudomonas, Agrobacterium, Aerobacter, Aeromonas, Brevibacterium, Bacillus, Flavobacterium, Serratia, Micrococcus, Arthrobacter, Alkaligenes, Achromobacter, Moraxella, or Paracoccus, and which are related to D-N-carbamoyl- α -amino-acid amidohydrolases. Specific examples of such strains are as follows: Aerobacter cloacae IAM 1221, Bacillus macroides ATCC 12905, Bacillus alvei IFO 3343, Brevibacterium ammoniagenes IFO 12071, Flavobacterium flavescens IFO 3086, Sarcina lutea IFO 1099, Serratia marcescens IFO 3054, Micrococcus luteus IFO 12708, Aeromonas hydrophilia IFO 3820, Agrobacterium radiobacter KNK 712 (FERM BP-1900), Pseudomonas-sp. KNK 003A (FERM BP-3181), Pseudomonas sp. KNK 505 (FERM BP-3182), Agrobacterium 1302 NRRL B11291, Alkaligenes aquamarinus AJ 11199, Achromobacter liquefaciens AJ 11198, Moraxella nonliquefaciens AJ 11221,

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Paracoccus denitrificans AJ 11222, Arthrobacter fragilis AJ 11223, and the like.

As the typical examples of the above bacteria, the micological characteristics of Agrobacterium radiobacter KNK 712 (FERM BP-1900), Pseudomonas sp. KNK 003A (FERM BP-3181), and Pseudomonas sp. KNK 505 (FERM BP-3182) are described below.

Agrobacterium radiobacter KNK 712 (FERM BP-1900)

(a) Morphology

- (1) Cell size and shape: 0.5-1.0 x 2.0-4.0 μ m, rods
- (2) Cellular polymorphism: none
- (3) Motility and flagellar arrangement: active, subpolar
- (4) Sporulation: none
- (5) Gram-staining: negative

(b) Cultural characteristics on various media

(1) Meat extract agar plate culture:

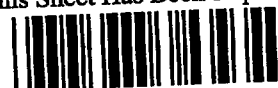
satisfactory growth, round, ridgy, smooth margin, smooth wet surface, white to cream-colored, glossy, opaque, liquid form

(2) Meat extract agar slant culture:

excellent growth, even growth on the inoculation line, thick ridge, smooth wet surface, smooth margin, white to cream-colored, glossy, opaque, no change in the medium

(3) Meat extract gelatin stab culture:

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feeble growth, growth along the stab line, growth around the stab line, no gelatin liquefaction, white to cream-colored, no change in transparency, no change in the medium

(4) Meat extract liquid culture:

medium growth, uneven turbidity, flock formation, no surface growth

(5) Litmus milk: weak acid

(c) Physiological Characteristics

(1) Nitrate reduction: +

(2) Denitrification: +

(3) MR test: +

(4) Vp test: -

(5) Indole formation: -

(6) Hydrogen sulfide formation: -

(7) Starch hydrolysis: -

(8) Citrate utilization: - (Simmons medium)

(9) Inorganic nitrogen source: + (nitrates, ammonium salts)

(10) Urease: -

(11) Oxidase: +

(12) Catalase: +

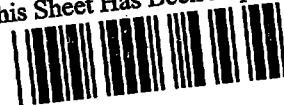
(13) Growth range: 20-37°C, pH 6.5-8.5

(14) Attitude toward oxygen: aerobic

(15) O-F test: O type

(16) Formation of acid and gas from saccharide:

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Acid formation, -; Gas formation, - (D-glucose)

- (17) Malonate utilization: -
- (18) Deaminase reaction of phenylalanine: -
- (19) Decarboxylase reaction: - (lysine)
- (20) Arginine dihydrolase reaction: -
- (21) Casein degradation: -
- (22) DNA degradation: -
- (23) Auxotrophy: none
- (24) Utilization of carbon compounds:

D-Glucose: +

L-Arabinose: +

Saccharose: +

D-Fructose: +

Malonate: -

Cellobiose: +

Ethanol: -

D-Xylose: +

D-Tartrate: -

Sorbitol: +

Citrate: -

Lactose: +

D-Mannitol: +

Meso-inositol: + -

Raffinose: + -

L-Rhamnose: +

Maltose: +

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α -Methyl-D-glucoside: +

D-Mannose: +

Salicin: -

N-Acetylglucosamine: +

Gluconate: -

Caprate: -

Adipate: -

Phenylacetate: -

Methanol: -

(25) Egg yolk reaction: -

(26) β -Galactosidase: +

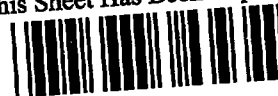
(27) Esculin hydrolysis: +

(28) Cytochrome oxidase: +

(29) Tween degradation: - (Tween 80)

(30) 3-Ketolactose formation: -

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Pseudomonas sp. KNK 003A (FERM BP-3181)

(a) Morphology:

(1) Cell size and shape: 0.5-0.7 $\times$ 1.2-2.5 μ m, rods

(2) Cellular polymorphism: none

(3) Motility: active

(4) Sporulation: none

(5) Gram-staining: negative

(6) Colony shape: round, regular, entire, flat, smooth,
shiny, semi-translucent, buff

(b) Physiological Characteristics

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- (1) Lysis by 3% KOH: +
- (2) Aminopeptidase: +
- (3) Oxidase: +
- (4) Catalase: +
- (5) Cell growth
 - Anaerobic condition: -
 - 37/40°C: +/+
 - pH 5.6: -
 - Mac-Conkey agar: -
 - SS agar: -
 - Cetrimid agar: -
- (6) Acid formation (O-F test)
 - Glucose aerobic condition: -
 - Glucose anaerobic condition: -
- (7) Gas formation from glucose: -
- (8) Acid formation (ASS)
 - Glucose: +
 - Fructose: -
 - Xylose: +
- (9) ONPG: -
- (10) ADH: -
- (11) ODC: -
- (12) VP: -
- (13) Indole formation: =
- (14) Nitrate reduction: -
- (15) Denitrification: -

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- (16) Phenylalanine deaminase: -
- (17) Levan formation from sucrose: -
- (18) Lecithinase: -
- (19) Urease: -
- (20) Hydrolysis
 - Starch: -
 - Geratin: -
 - Casein: -
 - DNA: -
 - Tween 80: -
 - Esculin: -
- (21) Tyrosine degradation: -
- (22) Utilization of various compounds
 - Acetate: weak
 - Adipate: -
 - Caprate: -
 - Citrate: -
 - Citraconate: -
 - Glycolate: -
 - Lactate: +
 - Levulinate: -
 - Malate: -
 - Malonate: -
 - Mesaconate: -
 - Phenylacetate: -
 - Suberate: -

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m-Tartrate: -
D-Tartrate: -
L-Arabinose: +
Fructose: +
Glucose: +
Mannose: +
Maltose: -
Xylose: +
Saccharose: -
Trehalose: -
Ribose: -
Saccharate: -
Hydroxybutylate: -
Benzoate: -
Mannitol: +
Gluconate: +
2-Ketogluconate: +
N-Acethylglucosamine: -
L-Serine: -
L-Histidine: -
L-Valine: -

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(23) Main respiratory quinone type: ubiquinone 10

Pseudomonas sp. KNK 505 (FERM BP-3182)

(a) Morphology:

(1) Cell size and shape: 0.5-0.7 x 1.2-2.5 μ m, rods

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- (2) Cellular polymorphism: none
- (3) Motility: active
- (4) Sporulation: none
- (5) Gram-staining: negative
- (6) Colony shape: round, regular, entire, flat, smooth, shiny, semi-translucent, buff

(b) Physiological Characteristics

- (1) Lysis by 3% KOH: +
- (2) Aminopeptidase: +
- (3) Oxidase: +
- (4) Catalase: +
- (5) Cell growth
 - Anaerobic condition: -
 - 37/40°C: +/+
 - pH 5.6: -
 - Mac-Conkey agar: -
 - SS agar: -
 - Cetrimid agar: -
- (6) Acid formation (O-F test)
 - Glucose aerobic condition: -
 - Glucose anaerobic condition: -
- (7) Gas formation from glucose: -
- (8) Acid formation (ASS)
 - Glucose: +
 - Fructose: -
 - Xylose: +

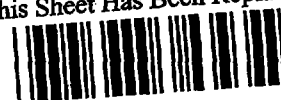
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- (9) ONPG: -
- (10) ADH: -
- (11) ODC: -
- (12) VP: -
- (13) Indole formation: -
- (14) Nitrate reduction: -
- (15) Denitrification: -
- (16) Phenylalanine deaminase: -
- (17) Levan formation from sucrose: -
- (18) Lecithinase: -
- (19) Urease: -
- (20) Hydrolysis
 - Starch: -
 - Geratin: -
 - Casein: -
 - DNA: -
 - Tween 80: -
 - Esculin: -
- (21) Tyrosine degradation: -
- (22) Utilization of various compounds
 - Acetate: weak
 - Adipate: -
 - Caprate: -
 - Citrate: -
 - Citraconate: -
 - Glycolate: -

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Lactate: +
Levulinate: -
Malate: -
Malonate: -
Mesaconate: -
Phenylacetate: -
Suberate: -
m-Tartrate: -
D-Tartrate: -
L-Arabinose: +
Fructose: +
Glucose: +
Mannose: +
Maltose: -
Xylose: +
Saccharose: -
Trehalose: -
Ribose: -
Saccharate: -
Hydroxybutylate: -
Benzoate: -
Mannitol: +
Gluconate: +
2-Ketogluconate: +
N-Acetylglucosamine: -
L-Serine: -

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L-Histidine: -

L-Valine: -

(23) Main respiratory quinone type: ubiquinone 10

To obtain a gene from these strains, which is related to an enzyme capable of converting D-N-carbamoyl- α -amino acids by removal of their carbamoyl groups into the corresponding D- α -amino acids, usually, genetic DNA is extracted from the chromosome of microorganisms according to the conventional procedure, after which a DNA fragment containing the desired gene is obtained and subjected to an analysis for its base sequence. Moreover, microorganisms which produce an enzyme capable of converting D-N-carbamoyl- α -amino acids by removal of their carbamoyl groups into the corresponding D- α -amino acids, as well as transformed microorganisms into which this enzyme gene has been incorporated, are cultivated, and the produced enzyme is purified, after which the molecular weight of its protein is determined and the amino acid sequence in the vicinity of its amino terminus is determined by a gas-phase protein sequencer or the like. Then, this DNA base sequence is compared with the amino terminal sequence of the protein, so that the initiation site for genetic translation into a protein, of the base sequence portion encoding an enzyme protein which is related to removal of carbamoyl groups is determined, and taking into consideration the relation to



the molecular weight of the protein, it is confirmed that the enzyme protein is 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, Chaps. 4 and 13). According to these procedures, DNA fragments of SEQ ID Nos. 1 and 2 in the accompanying Sequence Listing were obtained from the strains Agrobacterium radiobacter KNK 712 and Pseudomonas sp. KNK 003A. The thus-obtained gene encoding the above enzyme-and/or DNA fragment containing this gene are equivalent to DNA fragments having another base sequence which encodes an amino acid sequence corresponding to the above gene and/or DNA fragment because one amino acid usually corresponds to a plurality of base codons, and this fact is obvious.

As the vector used in the present invention, plasmids, phages, or derivatives thereof can be used, which are derived from microorganisms and can be autonomously grown in cells of a bacteria belonging to the genus Escherichia, Pseudomonas, Flavobacterium, Bacillus, Serratia, Croynebacterium, or Brevibacterium. For example, host-vector systems described in "Guidelines on Recombinant DNA Experiments" (Ed., the Life Science Section of the Research Development Office in the Science and Technology Agency: revised on September 16, 1987), page 55, can be used. Moreover, vectors, which have been modified to have a



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strong structural promoter for the purpose of increasing the amount of enzyme to be produced, can also be used.

The preparation of a recombinant comprising a vector DNA and a gene-containing DNA fragment can be conducted by freely using the known in vitro recombinant DNA technique. The in vitro DNA recombination is usually conducted by cleavage and ligation (ligase reaction) of a vector DNA and a donor DNA containing the desired gene (e.g., see the specifications of Japanese Patent Application No. 56-211908 and United States Patent No. 4,237,224). Many kinds of recombinant DNA, in addition to the desired recombinant DNA, are produced by ligase reaction, and therefore, for the purpose of selectively obtaining the desired recombinant DNA, microorganisms belonging to the genus Escherichia, Pseudomonas, Flavobacterium, Bacillus, Serratia, Croynebacterium, or Brevibacterium may be directly transformed with a ligase reaction mixture, and the resulting transformants on which the inherited character from the genetic information of the desired gene has been conferred may be selectively separated, and the desired recombinant DNA may be extracted and isolated from their cultured bacterial cells. -

For example, the recombinant can also be obtained without direct transformation of bacterial species belonging to any one of the above genera; that is, the desired gene is once cloned in a host vector system using other micro-

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organisms, such as Escherichia coli, after which recombinant DNA with an appropriate vector is in vitro produced, and then the above bacterial species are transformed, followed by selective separation of transformants in the same manner as described above.

The description of the following documents can be widely applied to the production of recombinants: the specification of United States Patent No. 4,237,224 to S.N. Cohen, et al.; "Idenshi-souse Jikken-hou (Experimental Method of Gene Manipulation)" [Ed., Yasuyuki Takagi, Kohdansha Scientific (1980)]; Method in Enzymology, 68, Recombinant DNA [Ed., Ray Mv, Academic Press (1979)]; the specification of Japanese Patent Application No. 56-211908, etc.

In the case of Escherichia coli transformed with various kinds of recombinant DNA, a method for selecting, from the transformed strains, particular transformed strains which have the desired gene, i.e., the gene of a particular D-N-carbamoyl- α -amino acid amidohydrolase, and in which that gene has been expressed, is conducted as follows: colonies of the transformed strains are first grown on the plate containing a selection marker, such as ampicillin. Then, various colonies of these transformed strains having the recombinant DNA are collected and suspended in physiological saline, after which this suspension is inoculated on the minimum liquid medium containing a particular D-N-carbamoyl- α -amino acid as a sole nitrogen source. On this medium,

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only transformed strains capable of utilizing the D-N-carbamoyl- α -amino acid, i.e., transformed strains on which D-N-carbamoyl- α -amino acid amidohydrolase activity has been now conferred, can be grown. The culture solution thus obtained is inoculated on the above minimum medium, and such an operation is repeated, resulting in an enrichment of the desired transformed strains. From this enriched culture solution, bacterial cells are separated according to the conventional method, and the separated transformed strains are cultivated to make a bacterial reaction with a D-N-carbamoyl- α -amino acid as a substrate, whereby the transformed strains containing the desired gene can be obtained by confirming the production of a D- α -amino acid.

From the transformed strains thus obtained, recombinant DNA is extracted according to the conventional method, such as a method using alkali denaturation (see Molecular Cloning A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Chap. 1); the structural gene of the desired enzyme in the DNA fragments containing the cloned desired gene is subcloned and unnecessary DNA is removed; recombinant DNA modified to have a strong promoter is prepared in a vector; the above host bacteria are transformed therewith; and the resulting transformed strains are used to increase the amount of the desired enzyme to be produced.

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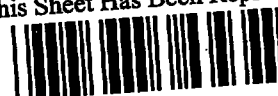
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The introduced character of the recombinant DNA can be expressed by cultivating the transformed strains on a conventional nutrient medium. In cases where the character from the gene DNA or vector DNA is conferred on the recombinant DNA, any drug may be supplemented to the medium depending upon that character.

To take the transformed strains thus obtained, as a source of enzyme supply, culture may be prepared with the use of a conventional medium, and when necessary, it is also possible to conduct a treatment for enzyme induction, such as an addition of hydantoin compounds, D-N-carbamoyl- α -amino acids, isopropyl-1-thio- β -D-galactosides (IPTG), or the like, and a temperature increase.

Usually, the medium used for cultivating the transformed strains may be a conventional medium containing carbon sources, nitrogen sources, and inorganic ions. If organic tracenutrients, such as vitamins and amino acids, are added thereto, satisfactory results may be obtained in many cases. As the carbon sources, carbohydrates, such as glucose and 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, are used. As the inorganic ions, phosphate ion, magnesium ion, potassium ion, iron ion, and the like, may be used.

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If culture is prepared under aerobic conditions for 1-10 days, while adjusting the pH and temperature to an appropriate range of 4-8 and 25-45°C, respectively, it is possible to obtain desirable results.

Examples of the embodiments acting as an enzyme produced by the transformed strains include culture solutions of the transformed strains, bacterial cells, treated bacterial cells, enzymes extracted from bacterial cells, immobilized bacterial cells, and the like.

As the bacterial cells, any of the culture solution as it is after completion of cultivation, bacterial cells separated from the culture solution, washed bacterial cells, and the like, can be used. As the treated bacterial cells, lyophilized bacterial cells, acetone-dried bacterial cells, bacterial cells brought into contact with toluene or detergents, lysozyme-treated bacterial cells, bacterial cells exposed to ultrasonic waves, mechanically ground bacterial cells, and the like, can be used, as well as enzyme extracts having the enzyme activity to convert D-N-carbamoyl- α -amino acids obtained from these treated bacterial cells, by removal of their carbamoyl groups into the corresponding D- α -amino-acids; these immobilized bacterial cells; insolubilized treated bacterial cells; enzyme proteins fixed on a support for immobilization (e.g., anion exchange resin), and the like. For an immobilization method, for example, it is possible to make reference to the



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specification of Japanese Patent Laid-open Publication No. 63-185382.

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, 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 bacterial cells, and cell-free extracts, can be used.

The preparation of immobilized enzymes can be conducted by using an enzyme solution and the conventional method in which, for example, enzymes are adsorbed on a support, followed by cross-linking treatment.

D-N-carbamoyl- α -amino acids, which are used as a substrate of the enzymatic reaction of the present

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invention, can be represented by the formula: $R-CH(NHCONH_2)-COOH$, and as a practical embodiment, in cases where the enzyme used has strict stereoselectivity to D-N-carbamoyl- α -amino acids, it can be used as the D-form or a mixture of the D- and L-forms. Moreover, in cases where the stereoselectivity is not strict because enzymes also act on L-carbamoyl amino acids, or where enzymes are used as an enzyme mixture also acting on the L-form thereof, it is preferred that only enzymes of the D-form are used to produce α -amino acids of the D-form.

The substituent R can be selected in a wide range, as described in the specifications of Japanese Patent Publication Nos. 57-18793, 63-20520, and 1-48758; in particular, to provide industrially useful compounds, such as intermediates of drugs, it is preferred that R is phenyl, phenyl substituted with hydroxy, alkyl, substituted alkyl, aralkyl, or thienyl. In the case of phenyl substituted with hydroxy, the number of hydroxy is one or more, and they may be attached at any of o-, m-, and p-positions, with the typical example thereof being p-hydroxyphenyl. The alkyl group is such a group of 1-4 carbon atoms that the corresponding amino acid becomes D-alanine, D-valine, D-leucine, D-isoleucine, or the like. The substituted alkyl group is such an alkyl having 1-4 carbon atoms substituted with hydroxy, alkylthio, carboxyl, amino, phenyl, phenyl substituted with hydroxy, amido, or the like, that the

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corresponding amino acid becomes D-serine, D-threonine, D-methionine, D-cysteine, D-asparagine, D-glutamine, D-tyrosine, D-tryptophane, D-aspartic acid, D-glutamic acid, D-histidine, D-lysine, D-arginine, D-citrulline, or the like. The aralkyl group is such a group having 7-8 carbon atoms, for example, benzyl or phenethyl, that the corresponding amino acid becomes D-phenylalanine or the like.

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, or the like, can also be added to the aqueous medium.

In cases where, while cultivating the bacterial cells of the above microorganisms in a water-soluble medium, the bacterial cells are brought into contact with a particular D-N-carbamoyl- α -amino acid, an aqueous medium containing not only a D-N- α -amino acid but also nutrients required for the growth of microorganisms, such as carbon sources, nitrogen sources, and inorganic ions, is used. Further, if organic tracenuitrients, such as vitamins and amino acids, are added thereto, satisfactory results can be obtained in many cases. As the carbon sources, carbohydrates, such as glucose and sucrose; organic acids, such as acetic acid; alcohols, and the like, are conveniently

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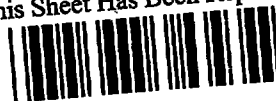
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used. As the nitrogen sources, ammonia gas, ammonia water, ammonium salts, and the like, are used. As the inorganic ions, phosphate ion, magnesium ion, potassium ion, iron ion, and the like, may be used.

The cultivation is conducted under aerobic conditions, while adjusting the pH and temperature to an appropriate range of 4-8 and 25-45°C, respectively. If the cultivation is conducted for 1-10 days, D-N-carbamoyl- α -amino acids can be converted into only D- α -amino acids with high efficiency.

To the contrary, in cases where the culture solution of the above microorganisms is allowed as it is, to react with cultivated bacterial cells, treated bacterial cells, enzyme extracts, immobilized bacterial cells, insolubilized bacterial cells, or fixed enzyme proteins, in an aqueous medium containing a particular D-N-carbamoyl- α -amino acid dissolved or suspended, the reaction mixture may be allowed to stand for some time or stirred, while maintaining at an appropriate temperature of 10-80°C and pH of 4-9.5. Thus, if 5-100 hours have run their course, the corresponding D- α -amino acid is produced in large quantities and enriched in the aqueous medium. Moreover, the D-N-carbamoyl- α -amino acid may be added in separate portions with the progress of reaction. The produced D- α -amino acid can be separated and purified by a conventional separation method.

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The D- α -amino acid thus obtained can be represented by the formula: R-CHNH₂COOH (R is as defined above).

Examples

The following will describe the embodiments of the present invention. The D- α -amino acid produced was detected and determined by high performance liquid chromatography (HPLC) or thin layer chromatography (TLC).

Example 1

Preparation of recombinant DNA comprising chromosome DNA from Agrobacterium radiobacter KNK 712 (FERM BP-1900) and vector DNA:-

Agrobacterium radiobacter KNK 712 (FERM BP-1900) was cultivated in 2 liters of L-broth (10 g of peptone/liter, 5 g of yeast extract/liter, 5 g of sodium chloride/liter; pH 7.0) at 33°C for 27 hours, after which cultures were harvested to give 20 g of bacterial cells. From the bacterial cells obtained, chromosome DNA was extracted according to the Marmur method. To 250 μ g of this chromosome DNA, 2 U of Sau3AI were added, and allowed to react at 37°C for 30 minutes, thereby causing partial digestion. From the partially digested DNA, 4-9 kbp DNA fragments were obtained by agarose gel electrophoresis. On the other hand, plasmid pUC₁₈ was completely digested with BamHI, and ligated with T4 DNA ligase to the above-obtained DNA fragments from chromosome, resulting in a mixed solution

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of various recombinant plasmids.

Example 2

Selection of plasmid derived from Agrobacterium radiobacter KNK 712 (FERM BP-1900) and containing gene related to conversion of D-N-carbamoyl- α -amino acid into the corresponding D- α -amino acid:-

Using the mixed plasmid solution of Example 1, Escherichia coli JM 109 was transformed according to the conventional method. This was inoculated on the medium of Table 1, containing ampicillin as a selection marker.

Table 1

Polypeptone	1 g
Yeast extract	0.5 g
Sodium chloride	0.5 g
Ampicillin	100 mg
Ager	15 g

Water was added to the volume of 1 liter (pH 7.0).

The grown colonies were collected and inoculated into the liquid medium of Table 2, containing D-N-carbamoyl-alanine as a sole nitrogen source, followed by cultivation.

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Table 2

Na ₂ HPO ₄	6 g
KH ₂ PO ₄	3 g
NaCl	0.5 g
MgSO ₄	0.12 g
CaCl ₂	11 mg
Glucose	2 g
Ampicillin	50 mg
Thiamin	1 mg
D-N-Carbamoyl-alanine	1 g

Water was added to the volume of 1 liter (pH 7.0).

Only the transformed strains expressing the desired gene can be grown on the medium of Table 2 by utilizing D-N-carbamoyl-alanine as a nitrogen source. The culture solution in the medium of Table 2 was inoculated into the same medium as used above, and this operation was repeated for enrichment of the desired transformed strains. From the enriched culture solution, the transformed strains were purely separated with the medium of Table 1. These purely separated cells were inoculated into 10 ml of L-broth (10 g of peptone/liter, 5 g of yeast extract/liter, 5 g of sodium chloride/liter; pH 7.0) containing 100 mg/liter of ampicillin, and incubated at 37°C for 16 hours, after which 1 ml of the culture solution was harvested and the supernatant was removed therefrom, followed by suspension of

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the bacterial cells in 0.5 ml of substrate solution (0.5% D-N-carbamoyl-parahydroxyphenylglycine, 0.05% Triton X-100, 0.1 M phosphate buffer (hereinafter referred to as KPB); pH 7.0) and allowing to react at 37°C for 3 hours. This reaction mixture was spotted on TLC and developed, followed by staining with ninhydrin, resulting in a spot of D-parahydroxyphenylglycine which was in agreement with the standard. From this fact, it was confirmed that the transformed strains purely separated have a plasmid containing a gene related to the desired enzyme. The plasmid contained in these strains was named pAHD 101.

Then, from the above transformed strains, pAHD 101 was prepared in large quantities according to the alkali denaturation method, followed by mapping, which revealed that it had the structure shown in Fig. 1.

Escherichia coli JM 109 transformed with pAHD 101 was named Escherichia coli JM 109 pAHD 101.

Example 3

Selection of plasmid derived from Pseudomonas sp. KNK 003A (FERM BP-3181) and containing gene related to conversion of D-N-carbamoyl- α -amino acid into the corresponding D- α -amino acid:-

In the same manner-as described in Example 1, a mixed solution of various recombinant plasmids formed from chromosome DNA fragments of Pseudomonas sp. KNK 003A (FERM BP-3181) and plasmid pUC18 was obtained. Using this mixed

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solution, Escherichia coli JM 109 was transformed according to the conventional method. From these various transformed strains, particular transformed strains having a plasmid which contains a gene related to an enzyme capable of converting D-N-carbamoyl- α -amino acids into the corresponding D- α -amino acids were obtained in the same manner as described in Example 2; except that the liquid medium of Table 2, containing 100 mg/liter of IPTG, was used in place of the liquid medium of Table 2. The plasmid contained in these strains was named pPHD 301.

Then, from the above transformed strains, pPHD 301 was prepared in large quantities, followed by mapping, which revealed that it had the structure shown in Fig. 2.

Escherichia coli JM 109 transformed with pPHD 301 was named Escherichia coli JM 109 pPHD 301.

Example 4

Subcloning of pAHD 101

The pAHD 101 obtained in Example 2 was completely digested with SmaI and EcoRI, and a 2.7 kbp SmaI-EcoRI fragment was obtained by agarose gel electrophoresis. On the other hand, plasmid pUC 19 was completely digested with SmaI and EcoRI, and ligated with T4 DNA ligase to the SmaI-EcoRI fragment of pAHD 101, after which Escherichia coli JM 109 was transformed with this ligated plasmid and the transformed strains were cultivated on an L-medium (1% peptone, 0.5% yeast extract, 0.5% sodium chloride, 1.5%

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agar; pH 7.0) containing isopropyl-1-thio- β -D-galactoside (IPTG), 5-chloro-4-bromo-3-indolyl- β -D-galactose (Xgal) and ampicillin.

The plasmid corresponding to pUC 19 containing the SmaI-EcoRI fragment of pAHD 101 formed white colonies without exhibiting blue color, and therefore, these white colonies were selected. To confirm that these selected bacteria have the desired enzyme activity, they were inoculated into 10 ml of L-broth (1% peptone, 0.5% yeast extract, 0.5% sodium chloride) containing 100 mg/liter of ampicillin after cultivation of which 1 ml of the culture solution was harvested and the supernatant was removed therefrom, and the bacterial cells were suspended in 0.5 ml of substrate solution (0.5% D-N-carbamoyl-parahydroxy-phenylglycine, 0.05% Triton X-100, 0.1 M phosphate buffer; pH 7.0), followed by reaction at 37°C for 3 hours. This reaction mixture was spotted on TLC and developed, followed by staining with ninhydrin, resulting in a spot of D-parahydroxyphenylglycine which was in agreement with the standard. The plasmid contained in these transformed strains was named pAD 107.

Then, the plasmid pAD 107 was prepared and partially hydrolyzed with SalI, followed by agarose gel electrophoresis to give a 4.5 kbp fragment containing the pUC 19 portion. This fragment was circularized with T4 DNA ligase, and this plasmid was used to transform Escherichia

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coli JM 109. The trasformed strains were obtained from the L-medium containing ampicillin and bacterial reaction was conducted in the same manner as described above, thereby obtaining transformed strains having the ability to convert D-N-carbamoyl-parahydroxyphenylglycine into D-parahydroxy-phenylglycine. The plasmid contained in these transformed strains was named pAD 108. The pAD 108 was prepared form the above transformed strains, followed by mapping, which revealed that it had the structure shown in Fig. 3.

Escherichia coli JM 109 transformed with pAD 108 was named Escherichia coli JM 109 pAD 108. The accession number given from the Fermentation Research Institute was FERM BP-3184.

Example 5

Subcloning of pPHD 301

The pPHD 301 obtained in Example 3 was completely digested with AccI, and a 5.2 kbp fragment containing the pUC 18 portion was obtained by agarose gel electrophoresis. This fragment was circularized with T4 DNA ligase, and this plasmid was used to transform Escherichia coli JM 109. The trasformed strains were obtained from an L-medium containing ampicillin and bacterial reaction was conducted in the same manner as described in Example 4, thereby obtaining transformed strains having the ability to convert D-N-carbamoyl-parahydroxyphenylglycine into D-parahydroxy-phenylglycine. The plasmid contained in these

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transformed strains was named pPD 302.

Then, the plasmid pPD 302 was prepared and completely digested with SphI and AccI, after which a 1.8 kbp SphI-AccI fragment was obtained by agarose gel electrophoresis. On the other hand, plasmid pUC 19 was completely digested with SphI and AccI, and ligated with T4 DNA ligase to the SphI-AccI fragment of pPD 302, after which Escherichia coli JM 109 was transformed with this ligated plasmid. The transformed strains were obtained from an L-medium containing ampicillin, and bacterial reaction was conducted in the same manner as described in Example 4, thereby obtaining transformed strains having the ability to convert D-N-carbamoyl-parahydroxyphenylglycine into D-parahydroxyphenylglycine. The plasmid contained in these transformed strains was named pPD 304. The plasmid pPD 304 was prepared from the above transformed strains, followed by mapping, which revealed that it had the structure shown in Fig. 4.

Escherichia coli JM 109 transformed with pPD 304 was named Escherichia coli JM 109 pPD 304. The accession number given from the Fermentation Research Institute was FERM BP-3183.

Example 6

Conversion of D-N-carbamoyl- α -amino acid into D- α -amino acid with enzyme obtained from transformed strains:-

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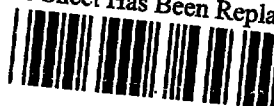
Using the plasmid pAD 108 obtained in Example 4, Escherichia coli JM 109 was transformed. This strain was cultivated in L-broth containing 100 µg/ml of ampicillin and 100 µg/ml of IPTG at 37°C for 16 hours. From 100 ml of this culture solution, bacterial cells were harvested and then suspended in 0.1 M KPB (pH 7.0) to the volume of 10 ml. This was subjected to ultrasonication while cooling with ice, followed by centrifugation to give the supernatant as a crude enzyme solution. Using this crude enzyme solution, reaction was conducted with various kinds of D-N-carbamoyl-α-amino acid shown in Table 3 as a substrate. To 2 ml of 40 mM D-N-carbamoyl-α-amino acid (Table 3) and 0.2 M KPB (pH 7.0), 100 µl of the above crude enzyme solution were added, thereby allowing to react at 40°C for 20 minutes, and the amount of D-α-amino acid produced was determined. The results are shown in Table 3. Moreover, there was a 10-fold improvement in the specific activity of a D-N-carbamoyl-α-amino acid amidohydrolase obtained from JM 109 pAD 108 at this time as compared with a crude enzyme solution from Agrobacterium radiobacter KNK 712 (FERM BP-1900).

Example 7

Conversion of D-N-carbamoyl-α-amino acid into D-α-amino acid with enzyme obtained from transformed strains:-

Using the plasmid pPD 304 obtained in Example 5, Escherichia coli JM 109 was transformed. From this strain,

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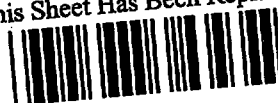
a crude enzyme solution was obtained in the same manner as described in Example 6. Using this crude enzyme solution, reaction was conducted in the same manner as described in Example 6. The results are shown in Table 3. There was a 40-fold improvement in the specific activity of a D-N-carbamoyl- α -amino acid amidohydrolase obtained from JM 109 pPD 304 at this time as compared with a crude enzyme solution from Pseudomonas sp. KNK 003A (FERM BP-3181).

Example 8

Conversion of D-N-carbamoyl-parahydroxyphenylglycine into D-parahydroxyphenylglycine with enzyme obtained from transformed strains:-

Using the plasmid pAD 108 obtained in Example 4, Escherichia coli JM 109 was transformed. This strain was cultivated in the same manner as described in Example 6. From 100 ml of the culture solution, bacterial cells were harvested, and washed with 0.1 M KPB (pH 7.0), after which they were suspended in 100 ml of 5% D-N-carbamoyl-parahydroxyphenylglycine, 0.05% Triton X-100, and 0.1 M KPB (pH 7.0), followed by stirring at 40°C for 20 hours to cause reaction. The reaction mixture thus obtained was subjected to centrifugation at 6000 rpm for 10 minutes to remove the bacterial cells, and pH was decreased to 2.7 by addition of concentrated hydrochloric acid, followed by adsorption on the cation exchange resin IR-120B (H⁺ type) and elution with 5% NH₄OH. Then, the eluates were desalinized with IRC-84

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(H⁺ type) and decolorized with an AF resin. The decolorized solution was concentrated to allow crystallization, and the deposited crystals were recrystallized from water to give 3.8 g of white powder. These crystals exhibited the specific rotation $[\alpha]_D^{20} = -158$ (C = 1, 1 N HCl), and gave a single spot on TLC, and the IR spectrum thereof was in agreement with that of the D-parahydroxyphenylglycine standard.

Example 9

Preparation of fixed D-N-carbamoyl- α -amino acid amidohydrolase:-

After 200 ml of the culture solution obtained, in the same manner as described in Example 6, from the strains of Escherichia coli JM 109 transformed with pAD 108 was harvested, they were washed with 0.1 M KPB (pH 7.0), and suspended in 20 ml of 0.1 M KPB (pH 7.0), followed by ultrasonication of the bacterial cells. This suspension of disintegrated bacterial cells was subjected to centrifugation at 12000 rpm for 20 minutes to give the supernatant as a crude enzyme solution. To this crude enzyme solution, 2 g of anion exchange resin Duolite A-568 equilibrated with 0.1 M KPB (pH 7.0) were added, and stirred at 4°C for 15 hours to make the enzyme adsorbed thereon. To this solution, glutaraldehyde was added to have a final concentration of 0.1%, and stirred for 1 hour, followed by cross-linking treatment, after which the resin was collected

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by filtration and washed with 0.1 M KPB, resulting in 2 g of fixed D-N-carbamoyl- α -amino acid amidohydrolase.

Example 10

Conversion of D-N-carbamoyl-parahydroxyphenylglycine into D-parahydroxyphenylglycine with immobilized enzyme:-

Two grams of immobilized D-N-carbamoyl- α -amino acid amidohydrolase obtained in Example 9 were added to 100 ml of 2% D-N-carbamoyl-parahydroxyphenylglycine and 0.1 M KPB (pH 7.0), and stirred at 40°C for 20 hours, while maintaining the pH at 7.0 by addition of 1 N HCl, thereby causing reaction. After the reaction, the mixture was allowed to stand, and the reaction mixture was collected by suction, after which the produced amino acid was purified in the same manner as described in Example 8 to give 1.5 g of D-parahydroxyphenylglycine.

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Table 3

Amount of amino acid produced (mmol/liter)

Reaction substrate	Amino acid produced	JM 109 pAD 108	JM 109 pPD 304	JM 109
D-N-Carbamoyl-parahydroxy-phenylglycine	D-Parahydroxy-phenylglycine	9.5	0.14	0
D-N-Carbamoyl-phenylglycine	D-Phenylglycine	9.7	0.06	0
D-N-Carbamoyl-homophenyl-alanine	D-Homophenyl-alanine	3.9	0.78	0
D-N-Carbamoyl-phenylalanine	D-Phenylalanine	4.2	0.98	0
D-N-Carbamoyl-leucine	D-Leucine	6.4	1.37	0
D-N-Carbamoyl-valine	D-Valine	6.6	0.98	0
D-N-Carbamoyl-alanine	D-Alanine	7.0	1.56	0

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Example 11

Analysis of DNA base sequence for the gene of enzyme derived from Agrobacterium radiobacter KNK 712 (FERM BP-1900) and capable of converting D-N-carbamoyl- α -amino acid into the corresponding D- α -amino acid:-

The plasmid pAD 108 containing the gene of a D-N-carbamoyl- α -amino acid amidohydrolase (hereinafter referred to as amidohydrolase) derived from Agrobacterium radiobacter KNK 712 (FERM BP-1900) was digested with restriction endonucleases EcoRI and HindIII (manufactured by Takara Shuzo Co., Ltd.), and a 1.8 kb DNA fragment was separated for preparation by agarose gel electrophoresis. This fragment was digested with various restriction endonucleases, and ligated with T4-DNA ligase (manufactured by Takara Shuzo Co., Ltd.) to M13mpl8 or M13mpl9, after which Escherichia coli JM 109 was infected therewith, resulting in the formation of a plaque. This single plaque was inoculated into 1.5 ml of 2YT medium (16 g/l of bactotrypton (Difco Co.), 10 g/l of yeast extract (Difco Co.), and 5 g/l of NaCl) into which 1% JM 109 had been inoculated, and subjected to shaking culture at 37°C for 5 hours. After centrifugation, 200 μ l of 20% polyethylene-glycol 6000 and 25 M NaCl solution were added to the supernatant, and allowed to stand at room temperature for 15 minutes, after which phage particles were recovered as a precipitate by centrifugation. This was dissolved in 100 μ l

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of TE solution [10 mM Tris HCl (pH 8.0), 1 mM EDTA], and extracted with 50 μ l of phenol (saturated with TE solution), after which 10 μ l of 3 M sodium acetate solution and 250 μ l of ethanol were added and allowed to stand overnight at -20°C, followed by centrifugation. After drying, the precipitate was dissolved in 50 μ l of TE solution. Then, 7 μ l of this solution was used for reaction, electrophoresis, and autoradiography with the aid of a DNA sequence kit (manufactured by United States Biochemical Corp.) using SEQUENASE (registered trade mark) ver. 2, according to its instruction manual. From the results obtained, the DNA base sequence of amidohydrolase gene for the strain KNK 712 was determined as shown by SEQ ID No. 1 in the accompanying Sequence Listing.

Example 12

Analysis of DNA base sequence for the gene of enzyme derived from Pseudomonas sp. KNK 003A (FERM BP-3181) and capable of converting D-N-carbamoyl- α -amino acid into the corresponding D- α -amino acid:

The plasmid pPD 304 containing amidohydrolase gene derived from the strain KNK 003A was digested with restriction endonucleases BamHI and HindIII (manufactured by Takara Shuzo Co., Ltd.), and a 1.8 kb DNA fragment was separated for preparation by agarose gel electrophoresis. This fragment was digested with various restriction endonucleases, and ligated with T4-DNA ligase (Takara Shuzo

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Co., Ltd.) to M13mpl8 or M13mpl9, after which Escherichia coli JM 109 was infected therewith, resulting in the formation of a plaque. This single plaque was inoculated into 1.5 ml of 2YT medium (16 g/l bactotrypton (Difco Co.), 10 g/l yeast extract (Difco Co.), and 5 g/l NaCl) into which 1% JM 109 had been inoculated, and subjected to shaking culture at 37°C for 5 hours. After centrifugation, 200 µl of 20% polyethyleneglycol 6000 and 25 M NaCl solution were added to the supernatant, and allowed to stand at room temperature for 15 minutes, after which phage particles were recovered as a precipitate by centrifugation. This was dissolved in 100 µl of TE solution [10 mM Tris HCl (pH 8.0), 1 mM EDTA], and extracted with 50 µl of phenol (saturated with TE solution), after which 10 µl of 3M sodium acetate solution and 250 µl of ethanol were added thereto, and allowed to stand overnight at -20°C, followed by centrifugation. After drying, the precipitate was dissolved in 50 µl of TE solution. Then, 7 µl of this solution was used for reaction, electrophoresis, and autoradiography with the aid of a DNA sequence kit (manufactured by United States Biochemical Corp.) using SEQUENASE (registered trade mark) ver. 2, according to its instruction manual. From the results obtained, the DNA base sequence of amidohydrolase gene derived from the strain KNK 003A was determined as shown by SEQ ID No. 2 in the accompanying Sequence Listing.

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Example 13

Purification of D-N-carbamoyl- α -amidohydrolase
derived from Agrobacterium radiobacter KNK 712 (FERM BP-
1900):-

Agrobacterium radiobacter KNK 712 (FERM BP-1900)
was cultivated in the medium of Table 4 at 33°C for 25
hours.

Table 4

Glycerin	25 g
Sucrose	5 g
KH ₂ PO ₄	5 g
Na ₂ HPO ₄	5 g
MgSO ₄ .7H ₂ O	1 g
MnCl ₂ .4H ₂ O	10 mg
Yeast extract	4 g
Urea	2 g
D-N-carbamoyl-P- hydroxyphenylglycine	1 g

Water was added to the volume of 1 liter (pH 6.5).

Twenty one liters of this culture solution were
harvested and the bacterial cells were ultrasonicated.
After removal of the residue by centrifugation, nucleic
acids were removed by protamine sulfate treatment (0.1 mg/mg
of protein). The centrifuged supernatant was subjected to

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heat treatment at 50°C for 20 minutes, and after removal of the precipitate, protein was precipitated by addition of ammonium sulfate, and a protein fraction having activity and being precipitated with 15% to 35% saturated ammonium sulfate was recovered. This fraction was dissolved and subjected to HPLC using DEAE-5pw column (manufactured by Toso Co., Ltd.), followed by elution with a concentration gradient of NaCl and recovered active fractions. At this stage, in comparison with a suspension of disintegrated bacterial cells, there was about 20-fold increase in the specific activity of amidohydrolase. When this fraction was analyzed by SDS-polyacrylamide gel electrophoresis, this amidohydrolase migrated near to the position corresponding to the molecular weight of about 35,000.

Example 14

Determination of amino acid sequence around protein amino terminus of D-N-carbamoyl- α -amino acid amidohydrolase derived from Agrobacterium radiobacter KNK 712 (FERM BP-1900):-

A purified amidohydrolase preparation produced by Agrobacterium radiobacter KNK 712 (FERM BP-1900) obtained in Example 13 was charged in a reverse-phase HPLC column (AP-303; manufactured by YMC Co.), and eluted with a concentration gradient of acetonitrile. This fraction containing the amidohydrolase was charged for analysis in a gas-phase protein sequencer (manufactured by Applied

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Biosystems Co., Ltd.), and it was found that the amidohydrolase is a protein having a sequence in the amino terminus portion, which consists of 1st to 20th amino acids of SEQ ID No. 1 in the accompanying Sequence Listing.

Example 15

Purification of D-N-carbamoyl- α -amino acid amidohydrolase derived from Pseudomonas sp. KNK 003A (FERM BP-3181):-

Pseudomonas sp. KNK 003A (FERM BP-3181) was cultivated in the medium of Table 5 at 45°C for 3 days.

Table 5

Glycerin	10 g
Glucose	5 g
KH ₂ PO ₄	3.5 g
Na ₂ HPO ₄	3.5 g
MgSO ₄ ·7H ₂ O	0.5 g
MnCl ₂ ·4H ₂ O	20 mg
FeSO ₄	10 mg
CaCO ₃	1 g
Meat extract	2 g
Yeast extract	2 g
Polypeptone	2 g
D-N-carbamoylalanine	1 g

Water was added to the volume of 1 liter (pH 7.0).

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Twenty six liters of this culture solution were harvested, and in the same manner as described in Example 13, the following operations were conducted: ultrasonication of the bacterial cells; removal of nucleic acids by protamine sulfate treatment; heat treatment (65°C, 20 min); fractionation by ammonium sulfate precipitation (separation of protein fractions having amidohydrolase activity and being precipitated with 50% to 70% saturated ammonium sulfate); and HPLC using DEAE-5pw column. These active fractions was allowed to adsorb in a Biogel-HT (Bio-Rad Laboratories Co., Ltd.) column, and eluted with a concentration gradient of ammonium sulfate, after which the active fractions were concentrated and subjected to gel filtration using a Sephacryl S-300 (Pharmacia LKB Biotechnology Co., Ltd.) column.

Then, when isoelectric focusing (pH 4 to 6.5) was conducted, the above amidohydrolase migrated near to the position at pH 5.7, and the gel of this band portion having activity was cut out, from which protein was extracted. At this stage, in comparison with a suspension of the disintegrated bacterial cells, there was about 100-fold increase in the specific activity of amidohydrolase. When this sample was analyzed by SDS-polyacrylamide gel electrophoresis, amidohydrolase migrated near to the position corresponding to the molecular weight of about 38,000. Moreover, when this sample was subjected to gel

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filtration using a Sephacryl S-200 column, it was eluted at the position corresponding to the molecular weight of about 67,000.

Example 16

Determination of amino acid sequence around protein amino terminus of D-N-carbamoyl- α -amino acid amidohydrolase derived from Pseudomonas sp. KNK 003A (FERM BP-3181):-

A purified amidohydrolase preparation from Pseudomonas sp. KNK 003A (FERM BP-3181) obtained in Example 15 was charged in a reverse-phase HPLC column, and eluted with a concentration gradient of acetonitrile. This fraction containing the amidohydrolase was charged for analysis in a gas-phase protein sequencer, and it was found that the amidohydrolase is a protein having a sequence in the amino terminus portion, which consists of 1st to 20th amino acids of SEQ ID No. 2 in the accompanying Sequence Listing.

Example 17

Purification of D-N-carbamoyl- α -amino acid amidohydrolase produced by transformed Escherichia coli:-

Escherichia coli JM 109.pAD 108 obtained in Example 4 and Escherichia coli JM 109.pPD 304 obtained in Example 5 were cultivated by the method as described in Example 6. The bacterial cells were collected from each culture solution by centrifugation, and ultrasonicated. After removal of the residue, these crude enzyme solutions were

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analyzed by SDS-polyacrylamide electrophoresis. With respect to a suspension of the cultivated and disintegrated bacterial cells of Escherichia coli JM 109.pAD 108, the amidohydrolase migrated to the position corresponding to the molecular weight of about 35,000, and an analysis by densitometer after staining revealed that the amidohydrolase amounted to about 50% of the whole soluble proteins of the bacterial cells. Moreover, with respect to a suspension of the cultivated and disintegrated bacterial cells of Escherichia coli JM 109.pPD-304, the amidohydrolase migrated to the position corresponding to the molecular weight of about 38,000, and an analysis by densitometer after staining revealed that the amidohydrolase amounted to about 15% of the whole soluble proteins of the bacterial cells.

Example 18

Determination of protein amino terminal sequence of D-N-carbamoyl- α -amino acid amidohydrolase produced by transformed Escherichia coli:-

A suspension of the cultivated and disintegrated bacterial cells of Escherichia coli JM 109.pAD 108 obtained in Example 17 was subjected to heat treatment at 50°C for 30 minutes, and after removal of the precipitate by centrifugation, ammonium sulfate was added to give 30% saturation, thereby causing precipitation of protein. The precipitated protein was removed by centrifugation and dissolved in deionized water, followed by desaltization

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using an NAP-10 column (Pharmacia LKB Biotechnology Co., Ltd.) and charging in a gas-phase protein sequencer. From the results obtained, it was found that there were mixed proteins in approximately the same quantities, one having a sequence in the amino terminus portion, which consists of 1st to 16th amino acids of SEQ ID No. 1 in the accompanying Sequence Listing, and the other having a sequence in the amino terminus portion, which contains an additional methionine residue attached to the amino terminus of the former's sequence.

Effect of the invention

As described hereinabove, the present invention makes it possible to produce D- α -amino acids from D-N-carbamoyl- α -amino acids with high efficiency by employing gene technology.

Brief Description of the drawings

Fig. 1 shows the restriction endonuclease map of plasmid pAHD 101 obtained by the present invention.

Fig. 2 shows the restriction endonuclease map of plasmid pPHD 301 obtained by the present invention.

Fig. 3 shows the restriction endonuclease map of plasmid pAD 108 obtained by the present invention.

Fig. 4 shows the restriction endonuclease map of plasmid pPD 304 obtained by the present invention.

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SEQUENCE LISTING

SEQ ID NO.: 1

SEQUENCE LENGTH: 1785

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: double

TOPOLOGY: linear

MOLECULE TYPE: genomic DNA

ORIGINAL SOURCE

ORGANISM: Agrobacterium radiobacter

STRAIN: KNK 712 (FERM BP-1900)

GTGACGGCG GGCTCGCGG AGAGCTTGTC AAGCAGCGCA AATTCCGGTT CCGCTCCGGT 60
TGACAGATCA AAAATTTTAC GCCTGTTATT GTCGTGCTGC ATGTAATATT TCGTACTTTA 120
TGTAGAATTT GCATTGCGCC GCGAGTCACA AAGCCGGTTT TCGGCGATGT GTTTCACAAC 180
GTTTTCCCGG CCGCTGGGCC GGACATCACC TAGGAAGGAG CAGAGGTTCA TG ACA CGT 238

Thr Arg

1

CAG ATG ATA CTT GCA GTG GGA CAA CAA GGT CCG ATC GCG CGC GCG GAG 286
Gln Met Ile Leu Ala Val Gly Gln Gln Gly Pro Ile Ala Arg Ala Glu

5

10

15

ACA CGC GAA CAG GTC GTC GTT CGT CTT CTC GAC ATG CTG ACG AAA GCC 334
Thr Arg Glu Gln Val Val Val Arg Leu Leu Asp Met Leu Thr Lys Ala

20

25

30

GCG AGC CGG GGC GCG AAT TTC ATT GTC TTC CCC GAA CTC GCG CTT ACG 382
Ala Ser Arg Gly Ala Asn Phe Ile Val Phe Pro Glu Leu Ala Leu Thr

35

40

45

50

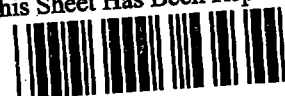
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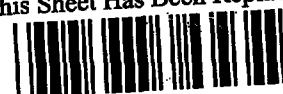
ACC TTC TTC CCG CGC TGG CAT TTC ACC GAC GAG GCC GAG CTC GAT AGC	430
Thr Phe Phe Pro Arg Trp His Phe Thr Asp Glu Ala Glu Leu Asp Ser	
55 60 65	
TTC TAT GAG ACC GAA ATG CCC GGC CCG GTG GTC CGT CCA CTC TTT GAG	478
Phe Tyr Glu Thr Glu Met Pro Gly Pro Val Val Arg Pro Leu Phe Glu	
70 75 80	
AAG GCC GCG GAA CTC GGG ATC GGC TTC AAT CTG GGC TAC GCT GAA CTC	526
Lys Ala Ala Glu Leu Gly Ile Gly Phe Asn Leu Gly Tyr Ala Glu Leu	
85 90 95	
GTC GTC GAA GGC GGC GTC AAG CGT CGC TTC AAC ACG TCC ATT TTG GTG	574
Val Val Glu Gly Gly Val Lys Arg Arg Phe Asn Thr Ser Ile Leu Val	
100 105 110	
GAT AAG TCA GGC AAG ATC GTC GGC AAG TAT CGT AAG ATC CAT TTG CCG	622
Asp Lys Ser Gly Lys Ile Val Gly Lys Tyr Arg Lys Ile His Leu Pro	
115 120 125 130	
GGT CAC AAG GAG TAC GAG GCC TAC CGG CCG TTC CAG CAT CTT GAA AAG	670
Gly His Lys Glu Tyr Glu Ala Tyr Arg Pro Phe Gln His Leu Glu Lys	
135 140 145	
CGT TAT TTC GAG CCG GGC GAT CTC GGC TTC CCG GTC TAT GAC GTC GAC	718
Arg Tyr Phe Glu Pro Gly Asp Leu Gly Phe Pro Val Tyr Asp Val Asp	
150 155 160	
GCC GCG AAA ATG GGC ATG TTC ATC TGC AAC GAT CGC CGC TGG CCT GAA	766
Ala Ala Lys Met Gly Met Phe Ile Cys Asn Asp Arg Arg Trp Pro Glu	
165 170 175	
GCC TGG CCG GTG ATG GGC CTC AGG GGC GCC GAG ATC ATC TGC GGC GGC	814
Ala Trp Arg Val Met Gly Leu Arg Gly Ala Glu Ile Ile Cys Gly Gly	
180 185 190	

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GTGCCCAGAT CAAAGAATGA GACCGACGCG CCGGCCGCGG CAAAGGATGA TCCTCAGGGT 1501
CGGATCTATC GCTCCGCCCT GAAGCAGGAG GGCACGCT GGCTGCTGAC GCGGAGGAA 1561
GGGTTGCTGG CAAAGCCCAA GCCGCCGCG CTTGTTCCGG CACTTGAGAA TCGATCGCC 1621
ATCGTCGATT ACATCAACGG TACACCGCCC CATATCGCGT CCCTGGCGGA GCTTTCAACG 1681
ACGCTCGGGA TATCCAAGAG CCACTGTCAC TCCATCCTCA AGACGCTGAC GCATTTCCGC 1741
TGGCTGAAAT TCGACAATCG CTCAAAGAGC TACGAGCTGA ATTC 1785

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SEQ ID NO.: 2

SEQUENCE LENGTH: 1820

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: double

TOPOLOGY: linear

MOLECULE TYPE: genomic DNA

ORIGINAL SOURCE

ORGANISM: Pseudomonas sp.

STRAIN: KNK 003A (FERM BP-3181)

GCATGCGCGG GGAAGTGAAG AACTTGCAAG ACGAACTCGG CATTACCTTC GTGCATGTAA	60
CCCATACCCA GCCTGAGGCG ATCGCGCTCG CCGACATGGT GGTGTGATG GATACGGGCC	120
GCATAGAGCA GGCAGCGAGC GCCAACGAAA TCTACAACCG GCCCGCGACG CCCTATGTGG	180
CGCGCTTCAT GGGCGGCCAA AACGTGTTGA CGGGGAGGGT GGAGAGCATC TCGCCCACCG	240
GCATGGTGCT GAAAAGCGAA AAGGGCGAGA TCTTCAATGC GCCTCTTACG GGTGCTGCGC	300
CGAAGCTGGG CGAACCCGTA TCGATATCCA TCGCCCGCGA CCGCATCAGC ATCAGCAAGC	360
CGCAAAACGG CAAGGGCGCG CAGCAGGCTG ACGCGGTAAC GGGTGTGGTC GATTCCACGG	420
AATACCAGGG CAGCTTCGTG AAGGTCAGCA TAGTGCTCGA CCGTGCGGAG ACCTTCGTGC	480
CAAACATGCC CGACCATGAA TTTTTCGCGG AACCGGTGGA TCACGGCGTC CCGGTGGTCG	540
CCCCTGGAA ACCGGAGCAT GTGCATGTCC TGTCCAAGTC TGACCGGGGC GCCGACCACA	600
CCGAAATCTA CCGCTTCCCT GCAGGCGAAA ATACCGTTTC AATGGGCAAG GGGCGGCAAA	660
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Thr Arg Ile Val Asn

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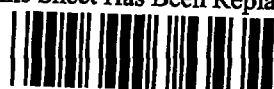


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GCA GCC GCC GCG CAG ATG GGG CCC ATC AGC CGG TCC GAA ACG CGC AAG	763
Ala Ala Ala Ala Gln Met Gly Pro Ile Ser Arg Ser Glu Thr Arg Lys	
10 15 20	
GAT ACG GTC CGG CGC CTG ATC GCG CTC ATG CGC GAG GCG AAG GCC CGC	811
Asp Thr Val Arg Arg Leu Ile Ala Leu Met Arg Glu Ala Lys Ala Arg	
25 30 35	
GGT TCC GAC CTT GTC GTC TTT ACC GAA CTC GCG CTC ACC ACC TTC TTT	859
Gly Ser Asp Leu Val Val Phe Thr Glu Leu Ala Leu Thr Thr Phe Phe	
40 45 50	
CCC CGC TGG GTG ATC GAG GAC GAA GCT GAG CTC GAC AGC TTC TAC GAG	907
Pro Arg Trp Val Ile Glu Asp Glu Ala Glu Leu Asp Ser Phe Tyr Glu	
55 60 65	
AAG GAG ATG CCA GGG CCC GAA ACC CAG CCG CTC TTC GAT GAG GCG AAG	955
Lys Glu Met Pro Gly Pro Glu Thr Gln Pro Leu Phe Asp Glu Ala Lys	
70 75 80 85	
CGC TTG GAG ATC GGC TTC TAT CTC GGT TAT GCC GAG CTG GCG GAG GAG	1003
Arg Leu Glu Ile Gly Phe Tyr Leu Gly Tyr Ala Glu Leu Ala Glu Glu	
90 95 100	
GGC GGC AGG AAG CGG CGC TTC AAC ACC TCT ATC CTT GTG GAC CGC AGC	1051
Gly Gly Arg Lys Arg Arg Phe Asn Thr Ser Ile Leu Val Asp Arg Ser	
105 110 115	
GGC CGG ATC GTC GGC AAG TAC CGC AAG GTG CAC CTG CCC GGC CAC AAA	1099
Gly Arg Ile Val Gly Lys Tyr Arg Lys Val His Leu Pro Gly His Lys	
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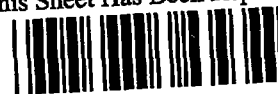
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Glu Pro Gln Pro Gly Arg Lys His Gln His Leu Glu Lys Arg Tyr Phe	
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GAG CCC GGC GAT CTC GGC TTC GGT GTC TGG CGC GCC TTC GAC GGC GTA	1195
Glu Pro Gly Asp Leu Gly Phe Gly Val Trp Arg Ala Phe Asp Gly Val	
150 155 200 205	
ATG GGC ATG TGC ATT TGC AAC GAC CGC CGC TGG CCG GAG ACC TAC CGG	1243
Met Gly Met Cys Ile Cys Asn Asp Arg Arg Trp Pro Glu Thr Tyr Arg	
210 215 220	
GTC ATG GGC TTG CAG GGA GTG GAG ATG GTC ATG CTG GGC TAC AAC ACG	1291
Val Met Gly Leu Gln Gly Val Glu Met Val Met Leu Gly Tyr Asn Thr	
225 230 235	
CCG TAT GAC CAT ACC GGT CAC GAC GAC ATC GAT TCA CTC ACC CAG TTT	1339
Pro Tyr Asp His Thr Gly His Asp Asp Ile Asp Ser Leu Thr Gln Phe	
240 245 250	
CAC AAT CAT CTC TCC ATG CAG GCG GGC GCC TAC CAG AAT TCG ACC TGG	1387
His Asn His Leu Ser Met Gln Ala Gly Ala Tyr Gln Asn Ser Thr Trp	
255 260 265	
GTG ATC GGC ACC GCC AAA TGC GGC ACC GAG GAG GGC TCC AAA ATG GTG	1435
Val Ile Gly Thr Ala Lys Cys Gly Thr Glu Glu Gly Ser Lys Met Val	
270 275 280 285	
GGG CAG AGC GTG ATC GTT GCG CCC TCG-GGC GAG ATC GTC GCT ATG GCC	1483
Gly Gln Ser Val Ile Val Ala Pro Ser-Gly Glu Ile Val Ala Met Ala	
290 295 300	



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TGC ACG ATC GAG GAC GAG ATC ATC ACC GCA CGC TGC GAT CTC GAC ATG	1531
Cys Thr Ile Glu Asp Glu Ile Ile Thr Ala Arg Cys Asp Leu Asp Met	
305 310 315	
GGC AAG CGC TAC CGC GAG ACC ATC TTC GAT TTC GCC CGC CAT CGC GAG	1579
Gly Lys Arg Tyr Arg Glu Thr Ile Phe Asp Phe Ala Arg His Arg Glu	
320 325 330	
CCC GAC GCC TAT CGC CTG ATC GTC GAA CGC AAA GGG GCT GTG CCG CCG	1627
Pro Asp Ala Tyr Arg Leu Ile Val Glu Arg Lys Gly Ala Val Pro Pro	
335 340 345	
CCG CAG TGA	1636
Pro Gln	
350	
TCGGAACCTG AAAACGAAAT ATCCGCGCGG ACGGTGGGAA GGTGAAAGGA GGAGTCTCCA	1696
TGACAACAGT TATCAAGGGT GGAACATCGT CGCCGCCGAT CGCAGCTATG AAGCCGATAT	1756
CCTGATCGAA GGCGAAAAGA TCGCCCAGAT CGGCAGGGAT CTGCAGGGCG ACAAGATTGT	1816
CGAC	1820

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CLAIMS

1. A process for the production of D- α -amino acids characterized in that D-N-carbamoyl- α -amino acids are converted into the corresponding D- α -amino acids in an aqueous medium, using a recombinant DNA comprising a vector DNA and a DNA fragment having a gene which is related to an enzyme capable of converting D-N-carbamoyl- α -amino acids by removal of their carbamoyl groups into the corresponding D- α -amino acids, with the aid of an action of said enzyme which is produced by a transformant which is obtained by transformation of host bacterial cells selected from microorganisms belonging to the genus Escherichia, Pseudomonas, Flavobacterium, Bacillus, Serratia, Corynebacterium, or Brevibacterium, after which the D- α -amino acids produced are collected.

2. A process according to claim 1, wherein D-N-carbamoyl- α -amino acid is a compound of the general formula:



(where R is phenyl, phenyl substituted with hydroxy, alkyl, substituted alkyl, aralkyl, or thienyl).

3. A process according to claim 1 or 2, wherein the enzyme produced by the transformant is used as a culture solution of said transformant, bacterial cells, treated

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bacterial cells, extracts from bacterial cells, immobilized bacterial cells, or immobilized enzyme.

4. A process according to any one of claims 1 to 3; wherein the DNA fragment containing said gene is derived from eucaryotes, procaryotes, viruses, bacteriophages, or plasmids.

5. A process according to claim 4, wherein said procaryotes are bacteria.

6. A process according to claim 5, wherein said bacteria are those selected from microorganisms belonging to the genus Pseudomonas, Agrobacterium, Aerobacter, Aeromonas, Brevibacterium, Bacillus, Flavobacterium, Serratia, or Micrococcus.

7. A process according to claim 6, wherein said bacteria are Pseudomonas sp. KNK 003A (FERM BP-3181) or Pseudomonas sp. KNK 505 (FERM BP-3182).

8. A process according to claim 6, wherein said bacteria are Agrobacterium radiobacter KNK 712 (FERM BP-1900).

9. A microorganism including a recombinant comprising a vector DNA and a DNA fragment having a gene related to an enzyme capable of D-N-carbamoyl- α -amino acids into the corresponding D- α -amino acids.

10. A microorganism according to claim 9, wherein said microorganism including the recombinant is one selected from microorganisms belonging to the genus Escherichia,

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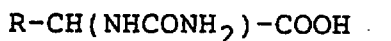
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Pseudomonas, Flavobacterium, Bacillus, Serratia,
Corynebacterium, or Brevibacterium.

11. A microorganism according to claim 10, wherein said microorganism is Escherichia coli JM 109 pAHD 101, Escherichia coli JM 109 pAD 108 (FERM BP-3184), Escherichia coli JM 109 pPHD 301, or Escherichia coli JM 109 pPD 304 (FERM BP-3183).

12. A process for the production of D-N-carbamoyl- α -amino acid amidohydrolases characterized by using a transformant which is obtained by transformation of host bacterial cells selected from microorganisms belonging to the genus Escherichia, Pseudomonas, Flavobacterium, Bacillus, Serratia, Corynebacterium, or Brevibacterium, with a recombinant DNA comprising a vector DNA and a DNA fragment having a gene related to an enzyme capable of converting D-N-carbamoyl- α -amino acids by removal of their carbamoyl groups into the corresponding D- α -amino acids.

13. A process according to claim 12, wherein D-N-carbamoyl- α -amino acid is a compound of the general formula:



(where R is phenyl, phenyl substituted hydroxy, alkyl, substituted alkyl, aralkyl, or thienyl).

14. A recombinant plasmid which is obtained by recombination of plasmid pUC 18 or pUC 19 with a DNA

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fragment having any one of the restriction endonuclease maps of Figs. 1-4 and containing an gene related to a D-N-carbamoyl- α -amino acid amidohydrolase derived from Pseudomonas sp. KNK 003A (FERM BP-3181) or Agrobacterium radiobacter KNK 712 (FERM BP-1900).

15. An immobilized enzyme in which an enzyme capable of converting D-N-carbamoyl- α -amino acids by removal of their carbamoyl groups into the corresponding D- α -amino acids is fixed on a support for immobilization capable of carrying the enzyme.

16. A gene for a protein having the enzyme activity to convert D-N-carbamoyl- α -amino acids by removal of their carbamoyl groups into the corresponding D- α -amino acids, said gene encoding all or a part of the amino acid sequence of 1st to 303rd amino acids shown in SEQ ID No. 1 in the accompanying Sequence Listing.

17. A DNA fragment in which the base sequence of 167th to 232nd bases of SEQ ID No. 1 in the accompanying Sequence Listing, or a base sequence equivalent thereto, is attached to the upstream of the 5' end of the DNA fragment according to claim 16.

18. A DNA fragment having all or a part of the base sequence of 1st to 1785th bases of SEQ ID No. 1 in the accompanying Sequence Listing, or having a base sequence equivalent thereto, and containing a gene of a protein which has the enzyme activity to convert D-N-carbamoyl- α -amino



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acids by removal of their carbamoyl groups into the corresponding D- α -amino acids.

19. A DNA fragment according to claim 18, which comprises a DNA fragment encoding the amino acid sequence of 1st to 20th amino acids of SEQ ID No. 1 in the accompanying Sequence Listing.

20. A gene of a protein having the enzyme activity to convert D-N-carbamoyl- α -amino acids by removal of their carbamoyl groups into the corresponding D- α -amino acids, said gene encoding all or a part of the amino acid sequence of 1st to 311th amino acids of SEQ ID No. 2 in the accompanying Sequence Listing.

21. A DNA fragment having the base sequence of 1st to 1820th bases of SEQ ID No. 2 in the accompanying Sequence Listing, or having a base sequence equivalent thereto, and containing a gene of a protein which has the enzyme activity to convert D-N-carbamoyl- α -amino acids by removal of their carbamoyl groups into the corresponding D- α -amino acids.

22. A DNA fragment according to claim 21, which comprises a DNA fragment encoding the amino acid sequence of 1st to 20th amino acids of SEQ ID No. 2 in the accompanying Sequence Listing.

23. A protein having all or a part of the amino acid sequence of 1st to 303rd amino acids of SEQ ID No. 1 in the accompanying Sequence Listing, and having the enzyme activity to convert D-N-carbamoyl- α -amino acids by removal



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of their carbamoyl groups into the corresponding D- α -amino acids.

24. A protein according to claim 23, which has the amino acid sequence of 1st to 20th amino acids of SEQ ID No. 1 in the accompanying Sequence Listing.

25. A protein having all or a part of the amino acid sequence of 1st to 311st amino acids of SEQ ID No. 2 in the accompanying Sequence Listing, and having the enzyme activity to convert D-N-carbamoyl- α -amino acids by removal of their carbamoyl groups into the corresponding D- α -amino acids.

26. A protein according to claim 25, which has the amino acid sequence of 1st to 20th amino acids of SEQ ID No. 2 in the accompanying Sequence Listing.



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