SPECIFICATION

Process for preparing D-α-amino acids from substituted hydatoins via the D-N-Carbanoyl-α Amino acids sing microbial enzymes

The present invention relates to a process for preparing D-α-amino acids, and more particularly to a process in which there is advantageously prepared hydantoinase which is an enzyme derived from microorganisms, having an ability of producing optically active D-N-carbamoyl-α-amino acids by asymmetrical hydrolysis of 5-substituted hydantoins, and the 5-substituted hydantoins are converted by the reaction in such a microbial enzyme system into D-N-carbamoyl-α-amino acids which may be then converted into D-α-amino acids by decarbamoylation.

It is known that optically active D-N-carbamoyl-α-amino acids are produced by subjecting 5-substituted hydantoins to the action of a cultured broth, cells or treated cells of microorganisms of various genera or an enzyme extracted from the cells, for instance, as disclosed in U.S. Patent No. 4,094,741, Japanese Patent Application No. 157713/1976 [Japanese Patent Unexamined Publication (Tokkyo Kokai) No. 91189/1978], Japanese Patent Application No. 48717/1977 [Japanese Patent Unexamined Publication (Tokkyo Kokai) No. 133688/1978], Japanese Patent Application No. 148556/1977 and British Patent 2019835.

These D-N-carbamoyl-α-amino acids can be converted into corresponding optically active D-α-amino acids, which are very important compounds as intermediates for medicines. Particularly, D-phenylglycine and D-(4-hydroxyphenyl)glycine are industrially available intermediates for preparing semi-synthetic penicillins and semi-synthetic cephalosporins.

The properties of the microbial enzyme capable of stereospecifially hydrolyzing 5-substituted hydantoins so as to cleave the hydantoin ring and to convert them into optically active D-N-carbamoyl-α-amino acids are made clear in Japanese Patent Application No. 148556/1977 and are designated as hydantoinase. In the preparation of the D-N-carbamoyl-α-amino acids utilizing the hydantoinase, a cultured broth containing cells of microorganisms is employed as it is, or the cells or treated cells taken out from the cultured broth are employed. At all events, it is important in industrial preparation to increase the accumulation of hydantoinase at the time of culturing microorganisms.

The object of the present invention is to provide a process for preparing D-α-amino acids.

A further object of the invention is to provide an industrially useful process for easily preparing D-α-amino acids in high yields.

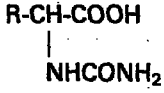
A still further object of the invention is to provide a process for preparing D-α-amino acids in which 5-substituted hydantoins are converted into D-N-carbamoyl-α-amino acids by employing microorganisms cultured in a manner capable of accumulating hydantoinase in large quantities.

Another object of the invention is to provide a process for preparing D-α-amino acids in which 5-substituted hydantoins are converted into D-N-carbamoyl-α-amino acids by employing micoorganisms having high hydantoinase activity and the D-N-carbamoyl-α-amino acids are then converted into D-α-amino acids by decarbamoylation.

These and other objects of the invention will become apparent from the description hereinafter.

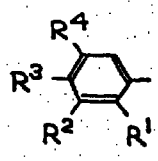
It has now been found that microorganisms having high hydantoinase activity can be obtained by culturing micoorganisms in a culture medium in the presence of metabolites of pyrimidine bases or their analogs as inducers. It has also been found that the accumulation of hydantoinase in cells is further increased, when a metal ion is present in the culture medium together with the inducer.

The present invention provides a process for preparing D-N-carbamoyl-α-amino acids having the following general formula [I]:

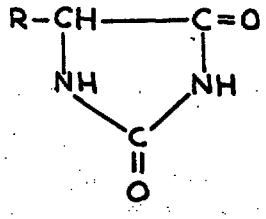


[I]

wherein R is an alkyl group having 1 to 12 carbon atoms, a substituted alkyl group, an aralkyl group having 1 to 12 carbon atoms, a substituted aralkyl group, an unsubstituted or substituted phenyl group of the formula:

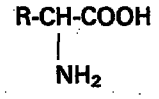


in which R^1, R^2, R^3 and R^4 are independently a hydrogen atom, a halogen atom, a hydroxyl group, a methoxy group, an ethoxy group or a methyl group, a thienyl group a furyl group, a pyridyl group, a thiazolyl group, an imidazolyl group or an indolyl group, which comprises subjecting 5-substituted hydantoins having the following general formula (II):



wherein R is as defined above, to the action of a cultured broth, cells or treated cells of a microorganism or an enzyme extracted from the cells, in an aqueous medium at pH 7-10, the concentration of said 5-substituted hydantoins in the aqueous medium being from 0.1 to 30 per cent by weight and said microorganism being one cultured at a temperature of 20-85 degree C and at pH 4-11 in a culture medium containing 0.05 to 1.0 per cent by weight of an inducer selected from metabolites of pyrimidine bases and their analogs and having an ability of hydrolyzing the 5-substituted hydantoins so as to substantially produce D-N-carbamoyl-a-amino acids, and recovering said D-N-carbamoyl-a-amino acids from the medium.

According to the present invention, the D-N-carbamoyl-α-amino acids of the above general formula [I] may be further converted to D-α-amino acids having the following general formula [III]:



[III]

wherein R is as defined above,
by a chemical reaction or a reaction of a microbial enzyme having a decarbamoylation activity.

The term "metabolites of pyrimidine bases" as used herein means products formed by biochemical metabolism of pyrimidine bases such as uracil, cytocine, tymine, dihydrouracil and dihydrotymine, or derivatives of the pyrimidine bases.

Typical examples of the metabolites of pyrimidine bases are β-alanine, β-ureidopropionic acid, β-amino-iso-butyric acid and β-ureido-iso-butyric acid. Typical examples of the analog of the metabolites of pyrimidine bases are β-amino-n-butyric acid and β-mercaptopropionic acid. Among these inducers, β-alanine is particularly practical for use on an industrial scale, since it is obtainable as an inexpensive chemical. The concentration of the inducer in a culture medium is selected from 0.05 % to 1.0 % by weight, preferably 0.1 % to 0.5 % by weight. The inducer may be added to a culture medium before starting culture, or during the culture continuously or by instalments. Industrially practical microorganisms having a high hydantoinase activity can be obtained by using the inducer. According to the present invention, accumulation of hydantoinase can be further increased by making a metal ion coexist with the inducer in a culture medium.

Examples of the metal ion added to the culture medium are bivalent metal ions such as manganese, cobalt, nickel, copper, magnesium and iron ions, trivalent metal ions such as aluminum ion, and hexavalent molybdenum ion. These metal ions are effective in increasing the accumulation of hydantoinase, and in particular bivalent manganese ion is preferably employed. These metal ions are usually added to a culture medium in the form of a compound capable of producing a metal ion by dissolution in a culture medium, e.g. an inorganic acid salt such as a hydrochloric acid salt or a sulfuric acid salt or an organic acid salt such as an acetic acid salt. The concentration of the metal ion in a culture medium is selected from 0.2 to 100 p.p.m., preferably at least 1 p.p.m.

The culture of microorganisms is usually effected in a liquid medium, and solid surface culture may also be employed. In general, carbon and nitrogen sources that microorganisms can assimilate and other nutrients essential for the growth of the microorganisms are included in the culture medium. In the present invention, in addition thereto, metabolites of pyrimidine bases or their analogs are further included in the culture medium as inducers. As occasion demands, the metal ion may be further employed in combination with the inducer. The culture conditions are selected from the temperature range of 20° to 85°C. and pH range of 4 to 11 in accordance with the optimum growth conditions of the employed miroorganism, and usually the microorganisms are cultured at a temperature of 20° to 40°C. at a pH of 5 to 9 for 10 to 75 hours. During the culture, the growth of the microorganism may be accelerated by aeration and agitation. With the progress of the culture, hydantoinase is produced and accumulated in cells of microorganisms.

The microorganisms employed in the present invention are selected by examining wild strains present in nature, strains deposited in public institutions and microorganisms obtained by artificial mutation from those strains for the presence of the hydantoinase activity. As a method of examining for this activity, for instance, a method as stated below may be employed:

First, cells are collected by subjecting 4 ml. of a cultured broth of a microorganism to centrifugation at 6,000 r.p.m. at 5°C. for 10 minutes, and are dispersed in 4 ml. of a carbonate buffer solution (0.05M $NaHCO_3$-Na_2CO_3, pH 8.7) containing 30 mM of DL-5-(4-hydroxyphenyl)-hydantoin. The reaction is then carried out at 37°C. for 30 minutes, and is stopped by adding 2 ml. of a 20 % by weight aqueous solution of trichloroacetic acid to the reaction mixture. Then, 1 ml. of a 10 % by weight concentrated hydrochloric acid

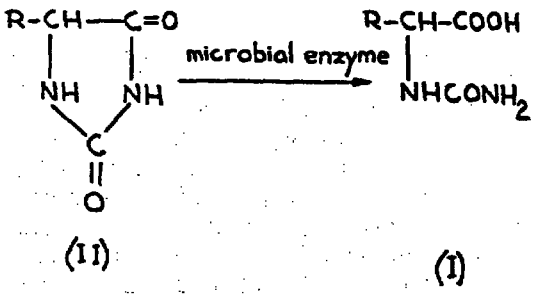
solution of p-dimethylaminobenzaldehyde is added to the reaction mixture to develop a color, and the resulting color-developed reaction mixture is subjected to centrifugation at 6,000 r.p.m. for 10 minutes to obtain the supernatant liquid. The amount of the produced N-carbamoyl-(4-hydroxyphenyl)glycine is then colorimetrically determined on the basis of color development due to carbamoyl group by measuring the absorbance of the supernatant liquid at 440 nm. The strains as are confirmed to have the ability of hydrolyzing the hydantoin ring to cleave it, namely to have the hydantoinase activity are adopted as the microorganisms employed in the present invention. In this examination method, it is of course posible to examine the activity by employing other 5-substituted hydantoins such as 5-(2-methylthioethyl)-hydantoin and 5-benzylhydantoin as a substrate instead of 5-(4-hydroxyphenyl)hydantoin.

The microorganisms employed in the present invention are those passing the above examination, being selected from bacteria, actinomycetes, molds, yeasts and deuteromycetes. Such microorganisms can be found in a very wide range of genera as disclosed in U.S. Patent No. 4,094,741, Japanese Patent Application No. 157713/1976 [Japanese Patent Unexamined Publication (Tokkyo Kokai) No. 91189/1978], Japanese Patent Application No. 48717/1977 [Japanese Patent Unexamined Publication (Tokkyo Kokai) No. 133688/1978] and Japanese Patent Application No. 148556/1977. For instance, examples of the bacteria are genera Achromobacter, Aerobacter, Aeromonas, Agrobacterium, Alcaligenes, Arthrobacter, Bacillus, Brevibacterium, Corynebacterium, Enterobacter, Erwinia, Escherichia, Klebsiella, Microbacterium, Micrococcus, Protaminobacter, Proteus, Pseudomonas, Sarcina, Serratia and Xanthomonas. Examples of the actinomycetes are genera Actinomyces, Actinoplanes, Mycobacterium, Nocardia and Streptomyces. Examples of the molds are genera Aspergillus, Paecilomyces and Penicillium. Examples of the yeasts are genera Candida, Pichia, Rhodotorula and Torulopsis. Among the above-mentioned microorganisms, there are specifically exemplified as practical strains having a relatively high hydantoinase activity, Aerobacter *cloacae* IAM 1221, Arthrobacter simplex IFO 12069, Agrobacterium *rhizogenes* IFO 13258, Bacillus *brevis* IFO 12333, Bacillus *badius* IAM 11059, Bacillus *sphaericus* IFO 3525, Brevibacterium *incertum* IFO 12145, Corynebacterium *erythrogenes* IFO 12974, Sarcina *marginata* IFO 3066, Microbacterium *flavum* ATCC 10340, Micrococcus *roseus* IFO 3764, Proteus *morganii* IFO 3848, Pseudomonas *chlororaphis* IFO 3904, Pseudomonas striata IFO 12996, Actinoplanes *philippiensis* IAM 0120, Mycobacterium *smegmatis* ATCC 607, Nocardia *corallina* IFO 3338 and Streptomyces *flaveolus* IFO 3408. The terms "IAM", "IFO" and "ATCC" indicate the depositories "Institute of Applied Microbiology, the University of Tokyo" (Japan), "Institute for Fermentation, Osaka" (Japan), and "American Type Culture Collection" (U. S. A.), respectively, and the strains marked with IAM, IFO or ATCC are deposited in these depositories under the shown catalogue numbers.

The hydantoinase produced in the process of the present invention is employed in the form of a cultured broth of microorganisms containing hydantoinase, cells, treated cells and cell extract of microorganisms, a crude enzyme or a purified enzyme. The cells may be employed in the form of intact cells or various dried cells such as lyophilized cells and the acetone powder, and also crushed cells and cells treated with a surface active agent are exemplified as the treated cells. Immobilized ones such as immobilized cells and immobilized enzymes are also employed in the method of using the hydantoinase in the present invention.

As is made clear in U.S. Patent No. 4,094,741, Japanese Patent Application No. 157713/1976 [Japanese Patent Unexamined Publication (Tokkyo Kokai) No. 91189/1978] and Japanese Patent Application No. 48717/1977 [Japanese Patent Unexamined Publication (Tokkyo Kokai) No. 133688/1978], the hydantoinase obtained by the process of the present invention acts on various DL-5-substituted hydantoins in an aqueous medium of pH 7 to 10 to produce D-forms of N-carbamoyl-α-amino acids, and accordingly can be utilized for the preparation of various D-α-amino acids important as intermediates for medicines.

The first process of the present invention may be illustrated by the following reaction formula:



(II) (I)

wherein R is as defined hereinbefore.

That is to say, the first process of the present invention relates to a process for preparing D-N-carbamoyl-α-amino acids shown by the general formula [I] by subjecting 5-substituted hydantoins shown by the general formula [II] to the action of hydantoinase derived from microorganisms cultured by the beforementioned method.

The alkyl group or the aralkyl group at the 5-position of the 5-substituted hydantoins employed in the present invention is selected from those having 1 to 12 carbon atoms. The substituent groups of the substituted alkyl group and substituted aralkyl group include a halogen atom, an alkylthio group, a sulfhydryl group, an alkoxyl group, a cyano group, an amino group, an acylated amino group, an indolyl group, a carboxyl group and an alkoxycarbonyl group.

Examples of the 5-substituted hydantoin in which a hydrogen atom at the 5-position of hydantoin is substituted by an alkyl group, a substituted alkyl group, an aralkyl group or a substituted aralkyl group are 5-methylhydantoin, 5-ethylhydantoin, 5-n-propylhydantoin, 5-isopropylhydantoin, 5-n-butylhydantoin, 5-(2-butyl)hydantoin, 5-isobutylhydantoin, 5-(2-methylthioethyl)hydantoin, 5-(hydroxymethyl)hydantoin, 5-sulfhydrylmethylhydantoin, 5-methoxymethylhydantoin, 5-(2-hydroxyethyl)hydantoin, 5-(1-hydroxyethyl)hydantoin, 5-benzylhydantoin, 5-(4-hydroxybenzyl)hydantoin, 5-(3,4,-dihydroxybenzyl)hydantoin, 5-(3-aminopropyl)hydantoin, 5-(2-cyanoethyl)hydantoin, 5-(4-aminobutyl)hydantoin, 5-(3-cyanopropyl)hydantoin, 5-(4-benzoylaminobutyl)hydantoin, 5-indolylmethyl-hydantoin, 5-carboxymethylhydantoin, 5-(2-carboxymethyl)hydantoin, 5-(2-methoxycarbonylethyl)hydantoin, 5-chloromethylhydantoin, 5-fluoromethylhydantoin and 5-trifluoromethylhydantoin.

Examples of the 5-substituted hydantoin in which a hydrogen atom at the 5-position of hydantoin is substituted by a phenyl group or a substituted phenyl group are 5-phenylhydantoin, 5-(4-hydroxyphenyl)hydantoin, 5-(4-methoxyphenyl)hydantoin, 5-(2-chlorophenyl)hydantoin, 5-(3-chlorophenyl)hydantoin, 5-(4-chlorophenyl)hydantoin, 5-(2-fluorophenyl)hydantoin, 5-(3-fluorophenyl)hydantoin, 5-(4-fluorophenyl)hydantoin, 5-(2-iodophenyl)hydantoin, 5-(3-iodophenyl)hydantoin, 5-(4-iodophenyl)hydantoin, 5-(2-hydroxyphenyl)hydantoin, 5-(3-hydroxyphenyl)hydantoin, 5-(2-methoxyphenyl)hydantoin, 5-(3-methoxyphenyl)hydantoin, 5-(2-methylphenyl)hydantoin, 5-(3-methylphenyl)hydantoin, 5-(4-methylphenyl)hydantoin, 5-(2,4-dichlorophenyl)hydantoin, 5-(3,4,-dichlorophenyl)hydantoin, 5-(3,5-dichlorophenyl)hydantoin, 5-(2,4-dihydroxyphenyl)hydantoin, 5-(2,5,-dihydroxyphenyl)hydantoin, 5-(3,4-dihydroxyphenyl)hydantoin, 5-(2,4-dimethoxyphenyl)hydantoin, 5-(2,5-dimethoxyphenyl)hydantoin, 5-(3,4-dimethoxyphenyl)hydantoin, 5-(3,5-dimethoxyphenyl)hydantoin, 5-(3-chloro-4-hydroxyphenyl)hydantoin, 5-(3-chloro-4-methoxyphenyl)-hydantoin, 5-(2-hydroxy-4-methoxyphenyl)hydantoin, 5-(2-hydroxy-5-methoxyphenyl)hydantoin, 5-(4-hydroxy-3-methoxyphenyl)hydantoin, 5-(4-hydroxy-3-ethoxyphenyl)hydantoin, 5-(4-hydroxy-3-methylphenyl)hydantoin, 5-(4-hydroxy-2-methylphenyl)hydantoin, 5-(3,5-dichloro-2-hydroxyphenyl)hydantoin, 5-(3,5-dichloro-4-hydroxyphenyl)hydantoin and 5-(3,5-dimethyl-4-methoxyphenyl)hydantoin.

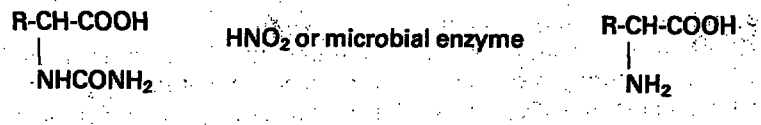
The reaction substrate, i.e. 5-substituted hydantoin is usually admixed with the cultured broth, cells or treated cells in an aqueous medium to make the hydantoinase act catalytically on the substrate. The concentration of the 5-substituted hydantoins in the reaction system is selected from 0.1 % to 30 % by weight.

When carrying out the hydrolysis reaction of the 5-substituted hydantoins in an aqueous reaction medium, the reaction mixture is maintained at pH 7 to 10. At pH 7 to 10 the conversion rate of the DL- or L-forms of the 5-substituted hydantoins into D-N-carbamoyl-α-amino acids is remarkably increased, since the optimum pH of the hydantoinase employed in the invention is near 8 to 9, the solubility of the substrate increases with increasing pH and the racemization of the hydantoin ring is effectively accelerated under alkaline conditions. Since the D-specific hydrolysis and racemization of the substrate proceed simultaneously, there is no necessity for using the D-forms of the 5-substituted hydantoins as the reaction substrates and the DL- or L-forms of the 5-substituted hydantoins can be employed. With the progress of the reaction the pH of the reaction system decreases and, therefore, it is preferable to add at an appropriate time a neutralizing agent such as ammonia, caustic soda, caustic potash and sodium carbonate to the reaction system to maintain it at the optimum pH.

The hydrolysis reaction is carried out at a temperature suitable to the hydantoinase of the employed microorganism, and usually at a temperature of 20°C to 85°C. The reaction time varies depending on the activity of the employed microorganism and the reaction temperature.

Usual methods such as pH adjustment and treatment with an ion-exchange resin are usable for isolating from the reaction mixture the D-N-carbamoyl-α-amino acids produced by the enzymatic hydrolysis of various 5-substituted hydantoins with the hydantoinase derived from microorganisms. For instance, when the product is relatively hydrophobic like D-N-carbamoylphenylglycine, D-N-carbamoylmethionine and D-N-carbamoylphenylalanine, the reaction mixture is adjusted to pH 5 to 6 and is subjected to centrifugation or filtration to remove insoluble materials such as unchanged substrate and cells, and then the resulting supernatant liquid or filtrate is adjusted to pH 2 to 4 to precipitate the desired product. When the product is relatively hydrophilic like D-N-carbamoylserine and D-N-carbamoylalanine, the product is suitably isolated by the ion-exchange resin treatment. The product is adsorbed by passing the reaction mixture through an ion-exchange resin, and then eluted from the ion-exchange resin with a solvent such as dilute hydrochloric acid. The eluate is then neutralized and concentrated under reduced pressure to recover the desired product.

According to the second process of the present invention, D-N-carbamoyl-α-amino acids produced by the enzymatic hydrolysis may be further converted into D-α-amino acids by decarbamoylation as shown by the following reaction formula:



[I] [III]

wherein R is as defined above.

That is to say, according to the present invention, D-α-amino acids can be advantageously prepared by subjecting the 5-substituted hydantoins of the general formula [II] to the action of a cultured broth, cells, treated cells or enzyme extracted from cells of microorganisms having an ability of hydrolyzing the 5-substituted hydantoins so as to substantialy produce D-N-carbamoyl-α-amino acids, which have been cultured in a culture medium containing metabolites of pyrimidine bases or their analogs as inducers, and if necessary, further containing metal ions, to produce D-N-carbamoyl-α-amino acids, and then removing carbamoyl group from the D-N-carbamoyl-α-amino acids.

The decarbamoylation may be attained by reacting the D-N-carbamoyl-α-amino acids with nitrous acid in an aqueous medium under an acidic condition. With respect to the optical activity at that time, D-form is maintained and, therefore, D-α-amino acids can be obtained. This chemical decarbamoylation reaction is preferably carried out by reacting D-N-carbamoyl-α-amino acid with an approximately equimolar nitrous acid in an aqueous medium, e.g. in water or a water-acetic acid mixed solvent, in the presence of a strong mineral acid such as hydrochloric acid or sulfuric acid of at least 0.4N. It is convenient to employ a water-soluble salt of nitrous acid such as sodium nitrite or potassium nitrite which forms nitrous acid in the reaction system by the reaction with an acid present in the reaction medium. Since this decarbamoylation accompanies generation of large quantities of heat and gas, an aqueous solution of nitrous acid is added gradually to the reaction medium in which a D-N-carbamoyl-α-amino acid and a catalyst, i.e. a strong mineral acid, are present, with agitation. The reaction temperature is usually selected from 0° to 40°C., and the reaction at a low temperature is preferred in order to decrease the loss of nitrous acid due to vaporization and to prevent a side reaction.

It has also been found that microorganisms belonging to bacteria such as genera Aerobacter, Aeromonas, Arthrobacter, Bacillus, Brevibacterum, Flavobacterium, Pseudomonas, Sarcina and Serratia have a decarbamoylation activity and the decarbamoylation of the D-N-carbamoyl-α-amino acids can be attained by an enzyme system derived from the microorganisms.

Typical examples of the strain having a relatively high decarbamoylation activity among these microorganisms are Aerobacter cloacae IAM 1221, Arthrobacter *simplex* ATCC 6946, Bacillus macroides ATCC 12905, Bacillus *alvei* IFO 3343, Brevibacterium *ammoniagenes* IFO 12071, Flavobacterium *flavescens* IFO 3086, Pseudomonas *putrefaciens* IAM 1002, Sarcina lutea IFO 1099, Serratia *marcescens* IFO 3054 and Aeromonas *hydrophila* IFO 3820.

These enzyme system showing the decarbamoylation activity can be prepared by culturing microorganisms in a usual manner. The culture is usually conducted in a liquid medium, and solid surface culture may also be conducted. In general, carbon and nitrogen sources that microorganisms can assimilate and inorganic salt nutrients essential for the growth of the microorganisms are included in the culture medium. It is preferable to further add to the culture medium a small amount of D-forms of N-carbamoyl-α-amino acids, e.g. N-carbamoyl-phenylglycine and N-carbamoyl-(4-hydroxyphenyl)glycine in order to induce and accumulate decarbamoylation enzymes. The concentration of such inducers in the culture medium is selected from 0.05 % to 1.0 % by weight.

The culture is carried out at a temperature of 20° to 85°C. at pH 4 to 11, and the growth of microorganisms may be accelerated by aeration and agitation.

In the enzymatic decarbamoylation, the thus cultured microorganism are employed as a source of an enzyme in the form of a cultured broth, cells and treated cells. It is of course possible to use the cells in the form of intact cells, and dried cells such as lyophilized cells, and treated cells such as crushed cells, cellular extract and surface active agent-treated cells are also usable as a source of microbial enzyme in the decarbamoylation. In particular, the use of treated cells obtained by ultrasonic treatment of the cells is preferred in order to increase the efficiency of the decarbamoylation so as to raise the reaction rate and the product yield. The ultrasonic treatment of cells is conducted in a phosphate buffer solution of about pH 8 at a low temperature, and thereby a suspension of cells having a high decarbamoylation activity can be obtained.

The enzymatic decarbamoylation is carried out in an aqueous medium at pH 6 to 11, preferably pH 7 to 9. The reaction temperature varies depending on the enzyme system of a strain used, and is usually selected from 20° to 85°C.

The isolation of the D-α-amino acids produced by the chemical or enzymatic decarbamoylation may be conducted by known methods such as concentration, neutralization and ion-exchange resin treatment of the resulting reaction mixture. For instance, in case of a relatively hydrophobic D-α-amino acid such as D-phenylglycine, D-(4-hydroxyphenyl)-glycine, D-leucine or D-phenylalanine, insoluble materials are removed from the reaction mixture under an acidic or alkaline condition and the mixture is then subjected to concentration or neutralization treatment to give the desired D-α-amino acid as a precipitate. Also, in case of a relatively hydrophilic D-α-amino acid such as D-thienylglycine, D-serine or D-alanine, the desired product is obtained by making the product absorb onto an ion-exchange resin, eluting the adsorbed product with

ammonia liquor, and neutralizing and concentrating the eluate.

Since D-N-carbamoyl-α-amino acids can be readily obtained from 5-substituted hydantoins in high yields by using the hydantoinase derived from microorganisms which has been accumulated in high concentrations in a culture medium containing a metabolite of pyrimidine bases or its analog, and if necessary, further containing a metal ion, and also, since D-α-amino acids can be readily obtained from the thus produced D-N-carbamoyl-α-amino acids by the chemical decarbamoylation or the reaction using a microbial enzyme having the decarbamoylation activity, the present invention can provide a very advantageous process for the preparation of D-α-amino acids.

The present invention is more particularly described and explained by means of the following Examples, in which all percents are percent by weight unless otherwise stated. These examples are intended to illustrate the invention and not to be construed to limit the scope of the invention.

Example 1

Microorganisms shown in Table 1 were previously cultured on bouillon agar slants containing 0.1 % of DL-5-(2-methylthioethyl)hydantoin at 33°C. for 24 hours.

A liquid culture medium of pH 7.0 containing 2.0 % of meat extract, 0.5 % of peptone, 0.5 % of yeast extract, 0.3 % of NaCl and 0.2 % of an inducer shown in Table 1 was prepared, and 100 ml. portions thereof were separately placed in 500 ml. shaking flasks and were steam-sterilized at 120°C. for 15 minutes. The above each microorganism previously cultured was inoculated into the culture medium in each flask, and then cultured at 33°C. for 24 hours with shaking.

The hydantoinase activity of the obtained each cultured broth was measured as follows:

Four milliliters of a cultured broth was taken out, and cells were collected at 5°C, by centrifugation at 6,000 r.p.m. for 10 minutes. The obtained cells were dispersed into 4 ml. of an aqueous substrate solution containing 30 mM of DL-5-(4-hydroxyphenyl)hydantoin as a substrate in a 0.05M $NaHCO_3$-Na_2CO_3 buffer solution of pH 8.7. The reaction was then carried out at 37°C. for 30 minutes, and was stopped by adding 2 ml. of a 20 % aqueous solution of trichloroacetic acid to the reaction mixture. To the reaction mixture was added 1 ml. of a 10 % aqueous solution of p-dimethylaminobenzaldehyde in a concentrated hydrochloric acid to develop a color.

The color-developed liquid was centrifuged at 6,000 r.p.m. for 10 minutes to remove cells, and the produced N-carbamoyl-(4-hyxdroxyphenyl)glycine in the resulting supernatant liquid was colorimetrically determined at 440 nm.

The results are shown in Table 1, wherein the hydantoinase activity is indicated by μ mole of N-carbamoyl-(4-hydroxyphenyl)glycine produced by the reaction for 30 minutes per milliliter of the employed cultured broth and N-carbamoyl-(4-hydroxyphenyl)glycine is referred to as "C-HPG".

TABLE 1

Inducer	Hydantoinase activity (μ mole C-HPG/30 minutes/ml. broth) Strain			
	Bacillus *brevis* IFO 12333	Arthrobacter *simplex* IFO 12069	Microbacterium *flavum* ATCC 10340	Agrobacterium *rhizogenes* IFO 13258
β-Alanine	5.0	2.1	2.7	1.8
β-Ureidopropionic acid	4.3	2.0	2.5	1.8
β-Aminoisobutyric acid	4.8	1.9	2.4	1.7
β-Ureidoisobutyric acid	4.2	1.9	2.3	1.8
None	0.8	0.3	0.3	0.2

Example 2

Microorganisms shown in Table 2 were previously cultured on bouillon agar slants containing 0.1 % of DL-5-(2-methylthioethyl)hydantoin at 33°C. for 24 hours.

A liquid medium of pH 7.0 containing 2.0 % of meat extract, 0.5 % of peptone, 0.5 % of yeast extract, 0.3 % of NaCl, 0.2 % of β-alanine and a metal salt shown in Table 1 was prepared, and 100 ml. portions thereof were placed in 500 ml. shaking flasks and were steam-sterilized at 120°C. for 15 minutes. The above each microorganism previously cultured was inoculated into the liquid medium in each flask, and then cultured at 33°C. for 24 hours with shaking.

The hydantoinase activity of the thus obtained each cultured broth was measured in the same manner as in Example 1 using DL-5-(4-hydroxyphenyl)hydantoin as a substrate.

The results are shown in Table 2, wherein C-HPG is as defined in Example 1.

TABLE 2

Metal salt (amount)	Hydantoinase activity (μ mole C-HPG/30 minutes/ml. broth) Strain			
	Bacillus *brevis* IFO 12333	Arthrobacter *simplex* IFO 12069	Microbacterium *flavum* ATCC 10340	Agrobacterium *rhizogenes* IFO 13258
$MnCl_2 \cdot 4H_2O$ (10 p.p.m.)	17.7	5.0	6.2	4.7
$CoCl_2 \cdot 6H_2O$ (5 p.p.m.)	7.3	3.2	5.4	3.0
$NiCl_2 \cdot 6H_2O$ (25 p.p.m.)	9.0	3.3	5.7	3.4
$Al_2(SO_4)_3 \cdot 16\text{-}18H_2O$ (25 p.p.m.)	7.8	2.5	3.8	2.8
$CuSO_4 \cdot 5H_2O$ (25 p.p.m.)	7.6	3.0	4.1	2.5
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (25 p.p.m.)	6.2	2.4	3.4	2.3
$MgSO_4 \cdot 7H_2O$ (200 p.p.m.)	6.0	2.4	2.9	2.0
$FeSO_4 \cdot 5H_2O$ (25 p.p.m.)	6.3	2.8	3.2	2.2
None	5.0	2.1	2.7	1.8

Example 3

Microorganisms shown in Table 3 were previously cultured on bouillon agar slants containing 0.1 % of DL-5-(2-methylthioethyl)hydantoin at 33°C. for 24 hours.

A liquid medium of pH 7.0 containing 2.0 % of meat extract, 0.5 % of peptone, 0.5 % of yeast extract, 0.3 % of NaCl, 10 p.p.m. of $MnCl_2 \cdot 4H_2O$ and 0.1 % of an inducer shown in Table 3 was prepared, and 100 ml. portions thereof were placed in 500 ml. shaking flasks and were steam-sterilized at 120°C. for 15 minutes.

The above each microorganism previously cultured was inoculated into the liquid medium in each flask, and was cultured at 33°C. for 24 hours with shaking. The hydantoinase activity of the thus obtained each cultured broth was measured in the same manner as in Example 1 using DL-5-(4-hydroxyphenyl)hydantoin as a substrate.

The results are shown in Table 3, wherein C-HPG is as defined in Example 1.

TABLE 3

Inducer	Hydantoinase activity (μ mole C-HPG/30 minutes/ml. broth) Strain			
	Bacillus *brevis* IFO 12333	Arthrobacter *simplex* IFO 12069	Microbacterium *flavum* ATCC 10340	Agrobacterium *rhizogenes* IFO 13258
β-Alanine	17.7	5.0	6.2	4.7
β-Ureidopropionic acid	14.2	4.8	5.4	4.5
β-Aminoisobutyric acid	16.0	5.1	5.2	4.9
β-Amino-n-butyric acid	10.8	3.8	4.0	3.6
β-Ureidoisobutyric acid	15.9	4.4	4.8	4.1
β-Mercaptopropionic acid	11.0	4.0	5.1	3.5
None	1.5	0.4	0.5	0.4

Example 4

Microorganisms shown in Table 4 were previously cultured on bouillon agar slants containing 0.1 % of DL-5-(2-methylthioethyl)hydantoin at 33°C. for 24 hours.

The following liquid culture mediums (A) and (B) were prepared, and 100 ml. portions thereof were separately placed in 500 ml. shaking flasks and steam-sterilized at 120°C. for 15 minutes.

Culture Medium (A)		Culture Medium (B)	
Meat extract	2.0 %	Meat extract	2.0 %
Peptone	0.5 %	Peptone	0.5 %
Yeast extract	0.5 %	Yeast extract	0.5 %
NaCl	0.3 %	NaCl	0.3 %
β-Alanine	0.2 %	β-Alanine	0.2 %
pH	7.0	$MnCl_2 \cdot 4H_2O$	10 p.p.m.
		pH	7.0

The above each microorganism previously cultured was inoculated into the liquid medium in each flask, and was cultured at 33°C. for 24 hours with shaking. The hydantoinase activity of the thus obtained each cultured broth was measured in the same manner as in Example 1 using DL-5-(4-hydroxyphenyl)hydantoin as a substrate.

The results are shown in Table 4, wherein C-HPG is as defined in Example 1.

TABLE 4

Strain	Hydantoinase activity (μ mole C-HPG/30 min./ml. broth)	
	Medium A	Medium B
Aerobacter *cloacae* IAM 1221	1.1	1.8
Brevibacterium *incertum* IFO 12145	0.5	0.7
Corynebacterium *erythrogenes* IFO 12974	0.3	0.5
Micrococcus *roseus* IFO 3764	0.4	0.7
Pseudomonas *chlororaphis* IFO 3904	0.9	1.4
Sarcina *marginata* IFO 3066	0.6	1.0
Proteus *morganii* IFO 3848	0.5	0.9
Nocardia *corallina* IFO 3338	0.7	1.3
Mycobacterium *smegmatis* ATCC 607	0.2	0.5
Bacillus *sphaericus* IFO 3525	0.9	3.6
Bacillus *badius* IAM 11059	0.3	0.5

Example 5

A liquid medium of pH 7.0 containing 2.0 % of glucose, 1.0 % of soybean meal, 0.25 % of yeast extract, 0.1 % of ammonium sulfate, 0.5 % of calcium carbonate, 0.4 % of potassium phosphate, 0.3 % of β-alanine and 20 p.p.m. of $MnCl_2$ $4H_2O$, and 100 ml. portions thereof were placed in 500 ml. shaking flasks and were steam-sterilized at 120°C. for 15 minutes.

Streptomyces *flaveolus* IFO 3408 and Actinoplanes *philippiensis* IAM 0120 were respectively inoculated into the liquid medium in each flask. At 30°C., Streptomyces *flaveolus* was cultured for 45 hours with shaking and Actinoplanes *philippiensis* was cultured for 64 hours with shaking. The hydantoinase activity of the thus obtained each cultured broth was measured in the same manner as in Example 1 using DL-5-(4-hydroxyphenyl)hydantoin as a substrate.

The results are shown in Table 5, wherein C-HPG is as defined in Example 1.

TABLE 5

Strain	Hydantoinase activity (μ mole C-HPG/30 min/ml. broth)
Streptomyces *flaveolus*	3.1
Actinoplanes *philippiensis*	2.9

On the other hand, the hydantoinase activity of a cultured broth obtained by culturing Streptomyces *flaveolus* without adding $MnCl_2 \cdot 4H_2O$ to the liquid medium was 1.6.

Example 6

A liquid culture medium of pH 7.5 containing 3.0 % of meat extract, 1.0 % of glucose, 0.3 % of β-alanine and 20 p.p.m. of $MnCl_2 \cdot 4H_2O$ was prepared, and 100 ml. portion thereof was placed in a 500 ml. shaking flask and was steam-sterilized at 120°C. for 15 minutes. Bacillus *brevis* IFO 12333 which had been previously cultured on a bouillon agar slant containing 0.1 % of DL-5-(2-methylthioethyl)hydantoin at 33°C. for 24 hours, was inoculated into the liquid culture medium and cultured at 33°C. for 20 hours with shaking. While the obtained cultured broth was maintained at pH 8.7, 2.0 g. of DL-5-(4-hydroxyphenyl)hydantoin was added to the broth, and the reaction was then carried out at 37°C. for 24 hours in a nitrogen steam with agitation. During the reaction, the reaction mixture was maintained at pH 8.7 with sodium hydroxide. After the completion of the reaction, the reaction mixture was centrifuged. The obtained supernatant liquid was color-developed by using Ehrlich's reagent, and the produced D-N-carbamoyl-(4-hydroxyphenyl)glycine was colorimetrically determined. The amount of the product was 2.0 g. and the conversion was 91.4 % by mole.

The reaction mixture obtained by this reaction was adjusted to pH 5 with hydrochloric acid and was centrifuged. Half of the resulting supernatant liquid was concentrated to about 20 ml., and after adjusting to pH 2 with hydrochloric acid, was cooled in a refrigerator to give a precipitate. The obtained precipitate was recrystallized from a water-ethanol mixed solvent to give 670 mg. of D-N-carbamoyl-(4-hydroxyphenyl)glycine. The melting point was 176° to 177°C. and the specific rotation $[\alpha]_D^{20}$ was −177.0° (C=0.5, 5 % ethanol).

Example 7

The reaction mixture was obtained in the same manner as in Example 6 by employing the hydantoinase derived from Bacillus *brevis* IFO 12333. To 100 ml. of the reaction mixture [content of D-N-carbamoyl-(4-hydroxyphenyl)glycine: 1.9 g.] was added 20 g. of 35 % hydrochloric acid, and thereto 6.2 g. of a 10 % aqueous solution of sodium nitrite was added at 10°C. with agitation over 30 minutes by employing a pump. The mixture was further agitated at 10°C. for 2 hours, and the resulting reaction mixture was analyzed by a high performance liquid chromatography. It was confirmed that 1.4 g. of D-(4-hydroxyphenyl)glycine was produced. The conversion was 92.7 % by mole.

The thus obtained decarbamoylation reaction mixture was filtered, and the filtrate was concentrated at a temperature of less than 50°C. under reduced pressure. After adjusting to pH 4.5 with sodium hydroxide, the mixture was further concentrated to 35 ml. to precipitate the product. The obtained pecipitate was dissolved in 70 ml. of hot water, and the solution was treated with active carbon and filtered. The filtrate was concentrated to give 810 mg. of white D-(4-hydroxyphenyl)glycine. The specific rotation $[\alpha]_D^{20}$ was −161.0° (C=0.5, 1N HCl).

Example 8

Microorganisms shown in Table 6 were cultured on bouillon agar slants containing 0.1 % of DL-5-(2-methylthioethyl)hydantoin at 33°C. for 24 hours. With respect to each microorganism cultured, a slant of cells was suspended in a saline water, and the suspension was added to 250 ml. of a steam-sterilized culture medium of pH 7.0 containing 2.0 % of meat extract, 0.5 % of peptone, 0.5 % of yeast extract, 0.3 % of NaCl, 10 p.p.m. of $MnCl_2 \cdot 4H_2O$ and 0.2 % of β-alanine. The mixture was placed in a 2 liter Sakaguchi's flask, and the culture was conducted at 33°C. for 24 hours with shaking. The cells were collected by centrifuging the obtained cultured broth at 6,000 r.p.m. at 5°C., and was suspended into 500 ml. of an aqueous substrate solution containing 30 mM of a 5-substituted hydantoin shown in Table 6 as a substrate in a 0.05M $NaHCO_3$-Na_2CO_3 buffer solution of pH 8.7. The reaction was carried out at 37°C. for 24 hours in a nitrogen stream with agitation. During the reaction, the reaction mixture was maintained at pH 8.7. After the completion of the reaction, the reaction mixture was centrifuged. The obtained supernatant liquid was color-developed with Ehrlich's reagent, and the produced D-N-carbamoyl-α-amino acid in the supernatant liquid was colorimetrically determined.

The results are shown in Table 6, wherein the molar conversions are shown.

TABLE 6

Strain	Conversion (% by mole) 5-Substituted hydantoin				
	DL-5-Methyl-hydantoin	DL-5-(2-Methyl-thioethyl)-hydantoin	DL-5-Benzyl-hydantoin	DL-5-Isobutyl-hydantoin	L-5-Hydroxymethyl-hydantoin
Bacillus *brevis* IFO 12333	85	90	72	70	68
Agrobacterium rhizogenes IFO 13258	82	87	68	66	62
Microbacterium *flavum* ATCC 10340	78	83	66	70	60
Aerobacter *cloacae* IAM 1221	83	85	70	-	65
Pseudomonas *striata* IFO 12996	90	88	73	80	63
Nocardia *corallina* IFO 3338	-	80	70	65	62
Arthrobacter *simplex* IFO 12069	82	84	75	77	-

TABLE 6 Continued

Strain	DL-5-Phenyl-hydantoin	DL-5-(4-Hydroxyphenyl)-hydantoin	DL-5-(2-Thienyl)-hydantoin	L-5-Carboxymethyl-hydantoin	DL-5-4-Amino-butyl)-hydantoin
Bacillus *brevis* IFO 12333	88	93	95	67	65
Agrobacterium *rhizogenes* IFO 13258	65	70	85	60	-
Microbacterium *flavum* ATCC 10340	65	73	80	-	61
Aerobacter *cloacae* IAM 1221	75	80	88	71	67
Pseudomonas *striata* IFO 12996	72	70	90	72	69
Nocardia *corallina* IFO 3338	72	74	84	70	-
Arthrobacter *simplex* IFO 12069	71	85	88	68	-

In the enzymatic hydrolysis of 5-substituted hydantoins shown in Table 6, respective N-carbamoyl-α-amino acids were isolated from the reaction mixtures obtained by employing Bacillus *brevis* and they were D-forms. Upon isolation, D-N-carbamoylmethionine, D-N-carbamoylserine and D-N-carbamoylalanine were isolated as follows: The reaction mixture was adjusted to pH 5.0 and centrifuged. The resulting supernatant liquid was concentrated to dryness under reduced pressure, and the solid matter was extracted with ethanol. After distilling away ethanol, the residue was treated with an anion-exchange resin (commercially available under the registered trademark "Amberlite IRA-400" of Rohm & Haas Co.; acetate type) followed by elution with acetic acid. The obtained fraction was dried under vacuum to give the product. D-N-Carbamoylphenylalanine, D-N-carbamoylleucine, D-N-carbamoylphenylglycine, D-N-carbamoyl-(4-hydroxyphenyl)glycine and D-N-carbamoyl-(2-thienyl)glycine were isolated as follows: The reaction mixture was adjusted to pH 5.0 and centrifuged. The obtained supernatant liquid was concentrated under reduced pressure and was adjusted to pH 2.0 to precipitate the product. The precipitate was taken out and was recrystallized from a water-ethanol mixed solvent.

The amounts and specific rotations of the obtained D-N-carbamoyl-α-amino acids are shown in Table 7.

TABLE 7

D-N-carbamoyl-α-amino acid	Amount (g.)	Specific rotation $[\alpha]_D^{25}$		
D-N-carbamoyl-alanine	0.84	−19.7°	(c=2.0,	1N NH_4OH)
D-N-carbamoyl-methionine	1.56	+24.1°	(c=2.0,	5N HCl)
D-N-carbamoyl-phenylalanine	1.46	−37.3°	(c=2.0,	1N NH_4OH)
D-N-carbamoyl-leucine	1.28	−0.7°	(c=2.0,	1N NH_4OH)
D-N-carbamoyl-serine	0.76	−33.4°	(c=2.0,	1N NH_4OH)
D-N-carbamoyl-phenylglycine	1.79	−135.5°	(c=2.0,	1N NH_4OH)
D-N-carbamoyl-(4-hydroxyphenyl)-glycine	1.91	−159.6°	(c=2.0,	1N HCl)
D-N-carbamoyl-(2-thienyl)glycine	1.43	−97.7°*	(c=1.0,	1N NH_4OH)

(Note) * $[\alpha]_D^{20}$

Example 9

By employing Bacillus brevis IFO 12333, various D-N-carbamoyl-α-amino acids were prepared in the same manner as in Example 8.

To 100 ml. of water was added 15 millimoles of a D-N-carbamoyl-α-amino acid, and thereto 25 g. of 35% hydrochloric acid was further added. While maintaining at 10°C. and agitating, 7.2 g. of a 15% aqueous solution of sodium nitrite was added to the mixture over 30 minutes by employing a chemical pump. The mixture was further agitated at 10°C. for 4 hours, and the resulting reaction mixture was analyzed by a high performance liquid chromatography or a colorimetric determination method for amino group using sodium trinitrobenzenesulfonate to determine the produced D-α-amino acid.

The molar conversions of the D-α-amino acids produced from respective D-N-carbamoyl-α-amino acids in the reaction mixtures and the specific rotations of the isolated and purified D-α-amino acids are shown in Table 8.

With respect to D-alanine, D-methionine, D-serine and D-(2-thienyl)glycine, the isolation was conducted by passing the reaction mixture through a cation-exchange resin (commercially available under the registered trademark "Amberlite IR-120B" of Rohm & Haas Co.; Na type), eluting the adsorbed amino acid with ammonia liquor, concentrating the active fraction, isolating the amino acid by means of isoelectric point neutralization and recrystallizing it from a water-ethanol mixed solvent.

TABLE 8

D-α-amino acid	Conversion (mole %)	Specific rotation $[\alpha]_D^{20}$	
D-alanine	95	−14.5°	(C=1, 6N HCl)
D-methionine	96	−22.5°	(c=1, 5N HCl)
D-phenylalanine	85	+34.5	(c=1, H_2O)
D-leucine	82	−15.1°	(c=1, 6N HCl)
D-serine	92	−14.5°	(c=1, 2N HCl)
D-phenylglycine	81	−156.1°	(c=1, 1N HCl)
D-(4-hydroxy-phenyl)glycine	93	−158.3°	(c=1, 1N HCl)
D-(2-thienyl)-glycine	80	−108.0°	(c=1, 1N HCl)

Example 10

A liquid culture medium of pH 7.0 containing 2.0 % of meat extract, 1.0 % of glycose, 0.2 % of β-alanine and 10 p.p.m. of $MnCl_2 \cdot 4H_2O$ was prepared, and 100 ml. portions thereof were placed in 500 ml. shaking flasks and were steam-sterilized at 120°C. for 15 minutes. Pseudomonas *striata* IFO 12996 previously cultured on a bouillon agar slant at 33°C. for 24 hours was inoculated in the liquid culture medium in each shaking flask, and was cultured at 33°C. for 24 hours with shaking. To the obtained culture broth was added 2.0 g. of DL-5-(3-chlorophenyl)hydantoin or DL-5-(4-methoxyphenyl)-hydantoin. The reaction was carried out at 37°C. for 24 hours with agitation in a nitrogen stream, while maintaining the reaction system at pH 8.7. After the completion of the reaction, D-N-carbamoyl-(3-chlorophenyl)-glycine or D-N-carbamoyl-(4-methoxyphenyl)glycine produced in the reaction mixture was colorimetrically determined with Ehrlich's reagent.

The molar conversions are shown in Table 9 together with the specific rotation of the products isolated from the reaction mixtures and purified.

The isolation was carried out by adjusting the reaction mixture to pH 5.0, centrifuging it, concentrating the resulting supernatant liquid under reduced pressure, adjusting the concentrated liquid to pH 5.0, filtering the resulting precipitate and recrystallizing the precipitate from ethanol.

TABLE 9

Product	Conversion (mole %)	Specific rotation $[\alpha]_D^{20}$	
D-N-carbamoyl-(3-chlorophenyl)-glycine	90	−124.0°	(c=0.5, 0.1 N NH_4OH)
D-N-carbamoyl-(4-methoxyphenyl)-glycine	93	−142.0°	(c=0.5, 0.1N NH_4OH)

Example 11

D-N-Carbamoyl-(3-chlorophenyl)glycine and D-N-carbamoyl-(4-methoxyphenyl)glycine were prepared in the same manner as in Example 10.

Into 100 ml. of water was suspended 20 millimoles of D-N-carbamoyl-(3-chlorophenyl)glycine or D-N-carbamoyl-(4-methoxyphenyl)glycine, and thereto 25 g. of 35% hydrochloric acid was added. To the mixture was added 9.2 g. of a 15% aqueous solution of sodium nitrite over 30 minutes by employing a pump, while maintaining at 10°C. and agitating the mixture. The mixture was further agitated at 10°C. for 2 hours, and the resulting reaction mixture was analyzed by a high performance liquid chromatography to determine the produced D-α-amino acid.

The conversions of the decarbamoylation are shown in Table 10 together with the specific rotatory powers of the products isolated and purified.

The isolation was carried out by filtering the decarbamoylation reaction mixture, concentrating the filtrate under the reduced pressure, adjusting it to pH 5.0 to 6.0, filtering the resulting precipitate and recrystallizing the precipitate from water.

TABLE 10

D-α-amino acid	Conversion (mole %)	Specific rotation $[\alpha]_D^{20}$	
D-(3-chlorophenyl)-glycine	87	−120.0°	(c=1, 1N HCl)
D-(4-methoxyphenyl)-glycine	91	−146.0°	(c=1, 1N HCl)

Example 12

A liquid culture medium of pH 7.0 containing 3 % of meat extract, 1 % of peptone, 0.3 % of NaCl and 50 p.p.m. of $MnCl_2 \cdot 4H_2O$ was prepared, and 400 ml. portions thereof were placed in 2 liter Sakaguchi's flasks and were steam-sterilized at 120°C. for 15 minutes. To the liquid culture medium in the flask was aseptically added 100 ml. of a 4 % aqueous solution of D-N-carbamoylphenylglycine passed through a milli-pore filter. Bacillus *macroides* ATCC 12905 previously cultured on a bouillon slant was inoculated into the liquid culture medium in the flask, and was cultured at 33°C. for 9 days with shaking. After removing cells from about 1.5 liters of the obtained cultured broth by centrifugation, the supernatant liquid was adjusted to pH 2.7 with a concentrated hydrochloric acid and was passed through a cation-exchange resin (commercially available under the registered trademark "Amberlite IR-120B" of Rohm & Haas Co.; H^+ type). The adsorbed product was eluted with a 5% NH_4OH. The eluent was further treated with an ion-exchange resin (commercially available under the registered trademark "Amberlite IRC-84" of Rohm & Haas Co.; H^+ type) to desalt and with an adsorbent ("AF resin" manufactured by Hokuetsu Tanso Kogyo Kabushiki Kaisha) to decolor. The decolored liquid was concentrated, and the obtained precipitate was recrystallized from water to give a white powder. The thus obtained crystal showed the specific rotation of $[\alpha]_D^{20} = -153.0°$ (c=1, 1N HCl), and also showed single spot in a thin layer chromatogram. Also, the infrared absorption spectrum agreed with that of the authentic D-phenylglycine.

Example 13

A liquid culture medium of pH 7.0 containing 3 % of meat extract, 1 % of peptone, 0.3 % of NaCl and 50 p.p.m. of $MnCl_2 \cdot 4H_2O$ was prepared, and 10 ml. portions thereof were placed in test tubes and were steam-sterilized at 120°C. for 15 minutes. To the liquid culture medium in each test tube was aseptically added 1 ml. of a 2 % aqueous solution of D-N-carbamoylphenylglycine passed through a Millipore filter as an inducer. Each microorganism shown in Table 11 which had been previously cultured on a bouillon agar slant was inoculated into the liquid medium in the test tube, and was cultured at 33°C. for 48 hours. Cells were collected by centrifuging the obtained each cultured broth at 6,000 r.p.m. for 10 minutes and were washed with a saline water once. The cells were then suspended into 10 ml. of a 0.1N phosphate buffer solution (KH_2PO_4-Na_2HPO_4, pH 8.0), and the suspension was subjected to ultrasonic treatment at 0°C. for 10 minutes.

To 5 ml. of the treated cell suspension was added 1 ml. of a 2 % liquor of D-N-carbamoylphenylglycine as a substrate in a phosphate buffer solution of pH 8.0, and the reaction was carried out at 33°C. for 24 hours. Also, 1 ml. of a 2 % liquor of D-N-carbamoyl-(4-hydroxyphenyl)-glycine as a substrate in a phosphate buffer solution of pH 8.0 was added to another 5 ml. of the treated cell suspension, and the reaction was carried out at 33°C. for 48 hours. After the completion of the reaction, the reaction mixture was centrifuged at 8,000 r.p.m. for 10 minutes to remove cells, the resulting supernatant liquid was subjected to a high performance liquid chromatography to determine the produced D-phenylglycine or D-(4-hydroxyphenyl)glycine.

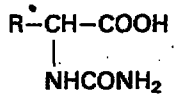
The conversions are shown in Table 11.

TABLE 11

Strain	Conversion (mole %) D-phenylglycine	D-(4-hydroxy-phenyl)glycine
Bacillus *macroides* ATCC 12905	82.5	30.5
Aerobacter *cloacae* IAM 1221	14.5	2.8
Brevibacterium *ammoniagenes* IFO 12071	11.0	4.4
Sarcina *lutea* IFO 1099	3.5	4.3
Aeromonas *hydrophila* IFO 3820	4.2	1.5
Flavobacterium *flavescens* IFO 3086	2.1	1.4
Bacillus *alvei* IFO 3343	4.8	1.8
Serratia *marcescens* IFO 3054	3.1	1.2
Arthrobacter *simplex* ATCC 6946	7.8	3.2
Pseudomonas *putre-faciens* IAM 1002	2.4	0.9

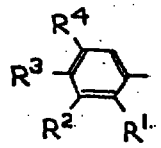
CLAIMS

1. A process for preparing D-N-carbamoyl-α-amino acids having the following general formula (I):



(I)

wherein R is an alkyl group having 1 to 12 carbon atoms, a substituted alkyl group, an aralkyl group having 1 to 12 carbon atoms, a substituted aralkyl group, an unsubstitued or substituted phenyl group of the formula:



in which R^1, R^2, R^3 and R^4 are independently a hydrogen atom, a halogen atom, a hydroxyl group, a methoxy group, an ethoxy group or a methyl group, a thienyl group, a furyl group, a pyridyl group, a thiazolyl group, an imidazolyl group or an indolyl group, which comprises subjecting 5-substituted hydantoins having the following general formula (II):

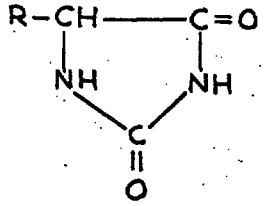
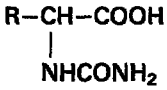


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Sarcina *lutea* IFO 1099	3.5	4.3
Aeromonas *hydrophila* IFO 3820	4.2	1.5
Flavobacterium *flavescens* IFO 3086	2.1	1.4
Bacillus *alvei* IFO 3343	4.8	1.8
Serratia *marcescens* IFO 3054	3.1	1.2
Arthrobacter *simplex* ATCC 6946	7.8	3.2
Pseudomonas *putre-faciens* IAM 1002	2.4	0.9

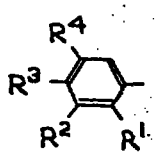
CLAIMS

1. A process for preparing D-N-carbamoyl-α-amino acids having the following general formula (I):

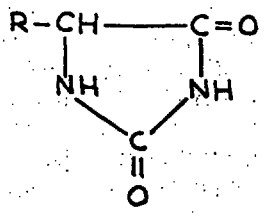


(I)

wherein R is an alkyl group having 1 to 12 carbon atoms, a substituted alkyl group, an aralkyl group having 1 to 12 carbon atoms, a substituted aralkyl group, an unsubstitued or substituted phenyl group of the formula:



in which R^1, R^2, R^3 and R^4 are independently a hydrogen atom, a halogen atom, a hydroxyl group, a methoxy group, an ethoxy group or a methyl group, a thienyl group, a furyl group, a pyridyl group, a thiazolyl group, an imidazolyl group or an indolyl group, which comprises subjecting 5-substituted hydantoins having the following general formula (II):



wherein R is as defined above, to the action of a cultured broth, cells or treated cells of a microorganism or an enzyme extracted from the cells, in an aqueous medium at pH 7-10, the concentration of said 5-substituted hydantoins in the aqueous medium being from 0.1 to 30 percent by weight and microorganism being one cultured at a temperature of 20-85 degree C and a pH 4-11 in a culture medium containing 0.05 to 1.0 percent by weight of an inducer selected from metabolites of pyrimidine bases and their analogs and having an ability of hydrolyzing the 5-substituted hydantoins so as to substantially produce D-N-carbamoyl-α-amino acids, and recovering said D-N-carbamoyl-α-amino acids from the medium.

2. The process of Claim 1, wherein said microorganism belongs to the genus Aerobacter, Arthrobacter, Agrobacterium, Bacillus, Brevibacterium, Corynebacterium, Sarcina, Microbacterium, Micrococcus, Proteus, Pseudomonas, Actinoplanes, Mycobacterium, Nocardia or Streptomyces.

3. The process of Claim 1 or 2, wherein said metabolites of pyrimidine bases and their analogs are selected from β-alanine, β-ureidopropionic acid, β-amino-iso-butyric acid, β-ureido-isobutyiric acid, β-amino-n-butyric acid and β-mercaptopropionic acid.

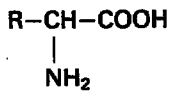
4. The process of Claim 1, 2 or 3, wherein a metal ion is added to the culture medium containing the inducer.

5. The process of Claim 4, wherein said metal ion is a member selected from bivalent manganese, cobalt, nickel, copper, magnesium and iron ions, trivalent aluminum ion, and hexavalent molybdenum ion.

6. The process of Claim 4 or 5, wherein the concentration of said metal ion in the culture medium is from 0.2 to 100 p.p.m.

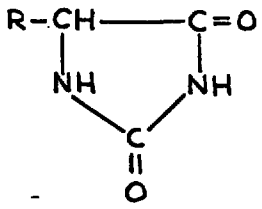
7. The process of Claim 1, 2 or 4, wherein the 5-substituted hydantoins are the DL-forms or L-forms.

8. A process for preparing D-α-amino acids having the following general formula (III):

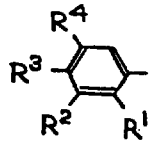


(III)

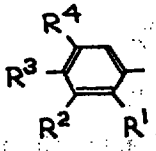
which comprises subjecting 5-substituted hydantoins having the following general formula (II):



wherein R is an alkyl group, a substituted alkyl group, an aralkyl group, a substituted aralkyl group, said groups having 1 to 12 carbon atoms, an unsubstituted or substituted phenyl group of the formula:



in which R^1, R^2, R^3 and R^4 are independently a wherein R is an alkyl group having 1 to 12 carbon atoms, a substituted alkyl group, an aralkyl group having 1 to 12 carbon atoms, a substituted aralkyl group, an unsubstituted or substituted phenyl group of the formula:



in which R^1, R^2, R^3 and R^4 are independently a hydrogen atom, a halogen atom, a hydroxyl group, a methoxy group, an ethoxy group or a methyl group, a thienyl group, a furyl group, a pyridyl group, a thiazolyl group, an imidazolyl group or an indolyl group, to the action of a cultured broth, cells or treated cells of a microorganism or an enzyme extracted from the cells, in an aqueous medium at pH 7-10, the concentration of said 5-substituted hydantoins in the aqueous medium being from 0.1 to 30 percent by weight and said microorganism being one cultured at a temperature of 20-85°C and at pH 4-11 in a culture medium containing 0.5 to 1.0 percent by weight at an inducer selected from metabolites of pyrimidine bases and their analogs and having an ability of hydrolyzing the 5-substituted hydantoins so as to substantially produce D-N-carbamoyl-α-amino acids having the following general formula (I):





$$R-CH-COOH$$
$$|$$
$$NHCONH_2$$

(I)

wherein R is as defined above, to give said D-N-carbamoyl-α-amino acids, and removing the carbamoyl group from said D-N-carbamoyl-α-amino acids.

9. The process of Claim 8, wherein said microorganism belongs to genus Aerobacter, Arthrobacter, Agrobacterium, Bacillus, Brevibacterium, Corynebacterium, Sarcina, Microbacterium, Micrococcus, Proteus, Pseudomonas, Actinoplanes, Mycobacterium, Nocardia or Streptomyces.

10. The process of Claim 8 or 9, wherein said metabolites of pyrimidine bases and their analogs are selected from β-alanine, β-ureidopropionic acid, β-amino-iso-butyric acid, β-ureido-isobutyric acid, β-amino-n-butyric acid and β-mercaptopropionic acid.

11. The process of Claim 8, 9 or 10, wherein a metal ion is added to the culture medium containing the inducer.

12. The process of Claim 11, wherein said metal ion is a member selected from bivalent manganese, cobalt, nickel, copper, magnesium and iron ions, trivalent aluminum ion, and hexavalent molybdenum ion.

13. The process of Claim 11 or 12, wherein the concentration of said metal ion in the culture medium is from 0.2 to 100 p.p.m.

14. The process of Claim 8, 9 or 11, wherein the 5-substituted hydantoins are the DL-forms or L-forms.

15. The process of Claim 8, wherein the removal of the carbamoyl group is carried out by reacting the D-N-carbamoyl-α-amino acids with nitrous acid in an acidic aqueous medium.

16. The process of Claim 15, wherein the removal of the carbamoyl group is carried out by employing an approximately equimolar amount of nitrous acid in an aqueous medium in which at least 0.4 normal of a strong mineral acid is present.

17. The process of Claim 8, 9 or 11, wherein the removal of the carbamoyl group is carried out in an aqueous medium at pH 6 to 11 by employing a cultured broth, cells or treated cells of a microorganism or an enzyme extracted from the cells, said microorganism having an ability of removing the carbamoyl group of the D-N-carbamoyl-α-amino acids.

18. The process of Claim 17, wherein said microorganism having an ability of removing the carbamoyl group of the D-N-carbamoyl-α-amino acids is cultured in a medium containing a D-N-carbamoyl-α-amino acid as an inducer.

19. The process of Claim 18, wherein the concentration of said inducer in the medium is from 0.05 % to 1 % by weight.

20. The process of Claim 17 or 18, wherein said microorganism having an ability of removing the carbamoyl group belongs to the genus Aerobacter, Aeromonas, Brevibacterium, Bacillus, Arthrobacter, Pseudomonas, Flavobacterium, Sarcina or Serratia.

21. A process for preparing D-N-carbamoyl-α-amino acids substantially as described with reference to any one of the examples described herein.

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