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SPECIFICATION

IMMOBILIZED ENZYME PREPARATION AND PROCESS FOR PRODUCING D-α-AMINO ACID

Field of the Invention

The present invention relates to an immobilized enzyme preparation and a process for producing D-α-amino acids using the same. In particular, the immobilized enzyme preparation of the present invention serves for the production of D-α-amino acids which are available as intermediate materials of antibiotics, such as D-(p-hydroxyphenyl)glycine used in the production of antibiotic amoxicillin.

Prior Art

It has already been known from JP-B 57-18793, JP-B 63-20520, JP-B 1-48758, etc., that there exist some enzymes (hereinafter referred to as "decarbamylases") capable of converting N-carbamyl-D-α-amino acid derivatives into D-α-amino acids.

With respect to the other numerous enzymes, it has already been known that the use of these enzymes or enzyme-producing cells in insolubilized or immobilized form makes it possible to prepare an enzyme reaction mixture having only a few impurities, which results in an easy recovery of the product, and also makes it possible to attain the

repeated use of these enzymes, which is advantageous from the view point of cost recovery. Moreover, there has been obtained a good effect by the use in the production on an industrial scale.

In the case of a decarbamylase, however, the possibility of using this enzyme as an immobilized enzyme is generally suggested as a mode of use only in JP-B 63-20520, although there are some examples of using this enzyme in the form of whole cells, toluene-treated bacterial cells, acetone-treated powder of cells or cell-free extracts.

These examples of using a decarbamylase will cause a problem of how to maintain its enzyme activity, although it is exhibited. In particular, they fall behind in repeated use, and they cannot be used on a commercial base.

Objects of the Invention

The present invention is to obtain an immobilized enzyme preparation of decarbamylases with stability sufficient to withstand the repeated use and attempts to use it in the production on an industrial scale.

Under these circumstances, the present inventors have intensively studied. As the result, they have found that an antioxydant is effective for the stabilization of decarbamylases and that a repeatedly usable immobilized preparation of decarbamylases can be obtained by bringing the carbamylases into contact with a solid support in the presence thereof, thereby completing the present invention.

Summary of the Invention

The present invention provides an enzyme capable of producing D-α-amino acids by hydrolysis of N-carbamyl-D-α-amino acids (decarbamylase); an immobilized enzyme preparation characterized in that it comprises an antioxydant and an appropriate support for immobilization; a process for producing the said immobilized enzyme preparation, characterized in that a decarbamylase is brought into contact with a support for immobilization in the presence of an antioxydant; and a process for producing D-α-amino acids, characterized in that the said immobilized enzyme preparation is allowed to act on N-carbamyl-D-α-amino acids.

Detailed Description of the Invention

The decarbamylase used in the present invention may be any one of the enzymes having an action to remove an N-carbamyl group from N-carbamyl-D-α-amino acids, and it may be either one having a stereospecific action on D-amino acids or one having an action on both of D- and L-amino acids. Depending upon the mode of use, enzymes specific to D-amino acids are usually useful in many cases.

The enzyme source, such as animals, plants and microorganisms, is not particularly limited, with microorganisms being preferred for the production on an industrial scale. Examples of the microorganisms are naturally occurring microorganisms such as those belonging to the genus, e.g., Agrobacterium, Pseudomonas,

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Arthrobacter, Alkaligenes, Achromobacter, Moraxella, Paracoccus, Aerobacter, Aeromonas, Brevibacterium, Bacillus, Flavobacterium and Serratia, as disclosed in JP-B 57-18793, JP-B 63-20520 and JP-B 1-48758, and artificial microorganisms which have being conferred, by gene manipulation methods, a given or enhanced ability to produce decarbamylases, as described in JP-B 2-400848. Typical examples of these microorganisms are Agrobacterium radiobacter KNK 712 (FERM BP-1900), Pseudomonas sp. KNK 003A (FERM BP-3181), Pseudomonas sp. KNK505 (FERM BP-3182), Escherichia coli JM109 pAD108 (FERM BP-3184) and Escherichia coli JM109 pPD304 (FERM BP-3183).

In the present invention, the said decarbamylase may be present in the immobilized enzyme preparation, as a purified enzyme, a partially-purified enzyme or a crude enzyme, or in some cases, as it is in the form of bacterial cells. If only this enzyme is under the conditions that it can exhibit the decarbamyl activity, there is no need for take care to its existence form and contaminants.

As the antioxydant, dithiothreitol, 2-mercaptoethanol, L-cysteine hydrochloride, cysteamine hydrochloride, dithioerythritol, mixtures of dithiothreitol and dithioerythritol, or reduced-type glutathione can be used.

The support used for immobilization may vary depending upon the mode of decarbamylase immobilization; in cases where crude enzyme solutions such as cell-free

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extracts or partially-purified enzyme solutions obtained by fractionation with ammonium sulfate are subjected to immobilization, a polymer support having ion exchange groups or covalent-bonding groups can be used.

As the polymer support having ion exchange groups, an anionic exchange resin such as a series of Duolite (registered trade mark) A and Amberlite IRA (registered trade mark), having primary, secondary, tertiary and quaternary amines as exchange groups, or a polystylene resin having diethanol-type functional groups, such as Diaion EX, can be used.

As those having covalent-bonding groups, a substituted polymethacrylate polymer having aldehydes as bonding groups, or high-density almina covered with a polyethyleneimine/glutaraldehyde composite can be used.

Moreover, for immobilization of whole cells such as viable bacterial cells or dried bacterial cells, a polymer such as polyacrylamide, polyurethane and calcium alginate, or a porous material such as pumice and almina, can be used.

The immobilized enzyme preparation of the present invention can be produced as follows.

First, a culture of microorganisms is prepared, and bacterial cells are collected therefrom. Then, a cell-free extract is prepared by ultrasonic disruption, mechanical disruption (e.g., homogenizer), enzyme treatment or the like. In this case, an antioxydant is allowed to exist at a

concentration of 0.1 to 20 mM, usually about 5 mM.

For the partial purification of an enzyme from the cell-free extract, any means usually used by those skilled in the art is employed. For example, nucleic acids are precipitated by addition of protamine sulfate in a 1/5 to 1/10 quantity to the amount of proteins, and after heating at 50° to 60°C for about 20 minutes, heat-instable proteins are precipitated by cooling to 4°C, after which the nucleic acids and the heat instable proteins are removed by centrifugation. Further, fractionation with ammonium sulfate is conducted, if necessary, to obtain an enzyme solution to be brought into contact with a support. During this step, an antioxydant is allowed to exist at a concentration of 0.1 to 20 mM, usually about 5 mM.

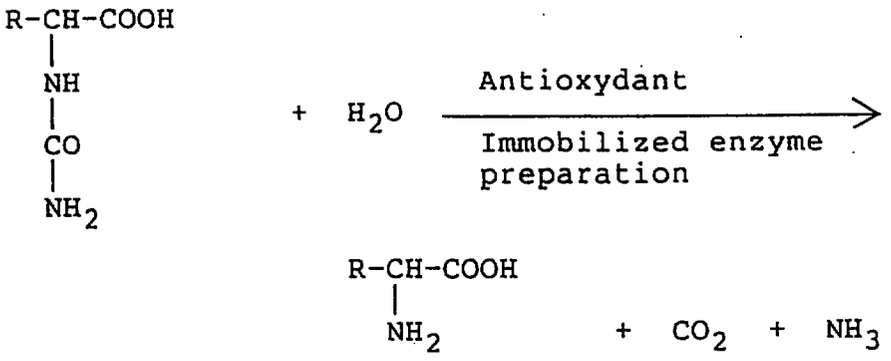
The support whose exchange groups has been activated with saline and then equilibrated in a buffer containing 0.1 to 20 mM, usually about 5 mM, of antioxydant is used. The support and the enzyme solution are brought into contact with each other in the presence of an anti-oxydant at a concentration of 0.1 to 20 mM, usually about 5 mM, followed by stirring at 4° to 30°C, preferably 25°C. After the amount of enzyme adsorbed reaches the desired amount (usually after 8 to 48 hours), the support is removed by filtration and treated with a cross-linking agent to attain the stability of enzymes by insolubilization. As the cross-linking agent, for example, those known in the art can

be used, such as not greater than 1% (preferably 0.2%) glutaraldehyde. It is also preferred that an antioxydant is allowed to exist at a concentration of 0.1 to 20 mM during this step. The cross-linked, immobilized enzyme preparation is washed with distilled water or a buffer (preferably containing an antioxydant), and stored under the moisturized conditions in a sealed vessel at low temperatures (about 4°C).

It is preferred that all procedures are conducted, if possible, in the atmosphere of an inert gas such as nitrogen. The amount of enzyme to be loaded, although it may vary depending upon the degree of purification of this enzyme, is usually in the range of 10 to 80 units/g of support (wherein one unit of decarbamylase refers to the amount of enzyme required to convert 1 μmol of N-carbamyl-D-(p-hydroxyphenyl)glycine into D-(p-hydroxyphenyl)glycine within one minute). It is preferred that an antioxydant is allowed to exist in the immobilized enzyme preparation at an amount of 5 to 10 mg/g of the preparation.

Then, the following will describe the process for producing D-α-amino acids from N-carbamyl-D-α-amino acids by use of an immobilized preparation of decarbamylases.

The reaction is conducted in the presence of an antioxydant, and it can be expressed by the reaction scheme:



wherein R is C_1-C_4 alkyl optionally substituted with a group such as hydroxy, alkylthio, carboxy, amino or amide; C_7-C_8 aralkyl (e.g., benzyl, fenetyl) having an aromatic ring optionally substituted with a group such as hydroxy; or phenyl optionally substituted with hydroxy or halogen.

The amount of antioxydant is in the range of 0.1 to 20 mM, preferably 2.0 to 5.0 mM.

The concentration of N-carbamyl-D-α-amino acid as a reaction substrate is selected in the range of 5 to 25 w/v%, and preferably used at 15 w/v%. The amount of immobilized enzyme preparation used is selected in the range of 10 to 20 units per substrate, and preferably used at 15 units/g of substrate, for those containing 10 to 80 units/g of support. The reaction is conducted, while controlling the pH in the range of pH 6.5 to 8.0, usually to pH 7.0. The temperature, although it may vary depending upon the kind of enzyme, is generally in the range of 30° to 60°C; in particular, higher temperatures can be employed for heat resistant enzymes. The reaction is conducted by a column technique or by

suspension in a reaction vessel; in the latter case, batch reaction is usually conducted for a period of reaction time in the range of about 6 to 48 hours per batch. The immobilized enzyme preparation of the present invention is quite stable; for example, it exhibited almost no decrease in its activity, even if used 15 times or more, and the repeated use at 30 times or more was made possible.

Preferably, the atmosphere in the reaction vessel is replaced by an inert gas and remains containing no oxygen.

The invention will be explained in more detail by way of the following Examples, which are only illustrative and not to be construed to limit the scope thereof.

Example 1

The decarbamylase was produced by bacterial fermentation using the following procedures.

Medium ingredients:

Sucrose	300 g
Urea	25.5 g
KH_2PO_4	30 g
Na_2HPO_4	30 g
$MgSO_4 \cdot 7H_2O$	15 g
$MnCl_2 \cdot 4H_2O$	150 mg
Yeast extract	75 g
N-carbamyl-D-phenylglycine	15 g
Anti-foaming agent	15 g

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First, 15 liters of fermentation medium containing these ingredients were provided and adjusted to pH 7.0. This medium was placed in a fermentation bath of 18 liters in volume and sterilized by autoclaving at 121°C for 30 minutes. Then, a solution of urea and a solution of N-carbamyl-D-phenylglycine were independently subjected to the removal of bacteria through a millipore filter, and each of these solutions was added in 500-ml portions to the fermentation bath under sterile conditions.

The fermentation bath was inoculated with a seed culture of Agrobacterium radiobacter KNK712 (FERM BP-1900) grown at 30°C for 30 hours in the same medium but containing no N-carbamyl-D-phenylglycine. During the fermentation, temperature was maintained at 33°C and aeration was performed. The pH was controlled to 7.0 using sulfuric acid and sodium hydroxide solutions.

After the fermentation for 30 hours, the cells were collected by centrifugation. This cell precipitate was resuspended in 1.5 liters of 0.2 M phosphate buffer (pH 7.0), to which dithiothreitol was added to give a concentration of 5 mM, followed by ultrasonic treatment to discharge a decarbamylase into the solution.

The partial purification of enzymes was conducted as follows.

First, 76 ml of 3% aqueous protamine sulfate was gradually added with stirring, thereby precipitating nucleic

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acids. The enzyme solution was heat treated at 50°C for 20 minutes, followed by cooling to 4°C. After allowed to stand overnight at 4°C, the nucleic acids, as well as proteins, were precipitated, and the cell debris was removed by centrifugation. This preparation was assayed to determine its decarbamyl activity. To 1% N-carbamyl-D-phenylglycine/0.2 M phosphate buffer (pH 7.0), the enzyme preparation was added, and the reaction was allowed to proceed under the conditions at 40°C, after which the formation of D-phenylglycine was examined.

On the other hand, to this enzyme preparation, dithiothreitol was added to give a concentration of 5 mM, and the mixture was subjected to fractionation with ammonium sulfate. Ammonium sulfate was added in small portions with ice-cooling and stirring, thereby precipitating proteins. Active fractions at 15% to 35% of saturation with ammonium sulfate were collected and dissolved in 100 ml of phosphate buffer (pH 7.0), after which the same activity assay as above was conducted.

Example 2

Each sample of three kinds of anion exchange resins purchased from commercial sources (Duolite, Amberlite and Diaion) was washed in 1M NaCl for 1 hour, and equilibrated overnight in 0.2 M phosphate buffer containing 5 mM of dithiothreitol. The equilibrated resins were filtered and stored until they were required for use. The partially-

purified enzyme prepared as described in Example 1 was used for immobilization. The enzyme of Example 1 after the heat treatment and that after the fractionation with ammonium sulfate at the respective amounts of 5 ml and 10 ml per 1 g (wet weight) of resin (10 to 80 units/g of resin) were mixed with each of the resins at 25°C for 24 hours, thereby allowing the enzymes to be adsorbed thereon. These enzyme-resin composites were filtered and treated with 1% glutaraldehyde at pH 7.0. After one hour, the resin samples were filtered once again, and washed three times with distilled water. Finally, the enzyme-resin composites were washed with 0.2 M phosphate buffer (pH 7.0), and stored as they remained wet in separate sealed vessels containing N_2 gas at 4°C.

Each of the enzyme-resin composites produced by this process was assayed to determine their activities as described in Example 1. The specific activities obtained from a series of commercially available ion exchange resins treated by this method are shown in Table 1.

Resin	Support	Functional group	Partially-purified enzyme	Specific activity
Duolite	Phenolform-aldehyde	Secondary amine	A	10.0
			B	80.2
Amberlite	Polystyrene	Tertiary amine	A	7.3
			B	52.2
Diaion	Polystyrene	Diethanol-type	A	12.2
			B	69.9

A. Enzyme after heat treatment
B. Enzyme after fractionation with ammonium sulfate
Specific activity: units/g of resin

The resins Duolite and Amberlite are products available from Rohm & Haas, Co., USA, and the resin Diaion is a product available from Mitsubishi Chemical Industries, Ltd., Japan.

Example 3

First, 100 ml of the partially-purified enzyme solution after the heat treatment (activity: 2.5 units/ml) prepared in Example 1 was added to 15 g of the resin Duolite A568. This mixture was moderately stirred at 25°C for 24 hours, thereby allowing the enzyme decarbamylase to be adsorbed thereon. Then, the enzyme-resin composite was separated by filtration, and stirred in 0.2% glutaraldehyde solution (pH 7.0) containing 5 mM dithiothreitol for 1 hour. The mixture was washed three times with distilled water, and finally washed with 0.2 M phosphate buffer (pH 7.0). This enzyme-resin was stored as it remained wet in a sealed vessel at 4°C. On the other hand, 30 ml of the enzyme solution after the fractionation with ammonium sulfate (activity: 9.3 units/ml) was added to 3 g of the resin Duolite A568, and then treated in the same manner as described above. The immobilized enzyme solutions were assayed as described in Example 1, and their specific activities were 9.8 units/g and 70 units/g of resin, respectively.

To 100 ml of distilled water in a vessel equipped with a jacket, 10 g of N-carbamyl-D-hydroxyphenylglycine and

8 mg of dithiothreitol (giving a concentration of 0.5 mM) were added, and the pH was adjusted to 7.0 by addition of 10 N NaOH with stirring. While blowing nitrogen gas thereinto, the temperature was controlled to 40°C. After this system was equilibrated, the reaction was conducted by addition of 147 units of the enzyme-resin composite (15 g). During this step, the mixture was adjusted to pH 7.0 by addition of a hydrochloric acid solution. After 24 hours, the yield of D-hydroxyphenylglycine was determined by HPLC to be 97%. It was found that the product produced by this process can be isolated by a conventional technique and it has high optical purity. On the other hand, for the enzyme-resin after the fractionation with ammonium sulfate, 15 g of N-carbamyl-D-hydroxyphenylglycine and 8 mg of dithiothreitol were added to 100 ml of distilled water in a vessel equipped with a jacket, followed by the same treatment, and the reaction was conducted in the same manner as described above, by addition of 210 units, i.e., 3 g of the enzyme-resin, resulting in a product having high optical purity.

The enzyme-resin used in this process was recycled 30 times by sucking out a solution of D-hydroxyphenylglycine after the completion of the reaction, while paying attention to maintain the enzyme in the atmosphere of nitrogen, and then newly setting, in place of that solution, a suspension of the substrate which had been equilibrated in

advance. Throughout these cycles, the degree of conversion into D-hydroxyphenylglycine was 95% or more.

Example 4

In the medium as described in Example 1, Pseudomonas sp. KNK003A (FERM BP-3181) was grown, and the cells harvested from 9 liters of fermentation broth by centrifugation. These cells were resuspended in 300 ml of 0.2 M Tris/HCl buffer (pH 7.0), and dithiothreitol was added to give a concentration of 5 mM, followed by ultrasonic treatment to discharge a decarbamylase into the solution. Then, 20 ml of 3% aqueous protamine sulfate was gradually added thereto with stirring, thereby precipitating nucleic acids. The enzyme solution was heat treated at 65°C for 20 minutes, and then cooled to 4°C. After stored overnight at 4°C, the nucleic acids, as well as proteins, were precipitated, and the cell debris was removed by centrifugation. Then, 100 ml of this partially-purified enzyme solution after the heat treatment (activity: 0.06 units/ml) was added to 10 g of the resin Duolite A568. This mixture was stirred at 25°C for 24 hours, thereby allowing the decarbamylase to be adsorbed on the support. The enzyme-resin composite was removed by filtration, and cross-linked by moderately stirring in 0.2% glutaraldehyde solution (pH 7.0) containing 5 mM dithiothreitol for 1 hour. The mixture was washed three times with distilled water, and finally washed with 0.2 M Tris/HCl buffer (pH 7.0). The specific

activity thereof was 0.38 units/g of resin, and the activity in the case where N-carbamyl-D-alanine was used as a substrate was 4.2 units/g.

Example 5

Using 5 g of the enzyme-resin produced by the process as described in Example 4, 2 g of N-carbamyl-D-alanine was hydrolyzed in 40 ml of distilled water containing 0.5 mM dithiothreitol. The hydrolysis was conducted at 45°C, at pH 6.7 to 7.0, for 24 hours, while blowing N_2 into this mixture to exclude oxygen therefrom. The yield of D-alanine after completion of the reaction was determined to be 96.5%.

Then, the hydrolysis of 5% N-carbamyl-D-alanine was conducted 35 times to give an average yield of 96.1%.

Example 6

First, 1 g of the enzyme-resin produced by the process as described in Example 4 was charged into a column, and 2% N-carbamyl-D-(p-hydroxyphenyl)glycine solution containing 1 mM antioxydant was applied to the above column for continuous hydrolysis. As the antioxydant, dithiothreitol, L-cysteine hydrochloride or cysteamine hydrochloride was used, and the continuous reaction was conducted at an application speed of 1 ml/min., at 40°C under N_2 sealing for 4 days.

With the use of these antioxydants, the degree of enzyme deactivation for 4 days was suppressed to 30% to 35%,

whereas the enzyme deactivation of 80% or more was found when no antioxydant was used.

Antioxydant	Ratio of remaining enzyme activity (%) after			
	1 day	2 days	3 days	4 days
Dithiothreitol	80	70	68	65
L-cysteine hydrochloride	78	71	68	66
Cysteamine hydrochloride	78	75	73	68
Dithioerythritol	78	72	68	-
No addition	50	29	20	14

As described above, according to the present invention, there is provided an immobilized preparation of decarbamylases capable of producing D-α-amino acids which are important as intermediate materials of antibiotics and the like. The use of this preparation makes possible the repeated use at 30 times or more, which gives the expectation of using it in the production on an industrial scale.

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CLAIMS

1. An immobilized enzyme preparation characterized in that it comprises an enzyme capable of producing D-α-amino acids by hydrolysis of N-carbamyl-D-α-amino acids, an antioxydant and a support for immobilization.

2. An immobilized enzyme preparation according to claim 1, wherein the support for immobilization is an anion exchange resin.

3. An immobilized enzyme preparation according to claim 1, wherein the enzyme capable of removing a carbamyl group by hydrolysis of N-carbamyl-D-α-amino acids is an enzyme derived from a microorganism.

4. An immobilized enzyme preparation according to claim 3, wherein the microorganism is Agrobacterium radiobacter KNK 712 (FERM BP-1900), Pseudomonas sp. KNK 003A (FERM BP-3181), Pseudomonas sp. KNK505 (FERM BP-3182), Escherichia coli JM109 pAD108 (FERM BP-3184) or Escherichia coli JM109 pPD304 (FERM BP-3183).

5. An immobilized enzyme preparation according to claim 2, wherein the anion exchange resin is a phenol-type ion exchange resin having an amine as an exchange group.

6. An immobilized enzyme preparation according to claim 1, wherein the antioxydant is dithiothreitol, 2-mercaptoethanol, L-cysteine hydrochloride, cysteamine hydrochloride, dithioerythritol, mixtures of dithiothreitol

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and dithioerythritol, or reduced-type glutathione.

7. A process for producing an immobilized enzyme preparation, characterized in that an enzyme capable of producing D-α-amino acids by hydrolysis of N-carbamyl-D-α-amino acids is brought into contact with a support for immobilization in the presence of an antioxydant.

8. A production process according to claim 7, wherein the support for immobilization is brought into contact with the enzyme, after equilibrated in a buffer containing the antioxydant.

9. A process for producing D-α-amino acids, characterized in that an immobilized enzyme preparation according to any one of claims 1 to 6 is allowed to act on N-carbamyl-D-α-amino acids.

10. A process for producing D-α-amino acids according to claim 9, wherein the immobilized enzyme preparation is allowed to act with the addition of an antioxydant to the reaction system.

11. A production process according to claim 9 or 10, wherein the immobilized enzyme preparation is repeatedly used.