Document Name: Specification

Title of the Invention:

PROCESS FOR PRODUCING D-AMINO ACIDS WITH COMPOSITE IMMOBILIZED ENZYME PREPARATION

Claims:

1. A process for the production of a D-amino acid from the corresponding DL-5-substituted hydantoin by one-step reaction which comprises using a composite immobilized enzyme at a pH about neutrality, said composite immobilized enzyme being obtained by immobilizing a hydantoinase having its optimal pH within an alkaline range (hereinafter abbreviated as Hase) and a D-N-carbamyl-α-amino acid amidohydrolase having its optimal pH about neutrality (hereinafter abbreviated as DCase) in a coexisting state on an immobilizing support, simultaneously.

2. A process for the production of a D-amino acid according to claim 1, wherein a composite enzyme to be used for immobilization is prepared by producing the Hase and the DCase separately and then mixing them.

3. A process for the production of a D-amino acid according to claim 1 or 2, wherein the Hase to be used is that derived from a microorganism belonging to the genus Bacillus.

4. A process for the production of a D-amino acid according to claim 1 or 2, wherein the Hase to be used is that

derived from Bacillus sp. KNK108 (FERM P-6056) or Bacillus sp. KNK245 (FERM BP-4863).

5. A process for the production of a D-amino acid according to claim 1 or 2, wherein the DCase to be used is that derived from Agrobacterium radiobacter KNK712 (FERM BP-1900), Pseudomonas sp. KNK003A (FERM BP-3181), Pseudomonas sp. KNK505 (FERM BP-3182), E. coli JM109pAD108 (FERM BP-3184), E. coli JM109pPD304 (FERM BP-3183).

6. A process for the production of a D-amino acid according to any one of claims 1 to 4, wherein the DCase to be used is that derived from a recombinant microorganism E. coli JM109pAD402 (FERM BP-3912), E. coli JM109pAD404 (FERM BP-3913), E. coli JM109pAD406 (FERM BP-3914), E. coli JM109pAD416 (FERM BP-3915), E. coli JM109pAD428, E. coli JM109pAD429 (FERM BP-4035), E. coli JM109pAD431, E. coli JM109pAD434, E. coli JM109pAD435, E. coli JM109pAD439, E. coli JM109pAD441, E. coli JM109pAD445, E. coli JM109pAD447, E. coli JM109pAD448, E. coli JM109pAD450, E. coli JM109pAD421, E. coli JM109pAD422, E. coli JM109pAD423, E. coli JM109pAD424 (FERM BP-4034), E. coli JM109pAD425, E. coli JM109pAD426, E. coli JM109pAD427, E. coli JM109pAD451, E. coli JM109pAD452, E. coli JM109pAD453, E. coli JM109pAD461, E. coli JM109pAD454, E. coli JM109pAD455 (FERM BP-4036), E. coli JM109pAD456, E. coli JM109pAD468, E. coli JM109pAD469, E. coli JM109pAD470 or E. coli HB101pNT4553 (FERM BP-4368), the stability of which is improved by amino acid

substitution of the DCase derived from Agrobacterium radiobacter KNK712 (FERM BP-1900).

7. A process for the production of a D-amino acid according to claim 1, wherein a composite enzyme used for immobilization is prepared by producing Hase and DCase simultaneously.

8. A process for the production of a D-amino acid according to claim 7, wherein the composite enzyme is produced by cultivating a host containing a plasmid prepared by ligating a Hase gene and a DCase gene to the same vector.

9. A process for the production of a D-amino acid according to claim 7, wherein the composite enzyme is produced by ligating the Hase gene and the DCase gene to different vectors having different modes of replication, introducing these vectors into the same host and cultivating the host.

10. A process for the production of a D-amino acid according to claim 7, wherein the composite enzyme is produced by cultivating a Hase producer microorganism and a DCase producer microorganism together.

Detailed Explanation of the Invention:

[0001]

Field of the Invention

The present invention relates to a composite immobilized enzyme preparation and a process for producing D-amino acids using the preparation. In particular, the

substitution of the DCase derived from Agrobacterium radiobacter KNK712 (FERM BP-1900).

7. A process for the production of a D-amino acid according to claim 1, wherein a composite enzyme used for immobilization is prepared by producing Hase and DCase simultaneously.

8. A process for the production of a D-amino acid according to claim 7, wherein the composite enzyme is produced by cultivating a host containing a plasmid prepared by ligating a Hase gene and a DCase gene to the same vector.

9. A process for the production of a D-amino acid according to claim 7, wherein the composite enzyme is produced by ligating the Hase gene and the DCase gene to different vectors having different modes of replication, introducing these vectors into the same host and cultivating the host.

10. A process for the production of a D-amino acid according to claim 7, wherein the composite enzyme is produced by cultivating a Hase producer microorganism and a DCase producer microorganism together.

Detailed Explanation of the Invention:

[0001]

Field of the Invention

The present invention relates to a composite immobilized enzyme preparation and a process for producing D-amino acids using the preparation. In particular, the

composite immobilized enzyme preparation of the present invention is useful for the production of D-α-amino acids which are intermediate compounds for the production of antibiotics, such as D-(p-hydroxyphenyl)glycine to be used for the production of the antibiotic, amoxycillin and the like.

[0002]

Prior Art and Problem to be Solved by the Invention

Optically-active D-amino acids are important compounds as intermediate compounds for drugs and it has been known that they can be produced efficiently by combining an asymmetric hydrolysis reaction of 5-substituted hydantoins into the corresponding D-N-carbamyl-α-amino acids with the enzymes, hydantoinases (hereinafter sometimes abbreviated as "Hase") (JP-B 62-30785), and an conversion reaction of the resultant D-N-carbamyl-α-amino acids into the corresponding D-α-amino acids with the enzymes, D-N-carbamyl-α-amino acid amidohydrolases (hereinafter sometimes abbreviated as "decarbamylase" or "DCase") (PCT/JP91/01696: WO 92/10579).

In addition, JP-A 63-185382, WO 92/00739 and the like disclose that the respective reactions are carried out more efficiently by using these enzymes in the form of so-called immobilized enzymes wherein they are immobilized on supports such as ion exchange resins and the like.

However, a two-step reaction has been employed for carrying out these reactions because the optimal and stable

pH's of both enzymes are considerably different from each other. Therefore, both immobilized enzymes should be prepared separately and complicated reaction operations are required.

[0003]

Means for Solving the Problem

The present invention relates to a technique for producing D-α-amino acids efficiently by using an immobilized enzyme resin obtained by immobilizing both hydantoinase and decarbamylase on one immobilizing resin support in a coexisting state of the enzymes (hereinafter referred to as a "composite enzyme"), simultaneously.

[0004]

In these two enzymatic reactions for converting 5-substituted hydantoins into D-α-amino acids, in general, the optimal pH of the hydantoinase reaction is pH 8 to 9 and the solubility of the substrate is increased as increase in pH. In addition, the racemic reaction of the hydantoin ring is promoted in an alkaline range. Therefore, it is desired to carry out the hydantoinase reaction in the pH ranging from 7 to 10, preferably in an alkaline range. On the other hand, in general, the optimal pH of the decarbamylase reaction is pH 6.5 to 9.0 but the hindrance of the reaction by ammonia formed is remarkably increased as increase in pH. Therefore, it is desired to carry out the decarbamylase reaction at pH about neutrality.

[0005]

If so-called one-step reaction can be employed for carrying out these reactions, i.e., these two enzymatic reactions can be carried out in one reaction vessel simultaneously, in comparison with so-called two-step reaction wherein two different enzymes react with the substrates separately, reaction operations become simple and the overall reaction time can be shortened. In addition, by combining the hydantoinase reaction which is a reversible reaction and the decarbamylase reaction which is a irreversible reaction, the conversion yield of hydantoin can be improved as well as the subsequent purification of D-amino acids can be simplified. Thus, it is expected to significantly reduce the production cost. However, when the respective enzymes are immobilized on different immobilizing supports and they are mixed upon using them, the reactivities become inferior because of the difference in optimal pH and there is a problem in the stability of the immobilized enzymes.

The present inventors have intensively studied to produce a composite immobilized enzyme preparation wherein both enzymes are immobilized on one immobilizing support simultaneously. If these two enzymes are immobilized simultaneously, two enzymatic reactions occur successively in micro-spaces of resins. Therefore, movement of the substrate for a decarbamylase, a D-N-carbamyl-α-amino acid, from immobi-

lized Hase to immobilized DCase by diffusion is not required and pH variation in the micro-spaces can be minimized. Thus, it is expected that, in addition to increase in reactivities of the respective enzymes and relief of the product hindrance due to ammonia, stability of enzymes upon using repeatedly is improved.

[0006]

As for the hydantoinase used in the present invention, the enzymes from animals, plants and microorganisms can be used. The hydantoinases from microorganisms are suitable for industrial production. As such a microorganism, those disclosed in JP-B 62-30758 are exemplified. The bacteria include Achromobacter, Aerobacter, Aeromonas, Agrobacterium, Alcaligenes, Arthrobacter, Bacillus, Brevibacterium, Corynebacterium, Enterobacter, Erwinia, Escherichia, Klebsiella, Microbacterium, Micrococcus, Protaminobacter, Proteus, Pseudomonas, Sarcina, Serratia, Xanthomonas and the like. The Actinomycetes include Actinomyces, Mycobacterium, Nocardia, Streptomyces, Actinoplanes and the like. The filamentous fungi include Aspergillus, Paecilomyces, and Penicillium and the like. The yeasts include Candida, Pichia, Rhodotorula, Torulopsis and the like.

[0007]

Among the above microorganisms, examples of strains which have relatively high hydantoinase activities and are

excellent in practical use include Aerobacter cloacae IAM 1221, Agrobacterium rhizogenes IFO 13259, Brevibacterium incertum IFO 12145, Corynebacterium sepedonicum IFO 3306, Microbacterium flavum ATCC 10340, Micrococcus roseus IFO 3764, Pseudomonas striata IFO 12996, Mycobacterium smegmatis ATCC 607, Nocardia corallina IFO 3338, Streptomyces flaveolus IFO 3241, Bacillus sp. KNK108 (FERM P-6056) and the like.

[0008]

In addition, the enzymes produced by artificial microorganisms in which hydantoinase productivity is imparted by gene recombinant technique, for example, E. coli HB101pTH104 (FERM BP-4864), or increased or the dihydropyrimidinase having similar activity as disclosed in JP-A 53-136583 can be used.

[0009]

As for the decarbamylase used in the present invention, the origins thereof is not specifically limited and those derived from animals, plants and microorganism can be used. However, for industrial production, the enzymes from microorganisms are suitable. Examples of such microorganisms include naturally occurring microorganisms such as Agrobacterium Pseudomonas, Arthrobacter, Alcaligenes, Achromobacter, Moraxella, Paracoccus, Aerobacter, Aeromonas, Brevibacterium, Bacillus, Flavobacterium and Serratia disclosed in JP-B 57-18793, JP-B 63-20520 and JP-B 1-48758,

or the artificial microorganisms described in WO 91/01696 in which decarbamylase productivity is imparted or increased by gene recombinant technique. Representative examples of such microorganisms include Agrobacterium radiobacter KNK712 (FERM BP-1900), Pseudomonas sp. KNK003A (FERM BP-3181), Pseudomonas sp. KNK505 (FERM BP-3182), Escherichia coli JM109pAD108 (FERM BP-3184), E. coli JM109pPD304 (FERM BP-3183) and the like.

[0010]

When a stabilized decarbamylase in which the amino acid responsible for heat resistance of the decarbamylase is replaced by another one is used, the following transformants disclosed in WO 94/03613 can be used. For example, the transformants include E. coli JM109pAD402 (FERM BP-3912), E. coli JM109pAD404 (FERM BP-3913), E. coli JM109pAD406 (FERM BP-3914), E. coli JM109pAD416 (FERM BP-3915), E. coli JM109pAD428, E. coli JM109pAD429 (FERM BP-4035), E. coli JM109pAD431, E. coli JM109pAD434, E. coli JM109pAD435, E. coli JM109pAD439, E. coli JM109pAD441, E. coli JM109pAD445, E. coli JM109pAD447, E. coli JM109pAD448, E. coli JM109pAD450, E. coli JM109pAD421, E. coli JM109pAD422, E. coli JM109pAD423, E. coli JM109pAD424 (FERM BP-4034), E. coli JM109pAD425, E. coli JM109pAD426, E. coli JM109pAD427, E. coli JM109pAD451, E. coli JM109pAD452, E. coli JM109pAD453, E. coli JM109pAD461, E. coli JM109pAD454, E. coli JM109pAD455 (FERM BP-4036), E. coli JM109pAD456, E. coli JM109pAD468, E. coli JM109pAD469, E. coli

JM109pAD470, E. coli HB101pNT4553 (FERM BP-4368) or the like. When such stabilized enzymes are used, better results can be obtained in repeated use of the composite immobilized enzyme preparation of the present invention.

[0011]

The enzymes used in the present invention can be produced simultaneously by using a microorganism which is capable of producing both enzymes. Alternatively, the enzymes can be produced separately or simultaneously using microorganisms which are capable of producing respective enzymes alone. As the microorganism which is capable of producing both enzymes, a microorganism isolated from a natural source such as an Agrobacterium disclosed in JP-B 63-205200 and the like can be used. In addition, a recombinant microorganism prepared by isolating genes of both enzymes from the microorganisms isolated from a natural source and introducing them into a host, for example, the microorganisms disclosed in WO 94/00577, can be used. Alternatively, genes of both enzymes can be isolated from the same or different microorganisms and inserted into the same vector in the expressible form of both genes, or inserted into different vectors having different replication modes, for example, pUC19 and pACYC184. Then, the recipient host such as E. coli can be transformed with the above vector or vectors so that a single microorganism can produce both enzymes. In these cases, by selecting the kinds

of promoters having various capabilities and the plasmids having different copy numbers, the ratio of production of each enzyme can be varied according to a particular purpose. However, it is preferred to adjust the ratio so as to obtain almost equal amounts of enzyme proteins. In the case that microorganisms which produce different enzymes separately are used, strains isolated from natural sources or recombinant microorganisms prepared by using gene recombinant technique can also be used. In such case, the production of the enzymes can be carried out either by cultivating the producer microorganisms separately, or cultivating in a mixed culture at various ratios, preferably, at such a ratio that almost equal amounts of the enzymes can be produced.

[0012]

The cultivation can be carried out aerobically, for example, by shaking culture using flasks or by spinner culture with aeration. As for culture medium used in the cultivation, normally, an nutrient medium which contains generally used natural nutrients such as meat extract and polypeptone and the like can be used. When hydantoinase is produced separately or simultaneously with decarbamylase, a good result can be obtained by carrying out the cultivation with addition of manganese ion in an amount of, for example, as manganese chloride, 1 to 100 mg/liter, preferably about 20 mg/liter.

[0013]

In the present invention, the enzymes can exist in the immobilized enzyme preparation in the purified, partially purified or crude form, or in some cases, in the form of microbial cells per se. And, in so far as the enzymes are under the conditions that the enzymatic activities can be exhibited the enzymes can exist in any form, and can be accompanied by any substance.

[0014]

In the preparation or use of the composite immobilized enzyme preparation of the present invention, better results can be obtained by adding an antioxidant for a repeated use. As such an antioxidant, there can be used dithiothreitol, 2-mercaptoethanol, L-cystein hydrochloride, cysteamine hydrochloride, dithioerythritol, a mixture of dithiothreitol and dithioerythritol, reduced glutathione and the like.

[0015]

An immobilized support used can be varied according to particular use conditions of the immobilized enzymes. When a crude enzyme solution such as a cell-free extract, or a partially purified enzyme solution treated by ammonium sulfate precipitation or the like are subjected to immobilization, polymer supports having ion exchanging groups or covalent bonding groups can be used.

[0016]

As for a polymer support having an ion exchanging group, for example, Duolite (registered trade mark) series, or Amberlite IRA (registered trade mark) series, the exchanging groups of which are primary, secondary, tertiary and quaternary amines; or a polystyrene resin having a diethanol type functional group, for example, Diaion EX can be used.

[0017]

As for a support with a covalent bonding group, a substituted polymethacrylate polymer having an aldehyde as a bonding group, a high density alumina covered with a complex of polyethyleneimine/glutaraldehyde and the like can be used.

[0018]

To immobilize whole microbial cells such as live cells or dried cells, a polymer such as polyacrylamide, polyurethane or calcium alginate, or a porous material such as alumina can be used.

[0019]

The composite immobilized enzyme preparation of the present invention is produced as follows. Firstly, each enzyme is produced by cultivating microbial cells. When a microorganism capable of producing the enzymes simultaneously is used, a cell-free extract can be prepared by collecting the cells and disrupting them with, for example, sonication, mechanical disruption (for example, homogenizer) or enzyme treatment. When a solution of the composite enzyme is

prepared by using different microorganisms, it can be prepared by cultivating the microorganisms separately, collecting the cells, preparing respective cell-free extracts and mixing them. Or, it can be prepared by cultivating respective producer microorganisms together and disrupting the cells at the same time to prepare a cell-free extract in which both two enzymes are mixed.

[0020]

The support is used after activation of its exchanging group with, for example, aqueous solution of sodium chloride and equilibrating, for example, in a buffer solution. The mixture is stirred at 4 to 30°C, preferably at 15°C, and the support is collected by filtration after the amount of enzyme adsorbed reaches the given amount (normally 8 to 48 hours, preferably this is carried out under the atmosphere of inert gas, such as nitrogen). Then, the support is washed and insolubilized by treating with a cross-linking agent to stabilize it. As for a cross-linking agent, a known agent such as at less than 1%, preferably 0.1 to 0.2% glutaraldehyde can be used. The cross-linked composite enzyme immobilized preparation is washed with distilled water and a buffer solution (preferably containing antioxidant) and stored in wet state in a sealed vessel at a low temperature (4°C).

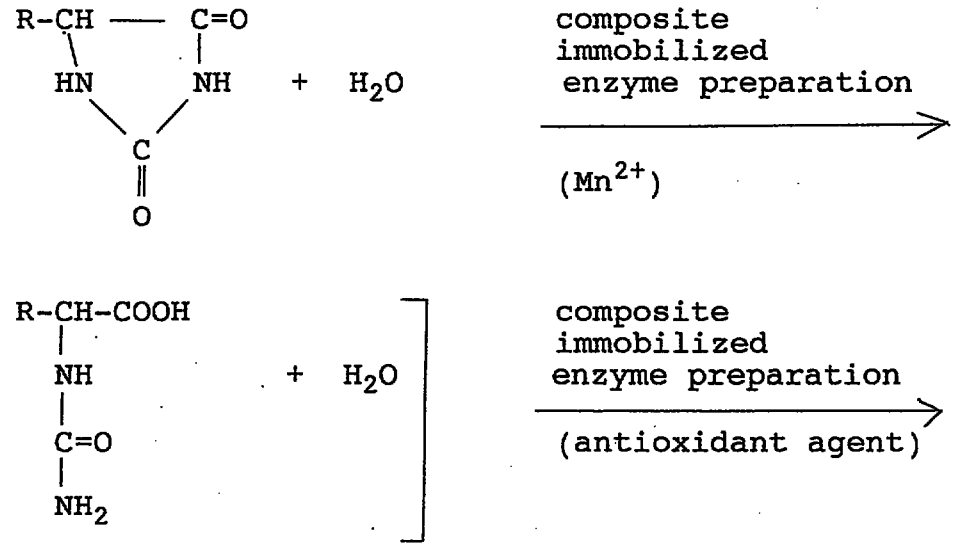
[0021]

The process for producing D-α-amino acids from 5-substituted hydantoins by using the composite immobilized enzyme preparation of the present invention will be described hereinafter.

[0022]

The reaction is carried out by reacting the substrate, 5-substituted hydantoin, with the composite immobilized enzyme preparation, if necessary, in the presence of an antioxidant agent and/or manganese ion. Normally, it is preferred that the reaction is carried out in the presence of 0.1 to 20 mM of an antioxidant agent and manganese ion. The reaction proceeds according to the following reaction scheme:

[Chemical formula 1]



wherein R represents phenyl group, phenyl group substituted with hydroxy group, alkyl group, substituted alkyl group, aralkyl group or thienyl group.

[0023]

The concentration of the substrate 5-substituted hydantoin to be used is 0.1 to 30% (w/v), preferably 1 to 5% (w/v). The enzyme activity of the composite immobilized enzyme preparation is generally, 5 to 80 units/g support for decarbamylase (wherein 1 unit of the enzyme is the amount of the enzyme required for converting the substrate D-N-carbamyl-p-hydroxyphenylglycine into D-p-hydroxyphenylglycine for 1

minutes at pH 7.0 and 40°C) and, for hydanotinase, depending upon conditions of adsorption, about 1 to 10 times per support (wherein 1 unit of the enzyme is the amount of the enzyme required for converting the substrate p-hydroxyphenylhydantoin into D-N-carbamyl-p-hydroxyphenylglycine for 1 minute at pH 8.7 and 40°C). The ratio of the enzymes to be adsorbed to the support is adjusted so that almost equal activities can be expressed, although this varies according to particular reaction conditions (e.g., pH). For example, when the reaction is carried out at pH 7.5, the desired immobilized enzyme preparation can be prepared by carrying out the immobilization reaction with an enzyme solution containing about 5 times larger amount of hydantoinase in terms of almost equal amount of protein and specific activity. The use amount of thereof is about 10 to 20 units/g substrate as decarbamylase activity. The reaction pH is adjusted to the range of 6.5 to 8.0, normally pH 7.5. The reaction temperature is varied according to a particular enzyme. However, in general, the reaction is carried out at 30 to 60°C. A higher temperature can be employed for a particular heat resistant enzyme. The reaction is carried out normally by column method or by suspending the composite immobilized enzyme preparation in a reaction vessel. In the latter case, a batch reaction is usually carried out and the reaction time is about 6 to about 48 hours per batch. By using the composite immobilized

enzyme preparation of the present invention, the corresponding D-amino acid can be produced from a 5-substituted hydantoin at a high yield in a one-step reaction.

[0024]

Examples

The following examples further illustrate the present invention in detail.

Example 1

Firstly, for preparing an enzyme solution of hydantoinase, Bacillus sp. KNK108 (FERM P-6056) was inoculated to a 250 ml of seed culture medium (meat extract 1.0%, polypeptone 1.0%, yeast extract 0.5% (pH 7.0)) and cultivated at 33°C for about 25 hours. This seed culture was inoculated to 2.5 liter of a culture medium (meat extract 1.0%, polypeptone 1.0%, yeast extract 0.5%, uracil 0.1%, $MnCl_2$ 20 ppm (pH 7.5)) and cultivated at 33°C for about 16 hours. The microbial cells were collected by centrifugation and suspended in 50 ml of 20 mM $MnSO_4$ aqueous solution. After adjusting the pH to 8.5, the cells were disrupted by sonication and the residue was removed to obtain a crude enzyme solution of hydantoinase.

[0025]

In addition, to prepare an enzyme solution of decarbamylase, E. coli HB101pNT4553 (FERM BP-4368) was cultivated in 20 ml of 2YT medium (Bacto peptone 1.6%, Bacto

yeast extract 1.0%, NaCl 0.5%) supplemented with 50 µg/ml ampicillin at 37°C for about 16 hours. This culture was inoculated to 1.4 liter of 2YT medium in an amount of 1%, and cultivated at 37°C for about 28 hours. The cells were collected by centrifugation and suspended to 140 ml of 5 mM dithiothreitol solution. After adjusting the pH to 7.0, the cells were disrupted by sonication and the residue was removed to obtain the supernatant as a crude enzyme solution of decarbamylase.

[0026]

Example 2

By using the crude enzyme solutions obtained in Example 1, immobilization of the enzymes was carried out. Duolite A-568 (Rohm & Haas) as a immobilization support was washed with firstly 1M NaCl and ion exchanged water and then put into the ion exchanged water and the pH was adjusted to 7.5. To 8.4 g of this resin were added 20 ml of the crude enzyme solution of hydantoinase and 11.5 ml of the crude enzyme solution of decarbamylase the pH of both of which had been adjusted to 7.5 and the mixture was stirred at 15°C for about 20 hours under nitrogen atmosphere. After washing this resin twice with 0.5 mM $MnSO_4$ solution, the resin was suspended in five time volumes of ion exchanged water. After the pH was adjusted to 7.5, the suspension was stirred for 35 minutes with addition of 544 µl of 2.5% glutaraldehyde with

portions. This suspension was treated with 50 mM Tris-HCl (pH 7.5), 5 mM DTT and 1 mM $MnSO_4$ overnight to obtain the composite immobilized enzyme preparation

[0027].

Then, as controls, resins to which two enzymes were separately immobilized were prepared.

Each of 20 ml of the crude enzyme solution of hydantoinase and 60 ml of the crude enzyme solution of decarbamylase was mixed with each of 4.2 g and 22.0 g of the above immobilization support and the same operations as described above was carried out to obtain each immobilized enzyme resin in which each enzyme was separately immobilized.

[0028]

Example 3

By using the composite immobilized enzyme preparation and control immobilized enzymes containing respective single enzymes obtained in Example 2, the reactions to produce the corresponding D-α-amino acid from a 5-substituted hydantoin were carried out.

After addition of 1g of 5-(p-hydroxyphenyl)hydantoin as the substrate to 100 ml of 0.1 M KPB (pH 7.5), 1 mM $MnSO_4$ and 5 mM DTT, nitrogen gas was sufficiently bubbled in and the reaction conditions were adjusted to 40°C and pH 7.5. In order to obtain the same enzymatic activities of both enzymes, 0.97 g of the composite immobilized enzyme preparation or 3.0

g of the hydantoinase immobilized enzyme and 0.26 g of the dacarbamylase immobilized enzyme were added. The reaction was carried out with bubbling of nitrogen gas and controlling the reaction pH to 7.5 with 2N H_2SO_4 or 6N NaOH. The reaction was carried out for 29 hours with periodical samplings. The amount of p-hydroxyphenylglycine produced at each sampling point was determined by high performance liquid chromatography (Nippon Bunko, Finepack SIL C-18 column).

The results are shown in Fig. 1.

As is seen from Fig. 1, it is found that the reaction can be carried out more efficiently when two enzymes were immobilized simultaneously on the same resin.

[0029]

Example 4

The stability of the enzyme activities was investigated by carrying out the reaction repeatedly with the composite immobilized enzyme preparation. Two grams of 5-(p-hydroxyphenyl)hydantoin was added to 100 ml of 1 mM $MnSO_4$ and 5 mM DTT and pH was adjusted to 7.5. To the mixture was added 8.9 g of the composite immobilized enzyme preparation obtained in Example 2 was added and the reaction was carried out at 40°C for 23 hours with bubbling of nitrogen gas and controlling the pH to 7.5. After filtering the reaction mixture with suction, another mixture was added to the composite immobilized enzyme according to the same manner as described above

and the reaction was carried out. This operation was repeated five times and both enzyme activities were determined at the end of each reaction.

The relative activities to those in the first reaction are shown in Fig. 2.

The decrease in the activities were scarcely observed in these five reactions.

[0030]

Effect of the Invention

By using of the composite immobilized enzyme preparation produced by immobilizing the hydantoinase and the decarbamylase simultaneously on the same resin in the production of the corresponding D-α-amino acids from 5-substituted hydantoins, it is possible to carry out the one-step reaction with much simpler operations than two-step reaction and more efficiently than the reaction by the mixture of the two immobilized enzymes obtained by immobilizing these enzymes separately to the different resins.

Brief Explanation of the Drawings:

Fig. 1 A graph illustrating the time courses of the reactions for the production of the corresponding D-p-hydroxyphenylglycine from 5-(p-hydroxyphenyl)hydantoin in Example 3 by using the composite immobilized enzyme preparation of the present invention and the mixture prepared by

immobilizing hydantoinase and decarbamylase separately to the different resins.

Fig. 2 A graph illustrating the stability of the enzymes in repeated reactions by using the composite immobilized enzyme preparation.