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(19)

(54) ENZYME PREPARATION HAVING L-α-AMINO ACYL AMIDASE ACTIVITY

(71) We, NOVO INDUSTRI A/S, a company organised under the laws of Denmark, of Novo Allé, DK-2880 Bagsvaerd, Denmark, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:-

This invention relates to the use as an L-α-amino acyl amidase of a preparation, having L-α-amino acyl amidase activity, obtained by cultivation of a micro-organism in the presence of a nutrient medium containing assimilable sources of carbon, nitrogen and phosphorus, and to processes of preparing L-α-amino acid and/or D-α-amino acid amide.

It is well known that some micro-organisms such as *Aspergillus oryzae, Aspergillus parasiticus, Mycobacterium phlei, Aeromonas proteolytica, Bacillus subtilis*, and *Bacillus stearothermophilus* can produce enzymes which are capable of hydrolyzing α-amino acid amides so as to form α-amino acid in an aqueous medium.

These prior art enzymes which in the following are referred to as α-amino acyl amidases show a highly stereo-specific activity and hydrolyze only L-α-amino acyl amides. Thus, D-α-amino acyl amides are substantially unaffected by these enzymes.

Therefore, these α-amino acyl amidases are suitable for effecting an optical resolution of DL-α-amino acids. In such a process, α-amino acyl amidase is contacted with DL-α-amino acid amide to effect a hydrolysis of the L-α-amino acid amide to form the corresponding amino acid, and the amino acid formed and/or the remaining D-α-amino acid amide is solated [Greenstein & Winitz: "Chemistry of the amino acids", vol. 3, pp. 1778-1781 (New York 1961)].

According to the first aspect of the present invention there is provided the use as an L-α-amino acyl amidase of a preparation, having L-α-amino acyl amidase activity, obtained by cultivation of a strain of *Pseudomonas putida* in the presence of a nutrient medium containing assimilable sources of carbon, nitrogen and phosphorus.

According to the second aspect of the present invention there is provided a process of preparing L-α-amino acid from L-α-amino acid amide, which process comprises contacting the L-α-amino acid amide with a preparation, having L-α-amino acyl amidase activity, obtained by cultivation of a strain of *Pseudomonas putida* in the presence of a nutrient medium containing assimilable sources of carbon, nitrogen and phosphorus.

According to the third aspect of the present invention there is provided a process of preparing L-α-amino acid and/or D-α-amino acid amide from DL-α-amino acid amide, which process comprises contacting the DL-α-amino acid amide with a preparation, having a L-α-amino acyl amidase activity, obtained by cultivation of a strain of *Pseudomonas putida* in the presence of a nutrient medium containing assimilable sources of carbon, nitrogen and phosphorus.

By cultivating a strain of *Pseudomonas putida* in a manner which is well known *per se*, preparations may be obtained having an exceptionally high amidase activity.

The micro-organism used in the method of the invention is described in Bergey's Manual of determinative bacteriology (Baltimore 1975).

Preferred strains are *Pseudomonas putida* ATCC 12633, ATCC 25571, ATCC 17390, ATCC 17426 and ATCC 17484.

These strains are available from American Type Culture Collection, Washing DC, U.S.A.

The strain ATCC 12633 is a particularly preferred micro-organism and mutants thereof are also suitable.

Pseudomonas putida is described in the literature as capable of producing mandelic acid racemase.

It is, therefore, surprising that *Pseudomonas putida* produces an enzyme having a stereo-specific amidase activity, and does not cause racemization of e.g. phenylglycine which is closely related to mandelic acid. This is no less surprising in view of a publication to the effect that *Pseudomonas putida* preparations are capable of oxidizing L(+)-mandalate to benzoyl formate (viz. that *P. putida* produces L(+)-mandalate dehydrogenase). The effects are exerted in *different places* in *different molecules*.

The micro-organism used in the method of the invention can be cultivated in ordinary nutrient media, e.g. as described by Hegeman in Journal of Bacteriology, 91, page 1140 (1966).

The micro-organism is preferably cultivated at a temperature within the range of from 30 to 35°C under aerobic conditions.

In most cases, the addition of growth factors or inductors is unnecessary. The addition of yeast extract appears to have a favourable influence on the production of the enzyme. After an incubation period of between about 2 and about 30 hours, the cells may be harvested, preferably during the period of exponential growth.

A preparation having α-amino acyl amidase activity may be obtained by precipitating the cells, optionally by using a flocculating agent. The cells may also be cross-linked or bonded to or absorbed on a carrier. In some cases, it may be desirable to modify the cell walls, e.g. by a heat treatment, to render the enzyme more accessible. A crude preparation may also be obtained by destroying the cells and recovering the enzyme by extraction, filtration and optionally spray-drying.

A preparation consisting of pure enzyme may be recovered in a conventional manner from the crude product described above. Pure enzyme or enzyme preparations may also be obtained from the culture medium by well known techniques.

The invention also relates to a process of preparing L-α-amino acid and/or D-α-amino acid amide from DL-α-amino acid amide by contacting said DL-α-amino acid amide with a preparation having L-α-amino acyl amidase activity.

This process is characterized in using a preparation obtained by cultivation of a strain of the micro-organism *Pseudomonas putida* in the presence of a nutrient medium containing assimilable sources of carbon, nitrogen, and phosphorus.

The preparation having L-α-amino acyl amidase activity is preferably contacted with the DL-α-amino acid amide in an aqueous medium at a temperature of between 0 and 60°C, and most preferred at a temperature of between 20 and 40°C, and at a pH value of between 6 and 10.5, and more preferred of between 7.5 and 9.5.

Outside these ranges, the activity and/or the stability of the enzyme is generally insufficient for practical use. The enzyme may be activated in a well known manner, e.g. by the addition of a metal compound such as magnesium, manganese, or zinc compound.

The weight ratio of the (unpurified) enzyme to the substrate may vary within wide ranges, e.g. between 1:25 and 1:750. If a pure enzyme is used, a higher ratio may be utilized.

When the hydrolysis of the L-α-amino acid amide has been completed, the free acid may be separated from the remaining D-α-amino acid amide, and the latter compound may then be hydrolized so as to form D-α-amino acid.

The process of the invention is suitable for isolating optically active natural or synthetic α-amino acids such as the D- and/or L-form of phenylalanine, 3,4-dihydroxyphenylalanine, tyrosine, methionine, leucine, alanine, phenylglycine, 4-hydroxyphenylglycine, 4-alkoxyphenylglycine, and other substituted phenylglycines.

The invention will now be described in further detail with reference to the following preparations and examples.

Preparation I

Pseudomonas putida ATCC 12633 was incubated at 28 - 30°C in a flask placed on a rotating shaker. The growth was measured with a spectrophotometer at $\lambda = 680$ nm. A nutrient medium of pH 6.85 was prepared by mixing: 1 litre of distilled water, 8.95 g secondary sodium phosphate dodecahydrate, 3.4 g primary potassium phosphate, 1.0g ammonium sulphate, 200 mg nitrilotriacetic acid, 580 mg magnesium sulphate heptahydrate, 67 mg calcium chloride dihydrate, 2.0 mg ferrous sulphate heptahydrate, 0.2 mg ammonium paramolybdate, and 1 ml "Hunter's metals 44" [a diluted solution of zinc, iron, manganese and copper sulphate, cobalt nitrate, sodium perborax, and E.D.T.A. - described in J. Cellular & Compar. Physiol. *49*, pp. 25-68 (1957)]. The mixture was then sterilized and subsequently

cooled, and finally 2 g asparagine and 2 g DL-mandelic acid were added.

The cells were harvested during the exponential growth phase by centrifugation (30 minutes at 10,000 rpm with cooling). The solid thus obtained was washed with 0.1 molar phosphate buffer at a pH of 6.8 and once again centrifuged (20 minutes at 10,000 rpm). The solid was suspended in the phosphate buffer (40 g wet cells in 100 ml buffer), whereafter the cell walls were destroyed with an ultrasonic cell disintegrator (20 kc/s for 20 minutes at 0°C). A crude extract was obtained by removing the solid particles by centrifugation (30 minutes, 10,000 rpm at 4°C). The yield of cell extract, calculated as dry substance, amounted to 0.8 g per litre of culture liquid.

Preparation II

The production of Preparation I was repeated, except that a medium containing 10 g of yeast extract (added before the sterilization) was used instead of asparagine. The yield of cell extract amounted to approximately 1.2 g of dry substance per litre of culture liquid.

Preparation III

The procedure of Preparation I was repeated, except that a culture medium containing 10 g of yeast extract was used instead of asparagine and mandelic acid. The yield of cell extract amounted to 1.25 g of dry substance per litre of culture liquid.

Example I

In a flask provided with a stirrer, 1.5 ml of 0.125 molar $MgCl_2$, 1/2 ml of 0.025 molar $MnCl_2$, and 0.1 ml crude cell extract (dry weight 7 mg) prepared as described in Preparation III were added to a solution of 2.0 g (13.3 mgmoles) of L-phenylglycineamide in 48 ml of water, with stirring, at 25°C. During the reaction the pH of the reaction mixture rose from 9.6 to 9.7.

After 20 hours, the reaction mixture was acidulated with 4N hydrochloric acid to a pH of 6.5. L-phenylglycine which then crystallized out was removed by filtration on a glass filter and washed on the filter with 2 portions of 10 ml of water and and subsequently with 2 portions of 10 ml of acetone. After drying, 1.6 g of L-phenylglycine (yield: 80%) were obtained.

The specific rotation of the L-phenylglycine was: $[\alpha]_D^{20} = 157.7°$ (C = 1.6; 2.6 % by weight of HCl).

From literature it is known (see Beilstein *14* III, page 1188) that $[\alpha]_D^{20} = 157.5$ (C = 1.6; 2.6 % by weight of HCl).

Example II

In a flask provided with a stirrer, 4.0 g (26.6 mgmoles) of DL-phenylglycineamide, 3 ml of 0.125 molar $MgCl_2$, 1 ml of 0.025 molar $MnCl_2$ and 1 ml of crude cell extract (dry weight 70 mg) prepared as described in Preparation III were added to 97 ml of water. This mixture was subsequently stirred for 20 hours at 25°C.

After said reaction time, the L-phenylglycine formed was removed by filtration, and the filtrate passed over 75 ml of Dowex 21 K exchanger in the OH^- form. Next, the exchanger was washed with 150 ml of water and the combined eluates concentrated by evaporation (40°C; 12 mm Hg). 1.8 g of D-phenylglycineamide (yield: 90%) were obtained. This product was pure according to thin-layer chromatography. The word "Dowex" is a Trade Mark.

In order to determine the optical purity and for a comparison with the literature, the amide was converted into the corresponding hydrochloric acid salt. To this end, 1.0 g of D-phenylglycineamide was dissolved in 20 ml of methanol, followed by filtration and addition of 1.5 ml of concentrated hydrochloric acid to the filtrate. 20 ml of acetone were then added, the D-phenylglycineamide, HCl formed then filtered on a glass filter and washed on the filter with 2 portions of 20 ml of acetone. 0.9 g of D-phenylglycineamide, HCl was obtained.

The specific rotation was:

$[\alpha]_D^{20} = 101.2°$ (C = 0.8; water).

It appears from the literature (Beilstein *14* III, page 1189) that $[\alpha]_D^{20} = 100.8°$ (C = 0.8; water).

Example III

In this example the rates of hydrolysis of L-phenylglycineamide and L-leucine amide are compared after 36 and 60 minutes when using an enzyme preparation obtained from *Pseudomonas putida*.

To a solution of 150.0 mg (1.0 mgmole) of L-phenylglycineamide in 15 ml of water, 0.5 mg of $MnCl_2$ and 2 mg of $MgCl_2$ and, subsequently, 0.10 ml of crude cell extract (7 mg dry weight) prepared as described in Preparation III were added. Following make-up with water to 25.0 ml samples were taken after 36 and 60 minutes, and the number of mgmoles of L-phenylglycine contained in each sample was determined by amino acid analysis.

In the same way and with a similar amount of cell extract, 130.0 mg (1 mgmole) of leucine amide were converted. Here, again, samples were taken after 36 and 60 minutes.

Results:

	36 minutes	60 minutes
L-phenylglycine-amide	0.63 mgmole/25 ml	0.88 mgmole/25 ml
L-leucineamide	0.16 mgmole/25 ml	0.28 mgmole/25 ml

Example IV

1.0 mgmole of each of the following α-amino acid amides was converted at 20°C with 0.1 ml of crude cell extract (dry weight 7 mg), prepared as described in Preparation III, in 5 ml of water in which 0.5 mg of $MnCl_2$ and 2.0 mg of $MgCl_2$ had been dissolved. After 3 and 18 hours, an amino acid analysis was conducted.

Results:

	% by weight of amino acid	
	after 3 hours	*after 18 hours*
L-phenylglycineamide	98 %	99 %
L-methionineamide	97 %	99 %
L-p-hydroxyphenylglycineamide	92 %	98 %
L-leucineamide	57 %	98 %
DL-α-amino-δ-cyanovaleramide	42 %	51 %
L-phenylalanineamide	34 %	96 %
L-tyrosineamide	29 %	96 %
Glycineamide	1 %	3 %
DL-α-aminocaprolactam	0 %	0 %

Example V

Substrate solutions each having the following composition were prepared: 5 ml water, 100 mg L-phenylglycineamide, 0.5 mg $MnCl_2$ and 2 mg $MgCl_2$.

Substrates of said composition were treated with 0.1 ml of crude cell extract (dry weight 2 mg) of each of the micro-organisms set forth in the following table. After 1/2 and 1 1/2 hours, an amino acid analysis was carried out.

	% by weight of amino acid	
	after 1/2 hour	*After 1½ hours*
Aspergillus oryzae	0 %	1 %
Aspergillus parasiticus	0 %	1 %
Bacillus subtilis	1 %	2 %
Pseudomonas putida	34 %	72 %
Bacillus stearothermophilus	2 %	3 %
Aeromonas proteolitica	21 %	48 %

As will appear from the above table, the enzyme preparation obtained by the cultivation of *Pseudomonas putida* has a significantly higher activity than the enzyme preparations obtained by the cultivation of the remaining micro-organisms.

Example VI

The selective hydrolysis to form L-4-hydroxyphenylglycine was determined by dissolving 0.071 g (0.43 millimoles) DL-4-hydroxyphenylglycineamide in 24 ml water and adding to this solution 25.1 mg of an enzyme preparation obtained by spray-drying the culture liquid from a culture of *Pseudomonas putida*. The pH of the mixture was 8.2. The mixture was maintained at 30°C with stirring for three hours. Every thirty minutes, a 2 ml sample was taken. The sample was diluted with 2 ml of 0.333 N sulfuric acid, and the content of L-4-hydroxyphenylglycine was determined by amino acid analysis. From these data, the amount of the L-amino acid amide which had been hydrolyzed could be calculated. The results are summarized below. No hydrolysis of the D-amino acid amide occurred.

time (hours)	mole % of L-4-hydroxyphenylglycineamide hydrolysed
0.5	65
1	88
1.5	93
2	99
2.5	99
3	99

Example VII

The hydrolysis of DL-4-methoxyphenylglycineamide was determined as described in Example VI using 1.36 g DL-4-methoxyphenylglycineamide, 43 ml water and 49.3 mg of the enzyme preparation obtained by spray-drying a *Pseudomonas putida* culture liquid.

Sampling and determination of the L-amino acid were also carried out as described above.

No hydrolysis of the D-amino acid amide occurred.

time (hours)	mole % of L-4-methoxyphenylglycineamide hydrolysed
0.5	18
1.0	41
1.5	61
2.0	77
2.5	91
3	99

WHAT WE CLAIM IS:

1. The use as an L-α-amino acyl amidase of a preparation, having L-α-amino acyl amidase activity, obtained by cultivation of a strain of *Pseudomonas putida* in the presence of a nutrient medium containing assimilable sources of carbon, nitrogen and phosphorus.

2. A process of preparing L-α-amino acid from L-α-amino acid amide, which process comprises contacting the L-α-amino acid amide with a preparation, having L-α-amino acyl amidase activity, obtained by the cultivation of a strain of *Pseudomonas putida* in the presence of a nutrient medium containing assimilable sources of carbon, nitrogen and phosphorus.

3. A process of preparing L-α-amino acid and/or D-α-amino acid amide from DL-$\alpha\partial$ amino acid amide, which process comprises contacting the DL-α-amino acid amide with a preparation, having L-α-amino acyl amidase activity, obtained by cultivation of a strain of *Pseudomonas putida* in the presence of a nutrient medium containing assimilable sources of carbon, nitrogen and phosphorus.

4. A process according to Claim 2 or 3, wherein the strain used is *Pseudomonas putida* ATCC 12633 or a mutant thereof.

5. A process of preparing L-α-amino acid, substantially as described in foregoing Example I.

6. A process of preparing L-α-amino acid and D-α-amino acid amide, substantially as described in foregoing Example II.

7. A process of preparing L-α-amino acid, substantially as described in foregoing Example III.

8. A process of preparing L-α-amino acid and D-α-amino acid amide, substantially as described in foregoing Example IV.

9. A process of preparing L-α-amino acid, substantially as described in foregoing Example V.

10. A process of preparing L-α-amino acid and D-α-amino acid amide, substantially as described in foregoing Example VI.

11. A process of preparing L-α-amino acid and D-α-amino acid amide, substantially as described in foregoing Example VII.

12. L-α-amine acid or D-α-amino acid amide whenever prepared by the process of any one of Claims 2 to 11.

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