

PATENT SPECIFICATION

NO DRAWINGS

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COMPLETE SPECIFICATION

Enzymes

5 We, BEECHAM GROUP LIMITED, a British Company, of Beecham House, Great West Road, Brentford, Middlesex 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:—

10 The present invention relates to an enzyme preparation, and particularly one prepared from deacylase enzymes known to split the amido bond in penicillins.

15 It is an object of the present invention to provide water-insoluble enzyme preparations that can be recovered from the reaction for re-use and are a convenient form for storing and transporting the enzyme. Also the resulting 6-aminopenicillanic acid is purer than when water-soluble penicillin amidase is used, 20 in its preparation from penicillins, since traces of the enzyme itself are not present in the product.

25 Accordingly the present invention provides a water-insoluble enzyme preparation comprising a deacylase enzyme known to split the amido bond in penicillins, and bonded to a water-insoluble inert polymeric material.

30 Preferably the deacylase enzyme is obtained from bacteria, such as strains of *Escherichia coli*, when used for the splitting of benzylpenicillin into 6-aminopenicillanic acid; or for example from fungi and actinomycetes when used for the splitting of phenoxymethylpenicillin.

35 Preferred polymeric materials include bromoacetylcellulose and the azido derivative of carboxymethylcellulose. The water-insoluble derivatives may, for example, be prepared by the methods described in British Patent 40 Specifications Nos. 916,931 and 1,062,596.

[Price 5s. 0d.]

The enzyme may be reacted for example with bromoacetylcellulose or coupled with CM-Cephadex ("Sephadex" is a registered trade mark) in presence of a suitable amide-forming reagent, such as a carbodiimide. Other suitable polymeric materials include certain activated ion-exchange resins, such as the acid chloride of Zeokarb type 226, and ethylene-maleic anhydride resins cross-linked with hydrazine. The water-insoluble derivatives retain the activity of the parent enzyme, but in use remain in the water-insoluble form, and so may be used in continuous processes in columns and other equipment.

55 The invention also provides a process for preparing 6-aminopenicillanic acid, which process comprises contacting benzylpenicillin or phenoxymethylpenicillin or a non-toxic salt thereof with a water-insoluble enzyme preparation as defined above, in presence of water, 60 separating the resulting solution from the solid material, and recovering the 6-aminopenicillanic acid therefrom.

65 With the deacylase enzyme produced by *E. coli*, the water-insoluble preparation may be obtained by first isolating and purifying the free enzyme and then coupling it with the polymeric material. Isolation of the enzyme may be effected by separating cells of *E. coli* from a fermented medium, killing the 70 cells with aqueous formaldehyde, and harvesting them by filtration or centrifugation. The cells are then disrupted and the enzyme is separated from cell debris by centrifugation, and purified by ammonium sulphate precipitation and subsequent chromatography. Enzyme thus obtained is found to be pure by electrophoresis and it has high specific activity in 75 splitting benzylpenicillin.

The following Examples illustrate the in- 80

vention. In these Examples an amidase unit is numerically equivalent to the quantity of 6-aminopenicillanic acid (expressed as $\mu\text{g}/\text{ml}$.) produced in the conditions of the assay which where, the enzymation of 3.3% benzylpenicillin for 1 and 1/2 hours at pH 7.9 and 37°C.

EXAMPLE 1

The enzyme obtained from *E. coli* was agitated in aqueous solution with bromoacetylcellulose at room temperature for 30 hours and at pH 5.8 to give preparations having activities of 30,000 to 250,000 deacylase units/g. These materials were then kept at 4°C and pH 8.9 for 12 hours to give preparations having activities of 13,000—100,000 deacylase units/g. The enzyme preparations were freed from unreacted bromine by treatment with 0.05 M ethanolamine in water at 4°C for 24 hours, then they were washed twice with 0.15 M aqueous sodium chloride, followed by water.

With one preparation obtained in this way having an activity of 40,000 units/g. dry bromoacetylcellulose, four successive enzymations of 2% aqueous benzylpenicillin were carried out under standard conditions. Each enzymation efficiency was satisfactory and the final preparation which retained activity was satisfactory for re-use; over 98% pure 6-aminopenicillanic acid was isolated from the reaction mixtures.

EXAMPLE 2

The azido derivative of carboxymethylcellulose was prepared by the known way.

Then 30g. of azido-carboxymethylcellulose was stirred in 3,000 ml. 0.05 M phosphate buffer at pH 8.7 and to this was added 5×10^8 units of pure amidase enzyme (from *E. coli*). The mixture was stirred at 4°C for 24 hours. The active preparation was centrifuged and the solid was washed successively with 1 M NaCl, 0.5 M NaHCO_3 , 1 M NaCl and finally with water. The solid after final centrifugation was found to have an activity of 100,000 units/g. dry azido-carboxymethylcellulose.

A further preparation using the above-described method and which had an activity of 34,300 units/g. dry azido-carboxymethylcellulose was used for two consecutive enzymations of 2% benzylpenicillin on a 400 ml. scale at pH 7.8, 37°C. The active preparation was recovered at the end of the first enzymation for re-use in the second; comparable enzymation efficiencies were obtained from the two experiments (67 and 70%, respectively).

EXAMPLE 3

200 mg. ethylene-maleic anhydride polymer resin (EMA 21) were stirred in 25 ml. phosphate buffer at pH 7.6 at 5°C. The cross linking agent, 2 ml. of 1% solution of hydrazine

hydrate was added, followed after 1—2 minutes by 10 mg. of pure amidase enzyme (from *E. coli* and having an activity of 5.7×10^7 units/g.) dissolved in a little phosphate buffer. The mixture was stirred in the ice bath for 2 hours and then kept overnight at 5°C. The active preparation was recovered and washed as described in Example 2 and found to have an activity of 360,000 units/g. dry resin.

Further experiments using the same method gave activities in the range of 100,000 to 600,000 units/g. dry resin.

EXAMPLE 4

Zeokarb—226 was converted to the acid chloride by refluxing with thionyl chloride and dimethylformamide in chloroform. The chlorine assay was 14%. To 30 ml. of phosphate buffer at pH 7.6 containing 250,000 units of pure amidase enzyme (from *E. coli*) was added 1.0 g. of the Zeokarb—226 acid chloride and the reaction mixture was stirred for 5 hours at 5°C. The active preparation was recovered and washed as described in Example 2 and found to have an activity 119,000 units/g. dry Zeokarb—226 acid chloride.

EXAMPLE 5

Zeokarb—225 was converted to the sulphonyl chloride by refluxing with phosphorus oxychloride. The chlorine assay was 12.5%. To 30 ml. of phosphate buffer at pH 8.5 containing 270,000 units of pure amidase enzyme (from *E. coli*) was added 1.0 g. of the Zeokarb—225 sulphonyl chloride and the reaction mixture was stirred for 5 hours at 5°C. The active preparation was recovered and washed as described in Example 2 and found to have an activity of 4,800 units/g. dry Zeokarb—225 sulphonyl chloride.

EXAMPLE 6

Carboxymethylcellulose was converted to the acid chloride by reaction with thionyl chloride and dimethylformamide in pyridine. The chlorine assay was 9.9%. To 30 ml. of phosphate buffer at pH 8.5 containing 270,000 units of pure amidase enzyme (from *E. coli*) was added 1.0 g. of the carboxymethylcellulose acid chloride and the reaction mixture was stirred for 2 and 1/2 hours at 5°C. The active preparation was recovered and washed as described in Example 2 and found to have an activity of 11,900 units/g. dry carboxymethylcellulose acid chloride.

WHAT WE CLAIM IS:—

1. A water-insoluble enzyme preparation comprising a deacylase enzyme known to split the amido bond in penicillins and bonded to a water-insoluble inert polymeric material.
2. An enzyme preparation as claimed in claim 1 wherein the deacylase enzyme is derived from bacteria.

vention. In these Examples an amidase unit is numerically equivalent to the quantity of 6-aminopenicillanic acid (expressed as $\mu\text{g}/\text{ml}$.) produced in the conditions of the assay which where, the enzymation of 3.3% benzylpenicillin for 1 and 1/2 hours at pH 7.9 and 37°C.

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WHAT WE CLAIM IS:—

1. A water-insoluble enzyme preparation comprising a deacylase enzyme known to split the amido bond in penicillins and bonded to a water-insoluble inert polymeric material.
2. An enzyme preparation as claimed in claim 1 wherein the deacylase enzyme is derived from bacteria.



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3. An enzyme preparation as claimed in claim 2 wherein the deacylase enzyme is derived from *Escherichia coli*.

5 4. An enzyme preparation as claimed in claim 1 wherein the deacylase enzyme is derived from fungi or actinomycetes.

10 5. An enzyme preparation as claimed in claims 1 to 4, wherein the water-insoluble polymeric material is bromoacetylcellulose, azido-carboxymethylcellulose, ethylene-maleic anhydride polymer resin cross-linked with hydrazine or carboxymethylcellulose acid chloride.

15 6. A process for preparing an enzyme preparation as claimed in claim 1 substantially as described with reference to any one of the specific Examples hereinbefore set forth.

7. A process for preparing 6-amino-

penicillanic acid which process comprises contacting benzylpenicillin or phenoxy methylpenicillin or a non-toxic salt thereof with an enzyme preparation as claimed in any one of claims 1 to 6 in presence of water separating the resulting solution from the solid material and recovering the 6-aminopenicillanic acid therefrom. 20

8. A process for preparing 6-aminopenicillanic acid substantially as described with reference to any one of the specific Examples hereinbefore set forth. 25

9. 6-Aminopenicillanic acid when prepared by a process as claimed in claim 7 or claim 8. 30

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