

PATENT SPECIFICATION

(11) 1357317



009

1357317

- (21) Application No. 41262/70 (22) Filed 27 Aug. 1970
 (21) Application No. 36601/71 (22) Filed 4 Aug. 1971
 (23) Complete Specification filed 25 Aug. 1971
 (23) Complete Specification published 19 June 1974
 (51) International Classification C07G 7/02 C07D 99/14
 (52) Index at acceptance

C3H 3
 C2A. 1C2C 2C1

- (72) Inventors THOMAS ADRIAN SAVIDGE
 LAWSON WILLIAM POWELL and
 MALCOLM DOUGLAS LILLY

(54) ENZYMES

(71) 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:—

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

It is an object of the present invention to provide water insoluble enzyme preparations that can be recovered from a reaction medium for re-use, and are a convenient form for storing and transporting the enzyme. Also when 6-aminopenicillanic acid is prepared by using the preparations, it is purer than when water-soluble penicillin deacylase is used, since traces of the enzyme itself are not usually present in the product.

Additionally, the enzyme preparation may be used for the enzymatic resolution of certain N-acyl-DL-amino acids.

Accordingly the present invention provides a water-insoluble enzyme preparation comprising a deacylase enzyme known to split the amido bond in penicillins absorbed on a water-insoluble absorbent material and rendered water-insoluble by means of a water-soluble dialdehyde, the said preparation having an activity as hereinafter defined.

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

Preferably the water-soluble dialdehyde is glyoxal or glutaraldehyde.

The water-insoluble absorbent material is preferably a finely divided polymeric material, since the enzyme preparation will

have a greater activity. The water-insoluble absorbent material must have properties such that the enzyme preparation of the invention has the desired activity which is defined as requiring that (a) at least 25 g. of the final enzyme preparation when packed in a 12-inch×1-inch column will resolve 5 ml. per hour of 0.5% w/v aqueous DL-phenylacetamido-p-hydroxyphenylglycine (PAPG) solution with at least 90% efficiency after at least 12 hours at 37°C; and (b) at least 50g. of the enzyme preparation must convert in a stirred tank reactor at least 70% of the potassium benzylpenicillin in 100 ml. of a 1% w/v aqueous solution at pH 7.8 to 6-aminopenicillanic acid after 6 hours at 37°C.

Suitable absorbent materials include cellulose powder and various cellulose derivatives, ion exchange resins, silica gel, colloidal silica and polymeric materials.

A particularly suitable polymeric material is nylon, which is preferably in finely divided form. A preferred form is nylon 6.6 having a low mean molecular weight, and having a particle size such that it will pass a 500 micron sieve and having a high content of amino end groups, for example, 200—500 gram-equivalents per 10⁶ grams. The nylon may be partially hydrolysed, for example with dilute mineral acid, to increase the number of free amino groups available for attachment to the enzyme by the dialdehyde.

The invention also provides a process for preparing a water-insoluble enzyme preparation as defined above, which process comprises contacting in aqueous solution a water-insoluble absorbent material with a penicillin deacylase and with a water-soluble dialdehyde.

Preferably the aqueous solution is at pH 5—9.

The water-absorbent material may be first contacted with the deacylase or with the dialdehyde or with both simultaneously.

When the absorbent material is first con-

[Price 25p]

tacted with the enzyme in aqueous solution, a residence time of 12 to 24 hours is desirable for good absorption. Then the product is contacted with the dialdehyde usually for 1 to 24 hours at 0 to 30°C. When the absorbent is contacted first with the dialdehyde and then with the enzyme, or with both simultaneously, similarly prolonged contact is desirable. The solid product may be washed with water to remove unbound protein and unreacted glutaraldehyde.

When the support does not have chemical groupings capable of reacting with the dialdehyde, the absorbed enzyme molecules will be cross-linked to each other by the dialdehyde. When the support does contain groupings capable of reacting with the dialdehyde, there will be covalent cross-linking of the enzyme molecules to the support by the dialdehyde in addition to the cross-linking of the enzyme molecules to each other.

The following Examples illustrate the invention, except for Examples 1 and 2, which describe the preparation of a penicillin deacylase.

EXAMPLE 1

A partially purified preparation of penicillin deacylase was prepared by releasing enzyme from the cells of a penicillin-deacylase producing strain of *Escherichia coli* by mechanical means in a homogeniser, centrifuging, treating the supernatant with ammonium sulphate to 40% saturation, rejecting the precipitate, adding further ammonium sulphate to 75% saturation, collecting the precipitate and dissolving it in water, and then dialysing against water and freeze drying.

EXAMPLE 2

A partially purified preparation of penicillin deacylase was prepared by releasing enzyme from the cells of a different though similar strain of *E. coli* to that used in Example 1, adjusting to pH 4.5 with acetic acid and removing the cell debris by filtration or centrifugation.

EXAMPLE 3

25 g. of diethylaminoethylcellulose (Whatman "Chromedia" DE-52, Whatman and Chromedia being Registered Trade Marks) was suspended in 100 ml. of 0.1M sodium borate buffer at pH 8.0 and partially purified penicillin deacylase (2.5g.) was added. The mixture was gently stirred at room temperature for 24 hours. Glutaraldehyde was then added to 5% (w/v) and stirring was continued for a further hour. The solid was then recovered by filtration, rinsed with a small amount of buffer solution and then exhaustively washed with M sodium chloride solution followed by 0.1M sodium hydroxide solution and M ammonium chloride buffer

solution at pH 8.6. The cellulose was left in the buffer solution overnight and then removed and washed with M sodium chloride solution and distilled water. The preparation was found to have sufficient activity to resolve 5 ml./hr. of 0.5% w/v PAPG solution at 100% efficiency for 20 hours.

EXAMPLE 4

The procedure of Example 3 was repeated with fibrous cellulose powder (Whatman "Chromedia" CF-1) in place of the diethylaminoethylcellulose. The preparation was found to have sufficient activity to enzymate 5 ml./hr of 0.5% w/v PAPG solution at 100% efficiency for 18 hours, and 10 g. of preparation converted at 100% efficiency 32-34 ml. of 5% w/v substrate per hour for 18 hours.

EXAMPLE 5

The procedure of Example 3 was repeated with carboxymethylcellulose (Whatman "Chromedia" CM-52) as support, and 2 g. of enzyme was used instead of 2.5g. The preparation was found to have sufficient activity to enzymate 5 ml./hour of 0.5% w/v PAPG solution at 100% efficiency for 19 hours.

EXAMPLE 6

The procedure of Example 3 was repeated with 25 g of "Ecteola" cellulose (epichlorhydrin triethanolamine cellulose) (Whatman "Chromedia" ET-11) as the support. In this experiment the pH of the buffer solution was 8.75 instead of pH 8.0. The preparation was found to have sufficient activity to resolve 5 ml./hour of 0.5% w/v PAPG solution at 100% efficiency for 19 hours.

EXAMPLE 7

5 g. of diethylaminoethyl-Sephadex A50 (Sephadex being a Registered Trade Mark) was allowed to swell for 3 days in 0.1M sodium borate buffer solution at pH 8.0. Excess buffer solution was then removed by decantation and 1 g. of the enzyme of Example 1 was added. The whole was stirred for 24 hours at room temperature. Glutaraldehyde was then added to 5% (w/v) and allowed to react with stirring for 1 hour. The product was washed as described in Example 3. The preparation was capable of resolving 5ml/hour of 0.5% w/v PAPG solution at 100% efficiency for 20 hours.

EXAMPLE 8

25 g. of Amberlite IR-45(OH) (Amberlite being a Registered Trade Mark) resin was suspended in 100 ml. of 0.1M phosphate buffer at pH 7.0 and 4 g. of partially-purified penicillin deacylase as prepared in Example 2 was added. The bonding, treatment with glutaraldehyde and subsequent

washing of the support were as described in Example 3. It was found that:

(I) 25 g. of preparation resolved 70 ml./hour of 5% w/v PAPG solution at 100% efficiency for 300 hours.

(II) 40 g. of preparation was used to carry out a series of 3 enzymations of 4% w/v potassium benzylpenicillin solution at pH 7.8 and 37°C on a 400 ml scale. Enzyme was recovered by filtration in each case. In all 3 enzymations substrate was converted to 6-aminopenicillanic acid after 6 hours.

EXAMPLE 9

200 g. of Amberlite IR-45(OH) was suspended in 1 l of a clarified *E. coli* homogenate prepared as described in Example 2, which had been buffered at pH 7 by the addition of 16.4 g. disodium hydrogen phosphate and 1.1 g. sodium dihydrogen phosphate. The whole was stirred for 24 hours at room temperature. Glutaraldehyde was then added to 5% (w/v) and allowed to react for 2 hours. The solid was recovered, washed with 5% w/v sodium bisulphite solution and then with 0.1M sodium chloride solution and water. The preparation resolved 80-100 ml./hour of a 5% w/v PAPG solution at 100% efficiency for 300 hours without detectable loss of activity.

EXAMPLE 10

200 g. of Amberlite IR-45(OH) was suspended in a clarified *E. coli* cell homogenate prepared as described in Example 2, and the pH was adjusted to 5.0. After stirring for 24 hours at room temperature the pH was adjusted to 8.0 and glutaraldehyde was added to 1% (w/v). Subsequent procedure was as described in Example 9. The preparation resolved 35 ml./hour of 5% w/v PAPG solution with 100% efficiency for over 300 hours. 20 g. of preparation was used to deacylate enzymatically 50 ml. of 2% w/v potassium benzylpenicillin solution at pH 7.8 and 37°C in two successive experiments. In both 80% conversion of the substrate to 6-aminopenicillanic acid was achieved after 3 hours.

EXAMPLE 11

The procedure of Example 3 was repeated, except that 3% (w/v) glutaraldehyde was used, with silica gel as adsorbent. The preparation was found to be capable of resolving 5 ml./hour of 0.5% w/v PAPG solution for 20 hours with 100% efficiency.

EXAMPLE 12

25 g. of aluminium oxide (Brockmann Grade II) was suspended in 100 ml. of distilled water and 1.5 g. of the enzyme of Example 1 was added. The pH was adjusted to 5.0 and the whole was agitated for 24 hours at room temperature. Glutaraldehyde

was then added to 2% (w/v) and the pH was adjusted to 8.0. After further agitation for 1 hour the solid was recovered and washed as described in Example 3. The preparation was found to be capable of resolving 5 ml./hour of 0.5% w/v PAPG solution with 100% efficiency for 15 hours; 21 g. of the preparation was used to carry out two successive enzymic deacylations of 1% w/v potassium benzylpenicillin solution at pH 7.8 and 37°C in a 400 ml. stirred reactor. In both enzymations, over 90% of the substrate was converted to 6-aminopenicillanic acid.

EXAMPLE 13

10 g. of colloidal silica ("Ludox" HS-40, Ludox being a Registered Trade Mark) was suspended in 50 ml. of 0.1M phosphate buffer solution at pH 7.0 containing 0.1 g./ml. of partially purified penicillin acylase as prepared in Example 2. The whole was shaken gently for 24 hours. Glutaraldehyde was then added to 5% (w/v) and shaking was continued for a further hour. The solid was then recovered by filtration and washed as described in Example 3. The enzyme preparation was found to enzymate 5 ml./hour of 0.5% PAPG solution at 100% efficiency for 20 hours.

EXAMPLE 14

To 32 ml. of distilled water were added 7.6 g. of acrylamide, 0.4 g. of N,N'-methylene-bisacrylamide, 18 mg. of ammonium persulphate and 20 μ l. of β -dimethylamino-propionitrile. The mixture was carefully de-aerated and a stiff gel formed, which was passed through a 30-mesh sieve.

20 g. of polyacrylamide gel and 2g. of partially-purified penicillin acylase as prepared in Example 1 were made up in 50 ml. of 0.1M sodium borate buffer at pH 8.0 and agitated for 24 hours at room temperature. Glutaraldehyde was then added to 5% (w/v) and allowed to react for 1 hour. The preparation was then washed as described in Example 3. The insoluble preparation bound sufficient enzyme to enzymate 5 ml./hour of 0.5% w/v PAPG solution at 100% efficiency for 20 hours.

EXAMPLE 15

The method described in Example 8 was used to attach the enzyme to Amberlite IR-45(OH) except that in place of glutaraldehyde, glyoxal was used at a concentration of 5% (w/v). The preparation resolved 5 ml./hour of 0.5% w/v PAPG solution at 100% efficiency for 20 hours.

EXAMPLE 16

10 g. of 'Amberlite' XAD 7 (a macroreticular acrylic ester resin) was stirred in 100 ml. of phosphate buffer at pH 5.0 con-

taining 1 g. of penicillin acylase for 16 hours at room temperature. The pH was then raised to 8.0 and 10 ml. of 50% v/v glutaraldehyde was added and the mixture was stirred for 2 hours at room temperature. The solid was recovered by filtration, washed with a 5% w/v solution of sodium bisulphite, then with 0.5M phosphate buffer at pH 7.8 and finally with water. This preparation was used for the enzymatic deacylation of 6% w/v potassium benzylpenicillin (160 ml. scale). In three successive experiments with the same material over 80% substrate was converted to 6-aminopenicillanic acid in 4 to 5 hours.

EXAMPLE 17

25g. of diethylaminoethylcellulose (Whatman "Chromedia" DE-52) was suspended in 100 ml. of 0.1M phosphate buffer at pH 5.0 and 2.5 g. of penicillin deacylase was added. The mixture was stirred for 20 hours at room temperature. The pH was then raised to 8.0 and glutaraldehyde was added to 5% (w/v) and allowed to react with stirring for 1 hour. The solid (40 g. moist wt) was recovered by filtration and washed by the method described in Example 9.

20 g. of this material was used to enzymatically deacylate 100 ml. of 2% w/v potassium benzylpenicillin solution in 2 hours at pH 7.8 and 37°C. In 5 successive experiments, 95% of the substrate was converted to 6-aminopenicillanic acid.

EXAMPLE 18

25 g. of Diethylaminoethyl cellulose (Whatman "Chromedia" DE-52) was suspended in 100 ml. of phosphate buffer at pH 8.0. Glutaraldehyde was added to 5% (w/v) and the mixture was stirred for 30 minutes. 2.5 g. of partially purified penicillin acylase was then added and the mixture was stirred for 2 hours. The solid product was then isolated by filtration and washed by the method described in Example 9.

20 g. of the treated enzyme were used to deacylate enzymatically 100 ml. of 2% w/v potassium benzyl penicillin solution in 2 hours at pH 7.8 and 37°C. In 2 successive experiments 95% of the substrate was converted to 6-aminopenicillanic acid.

EXAMPLE 19

The method described in Example 17 was repeated using aminoethyl cellulose (Cellex-AE, Cellex being a Registered Trade Mark) as the support in place of diethylaminoethylcellulose.

20 g. of the preparation was used to deacylate enzymatically 100 ml. of 1% w/v potassium benzylpenicillin solution in 2 hours at pH 7.8 and 37°C. In 2 successive experiments 95% of the substrate was converted to 6-aminopenicillanic acid.

EXAMPLE 20

2 g. of nylon (particle size 53—75 microns; 472 grams—equivalent of NH₂ groups per 10⁴ grams) was stirred with 200 ml. of enzyme solution (prepared by dissolving 240 mg. of enzyme prepared as described in Example 1 in 200 ml. of 0.05 M sodium phosphate buffer at pH 7.5) and 2 ml. of 25% aqueous glutaraldehyde at 3°C for 16 hours. The solid was collected by centrifugation and washed well with water.

The activity of the solid enzyme preparation using phenyl acetamide as the substrate was 13,950 μ mols per hour per gram.

4 g. of solid enzyme preparation made as described above was used to split 750 ml. of 15 m M solution of benzylpenicillin in 0.05 M sodium phosphate buffer at pH 8 and at 37°C. After 17 minutes 70% of the substrate had been converted to 6-aminopenicillanic acid, after 25 mins 90% and after 33 minutes 95%.

EXAMPLE 21

10 g. nylon (particle size 53—75 microns; 472 gram-equivalents of NH₂ groups per 10⁴ grams) was stirred with 50 ml. of 25% aqueous glutaraldehyde for 1 hour at 3°C. The solid was collected and washed with water to remove excess glutaraldehyde and 100 ml. of enzyme solution (prepared by dissolving 240 mg. of enzyme prepared as described in Example 1 in 100 ml. of 0.05 M sodium phosphate at pH 8.0) was added and stirred for 16 hours at 3°C. The solid was collected by centrifugation and washed well with water. The activity of the solid enzyme preparation using phenylacetamide as the substrate was 1,260 μ moles per hour per gram.

WHAT WE CLAIM IS:—

1. A water-insoluble enzyme preparation comprising a deacylase enzyme known to split the amido bond in penicillins absorbed on a water-insoluble absorbent material and rendered water-insoluble by means of a water-soluble dialdehyde, the said preparation having an activity as hereinbefore defined.

2. A water-insoluble enzyme preparation as claimed in claim 1, wherein the deacylase enzyme is derived from bacteria.

3. A water-insoluble enzyme preparation as claimed in claim 2, wherein the bacteria are *Escherichia coli*.

4. A water-insoluble enzyme preparation as claimed in any one of the preceding claims, wherein the water-insoluble absorbent material is a cellulose powder, or cellulose derivative, an ion-exchange resin, silica gel, colloidal silica or a polymeric material.

5. A water-insoluble enzyme preparation as claimed in claim 4, wherein the polymeric material is nylon.

4 taining 1 g. of penicillin acylase for 16 hours
at room temperature. The pH was then
raised to 8.0 and 10 ml. of 50% v/v glutar-
aldehyde was added and the mixture was
5 stirred for 2 hours at room temperature.
The solid was recovered by filtration, washed
with a 5% w/v solution of sodium bisul-
phite, then with 0.5M phosphate buffer at
pH 7.8 and finally with water. This prepa-
10 ration was used for the enzymatic deacyla-
tion of 6% w/v potassium benzylpenicillin
(160 ml. scale). In three successive experi-
ments with the same material over 80%
15 substrate was converted to 6-aminopenicillanic
acid in 4 to 5 hours.

EXAMPLE 17

25g. of diethylaminoethylcellulose (What-
man "Chromedia" DE-52) was suspended in
100 ml. of 0.1M phosphate buffer at pH 5.0
and 2.5 g. of penicillin deacylase was added.
The mixture was stirred for 20 hours at room
temperature. The pH was then raised to 8.0
and glutaraldehyde was added to 5% (w/v)
and allowed to react with stirring for 1 hour.
25 The solid (40 g. moist wt) was recovered
by filtration and washed by the method de-
scribed in Example 9.

20 g. of this material was used to enzy-
matically deacylate 100 ml. of 2% w/v
potassium benzylpenicillin solution in 2 hours
at pH 7.8 and 37°C. In 5 successive experi-
ments, 95% of the substrate was converted
to 6-aminopenicillanic acid.

EXAMPLE 18

35 25 g. of Diethylaminoethyl cellulose
(Whatman "Chromedia" DE-52) was sus-
pended in 100 ml. of phosphate buffer at
pH 8.0. Glutaraldehyde was added to 5%
(w/v) and the mixture was stirred for 30
40 minutes. 2.5 g. of partially purified peni-
cillin acylase was then added and the mixture
was stirred for 2 hours. The solid product
was then isolated by filtration and washed
by the method described in Example 9.

45 20 g. of the treated enzyme were used
to deacylate enzymatically 100 ml. of 2%
w/v potassium benzyl penicillin solution in
2 hours at pH 7.8 and 37°C. In 2 successive
50 experiments 95% of the substrate was con-
verted to 6-aminopenicillanic acid.

EXAMPLE 19

The method described in Example 17 was
repeated using aminoethyl cellulose (Cellex-
AE, Cellex being a Registered Trade Mark)
55 as the support in place of diethylaminoethyl-
cellulose.

20 g. of the preparation was used to
deacylate enzymatically 100 ml. of 1% w/v
potassium benzylpenicillin solution in 2 hours
at pH 7.8 and 37°C. In 2 successive experi-
60 ments 95% of the substrate was converted
to 6-aminopenicillanic acid.

EXAMPLE 20

2 g. of nylon (particle size 53—75 microns;
472 grams—equivalent of NH_2 groups per
 10^6 grams) was stirred with 200 ml. of
enzyme solution (prepared by dissolving 240
mg. of enzyme prepared as described in
Example 1 in 200 ml. of 0.05 M sodium
phosphate buffer at pH 7.5) and 2 ml. of
70 25% aqueous glutaraldehyde at 3°C for 16
hours. The solid was collected by centrifug-
ation and washed well with water.

The activity of the solid enzyme prepara-
tion using phenyl acetamide as the substrate
was 13,950 μ mols per hour per gram.

4 g. of solid enzyme preparation made as
described above was used to split 750 ml. of
15 M solution of benzylpenicillin in 0.05
M sodium phosphate buffer at pH 8 and at
37°C. After 17 minutes 70% of the sub-
strate had been converted to 6-aminopenicil-
lanic acid, after 25 mins 90% and after 33
minutes 95%.

EXAMPLE 21

10 g. nylon (particle size 53—75 microns;
472 gram-equivalents of NH_2 groups per 10^6
grams) was stirred with 50 ml. of 25%
aqueous glutaraldehyde for 1 hour at 3°C.
The solid was collected and washed with
water to remove excess glutaraldehyde and
100 ml. of enzyme solution (prepared by
dissolving 240 mg. of enzyme prepared as
described in Example 1 in 100 ml. of 0.05 M
sodium phosphate at pH 8.0) was added and
stirred for 16 hours at 3°C. The solid was
collected by centrifugation and washed well
with water. The activity of the solid enzyme
preparation using phenylacetamide as the
substrate was 1,260 μ moles per hour per
gram.

WHAT WE CLAIM IS:—

1. A water-insoluble enzyme preparation
comprising a deacylase enzyme known to
split the amido bond in penicillins absorbed
on a water-insoluble absorbent material and
rendered water-insoluble by means of a
water-soluble dialdehyde, the said prepara-
tion having an activity as hereinbefore de-
fined.

2. A water-insoluble enzyme preparation
as claimed in claim 1, wherein the deacylase
enzyme is derived from bacteria.

3. A water-insoluble enzyme preparation
as claimed in claim 2, wherein the bacteria
are *Escherichia coli*.

4. A water-insoluble enzyme preparation as
claimed in any one of the preceding claims,
wherein the water-insoluble absorbent
material is a cellulose powder, or cellulose
derivative, an ion-exchange resin, silica gel,
colloidal silica or a polymeric material.

5. A water-insoluble enzyme preparation
as claimed in claim 4, wherein the polymeric
material is nylon.



6. A water-insoluble enzyme preparation as claimed in claim 4 or claim 5, wherein the water-insoluble absorbent material is in finely divided form. 20
- 5 7. A water-insoluble enzyme preparation as claimed in any one of the preceding claims, wherein the water-soluble dialdehyde is glutaldehyde or glyoxal.
- 10 8. A process for preparing a water-insoluble enzyme preparation as claimed in claim 1, which process comprises contacting in aqueous solution a water-insoluble absorbent material with a penicillin deacylase and with a water-soluble dialdehyde.
- 15 9. A process as claimed in claim 8, wherein the aqueous solution is at pH 5—9.
- 10 11. A water-insoluble enzyme preparation when prepared by a process as claimed in any one of claims 8 to 10. 25
12. A process for preparing 6-aminopenicillanic acid, which process comprises treating benzylpenicillin or a salt thereof in aqueous solution with a water-insoluble enzyme preparation as claimed in any one of claims 1 to 7. 30

RONALD SMITHER,
Chartered Patent Agent.
Agent for the Applicants.

Printed for Her Majesty's Stationery Office, by the Courier Press, Leamington Spa, 1974.
Published by The Patent Office, 25 Southampton Buildings, London, WC2A 1AY, from which copies may be obtained.

