

PATENT SPECIFICATION

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DRAWINGS ATTACHED

- (21) Application No. 2170/69 (22) Filed 14 Jan. 1969
- (31) Convention Application No. P 17 92 043.3
- (32) Filed 16 July 1968 in
- (33) Germany (DT)
- (31) Convention Application P 18 08 042.7
- (32) Filed 9 Nov. 1968 in
- (33) Germany (DT)
- (45) Complete Specification published 11 Nov. 1970
- (51) International Classification C 07 g 7/028 A 61 k 19/00
- (52) Index at acceptance

C3H 3

A5B 311 31Y 351 35Y 38Y 39X

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(54) ENZYME PURIFICATION

(71) We, **FARBENFABRIKEN BAYER AKTIENGESELLSCHAFT**, a body corporate organised under the laws of Germany, of 509 - Leverkusen - Bayerwerk, Germany, 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 a process for the production of pyrogen - free L - asparaginase.

Our copending Patent Application No. 61394/68 (Serial No. 1,212,136) discloses 15 processes for the bacterial production of L - asparaginase and for its purification to a high state of purity. Although the L - asparaginase produced thus is very pure by the standards of the prior art, it contains pyrogens, which inhibit its clinical use. The present invention relates to a process for the removal 20 of pyrogens from L - asparaginase.

Although the process described herein is primarily intended for use with L - asparaginase produced according to the process described in our above mentioned Patent Application, we regard it as applicable to L - asparaginase produced by any method from any source.

30 The pyrogens in L - asparaginase prepared from bacterial cells may be carried over from these cells. Impurities having a pyrogenic action may also get into the L - asparaginase preparation during the isolation and purification of the enzyme. Despite many attempts, L - asparaginase free of pyrogens has not yet been obtained from bacterial sources; unfortunately these sources are the most promising for the production of L - asparaginase

on an industrial scale. The conventional methods for removing pyrogens, such as 40 filtration over clarifying layers, and adsorption on hydroxyl apatite, aluminium hydroxide, silicates and synthetic resins were unsuccessful.

Our copending Patent Application No. 2169/69 (Serial No. 1,212,138) discloses a method of stabilization of L - asparaginase by the use of amino acids. 45

Our copending Patent Application No. 61846/68 (Serial No. 1,212,137) discloses a process for the production of pyrogen-free L - asparaginase by adsorption on, and elution from swollen DEAE - dextran gel. 50

The present invention provides a process 55 for the production of pyrogen - free L - asparaginase as hereinafter defined which comprises heating a solution of L - asparaginase containing pyrogens to from 40 to 61°C. at a pH of from 7.0 to 9.2 in the presence of an aliphatic alcohol having up to four carbon atoms and/or of an amino acid, removing any precipitated material from the solution, and precipitating the L - asparaginase at pH 4.5 to 5.5 by the addition of polyethylene glycol. 60 65

The L - asparaginase may subsequently, if desired, be separated from the polyethylene glycol.

This process provides more efficient pyrogen removal than any previous method known to us, and in addition substantially increases the specific activity of the enzyme. The inert accompanying proteins found in impure preparations of L - asparaginase, which have a high antigenic action, and are difficult to remove by any other method, can be eliminated by this process. 70 75

The process of the invention is preferably applied to L - asparaginase which has already been brought to a purity of from 160 to 220 U/mg. or more as defined below. The starting solution of the enzyme preferably contains 0.5 to 15% enzyme. The alcohol is preferably present in a concentration of 4-40% of the solution, most preferably about 10%. When an amino acid is used it may be added at any temperature from -10°C. to +65°C. The amino acid is preferably present in a concentration of 1-10% of the solution, most preferably about 3%. The starting solution should preferably be adjusted to a pH of 8 to 9. Heating is preferably continued at 50°C. for from 1 to 5 days, after which any precipitated material is removed, for example by centrifugation. Such material comprises the denatured inert proteins. The pH of the clear solution is then adjusted to pH 4.5 to 5.5, preferably about 5.2, and polyethylene glycol added. A precipitate containing polyethylene glycol and L - asparaginase is formed, which is separated from the supernatant solution. The pyrogen - free L - asparaginase is then separated from the polyethylene glycol. Some methods for such separation are disclosed in our copending Patent Applications Nos. 61394/68 and 2169/69, (Serial Nos. 1,212,136 and 1,212,138) but any method may be used.

This invention enables pyrogen - free L - asparaginase as hereinafter defined having a purity of more than 200 U/mg. to be obtained for the first time. Preferably the L - asparaginase has a purity of at least 220 U/mg. Such L - asparaginase is within the scope of the present invention.

The L - asparaginase unit (herein abbreviated to "U") is defined in our copending Patent Application No. 61394/68. Throughout this specification the "1 minute unit" is used.

The term "pyrogen - free" L - asparaginase in the present specification means that with 200 units (1 minute) per kg. body-weight the mean temperature rise of at least three test rabbits is less than 0.5°C.

The invention also provides as a new pharmaceutical composition, a parenterally injectable solution of pyrogen - free L - asparaginase produced by the process of the invention.

The invention further provides, as a new medicament in dosage unit form, ampoules of a parenterally injectable solution of pyrogen - free L - asparaginase produced by the process of the invention.

The term "medicament in dosage unit form" as used in the present specification means a medicament as defined above in the form of discrete portions each containing a unit dose, or a multiple or sub-multiple of a unit dose of the pyrogen - free enzyme. Such portions are, in the case of the present invention, ampoules, each containing a unit dose,

or a multiple or sub-multiple of a unit dose of the enzyme in the form of a solution suitable for parenteral injection.

The following Examples illustrate the invention.

EXAMPLE 1

100 g asparaginase with a specific activity of 100 U/mg are dissolved in one litre of water. About 40 g glycine are then added and the solution is adjusted to pH 8.9. The solution is kept at +50°C for 90 hours, and the subsequently precipitated protein is centrifuged at 6000 r/minutes for 20 minutes. The clear centrifuged product is adjusted to pH 5.2 with 2N HCl and fractionally precipitated with a 50% polyethylene glycol solution. The fraction precipitated after the addition of about 200 ml polyethylene glycol is centrifuged, washed with acetone and dried. Yield: about 35 g with 200 to 220 U/mg substance.

The drawing shows the results of an experiment to demonstrate the small pyrogen content of the enzyme purified as described above.

Three rabbits (Nos. 4, 5 and 6) were each injected intravenously with 1 ml. of a solution containing sufficient of the enzyme purified as described above to give about 240 units of L - asparaginase per kg. body weight. The injections were performed at 10.30 a.m. and the variations of the body temperature of the rabbits recorded until 2.30 p.m. In the drawing, the horizontal axis denotes the passage of time, the vertical axis the temperature of the animals, the column of figures (ΔT) the maximum temperature rise in each case. The mean rise was 0.4°C.

The Deutsches Arzneibuch prescribes as a criterion of pyrogenicity, that the sum of the squares of the temperature rise for three animals should not exceed 0.59. The corresponding value of this experiment ("D.A.B.") was 0.51.

EXAMPLE 2

The procedure is the same as that in Example 1, but the solution of the L - asparaginase is heated at 60°C for 24 hours.

EXAMPLE 3

The procedure is the same as that in Example 1, but the pH value is adjusted to about 8.2.

EXAMPLE 4

The procedure is the same as that in Example 2, but only 20 g glycine are added to the solution of L - asparaginase.

EXAMPLE 5

100 g L - asparaginase with a specific activity of 100 U/mg are dissolved in one litre water. 100 ml methanol and 40 g glycine are

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added and the solution is adjusted to pH 8.9. The solution is kept at +50°C for 90 hours, the subsequently precipitated protein is centrifuged for 20 minutes at 6000 r/minute. The clear centrifuged product is adjusted to pH 5.2 with 2N HCl and fractionally precipitated with a 50% polyethylene glycol solution. The portion which is precipitated after the addition of about 200 ml polyethylene glycol, is centrifuged, washed with acetone and dried. Yield: about 33 g with 200 to 235 U/mg substance.

EXAMPLE 6

- 15 The procedure is the same as that described in Example 1, but 200 ml methanol are added.

EXAMPLE 7

- 20 The procedure is the same as that described in Example 1, but 100 ml ethanol are added to the solution.

- L - Asparaginases that have been treated according to Examples 2 to 7 are pyrogen-free in tests on rabbits as described in Example 1.

WHAT WE CLAIM IS:—

1. A process for the production of pyrogen-free L - asparaginase as hereinbefore defined, which comprises heating a solution of L - asparaginase containing pyrogens to from 40 to 61°C at a pH of from 7.0 to 9.2 in the presence of an aliphatic alcohol having up to four carbon atoms and/or of an amino acid, removing any precipitated material from the solution, precipitating the L - asparaginase at pH 4.5 to 5.5 by the addition of polyethylene glycol.
2. The process of claim 1 wherein the solution contains from 4 to 40% of the alcohol.
3. The process of claim 2 wherein the solution contains 10% of the alcohol.
4. The process of claim 1, 2 or 3 wherein the alcohol is methanol.
5. The process of any preceding claim wherein the solution contains 1 to 10% of the amino acid.
6. The process of claim 5 wherein the solution contains about 3% of the amino acid.
7. The process of any preceding claim wherein the amino acid is glycine.
8. The process of any preceding claim wherein heating is continued for from 1 to 5 days at 50°C.
9. The process of any preceding claim wherein the heating is carried out with the solution at a pH of from 8 to 9.
10. The process of any preceding claim

wherein the solution initially contains from 0.5 to 15% of L - asparaginase.

11. The process of any preceding claim wherein the L - asparaginase initially has a purity of from 160 to 220 U/mg as hereinbefore defined.

12. The process of any preceding claim wherein the pH of the solution is adjusted to about 5.2 before addition of the polyethylene glycol.

13. A process for the production of pyrogen-free L - asparaginase as hereinbefore defined which comprises heating a solution of L - asparaginase containing pyrogens to from 40 to 61°C, at a pH of from 7.0 to 9.2, in the presence of an amino acid, removing any precipitated material from the solution, precipitating the L - asparaginase at pH 4.5 to 5.5 by the addition of polyethylene glycol, and separating the L - asparaginase from the polyethylene glycol.

14. A process for the production of pyrogen-free L - asparaginase which comprises heating a solution of L - asparaginase containing pyrogens to from 40 to 61°C, at a pH of from 7.0 to 9.2, in the presence of an aliphatic alcohol having up to four carbon atoms, removing any precipitated material from the solution, precipitating the L - asparaginase at pH 4.5 to 5.5 by the addition of polyethylene glycol, and separating the L - asparaginase from the polyethylene glycol.

15. The process of any preceding claim substantially as hereinbefore described in any of the Examples.

16. The process of claim 13 substantially as hereinbefore described in any of Examples 1 to 4.

17. Pyrogen-free L - asparaginase as hereinbefore defined having a purity of more than 200 U/mg.

18. Pyrogen-free L - asparaginate whenever prepared by the process of any of claims 1—16.

19. Pyrogen-free L - asparaginase whenever prepared by the process of claim 13.

20. A pharmaceutical composition consisting of a parenterally injectable solution of the L - asparaginase of claim 17, 18 or 19.

21. A medicament is dosage unit form consisting of ampoules containing a parenterally injectable solution of the L - asparaginase of claim 17, 18 or 19.

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8. The process of any preceding claim wherein heating is continued for from 1 to 5 days at 50°C.
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13. A process for the production of pyrogen-free L - asparaginase as hereinbefore defined which comprises heating a solution of L - asparaginase containing pyrogens to from 40 to 61°C, at a pH of from 7.0 to 9.2, in the presence of an amino acid, removing any precipitated material from the solution, precipitating the L - asparaginase at pH 4.5 to 5.5 by the addition of polyethylene glycol, and separating the L - asparaginase from the polyethylene glycol.

14. A process for the production of pyrogen-free L - asparaginase which comprises heating a solution of L - asparaginase containing pyrogens to from 40 to 61°C, at a pH of from 7.0 to 9.2, in the presence of an aliphatic alcohol having up to four carbon atoms, removing any precipitated material from the solution, precipitating the L - asparaginase at pH 4.5 to 5.5 by the addition of polyethylene glycol, and separating the L - asparaginase from the polyethylene glycol.

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Printed for Her Majesty's Stationery Office, by the Courier Press, Leamington Spa, 1970.
Published by The Patent Office, 25 Southampton Buildings, London, WC2A 1AY, from which copies may be obtained.

