

PATENT SPECIFICATION

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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:—

The present invention relates to a process for the production of pyrogen-free L-asparaginase.

Our copending Patent Application No. 61394/68 (Serial No. 1212136) discloses processes for the bacterial production of L-asparaginase and for its purification to a high state of purity. Although the L-asparaginase produced by this process is very pure by the standards of the prior art, it may contain pyrogens which may inhibit its clinical use. The present invention relates to a process for the removal of pyrogens in 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 copending application, we regard it as applicable to L-asparaginase produced by any method or from any source.

The pyrogens in L-asparaginase prepared from bacterial cells may be carried over from those cells. Impurities having a pyrogenic action may also get into the L-asparaginase preparation during the isolation and purification of the enzyme. In spite of many endeavours, 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 fil-

tration over clarifying layers, and adsorption on hydroxyl apatite, aluminium hydroxide, silicates and synthetic resins did not succeed. Even chromatography on dextran gels without ionized groups or on diethylaminoethyl cellulose was ineffective.

The present invention provides a process for the production of pyrogen-free L-asparaginase which comprises dissolving L-asparaginase containing pyrogens in a buffer adsorbing the enzyme on a swollen DEAE-dextran gel, and eluting the enzyme with a solution of higher ionic strength than the original buffer.

The buffer used as solvent for the enzyme is preferably of low ionic strength i.e. 0.02 to 0.05M. The dextran gel is preferably previously swollen in this buffer, and equilibrated by repeatedly changing the buffer.

There are two preferred ways in which the process of the invention can be carried out. Firstly, the gel may be packed into a column onto which the buffer containing the enzyme is poured. The enzyme is adsorbed on the gel and is later eluted with a solution of higher ionic strength. Secondly, a batch process may be operated in which the enzyme solution is mixed with the dextran gel. When the enzyme is adsorbed, the gel is separated from the residual solution and the enzyme eluted with a solution of higher ionic strength.

When the column method is used, the crude enzyme preparation is preferably dissolved in an about 0.02 M-buffer. The buffer is preferably ammonium acetate or formate and preferably has a neutral or weakly alkaline pH of 7 to 8.5. The concentration of the solution formed should not be too high. Preferably 1 to 2% solutions of the enzyme are used.

After the dextran gel is swollen it is put

[Price 5s. 0d. (25p)]

in chromatography columns, the ratio of the diameter and bed height being preferably 1:3 to 1:30. The enzyme solution is poured into the column, the rate of flow preferably amounting to 2 to 4 ml per hour per cm² cross-section of the column. The elution is carried out while increasing the salt content, preferably with a linear gradient; however, it is also possible to increase the concentration of the eluant stepwise. The enzyme is eluted by acetate ions at concentration of 0.08 M and more.

In the batch process, the enzyme solution is preferably added to the swollen DEAE-dextran gel, and the pH adjusted to about 7.5. The enzyme is thus adsorbed on the gel and can be eluted, after thoroughly washing out the gel, on a filter or with the aid of a centrifuge successively by salt solutions of increasing ionic strength. In comparison with the column method, the advantage of this method is that it is possible to work with large quantities. The disadvantage consists in the lesser possibility of exactly separating accompanying substances and in the larger amount of liquid required for the elution.

Isolation of L-asparaginase in solid form from the solution can be carried out according to any available method, such as freeze-drying, optionally after dialysis to remove low molecular weight impurities, or precipitation with polyethylene glycol or acetone, as described in our three copending Patent Applications Nos. 61394/68, 2169/69 and 2170/69 (Serial Nos. 1212136, 1212138 and 1212139).

According to the process of the invention there are obtained, starting from preparations with about 20 to 100 or more U/mg, pure preparations with 180 to 220 or more U/mg protein which are pyrogen-free as herein-after defined.

The L-asparaginase unit is defined in our copending Patent Application No. 61394/68. Through the present specification "1 minute units" are used.

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

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

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, contained in ampoules as a sterile solution suitable for parenteral injection. Each

ampoule contains a unit dose, or a multiple or a sub-multiple of a unit dose of the solution.

The following Examples illustrate the invention.

The term "pyrogen-free" used in the present specification means that with doses of 200 units per kg bodyweight the mean temperature rise of at least three test rabbits is less than 0.5°C.

EXAMPLE 1

30 g DEAE-dextran gel (diethylaminoethyl dextran gel=DEAE-SEPHADEX A 50, commercial product of Aktiebolaget Pharmacia, Uppsala, Sweden; the word SEPHADEX is a Registered Trade Mark) are pre-treated in usual manner, equilibrated with a 0.02 M-glycine, 0.02 M-ammonium formate buffer of pH 7.8 and placed in a column of 3.2 cm diameter, and 90 cm bed height. 500 mg crude L-asparaginase with 100 U/mg are dissolved in 50 ml of the above buffer and placed on the column. The enzyme is subsequently eluted with a linear gradient of 2 litres of the buffer to 2 litres 0.02 M-glycine, 1 M-ammonium formate, pH 7.5. The asparaginase-containing fractions are combined and freeze-dried. Yield: 1.03 g of enzyme containing some glycine of the buffer with 27,800 U=56% of the initial activity. The specific activity, referred to protein, is 210 U/mg.

Pyrogen test on rabbits: 200 U/kg animal weight were administered to 5 rabbits. The mean value of the temperature increase was 0.48°C.

The crude pyrogen-containing enzyme, that had not been purified as described showed a temperature increase of 1.6°C under the same conditions.

EXAMPLE 2

30 g DEAE-dextran gel are pre-treated in usual manner and equilibrated with 0.05 M-ammonium acetate of pH 7.5 and placed into a column of 3.2 cm diameter; bed height: 95 cm. 500 mg crude L-asparaginase with 39.7 U/mg are dissolved in 50 ml 0.05 M-ammonium acetate of pH 7.5 and poured onto the column. Elution is effected with a linear gradient of 2 litres 0.05 M-ammonium acetate of pH 7.5 to 2 litres 1 M-ammonium acetate of pH 7.5. The asparaginase-containing fractions are combined. Yield: 18,600 U=94% of the initial activity; specific activity: 200 U/mg protein.

Pyrogen test on rabbits: with 300 U/kg animal weight the mean value of the temperature increase was 0.4°C.

The crude enzyme showed a temperature increase of 1.6°C with 200 U/kg animal weight.

For comparison, a purification experiment with diethylaminoethyl cellulose may be described:

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30 g DEAE-cellulose were equilibrated with 0.05 M-ammonium acetate of pH 7.5 and placed into a column. 500 ml crude L-asparaginase (34 U/mg) were dissolved in 50 ml of the above buffer, placed on the column and eluted with a linear gradient of 1 litre 0.05 M-ammonium acetate of pH 7.5 to 1 litre 0.5 M-ammonium acetate of pH 7.5.

Yield: 14,000 U=82% of the activity with 89 U/mg protein.

Pyrogen test on rabbits: the mean value of the temperature increase was 1.0°C with 200 U/kg animal weight.

The crude enzyme showed a temperature increase of 1.8°C under the same conditions.

Other experimental conditions also did not result in any further removal of the pyrogens and this fact shows clearly the superiority of the process according to the invention.

EXAMPLE 3

490 g DEAE-dextran gel were pre-treated in the usual manner and equilibrated with 0.05 M-ammonium acetate of pH 7.5, to which merthiolate at a concentration of 20 mg/litre was added. The suspension was introduced into a column cooled to 4°C with a diameter of 20 cm and a bed height of 65 cm. 9.8 g of a crude asparaginase with 46 U/mg were dissolved in 880 ml 0.02 M-

ammonium acetate of pH 7.5 and poured on the column. Elution was carried out with a linear gradient of 10 litres 0.05 M-ammonium acetate of pH 7.5 to 10 litres of 0.5 M-ammonium acetate of pH 7.5. The asparaginase-containing fractions were combined.

Yield: 365,000 U=81% of the initial amount.

Pyrogen test on rabbits: the mean value of the temperature increase was 0.33°C with 200 U/kg animal weight.

The crude enzyme showed a temperature increase of 1.8°C under the same conditions.

EXAMPLE 4

A solution of 500 mg L-asparaginase with 39.7 U/mg in 100 ml of a 0.05 M-ammonium acetate solution of pH 7.5 is added to 10 g DEAE-dextran gel, equilibrated in a 0.05 M-ammonium acetate solution of pH 7.5. The mixture is stirred for 15 minutes and then filtered off with suction. The residue is successively eluted with buffers of varying ion strength (see Table). The portions of L-asparaginase eluted at a low ion strength contain pyrogen, whereas the portions eluted with stronger buffers are free from pyrogen. The yields can be seen from the Table.

Activity U	Yield %	Pyrogenity $\Delta T/\text{animal } (^{\circ}\text{C})$
5010	24	1.1
3820	18	1.2
7850	37	0.9
4060	19	0.3
520	2	0.3

Buffer

0.05 M-ammonium acetate pH 7.5
0.10 M-ammonium acetate pH 7.5
0.20 M-ammonium acetate pH 7.5
0.40 M-ammonium acetate pH 7.5
0.80 M-ammonium acetate pH 7.5

WHAT WE CLAIM IS:—

1. A process for the production of pyrogen-free L-asparaginase as hereinbefore defined which comprises dissolving L-asparaginase containing pyrogens in a buffer, adsorbing the enzyme on a swollen DEAE-dextran gel, and eluting the enzyme with a solution of higher ionic strength than the original buffer.

2. The process of claim 1 wherein the buffer is of concentration from 0.02 M to 0.05 M.

3. The process of claim 1 or 2 wherein the buffer is approximately 0.02 M.

4. The process of claim 1, 2 or 3 wherein the buffer comprises ammonium formate or ammonium acetate.

5. The process of any preceding claim wherein a 1 to 2% solution of enzyme in buffer is used.

6. The process of any preceding claim wherein the gel is in the form of a column onto which the buffer containing the enzyme is poured.

7. The process of claim 6 wherein the

ratio of diameter and height of the gel column is from 1:3 to 1:30.

8. The process of claim 6 or 7 wherein the rate of flow of the enzyme solution through the column is from 2 to 4 ml. per hour per sq. cm. cross-section.

9. The process of claim 6, 7 or 8 wherein elution of the enzyme from the column is carried out by linearly increasing the ionic concentration of the eluting solution.

10. The process of any of claims 1 to 5 wherein the enzyme is adsorbed on the gel by mixing the enzyme solution with the gel.

11. The process of claim 10 wherein the enzyme solution is added to the gel.

12. The process of claim 10 or 11 wherein the pH of the mixture is adjusted to about pH 7.5.

13. The process of claim 10, 11 or 12 wherein the gel carrying the adsorbed enzyme is separated from the residual solution by filtration.

14. The process of any of claims 10 to 110



30 g DEAE-cellulose were equilibrated with 0.05 M-ammonium acetate of pH 7.5 and placed into a column. 500 ml crude L-asparaginase (34 U/mg) were dissolved in 50 ml of the above buffer, placed on the column and eluted with a linear gradient of 1 litre 0.05 M-ammonium acetate of pH 7.5 to 1 litre 0.5 M-ammonium acetate of pH 7.5.

Yield: 14,000 U=82% of the activity with 89 U/mg protein.

Pyrogen test on rabbits: the mean value of the temperature increase was 1.0°C with 200 U/kg animal weight.

The crude enzyme showed a temperature increase of 1.8°C under the same conditions.

Other experimental conditions also did not result in any further removal of the pyrogens and this fact shows clearly the superiority of the process according to the invention.

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490 g DEAE-dextran gel were pre-treated in the usual manner and equilibrated with 0.05 M-ammonium acetate of pH 7.5, to which merthiolate at a concentration of 20 mg/litre was added. The suspension was introduced into a column cooled to 4°C with a diameter of 20 cm and a bed height of 65 cm. 9.8 g of a crude asparaginase with 46 U/mg were dissolved in 880 ml 0.02 M-

ammonium acetate of pH 7.5 and poured on the column. Elution was carried out with a linear gradient of 10 litres 0.05 M-ammonium acetate of pH 7.5 to 10 litres of 0.5 M-ammonium acetate of pH 7.5. The asparaginase-containing fractions were combined.

Yield: 365,000 U=81% of the initial amount.

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EXAMPLE 4

A solution of 500 mg L-asparaginase with 39.7 U/mg in 100 ml of a 0.05 M-ammonium acetate solution of pH 7.5 is added to 10 g DEAE-dextran gel, equilibrated in a 0.05 M-ammonium acetate solution of pH 7.5. The mixture is stirred for 15 minutes and then filtered off with suction. The residue is successively eluted with buffers of varying ion strength (see Table). The portions of L-asparaginase eluted at a low ion strength contain pyrogen, whereas the portions eluted with stronger buffers are free from pyrogen. The yields can be seen from the Table.

Buffer	Activity		Pyrogenity $\Delta T/\text{animal } (^{\circ}\text{C})$
	U	%	
0.05 M-ammonium acetate pH 7.5	5010	24	1.1
0.10 M-ammonium acetate pH 7.5	3820	18	1.2
0.20 M-ammonium acetate pH 7.5	7850	37	0.9
0.40 M-ammonium acetate pH 7.5	4060	19	0.3
0.80 M-ammonium acetate pH 7.5	520	2	0.3

WHAT WE CLAIM IS:—

1. A process for the production of pyrogen-free L-asparaginase as hereinbefore defined which comprises dissolving L-asparaginase containing pyrogens in a buffer, adsorbing the enzyme on a swollen DEAE-dextran gel, and eluting the enzyme with a solution of higher ionic strength than the original buffer.

2. The process of claim 1 wherein the buffer is of concentration from 0.02 M to 0.05 M.

3. The process of claim 1 or 2 wherein the buffer is approximately 0.02 M.

4. The process of claim 1, 2 or 3 wherein the buffer comprises ammonium formate or ammonium acetate.

5. The process of any preceding claim wherein a 1 to 2% solution of enzyme in buffer is used.

6. The process of any preceding claim wherein the gel is in the form of a column onto which the buffer containing the enzyme is poured.

7. The process of claim 6 wherein the

ratio of diameter and height of the gel column is from 1:3 to 1:30.

8. The process of claim 6 or 7 wherein the rate of flow of the enzyme solution through the column is from 2 to 4 ml. per hour per sq. cm. cross-section.

9. The process of claim 6, 7 or 8 wherein elution of the enzyme from the column is carried out by linearly increasing the ionic concentration of the eluting solution.

10. The process of any of claims 1 to 5 wherein the enzyme is adsorbed on the gel by mixing the enzyme solution with the gel.

11. The process of claim 10 wherein the enzyme solution is added to the gel.

12. The process of claim 10 or 11 wherein the pH of the mixture is adjusted to about pH 7.5.

13. The process of claim 10, 11 or 12 wherein the gel carrying the adsorbed enzyme is separated from the residual solution by filtration.

14. The process of any of claims 10 to 110

13 wherein the enzyme is eluted from the gel by contacting the gel successively with solutions of increasing ionic strength.

5 15. The process of any preceding claim wherein the eluted enzyme solution is subsequently freeze-dried.

10 16. The process of claim 15 wherein low molecular weight impurities are removed from the eluted enzyme solution by dialysis before freeze-drying.

17. The process of any of claims 1 to 14 wherein the enzyme is precipitated from the solution resulting from elution by the addition of polyethylene glycol or acetone.

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

19. Pyrogen-free L-asparaginase, whenever

produced by the process of any preceding claim.

20. Pyrogen-free L-asparaginase having a purity of from 180 to 220 Units/mg (1 min.) of protein.

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

22. A medicament in dosage unit form consisting of ampoules of a parenterally injectable solution of the L-asparaginase of claim 19 or 20.

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