

PATENT SPECIFICATION

(11) 1212 138

NO DRAWINGS



1212 138

- (21) Application No. 2169/69 (22) Filed 14 Jan. 1969
- (31) Convention Application No. P 17 67 617.4
- (32) Filed 29 May 1968 in
- (33) Germany (DT)
- (31) Convention Application No. P 18 07 447.0
- (32) Filed 7 Nov. 1968 in
- (33) Germany (DT)
- (45) Complete Specification published 11 Nov. 1970
- (51) International Classification C 07 g 7/02
- (52) Index at acceptance
C3H 3
ASB 312 31Y 38Y 39X
- (72) Inventors OTTO WAGNER, KLAUS BAUER, WILFRIED KAUFMANN, ERICH RAUENBUSCH, ALFRED ARENS and ECKART IRION

(54) ENZYME STABILIZATION

- (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 the stabilization of L-asparaginase.
- The enzyme L-asparaginase (L-asparagine-amido-hydrolase, E.C.3.5.1.1.) is known in the form of impure preparations obtained from animal and vegetable sources. Recent research has shown that this enzyme is apparently effective as a therapeutic agent against certain cancers, that have a growth requirement for the amino-acid L-asparagine, which is not essential for normal cells. L-asparaginase is however difficult to produce in large quantities in the purity necessary for clinical use. In particular difficulties have been encountered in stabilizing the enzyme to prevent denaturation during purification and storage.
- 15 Our copending Patent Application No. 61394/68 (Serial No. 1212136) describes a process for the isolation of L-asparaginase from microbial cells, and for its purification to a degree of purity in which it can be crystallized. Our copending Applications Nos. 61846/68 and 2170/69 (Serial Nos. 1212137 and 1212139) describe processes by which pyrogenic impurities, which inhibit the clinical use of the enzyme, can be removed.
- 20 The present application describes a process for the stabilization of L-asparaginase.
- This invention provides a process for the stabilization of L-asparaginase which comprises adding an amino acid/or polyethylene glycol to a solution of L-asparaginase.
- The amino acid added is preferably glycine. The amino acid is preferably added in an amount of 0.01 to 5.00%, and at a temperature between - 10°C and +65°C.
- 25 Although the addition of the amino acid alone, or of the polyethylene glycol alone, both give good stabilization, even better results are achieved by the addition of amino acid and polyethylene glycol.
- The invention further provides, as a new substance, L-asparaginase stabilized by a content of amino acid (preferably glycine) and/or polyethylene glycol.
- The process of the invention allows the enzyme to be stabilized against treatments or sets of conditions which would otherwise result in denaturation. Such treatments and conditions are known to those skilled in protein chemistry. For instance, polyethylene glycol and glycine are both effective to stabilize L-asparaginase against heat and lyophilization. Polyethylene glycol is effective against surface denaturation, such as tends to occur during shaking or stirring.
- 30 Thermal denaturing usually occurs when proteins are heated to temperatures above 40°C. Owing to the varying solidity of structure, differences exist for individual proteins in respect of the critical temperature at which denaturing starts. Our copending Patent Application No. 2170/69 (Serial No. 1212139) describes the purification of L-asparaginase in solution by heating the solution to a temperature at which the inert accompanying proteins present are denatured, the

L-asparaginase being stabilized by the addition of an amino acid or an aliphatic alcohol. If the accompanying proteins are to be denatured at about 60°C, the heating time amounts to 10 minutes to 4 hours. The supernatant solution of such a denaturing mixture contains the active 2-asparaginase, and is sufficiently pure to serve as starting material for the subsequent preparation of L-asparaginase in pure form. It is surprising that the purification process according to the said invention leads only to the denaturing of the accompanying proteins but not of L-asparaginase, since most enzymes are known to be thermolabile.

The stabilization effect is independent of heating time.

The process of the present invention is not, however, to be regarded as limited to the stabilization of L-asparaginase in a process such as that of our copending Application No. 2170/69.

Stabilization according to the invention with polyethylene glycol is also effective for stabilizing L-asparaginase against surface denaturation in solution. This is surprising, as polyethylene glycol has no specific surface-active effect which might prevent surface denaturing by suppressing foam formation. Surface denaturation sets in when the hydrogen bonds of the protein are split by interaction with a surface or by spreading at a phase boundary surface. The latter can be caused very easily, for example by foam formation during shaking or by blowing in inert gases. Even a single pouring of an enzyme solution from one vessel into another can cause a reduction of activity. Surface denaturation becomes particularly marked at the isoelectric point. Moreover the sensitivity of enriched proteins increases as the degree of purity increases, and highly purified preparations are especially sensitive to denaturing phenomena.

The stabilizing effect of polyethylene glycol is independent of the purity of the enzyme. The concentration of the polyethylene glycol added may vary within wide limits, but the stabilizing effect is clearly marked with a content of only 10% of polyethylene glycol referred to the enzyme present.

The polyethylene glycol may be added in solution, preferably aqueous solution, but also alcoholic solution, or in solid form.

The present invention may also be used to stabilize the enzyme when freeze-dried or lyophilized. The solubility of the freeze-dried or lyophilized enzyme is thus increased, because a partial denaturation of the enzyme is completely prevented.

The invention also provides, as a new substance, L-asparaginase stabilized by a content of an amino acid (preferably glycine) and/or polyethylene glycol. This substance may be either in the form of a solution or a solid. It has great commercial significance because up

to now the sensitivity of pure L-asparaginase has made it difficult to handle and store under convenient conditions.

The invention also provides, as a new pharmaceutical composition, a parenterally injectable solution of L-asparaginase stabilized according to the invention.

The invention further provides, as a new medicament in dosage unit form, ampoules of a parenterally injectable solution of L-asparaginase stabilized according to the invention.

The term "medicament in dosage form" as used in the present specification means ampoules of parenterally injectable solution each containing a unit dose, or a multiple or sub-multiple of a unit dose of the stabilized enzyme.

The L-asparaginase unit is defined in our co-pending Patent Application No. 61394/68 (Serial No. 1,212,136). Throughout the present specification, "1 minute units" are given unless otherwise stated.

The following Examples illustrate the invention.

EXAMPLE 1.

50 g of crude asparaginase with an activity of 97 U/mg (30 min.) are dissolved in glycine buffer of pH 8.5 and heated at 60°C for 1 hour. The mixture, which contains plenty of denatured inactive protein, is centrifuged at 6000 r.p.m. until clear, and fractionated with acetone. The fraction which is precipitated upon the addition of 40 to 45% acetone, amounts to 3.9 g. It contains 950 U/mg. (30 min.)

18.6 g of enriched L-asparaginase which are collected from several denaturing mixtures are dissolved in 380 ml of a 3-molar urea solution, adjusted to pH 8.5 by means of a 1N sodium hydroxide solution, and fractionated with a 50% polyethylene glycol solution. The fraction precipitated after the addition of 45 to 60 ml is centrifuged off, washed with acetone, and dried. Yield 4.2 g; 3130 U/mg (30 min.)

A solution of 4.0 g of this sample in 80 ml of bidistilled water is adjusted to pH 5.2 by means of 1N hydrochloric acid, and fractionated with a polyethylene glycol solution. The fraction precipitated after the addition of 25 to 30 ml is allowed to stand for 20 minutes at room temperature and then centrifuged off; after standing under acetone for 2 hours, it is washed with more acetone. Yield 1.2 g; U/mg 6110 (30 min.)

This sample was found to be a uniformly crystallised substance. Recrystallisation was achieved

1. by precipitation of a solution of the crystals in water at pH 4.9 with a polyethylene glycol solution, and
2. by precipitation of the same aqueous solution with acetone.

Flat
289°C,

5 g of
(30 min
alanine
pH 8.5
tion, and
15 min
much d
centrifug
full aspa
By fu
ample 1
lised L
mixture.

5 g
activity
dissolved
perature
centrifug
into the
perature
until th
The pH
subsequ
sample

A pre
tionated
and the
was dial
U with
protein.
The f
dried. A
U (81%
activity
The s
mixed w
ethylene
yield arr
activity)
protein.

Portic
L-aspar
in 0.2 N
as follow
(a) P
so
oc
(b) T
10
15
tic
(c) T
vc

Flat prisms; decomposition starting at 289°C, complete decomposition at 292°C.

EXAMPLE 2.

5 g of crude asparaginase with 97 U/mg (30 min.) were dissolved in 100 ml of a 1% alanine solution which had been adjusted to pH 8.5 by means of a sodium hydroxide solution, and the solution was heated at 60°C for 15 minutes. The mixture, which contained much denatured protein, was clarified by centrifuging at 6000 r.p.m. It contained the full asparaginase activity.

By further working up according to Example 1, it is also possible to obtain crystallised L-asparaginase from this denaturing mixture.

EXAMPLE 3.

5 g of L-asparaginase with a specific activity of about 170 U/mg substance are dissolved in 1 litre of water at room temperature, and the undissolved components are centrifuged off. 10 g glycine are introduced into the solution so obtained at room temperature while stirring. Stirring is continued until the material has completely dissolved. The pH value is 5.2. The solution is frozen and subsequently lyophilized. The activity of the sample was completely preserved.

EXAMPLE 4.

A preparation of L-asparaginase was fractionated through a column with dextran gel, and the fraction containing L-asparaginase was dialysed. The solution contained 12,400 U with a specific activity of 251 U/mg protein.

The first half of this solution was freeze-dried. A preparation with an activity of 5050 U (81% of the initial activity) and a specific activity of 202 U/mg protein was obtained.

The second half of the above solution was mixed with 0.1 ml of a 50% solution of polyethylene glycol and then freeze-dried. The yield amounted to 6100 U (98% of the initial activity) with a specific activity of 244 U/mg protein.

EXAMPLE 5.

Portions of 5 ml of a 0.1% solution of L-asparaginase with a content of 146 U/mg in 0.2 N acetate buffer of pH 5.0 were tested as follows:

(a) Pure argon was blown in for 90 minutes so that an intense formation of foam occurred.

(b) The procedure of (a) was followed, but 100 mg polyethylene glycol of MW 1500 were previously added to the solution.

(c) The solution was shaken in a closed vessel for 3 hours.

(d) The procedure of (c) was followed, but 100 mg polyethylene glycol were previously added to the solution.

(e) The solution was stored in a refrigerator for 3 hours (control test).

Determination of the content of the solutions (a) to (e) gave the following values:

(a) U/ml = 15

(b) U/ml = 140

(c) U/ml = 35

(d) U/ml = 138

(e) U/ml = 146

EXAMPLE 6.

Portions of 5 ml of a 0.1% solution of L-asparaginase with a content of 146 U/mg in 1/15-molar phosphate buffer of pH 7.0 were treated in analogy with Example 5, tests (a) and (b).

Determination of the content gave:

(a) U/ml = 76

(b) U/ml = 145

EXAMPLE 7.

A preparation of L-asparaginase with 100 U/mg dry weight was dissolved in 0.1-molar tris-buffer (pH 8.9) at a concentration of 1 mg/ml. 6.0 ml of this solution were diluted with 0.5 ml of water and heated in a thermostat at 55°C. At the same time, 6.0 ml of the same L-asparaginase solution were mixed with 0.3 ml of water and 0.2 ml of a 50% solution of polyethylene glycol in water and subjected to the same temperature. After certain periods of time, samples were taken from the mixtures and tested for their activity. The results are given in Table 1.

An analogous test was carried out with the same L-asparaginase preparation at 60°C. The results are also contained in Table 1.

EXAMPLE 8.

A preparation of L-asparaginase with about 100 U/mg was dissolved in 0.1N tris-buffer (pH 8.0) at a concentration of 1 mg/ml. Samples of 6.0 ml of this solution were admixed either with 0.5 ml of water or with 0.5 ml of aqueous solutions containing 50, 100 or 200 mg polyethylene glycol. The solutions were heated at 55°C for 80 minutes. The results are given in Table 2.

EXAMPLE 9.

A preparation of L-asparaginase with 100 U/mg was dissolved in 0.1N tris-buffer (pH 8.0) at a concentration of 1 mg/ml. Portions of 6 ml of this solution were admixed with:

(a) 0.3 ml of water

(b) 0.2 ml of water and 0.1 ml polyethylene glycol (50% in water)

(c) 0.2 ml of a glycine solution (100 mg/ml)

(d) 0.1 ml polyethylene glycol (50% in water) and 0.2 ml of a glycine solution (100 mg/ml).

The solutions were heated at 60°C for 80 minutes and then tested for their content of L-asparaginase. The results are given in Table 3.

TABLE 1

Heat stabilization of L-asparaginase in the presence of polyethylene glycol

Duration of heating (Minutes)	Temperature (°C)	Activity of L-asparaginase (U/ml)	
		without additive	with polyethylene glycol
0	55	86	104
20	55	74	103
40	55	62	101
60	55	61	94
80	55	43	94
0	60	91	96
20	60	49	69
40	60	33	48
60	60	26	40
80	60	16	32

L-asparaginase with 100 U/mg dissolved in 0.1N tris-buffer (pH 8.0); Concentration 0.92 mg/ml. 15.1 mg/ml. of polyethylene glycol was added.

TABLE 2

Stability of L-asparaginase as a function of the amount of polyethylene glycol

Concentration of polyethylene glycol mg/ml	Ratio of polyethylene glycol to enzyme	Activity before heating U/ml	Activity after 80 minutes at 55°C U/ml	Activity as % of initial value
0	0	92	43	47
7.5	8.2	98	86	88
15.0	16.3	98	94	96
30.0	32.6	98	91	93

The preparation of L-asparaginase had a specific activity of 100 U/mg dry weight. The concentration in the solution amounted to 0.92 mg/ml. Upon addition of polyethylene glycol, an increase of activity from 92 U/ml to 98 U/ml (=106%) was observed.

L-asparag:
L-asparag
L-asparag
L-asparag
glycine

Our
claims
free L-
a solut
gens to
7.0 to
alcohol
and/or
cipitate
rating
by the
separat
ethylene
claimed
in the
Sub
WE C
1. /
20 L-aspa
amino
solutic
2.
amino
25 3.
the a
0.01 i
4.
the a
30 tempe
5.
where
an ac
6.
35 1—1

TABLE 3

Stability of L-asparaginase in the presence of several stabilizers

	Activity of L-asparaginase after heating at 60°C for 80 minutes	
	U/ml	% of initial value
L-asparaginase only	14	15
L-asparaginase with polyethylene glycol	44	48
L-asparaginase with glycine	52	57
L-asparaginase with polyethylene glycol and glycine	83	90

Our copending Application No. 2170/69 claims 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 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, and separating the L-asparaginase from the polyethylene glycol. This process is hereby disclaimed from the present invention as defined in the following claims.

Subject to the above disclaimer, WHAT WE CLAIM IS:—

1. A process for the stabilization of L-asparaginase which comprises adding an amino acid and/or polyethylene glycol to a solution of L-asparaginase.
2. The process of claim 1 wherein the amino acid is glycine.
3. The process of claim 1 or 2 wherein the amino acid is added in an amount of 0.01 to 5.00%.
4. The process of claim 1, 2 or 3 wherein the amino acid is added to the solution at a temperature between -10°C and +65°C.
5. The process of any preceding claim wherein the polyethylene glycol is added as an aqueous solution.
6. A process according to any of claims 1—4 wherein the amino acid is used.

7. A process according to claim 1 or 5 wherein the polyethylene glycol is used.

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

9. The process of claim 6 substantially as hereinbefore described in any of Examples 1, 2 and 3.

10. L-asparaginase whenever stabilized by a content of an amino acid.

11. L-asparaginase whenever stabilized by a content of glycine.

12. L-asparaginase whenever stabilized by a content of polyethylene glycol.

13. L-asparaginase as claimed in claim 10, 11 or 12 whenever stabilized by the process of claim 1.

14. L-asparaginase as claimed in claim 10 or 11 whenever stabilized by the process of claim 6 or 9.

15. A pharmaceutical composition, consisting of a parenterally injectable solution of the L-asparaginase of any of claims 10 to 14.

16. A medicament in dosage unit form consisting of a parenterally injectable solution of the L-asparaginase of any of claims 10 to 14.

For the Applicants,
CARPMAELS & RANSFORD,
Chartered Patent Agents,
24 Southampton Buildings,
Chancery Lane, London, W.C.2.

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.

TABLE 3

Stability of L-asparaginase in the presence of several stabilizers

	Activity of L-asparaginase after heating at 60°C for 80 minutes	
	U/ml	% of initial value
L-asparaginase only	14	15
L-asparaginase with polyethylene glycol	44	48
L-asparaginase with glycine	52	57
L-asparaginase with polyethylene glycol and glycine	83	90

Our copending Application No. 2170/69 claims 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 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, and separating the L-asparaginase from the polyethylene glycol. This process is hereby disclaimed from the present invention as defined in the following claims.

Subject to the above disclaimer, WHAT WE CLAIM IS:—

1. A process for the stabilization of L-asparaginase which comprises adding an amino acid and/or polyethylene glycol to a solution of L-asparaginase.
2. The process of claim 1 wherein the amino acid is glycine.
3. The process of claim 1 or 2 wherein the amino acid is added in an amount of 0.01 to 5.00%.
4. The process of claim 1, 2 or 3 wherein the amino acid is added to the solution at a temperature between -10°C and +65°C.
5. The process of any preceding claim wherein the polyethylene glycol is added as an aqueous solution.
6. A process according to any of claims 1—4 wherein the amino acid is used.

7. A process according to claim 1 or 5 wherein the polyethylene glycol is used.

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

9. The process of claim 6 substantially as hereinbefore described in any of Examples 1, 2 and 3.

10. L-asparaginase whenever stabilized by a content of an amino acid.

11. L-asparaginase whenever stabilized by a content of glycine.

12. L-asparaginase whenever stabilized by a content of polyethylene glycol.

13. L-asparaginase as claimed in claim 10, 11 or 12 whenever stabilized by the process of claim 1.

14. L-asparaginase as claimed in claim 10 or 11 whenever stabilized by the process of claim 6 or 9.

15. A pharmaceutical composition, consisting of a parenterally injectable solution of the L-asparaginase of any of claims 10 to 14.

16. A medicament in dosage unit form consisting of a parenterally injectable solution of the L-asparaginase of any of claims 10 to 14.

For the Applicants,
CARPMAELS & RANSFORD,
Chartered Patent Agents,
24 Southampton Buildings,
Chancery Lane, London, W.C.2.

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.

