



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification⁴ : C12N 9/82	A1	(11) International Publication Number: WO 87/ 03618 (43) International Publication Date: 18 June 1987 (18.06.87)
(21) International Application Number: PCT/US86/02665 (22) International Filing Date: 11 December 1986 (11.12.86) (31) Priority Application Number: 808,729 (32) Priority Date: 13 December 1985 (13.12.85) (33) Priority Country: US (71) Applicant: UNITED STATES OF AMERICA, represented by THE UNITED STATES DEPARTMENT OF COMMERCE [US/US]; Washington, DC 20230 (US). (72) Inventors: LEE, Shwu-Maan ; 330 S. Jefferson Street, Frederick, MD 21701 (US). ROSS, John, T. ; 11310-A Pleasant Walk Lane, Myersville, MD 21773 (US). WROBLE, Marie ; 13214 Gerlach Court, Mt. Airy, MD 21771 (US).		(74) Agent: OLIFF, James, A.; Parkhurst & Oliff, 277 S. Washington Street, Alexandria, VA 22314 (US). (81) Designated States: AU, JP. Published <i>With international search report.</i>
(54) Title: PROCESS FOR MANUFACTURE OF L-ASPARAGINASE FROM ERWINIA CAROTOVORA (57) Abstract A process for the recovery and purification of L-asparaginase from <i>Erwinia carotovora</i> involves preparation of cellular acetone powder extract followed by either an ion exchange and affinity chromatography purification or by affinity chromatography alone. The column eluent is then dialyzed to produce substantially pure L-asparaginase.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	ML	Mali
AU	Australia	GA	Gabon	MR	Mauritania
BB	Barbados	GB	United Kingdom	MW	Malawi
BE	Belgium	HU	Hungary	NL	Netherlands
BG	Bulgaria	IT	Italy	NO	Norway
BJ	Benin	JP	Japan	RO	Romania
BR	Brazil	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	LI	Liechtenstein	SN	Senegal
CH	Switzerland	LK	Sri Lanka	SU	Soviet Union
CM	Cameroon	LU	Luxembourg	TD	Chad
DE	Germany, Federal Republic of	MC	Monaco	TG	Togo
DK	Denmark	MG	Madagascar	US	United States of America
FI	Finland				

PROCESS FOR MANUFACTURE OF L-ASPARAGINASE
FROM ERWINIA CAROTOVORA

The invention described herein was made in the course of work under a contract or award from the United States Department of Health and Human Services.

This invention involves a process for the production of L-asparaginase from bacterial cell cultures of Erwinia carotovora which involves a multi-step separation procedure highlighted by a dual chromatographic separation, one for ion exchange and then one for affinity, or a single chromatographic separation using affinity chromatography. The generalized process is set out below. The enzyme is used therapeutically to benefit the treatment of leukemia.

A process for the recovery and purification of L-asparaginase from Erwinia carotovora is disclosed. The process, as shown in the flow diagram, involves sequential ion exchange chromatography and affinity chromatography purification steps, or affinity chromatography by itself, followed by a dialysis step in order to produce substantially pure L-asparaginase.

General Description

A process is described for the recovery and purification of gram quantities of a therapeutic enzyme, L-asparaginase, from submerged cultures of Erwinia carotovora. Briefly, cells from Erwinia carotovora fermentation broth are harvested and washed. A cellular acetone powder is prepared and extracted with pH 9.5 borate buffer. After centrifugation and filtration to remove cell debris, the acetone powder extract is adjusted to pH 7.7 and adsorbed onto an agarose column, such as CM-Sepharose Fast Flow (Pharmacia). Sepharose Fast Flow is a cross-linked agarose gel containing 6% agarose. This column is a low acid type suitable for ion exchange purposes. In addition, there is a pre-column packed with cell debris remover modified cellulose. The enzyme is desorbed from the ion-exchange column at pH 9.0 and further purified by an affinity column, such as L-asparagine (L-Asn) Sepharose CL-

- 2 -

4B, which column is tailored to select or allow molecular components. This affinity column is neutral as to ion selectivity, as contrasted with the ion exchange column above. CL-4B is a cross-linked agarose gel containing about 4% agarose. L-Asn Sepharose CL-4B is made by linking L-Asn to Sepharose CL-4B through an epoxy-activation procedure. After dialysis-concentration to remove buffer salt, the L-asparaginase enzyme is depyrogenated, formulated, sterile filled, and lyophilized as a single-dose final product.

The ion exchange chromatography step can be eliminated and substituted with at least one affinity chromatography step(s) using L-Asn Sepharose Fast Flow. L-Asn Sepharose Fast Flow is made by linking L-Asn to Sepharose Fast Flow beads through an epoxy activation procedure.

BACKGROUND OF THE INVENTION

L-asparaginase (L-asparagine amidohydrolase) catalyzes the hydrolysis of L-asparagine (L-Asn) to L-aspartic acid and ammonia. Interest in this enzyme arose a few decades ago when it was discovered that the antilymphoma activity of whole guinea pig serum was due to the enzyme L-asparaginase. Most normal tissues synthesize L-asparagine in amounts sufficient for their metabolic needs. Certain neoplastic tissues, in particular, acute lymphoblastic leukemic cells, require an exogenous source of this amino acid. L-asparaginase, by enzymatically catalyzing the hydrolysis of asparagine to aspartic acid and ammonia, deprives these malignant cells of the asparagine available from extracellular fluid, resulting in cellular death. L-asparaginase produced from Escherichia coli has a tumor inhibitory effect and the enzyme isolated from Erwinia carotovora is also pharmacologically active. Since the L-asparaginases from E. coli and Erwinia carotovora possess different immunological specificities, the availability of both provides an important alternative therapy if a patient happens to develop hypersensitivity to one of the enzymes (a common phenomenon associated with the admin-

- 3 -

istration of L-asparaginase).

The process used by the only known manufacturer includes alkali lysis, multiple ion-exchange adsorptions, ammonium sulfate precipitation, multiple dialysis-concentration steps, and ethanol crystallization (Buck, et al., J. Gen. Microbiol., Vol. 65, page i (1971)). The present invention is an improved process to produce Erwinia L-asparaginase in gram quantities for therapeutic use.

As shown in U.S. Patent No. 3,511,754 to Berk et al, and U.S. Patent No. 3,511,755 to Ratych et al, the use of acetone in the production of E. coli-derived L-asparaginase is known to practitioners of the art. Part of the difficulty of large-scale production of this enzyme lies in the purification process. The only major manufacturer of asparaginase purifies the enzyme by a process which includes alkali lysis, multiple ion-exchange adsorptions, ammonium sulfate precipitation, multiple dialysis-concentration steps, and ethanol crystallization (Buck et al, J. Gen. Microbiol., Vol. 65, page i (1971)). On the other hand, the present application discloses a method by which significant purification of asparaginase may be achieved by initial ion-exchange chromatography. The material eluted from the ion exchange column (the starting material for the affinity column) contains only one extra protein contaminant as compared to the product available in the market. This extra protein contaminant is successfully removed by affinity chromatography. Moreover, the same purification can be achieved using affinity chromatography by itself.

Furthermore, compared to the multiple purification and dialysis steps used by Buck et al, the process of the present invention includes only one or two chromatographic steps and one dialysis step. The enzyme is eluted from the ion-exchange column with a change in the buffer pH rather than a change in ionic strength. The enzyme coming off the column is very concentrated and contains low salt, thus avoiding a dialysis-concentration step between the

- 4 -

ion-exchange and affinity chromatographic steps.

Specific Disclosure

In the process of the present invention, L-asparaginase is purified from submerged cultures of Erwinia carotovora. See Examples 1-5 for detailed descriptions of the processes. The process begins with harvesting and washing Erwinia cells using a plate and frame type filter press or centrifugation. A cellular acetone powder is prepared from the buffer washed cells by one or more treatments with acetone. The cellular acetone powder is then extracted to form an enzyme-rich extract. The cell extract is again filtered or centrifuged to remove cell debris and form a cell-free enzyme extract (see Examples 1 and 2). The cell-free extract is applied to an ion exchange column, enzyme-rich fractions are pooled, and then applied to an affinity column. Alternately, the cell-free extract can be applied to the affinity column without going through the ion exchange column. Sequential washing and elution of the affinity column produces an enzyme which is substantially pure.

In the following specific method, as has been stated above, alternative materials to the ion exchange CM Sepharose Fast Flow and affinity chromatography L-Asn Sepharose CL-4B column may be utilized.

The critical steps of this process are the preparation of a cellular acetone powder extract and the purification of the cell-free extract by means of chromatography--an ion exchange column and an affinity column or an affinity column. The following describes a preferred method of purification.

Ion Exchange Column Purification

CM-Sepharose Fast Flow (an ion exchanger sold by Pharmacia) is suspended in water and packed in a 16 L column (Pharmacia, 37 x 15 cm) with compressed air pressure of 1 bar. Cell debris remover (CDR) modified cellulose is pre-equilibrated with 0.1M sodium phosphate buffer, pH 7.7, basket centrifuged to remove the buffer, and re-equili-

brated twice with 5mM sodium phosphate buffer, pH 7.7. The CDR is packed in a 16 L column with the same buffer containing 0.1% benzalkonium chloride as a preservative. Before being used for the production run, the CM column and the CDR pre-column are each washed with at least 2 bed volumes of 5 mM sodium phosphate buffer, pH 7.7, to remove preservatives. The two column sections are serially joined and the flow (upward for CM column and downward for the pre-column) is maintained with a peristaltic pump. The acetone powder extract is adjusted to pH 7.7 and applied to the columns with a flow rate of 0.5 L/min, which is equivalent to a linear flow rate of 28 cm/h. The flow rate can be reduced if necessary during the run to keep the running pressure at or below 1 bar. Following loading, the CDR pre-column is detached and the CM column is extensively washed with the buffer until the effluent is devoid of protein. The enzyme is then eluted from the column with 10 mM glycine buffer, pH 9.0. The enzyme-rich fractions are pooled, adjusted to pH 7.5 with dilute HCl and applied to the L-Asn Sepharose CL-4B or L-Asn Sepharose Fast Flow affinity column.

Affinity Chromatography Purification

The L-Asn Sepharose CL-4B is prepared using methods adapted from Sundberg and Porath (J. Chromatogr., Vol. 90, p. 87 (1974)). Sepharose CL-4B (Pharmacia, 4 Kg) is activated by reaction with 4 L of 1,4-butanediol diglycidyl ether and 4 L of 0.6 N sodium hydroxide solution containing 8 g of sodium borohydride. The activation reaction is allowed to proceed at room temperature for 15 hours in a 50-L glass rotary evaporator. The activated gel is recovered by suction filtration, washed seven times with 150 L of water each time and coupled to L-Asn by reaction of the gel with L-Asn (2 Kg) in 0.5M sodium carbonate buffer (35 L final pH 8.5). The coupling reaction is allowed to proceed at room temperature for 15 hours with slow rotation in the same rotary evaporator. After the coupling step, the gel is washed seven times with 150 L of

water each time and stored in 0.02% sodium azide.

The gel is packed in a column and the column is washed with at least 2 bed volumes of 5 mM sodium phosphate buffer, pH 7.5, to remove preservatives. A linear flow rate of 34 cm/h is used, which is equivalent to 2.7 L/h. At the end of loading, the column is washed with at least 5 bed volumes of the same buffer, and the enzyme is eluted with 10 mM glycine buffer, pH 9.0. The enzyme-rich fractions are pooled, adjusted to pH 7 with dilute HCl, and frozen at -20°C for final processing.

The affinity column eluent pool is thawed at 2° to 8°C, concentrated using a 2 L ultrafiltration pressure cell with C-30 membrane (cellulosic membrane, 30,000 dalton nominal molecular weight cut-off), and dialyzed at least 10⁸-fold with depyrogenated water for 15 hours. The dialyzed-concentrated enzyme is transferred to a 200 mL ultrafiltration pressure cell and further concentrated to a minimum titer of 30,000 IU/mL. The dialyzed concentration is depyrogenated, formulated, sterile-filled and lyophilized as a single dose final product.

The overall purification factor achieved from the ion-exchange and affinity chromatography is about 46-fold, as shown in Table 1. Table 2 shows the purification of L-asparaginase using filtration and affinity chromatography.

Erwinia is a gram negative microorganism and as such the cell-free extract contains high quantities of endotoxins. As shown in Table 1, the endotoxin level drops from 11.5 µg/mL to 0.676 ng/mL through ion-exchange chromatographic and further decreases to 0.45 ng/mL through affinity chromatographic. When depyrogenated water is used in all chromatography operations, the enzyme-rich affinity column eluent contains endotoxins in the range of 0.1 to 0.5 ng/mL.

Aluminum oxide gel has been demonstrated to be an effective endotoxin adsorbent for certain proteins. It also has been demonstrated to be effective for pyrogen

- 7 -

removal from L-asparaginase. In order to determine optimum conditions for depyrogenation, concentration of pH effects were studied. Purified L-asparaginase (protein concentration 2.45 mg/mL) containing an endotoxin concentration of 17 ng/mL was treated with an equal volume of varying concentrations of aluminum oxide while varying pH. As shown in Table 3, aluminum oxide appeared to be equally effective for depyrogenation at pH 5, 6, and 7 at 1% and 0.5%. At pH 7, however, some protein loss occurred.

Table 1
Recovery and Purification of L-Asparaginase from Erwinia carotovora
Using Centrifugation and Ion Exchange

Steps	Total activity ^a (IU) x 10 ⁻⁶	Total protein ^b (g)	Specific activity (IU/mg)	Endotoxin (ng/mL)	Purification (fold)	Recovery (%)
1) Whole broth	9.0	N.D. ^c	N.D. ^c	N.D. ^c	N.D. ^c	100
2) Cell cake	7.0	N.D. ^c	N.D. ^c	N.D. ^c	N.D. ^c	78
3) Acetone powder (1st)	5.8	N.D. ^c	N.D. ^c	N.D. ^c	N.D. ^c	64
4) Acetone powder (2nd)	4.8	N.D. ^c	N.D. ^c	N.D. ^c	N.D. ^c	53
5) Acetone powder extract	2.9	430	6.7	11,500	1	32
6) CM-Sepharose Fast Flow	2.3	7.4	310	0.676	46	26
7) L-Asn Sepharose CL-4B	2.1	6.8	310	0.45	46	23

- a) Determined by ammonia probe assay
b) Determined by Bio-Rad dye binding assay
c) Not determined

Table 2
Recovery and Purification of L-Asparaginase
Using Filtration and Affinity Chromatography

Steps	Enzyme Activity (I.U./mL)	Protein Concen- tration (mg/mL)	Volume (L)	Specific Activity (I.U./mg)	Total Activity (I.U.) 10^{-6}	Total Protein (g)	Purifi- cation (fold)	Recovery (%)	Endotoxin (ng/mL)
1) Whole broth	42	-	880	-	37	-	-	100	-
2) Acetone powder extract	15	0.32	1400	47	21	448	1.0	57	-
3) L-Asn Sepharose Fast Flow affinity column (9 l)	1300	3.3	10.5	394	14	35	8.4	38	1.34

Table 3

Dose and pH Effect of Aluminum Oxide Depyrogenation

	Aluminum oxide (%)	pH	Protein Concentration* After Treatment (mg/mL)	Endotoxin Level After Treatment (ng/mL)
5	1	5	1.3	< 0.006
	1	6	1.3	< 0.006
	1	7	0.87	< 0.006
	0.5	5	1.4	< 0.006
10	0.5	6	1.3	< 0.006
	0.5	7	1.1	< 0.006

* Purified asparaginase (2.45 mg/mL) with endotoxin concentration 17 ng/mL was mixed with equal volumes of aluminum oxide solution at different percentages, which increased total volume to two fold. Protein concentration was expected to be 1.2 mg/mL after treatment, assuming protein was not adsorbed by aluminum oxide gel.

In this process, the cells were treated with acetone and the enzymes were recovered by borate extraction. The average step yield for acetone powder preparation was about 60% and for extraction was about 80%. The yields were slightly lower than the other methods reported (toluene-urea lysis: 85%, alkali lysis: 60-100%). Alkali lysis, in our experience, however, gave very poor yield (6%). Toluene-urea treatment, osmotic shock and mechanical fracture gave unsatisfactory results as well.

The use of the word chromatography is intended to include processes by which complex mixtures of molecules are separated by repeated partitionings between a flowing phase and a stationary phase. In ion exchange chromatography, a synthetic resin containing fixed charged groups attracts and binds charged substituents in a sample mixture. In affinity chromatography, proteins in a mixture are removed by exposing the mixture to a column containing a specific ligand for the protein. When the column is washed with buffer, the only protein(s) that remain on the

- 11 -

column are those with a high affinity for the ligand.

The present application also discloses the use of a filter press in the production of an enzyme-rich extract. Such filter presses are well known in the art of biomass separation and include flush-plate or plate-and-frame presses. See Chemical Engineers Handbook, Robert H. Perry and Cecil H. Chilton, eds., page 19-65 (1973).

Example 1

A production-scale process was developed to purify a therapeutic enzyme, L-asparaginase, from submerged cultures of Erwinia carotovora. Cells from 880 L of fermentation broth were harvested and washed using a 208 sq. ft. plate and frame-type filter press. A cellular acetone powder was prepared from the washed cells by suspending the cells in acetone at 10°C, then the acetone powder was recovered using the filter press. After a second acetone treatment, the residual acetone was removed by washing the acetone powder in the filter press with 10 mM phosphate buffer (pH 7.0). The acetone powder was then extracted with 10 mM borate buffer at pH 9.5. The enzyme-rich borate extract was recovered by filtration through the filter press and clarified by in-line passage through a bag filter loaded with Cell Debris Remover. The filtrate was adjusted to about pH 7.5 and then filtered through a 1 μ bag filter pre-coated with filter aid (Celite) followed by a sterile 0.22 μ final filter.

Example 2

Preparation of a cell extract: Cells were harvested from chilled whole cell broth (150 L) using two refrigerated vertical bowl centrifuges operating at 9,000xg and a flow rate of 1 L/min. The 4 kg of wet cell paste recovered was resuspended in 150 L of 10 mM potassium phosphate buffer, pH 7.0, and mixed for 30 min. The washed cells were again recovered by centrifugation. Acetone powder was prepared by resuspending the cell paste in 150 L of anhydrous acetone at 10°C and agitating for 30 min. To the mixture of acetone/cells was added 1.5 Kg of a fine

diatomaceous earth filter aid (Supercel, Johns-Manville Company), and the suspension was basket centrifuged using a 2 kg precoat of coarse filter aid (Celite type 545). The recovered solids (first acetone powder) were treated the same way a second time. This second acetone powder was extracted with 150 L of 10 mM sodium borate buffer, pH 9.5, for 40 min and the extract was clarified by basket centrifugation (1,100xg). The enzyme-rich extract was adjusted to about pH 7.7, then serially filtered through a Celite precoated μ bag filter and a 0.45 μ nominal hydrophilic cartridge filter.

Example 3

The sterile cell-free acetone powder extract at about pH 7.5 (1400 L) was applied to an L-Asn Sepharose Fast Flow affinity column (25.2 x 18 cm, 9 L bed volume) at a flow rate of 80 L/h using a bag filter loaded with Cell Debris Remover as an in-line pre-filter. The affinity gel was prepared by coupling L-Asn at pH 9.0 to epoxy-activated Sepharose Fast Flow beads. After washing the column with at least 10 bed volumes of 5mM sodium phosphate buffer, pH 7.5, the column was eluted with 10 mM glycine buffer at pH 9.0. A total of 14×10^6 IU (35 g protein) of purified enzyme was eluted from the column in 10.5 L volume. A 9-fold purification of enzyme with a 10,000-fold reduction of endotoxins was achieved with affinity chromatography. The eluted enzyme was determined to be greater than 90% pure when assayed by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Example 4

The cell-free acetone powder extract (150 L) at about pH 7.7 is adsorbed onto a 16 L CM-Sepharose Fast Flow column using a pre-column packed with Cell Debris Remover modified cellulose. The column was washed at pH 7.7, the enzyme was desorbed from the ion exchange column at pH 9.0 and further purified by an affinity column of L-Asparagine Sepharose at pH 7.5. After washing the column with at least 5 bed volumes of 5 mM phosphate buffer, pH 7.5, the

column was eluted with 10 mM glycine buffer at pH 9.0. A total of 7 grams of substantially purified enzyme was eluted from the column. A 46-fold purification of enzyme with 10,000-fold reduction of endotoxin was achieved. The eluted enzyme was determined to be greater than 90% pure when assayed by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

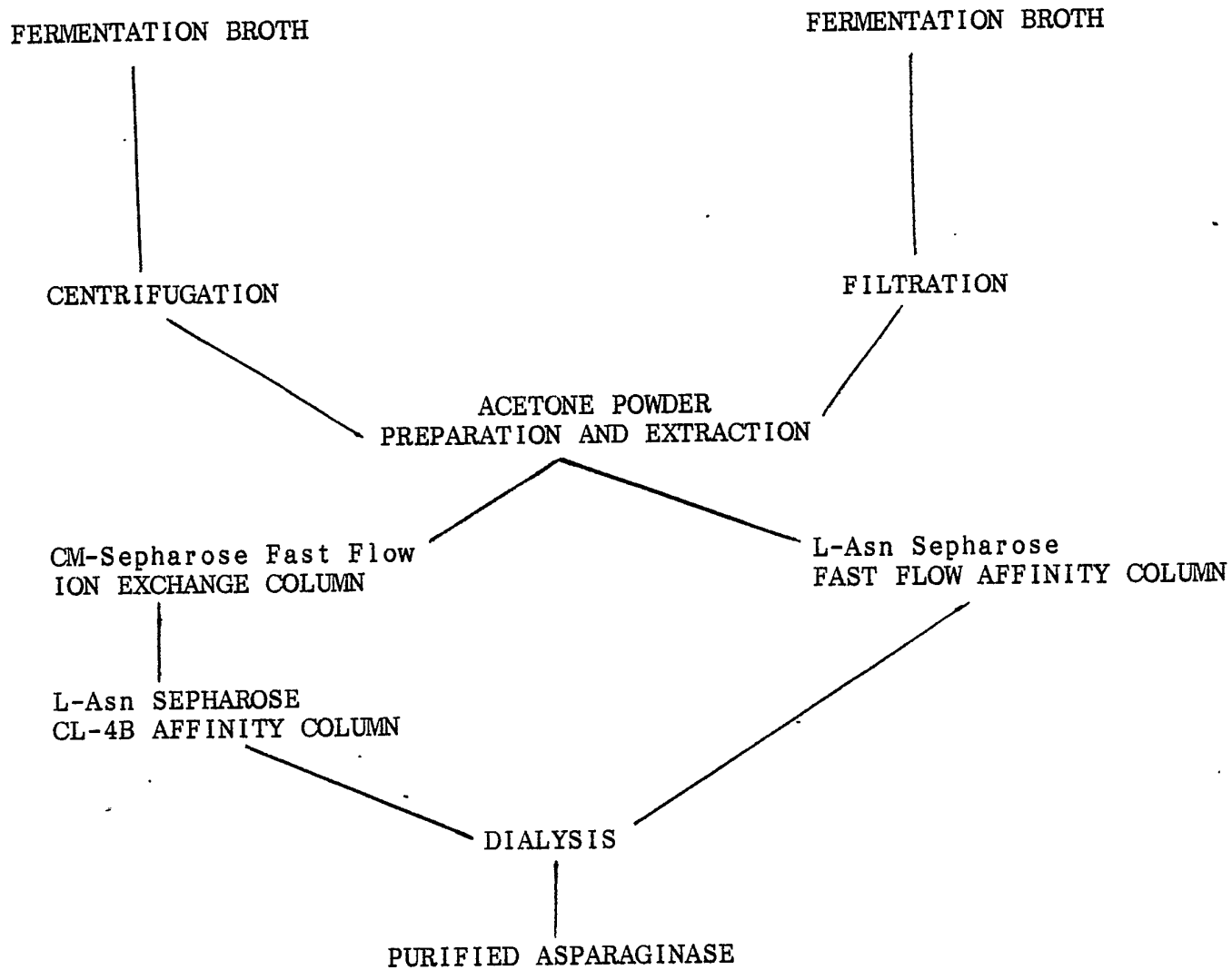
Example 5

Table 4 summarizes a typical final processing lot from the affinity column eluate pool to the sterile formulated enzyme. The overall yield of the product from fermentation broth to final formulated product as produced for this lot by this invention was 19%. See Table 4.

Table 4
L-Asparaginase Lot XX004B Final Processing

Steps	Enzyme Activity (IU/mL)*	Total Volume (mL)	Total Activity (IU)-6	Recovery (%)
1) Affinity column pool	2,610	1,000	2.61	100
2) Dialyzed concentrated enzyme	54,600	46	2.51	96
3) Aluminum oxide treatment	26,700	85	2.26	87
4) Diluted enzyme before formulation	13,300	167	2.22	85
5) Formulated sterile filtered enzyme	10,800	197	2.13	82

* Determined by Nessler reaction



IN THE CLAIMS:

1. A process for purifying L-asparaginase from Erwinia carotovora, characterized by:

forming a cell-free extract containing asparaginase enzyme;

purifying said cell-free extract through an affinity chromatography column.

2. The process of Claim 1, further characterized in that said cell-free extract is purified through an ion exchange chromatography column prior to purification through said affinity chromatography column.

3. The process of Claim 1, further characterized in that a modified cellulose product is used to remove cell debris prior to affinity chromatography.

4. The process of Claim 2, further characterized in that a modified cellulose product is used to remove cell debris prior to ion exchange chromatography.

5. The process of Claim 1, further characterized in the L-asparaginase is dialyzed after affinity chromatography.

6. A process for purifying L-asparaginase from Erwinia carotovora, characterized in subjecting said L-asparaginase to at least one chromatographic separation step.

7. The process according to Claim 6, further characterized in that one chromatographic separation step is ion exchange and another chromatographic separation step is affinity chromatography.

8. The process of Claim 6, further characterized in that the chromatographic separation is affinity chromatography.

9. A process of purifying L-asparaginase from cell cultures of Erwinia carotovora, characterized by a step-wise process of:

(a) producing cell-free extract containing L-asparaginase enzyme by preparation of a cellular acetone powder and subsequent extraction in buffer; and

- 17 -

(b) purifying said extract by affinity chromatography.

10. The process of Claim 9, further characterized in that said cell-free extract is purified through an ion exchange chromatography column prior to the affinity chromatography purification step.

11. The process of Claim 9, further characterized in that step (a) comprises a cell wash step at about pH 7.0 followed by acetone powder preparation and extraction at pH 9.5.

12. The process of Claim 10, further characterized in that the ion exchange purification is carried out at about pH 7.7 and said enzyme is eluted from said column at about pH 9.0.

13. The process of Claim 10, further characterized in that the affinity chromatography is carried out at about pH 7.5 and said enzyme is eluted from said column at about pH 9.0.

14. A process for the production of purified L-asparaginase from Erwinia carotovora, characterized by:
separating crude Erwinia carotovora extract through at least one chromatography column to obtain crude asparaginase enzyme; and

dialyzing said purified enzyme.

15. The process of Claim 14, further characterized in that the chromatography step is a single affinity chromatography step.

16. The process of Claim 14, further characterized in that said at least one the chromatography step comprises an ion exchange chromatography step followed by an affinity chromatography step.

17. A process for the production of purified L-asparaginase from Erwinia carotovora, characterized by:

harvesting and washing Erwinia carotovora cells;

producing a cellular acetone powder by contacting said washed cells at least once with acetone;

- 18 -

extracting the acetone-treated cells with borate buffer to produce an enzyme rich borate extract;

clarifying and filtering said extract by removing cell debris to produce a sterile cell-free extract;

separating said purified enzyme through an affinity chromatography column to produce substantially pure L-asparaginase.

18. The process of Claim 17, further characterized by an ion exchange separation step between said clarifying and filtering step and said affinity chromatography step.

19. The process of Claim 18, further characterized in that said substantially pure L-asparaginase is dialyzed.

20. A stepwise process for the purification of L-asparaginase enzyme from Erwinia carotovora, characterized by:

(a) initially harvesting and washing cells from a fermentation broth of Erwinia carotovora utilizing a filter press or centrifugation;

(b) treating the washed cells with dry acetone at least one time;

(c) extracting cellular acetone powder with 10 mM borate buffer at a pH of about 9.5 and recovering a cell-free enzyme-enriched extract;

(d) adjusting the pH of said extract to a pH of about 7.7, loading said extract on an ion exchange chromatography column, eluting the ion exchange column with 10 mM glycine buffer at a pH of about 9.0, adjusting the ion exchange eluent to a pH of about 7.5, loading the ion exchange eluent onto an affinity column, and eluting substantially pure enzyme with 10 mM glycine buffer at a pH of about 9.0.

21. A stepwise process for the purification of L-asparaginase enzyme from Erwinia carotovora, characterized by:

- 19 -

(a) initially harvesting and washing cells from a fermentation broth of Erwinia carotovora utilizing a filter press or centrifugation;

5 (b) treating the washed cells with dry acetone at least one time;

(c) extracting cellular acetone powder with 10 mM borate buffer at a pH of about 9.5 and recovering a cell-free enzyme-enriched extract;

10 (d) adjusting the pH of said extract to a pH of about 7.5, loading the extract on an affinity chromatography column, and eluting the substantially pure enzyme with 10 mM glycine buffer at a pH of about 9.0.

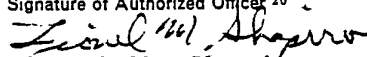
22. A method of preparing L-asparaginase from Erwinia carotovora comprising at least two processing
15 stages, characterized in that said processing stages include at least one filter processing or centrifugation stage and at least one chromatography stage.

23. The method of Claim 22, further characterized in that the chromatography stage includes affinity
20 chromatography.

24. The method of Claim 22, further characterized in that the chromatography stage includes ion exchange chromatography followed by affinity chromatography.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 86/02665

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int. C1 4 C12N 9/82		
U.S. C1 435/229		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
U.S.	435/229, 815, 847	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁵		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
X	Biotechnology and Bioengineering, Vol. XX, issued 1978, (John Wiley & Sons, Inc. New York, USA) (KARUBE et al), "Photocontrol of Affinity Chromatography" Purification of Asparaginase by Photosensitive AHA-Gel", pages 1775-1783, See particular pages 1777-1778.	1,3,5,6 8,9,11,14, 15,21,22 & 23
X	Chemical Abstracts, Vol. 83, No. 1, issued 7 July 1975 (Columbus, Ohio, USA) (MARDASHEV et al) "Purification of Microbial asparaginases using affinity chromatography", page 298, the abstract No. 3257p, Biokhimiya 1975, 40(1), 78-82 (Russ)	1,3,5,6 8,9,11,14 15,21,22 & 23
* Special categories of cited documents: ¹⁵ "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ²	Date of Mailing of this International Search Report ²	
15 January 1987	10 FEB 1987	
International Searching Authority ¹	Signature of Authorized Officer ²⁰	
ISA/US	 Lionel M. Shapiro	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No ¹⁸
X	Chemical Abstracts, Vol. 88, No. 21 issued 22 May 1978 (Columbus Ohio, USA) (KARASKEVICH et al) page 220, the abstract No. 148009x "Affinity Chromatography of L-asparaginase from Escherichia coli on silicate carriers", Khim Priir Soedin 1978, (1), 118-121 (Russ)	1,3,5,6,8 9,11,14,15 21,22 & 23
Y	Journal of General Microbiology, Vol. 65, Part 3, page 1, issued March 1971 (Cambridge University Press, London England) (BUCK et al) "The Batch Production of L-asparaginase from Erwinia carotovora"	2,4,7,9,10 12,13,16-24
Y	US, A, 3,597,323 issued 3 August 1971 Roberts, note column 3 lines 1-23	2,4,7,9, 10,12,13, 16-24
Y	US, A, 3,684,659 issued 15 August 1972 BERGMEYER et al, note column 3 lines 1-55 and examples 1-3	2,4,7,9, 10,12,13, 16-24
Y	US, A, 4,473,646 issued 25 September 1984, GUY et al, note column 7, Table 2	
Y	US, A, 3,511,754 issued 12 May 1970 BERK et al, note column 1 line 34 to column 2 line 12	2,4,7,9, 10,12,13, 16-24
A	US, A, 3,511,755 issued 12 May 1970 RATYCH et al, note the Abstract of the Disclosure.	
A	US, A, 3,589,982 issued 29 June 1971 PETERSON et al, note column 2 lines 47-63.	
A	US, A, 3,660,238 issued 2 May 1972 WADE et al, note column 1 lines 44-55.	
A	US, A, 3,686,072 issued 22 August 1972 HERBERT et al, note the Abstract of the Disclosure.	
A	US, A, 3,783,102 issued 1 January 1974 CALLOW et al, note the Abstract of the Disclosure.	
A	US, A, 3,843,445 issued 22 October 1974 CHRISTIE et al, note column 5 lines 56-61.	

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X	Chemical Abstracts, Vol. 97, No. 13, issued 27 September 1982 (Columbus, Ohio, USA) (SUZUKI et al) "Photocontrol of Affinity Chromatography", page 234, the abstract No. 105968C, Recent Dev. Spe. Sci. 1981, 6, 117-135 (Eng).	1,3,5,6,8 9,11,14,15, 21,22 & 23
---	---	--

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹⁰

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers because they relate to subject matter ¹² not required to be searched by this Authority, namely:

2. ☐ Claim numbers because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ¹¹

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
☐ No protest accompanied the payment of additional search fees.