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Large-Scale Recovery and Purification of L-Asparaginase from *Erwinia carotovora*

SHWU-MAAN LEE,* JOHN T. ROSS, MARK E. GUSTAFSON,† MARIE H. WROBLE, AND GARY M. MUSCHIK

Program Resources, Inc., National Cancer Institute, Frederick Cancer Research Facility, P.O. Box B, Frederick, MD 21701

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ABSTRACT

A large-scale process was developed to purify gram quantities of a therapeutic enzyme, L-asparaginase, from submerged cultures of *Erwinia carotovora*. Cells were harvested from 150 L of fermentation broth and washed. A cellular acetone powder was prepared and extracted with pH 9.5 borate buffer. After continuous centrifugation and filtration to remove cell debris, the acetone powder extract was adjusted to pH 7.7 and adsorbed onto a 16-L CM-Sepharose Fast Flow column, with a precolumn packed with Cell Debris Remover. The enzyme was desorbed from the catin-exchange column at pH 9.0 and further purified with an affinity column of L-asparagine Sepharose CL-4B. After dialysis-concentration to remove buffer salt, the enzyme was depyrogenated, formulated, sterile filled, and lyophilized as a single-dose final product. the final-product evaluation included analysis of the content of protein, sodium chloride, glycine, sodium, glucose hydrate, phosphate, and endotoxin, as well as reconstitution, potency, pH, specific activity, uniformity of fill, and sterility. The product was further subjected to visual examination, sodium dodecyl sulfate polyacrylamide gel electrophoresis, native gel electrophoresis, isoelectric focusing, amino acid analysis, *N*-terminal sequencing, peptide mapping, and immunological comparison.

†Present address: Monsanto Company, 800 North Lindbergh Boulevard, St. Louis, MO 63167.

*Author to whom all correspondence and reprint requests should be addressed.

Index Entries: L-Asparaginase; *Erwinia carotovora;* affinity chromatography; downstream processing; protein purification; protein recovery.

INTRODUCTION

L-Asparaginase [L-asparagine (L-Asn) amidohydrolase, EC 3.5.1.1] catalyzes the hydrolysis of 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 a result of the enzyme L-asparaginase (*1*). Unlike normal cells, the malignant cells can only synthesize L-Asn slowly and are dependent on an exogenous supply. The antineoplastic activity results from depletion of the circulating pools of L-Asn by L-asparaginase (*2*). L-Asparaginase from *Escherichia coli* has a tumor inhibitory effect (*3*), and the enzyme isolated from *Erwinia carotovora* was also found to be pharmacologically active (*4*). Because the L-asparaginases from *E. coli* and *E. carotovora* possess different immunological specificities, they offer an important alternative therapy if a patient becomes hypersensitive to one of the enzymes (*2*). The literature on the fermentation and purification processes for L-asparaginase production has been reviewed (*5*). L-Asparaginase from *E. carotovora* is an intracellular enzyme (*6*). One purification process involved alkali lysis, multiple ion-exchange adsorptions, ammonium sulfate precipitation, multiple dialysis-concentration steps, and ethanol crystallization (*7*). The purpose of our study was to develop an improved process to produce *Erwinia* L-asparaginase in gram quantities for animal toxicology testing.

MATERIALS AND METHODS

Asparaginase Assay

Two assays were used, one measuring the ammonia liberated during the enzymatic reaction of L-asparaginase with L-Asn using an ammonia electrode and a second measuring the ammonia using a colorimetric Nessler reaction (*8,9*).

Ammonia Probe Assay

Whole-broth analysis was initiated by centrifugation of a 1-mL aliquot at 12,000*g*. The pelleted cells were washed twice with 1 mL of 0.1*M* borate buffer containing 0.01% bovine serum albumin (BSA). The washed cells were resuspended in the same buffer and assayed. Cell pellet was washed the same way and assayed as a suspension. Acetone powder was assayed as a suspension without washing. All soluble enzyme samples were assayed directly. The assay mixture consisted of 1.9 mL of 0.1*M* L-Asn in borate/BSA buffer and 0.1 mL of sample. The reac-

tion was allowed to proceed at 37° for 10 min, and the incubation was stopped by adding 0.5 mL of 15% (w/v) trichloroacetic acid (TCA). The ammonia generated was analyzed with an ion-selective electrode (HNU systems, Inc.), using an ionalyzer (model 407A, Orion Research). The assay was linear between 0.04 and 5 IU. One IU of L-asparaginase was defined as that amount of enzyme that catalyzed the formation of 1 μmol of ammonia/min.

Nessler Assay

The assay is based on procedures described by Wriston (*8*) and Wade and Phillips (*9*). The assay buffer was 0.05*M* sodium borate buffer (0.05*M* in sodium), pH 8.5, containing 0.005% BSA. The reaction was started by adding 0.25 mL of 0.04*M* L-Asn to 0.75 mL of enzyme solution and allowed to proceed at 37°C for 5 min. The reaction was stopped with 0.25 mL of 15% TCA. A total of 3.25 mL of water and 0.5 mL of Nessler's reagent (Sigma) were subsequently added; the reaction mixture was allowed to stand for 15 min at room temperature (RT), and the absorbance at 500 nm was measured. The amount of ammonia released was determined from an ammonium sulfate standard curve. The assay was linear between 0.05 and 0.8 IU.

Protein Determination

Protein was determined using either the Bio-Rad dye binding assay (*10*) with bovine γ-globulin as a standard or the Lowry protein assay (*11*) with BSA (Sigma) as a standard.

Affinity Gel Preparation

The L-Asn Sepharose CL-4B was prepared using an adaptation of the Sundberg and Porath method (*12*). Sepharose CL-4B (Pharmacia, 4 kg) was activated by reaction with 4 L of 1,4-butanediol diglycidyl ether (Sigma) and 4 L of 0.6*N* sodium hydroxide solution containing 8 g of sodium borohydride. The activation reaction was allowed to proceed at RT for 15 h in a 50-L glass rotary evaporator (Brinkman). The activated gel was 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 0.5*M* sodium carbonate buffer (35 L, final pH 8.5). The coupling reaction was allowed to proceed at RT for 15 h with slow rotation in the same rotary evaporator. After the coupling step, the gel was washed seven times with 150 L of water each time and stored in 0.02% sodium azide.

Enzyme Purification

All procedures were carried out at 2–10°C, unless otherwise mentioned.

Preparation of a Cell-Free Extract

Cells were harvested from 150 L of chilled whole-cell broth using two refrigerated, vertical-bowl centrifuges (Sharples, M-16) operating at 9000*g* and a flow rate of 1 L/min. The 4 kg of wet cell paste recovered was resuspended in 150 L of 10 m*M* 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 agitated for 30 min. To the mixture of acetone and cells was addd 1.5 kg of Supercel (Johns-Manville Co.), and the suspension was centrifuged (Western States 12-in basket centrifuge) using a precoat of celite type 545 (2 kg, Johns-Manville Co.). The recovered solids (first acetone powder) were treated the same way with acetone a second time and centrifuged without adding Supercel. This second acetone powder was extracted with 150 L of 10 m*M* sodium borate buffer, pH 9.5, for 40 min, and the extract was clarified by basket centrifugation (1100*g*). The enzyme-rich extract was serially filtered through a celite-precoated 1-μm bag filter (GAF Corporation) and a 0.45-μm nominal hydrophilic cartridge filter (Sartorius).

Cation-Exchange Column Chromatography

The CM-Separose Fast Flow gel (Pharmacia) was suspended in water and packed in a 16-L column (Pharmacia, 37 × 15 cm) with compressed air pressure of 1 bar. The CM column was stored in 0.02% sodium azide when not in use. Cell Debris Remover (CDR, Whatman) was preequilibrated with 0.1*M* sodium phosphate buffer, pH 7.7, basket centrifuged to remove the buffer and reequilibrated twice with 5 m*M* sodium phosphate buffer, pH 7.7. The CDR was packed in a 16-L column (37 × 15 cm, Pharmacia) with the same buffer, containing 0.1% benzalkonium chloride (Sigma) as a preservative. Before the production run, the CM column and the CDR precolumn were washed separately with at least 2 bed vol of 5 m*M* sodium phosphate buffer, pH 7.7, to remove preservatives. The two column sections were joined, and the flow (upward for the CM column and downward for the precolumn) was maintained with a peristatic pump (Millipore). The acetone powder extract (150 L) was adjusted to pH 7.7 and applied to the columns with a flow rate of 0.5 L/min, which was equivalent to a linear flow rate of 28 cm/h. The flow rate was reduced during the run, if necessary, to keep the running pressure at or below 1 bar. After loading, the CDR precolumn was detached and the CM column extensively washed with the buffer until the effluent was devoid of protein, as determined with the Bio-Rad protein microassay (*10*). The enzyme was eluted from the column with 10 m*M* glycine buffer, pH 9.0. The enzyme-rich fractions were pooled, adjusted to pH 7.5 with dilute HCl, and applied to the L-Asn Sepharose CL-4B affinity column. The CM column regeneration was accomplished by sequential washing with 2*M* NaCl and 0.1*N* NaOH.

Affinity Chromatography

The L-Asn Sepharose CL-4B affinity column (10 × 20 cm, 1.6-L bed vol, Pharmacia) was packed and stored in 0.02% sodium azide. Before use, the column was washed with at least 2 bed vol of 5 m*M* sodium phosphate buffer, pH 7.5, to remove sodium azide. The linear flow rate was 34 cm/h, which was equivalent to a flow rate of 2.7 L/h. After loading, the column was washed with at least 5 bed vol of the same buffer, and the enzyme was eluted with 10 m*M* glycine buffer, pH 9.0. The enzyme-rich fractions were pooled, adjusted to pH 7 with dilute HCl, and frozen at −20°C for final processing. The affinity column was regenerated by sequential washing with 0.1*M* Tris-HCl, pH 9.0, containing 0.5*M* NaCl and 0.1*M* sodium acetate buffer, pH 4.5, containing 0.5*M* NaCl.

Final Processing

The affinity column eluent pool (1 L) was thawed at 2–8°C, concentrated using a 2-L ultrafiltration pressure cell (Amicon, High-Output Stirred Cell 2000) with C-30 membrane (cellulosic membrane, 30,000 nominal mol wt cut-off, Dorr-Oliver), and dialyzed at least 10^8-fold with depyrogenated water for 15 h. The dialyzed-concentrated enzyme was transferred to a 200-mL ultrafiltration pressure cell (Amicon) and further concentrated to a minimum titer of 30,000 IU/mL. Sterile aluminum oxide (Rehsorptar II, Reheis Chemical Company) was diluted with depyrogenated water and mixed with an equal volume of concentrated enzyme. The mixture was adjusted to pH 6.0 with dilute acetic acid or dilute sodium hydroxide, stirred for 30 min at RT, and centrifuged (45,000*g*, 30 min) to pellet the aluminum oxide. Beyond this state, all glassware was treated with 1% nitric acid after detergent washing and subsequently autoclaved to avoid endotoxin contamination. Following centrifugation, the enzyme solution was diluted to 13,000 IU/mL with depyrogenated water preadjusted to neutral pH. Based on chloride analysis (*13*), the enzyme solution was adjusted to a final chloride concentration of 12.5 m*M* with sodium chloride. Glucose hydrate (USP grade) was added to a final concentration of 0.625%. The formulated enzyme solution was filtered through a 0.8-μm prefilter and 0.22-μm final filter (Nalgene) into a sterile container. Residual enzyme was washed though the filtration units with neutralized depyrogenated water (25% of the original enzyme solution volume was used) and combined with the enzyme solution to bring the enzyme titer to 10,750 IU/mL, the glucose hydrate concentration to 0.5%, and the NaCl concentrtion to 10 m*M*. This solution was dispensed as 1-mL aliquots into sterile serum vials (Wheaton) using a calibrated sterile wyringe (Cornwall). The vials were lyophilized in a Consol 12 (Virtis) shelf lyophilizer and subsequently capped under sterile nitrogen.

Standard

Standard *Erwinia* L-asparaginase (lots MRE 31, 33, 34, and 35) was provided by Dr. Matthew Suffness, Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland.

Final Product Analysis

The following tests were performed on the finished enzyme product and the results were compared to those obtained in assays of the standard.

Visual Examination

The final product and the standard were examined for color (uniformly white powder) as well as the absence of foreign particulate matter.

Reconstitution

Two milliliters of 0.9% sodium chloride/vial dissolution yielded a colorless solution 2 min after it was added to the product.

Analyses of Potency, Protein, and Specific Activity

Each sample vial was reconstituted with 2 mL of 0.9% sodium chloride; the Nessler and Lowry assays were used to determine potency and protein content, respectively. Potency of 9500–12,000 IU/vial was considered acceptable. The specific activity of individual vials was calculated from the potency and protein data. A specific activity of 600–750 IU/mg was acceptable. Results of lots XX04A and XX04B and the standard were reported as the mean value obtained in assays of five, seven, and four vials, respectively. The four vials of the standard were from four different lots.

pH

pH value was determined by measuring the sodium chloride-reconstituted enzyme solution. A pH range of 6.0–7.5 was acceptable.

Uniformity

The uniformity of fill was assessed by assaying the protein content of the final product reconstituted in sodium chloride solution. A standard deviation (n = 5 for XX04A, n = 7 for XX04B) of 5% or less was acceptable.

Sodium Chloride Content

The final product was reconstituted with water and deproteinized with nitric acid. Chloride content was determined using the colorimetric mercuric thiocyanate–ferric ion method (*13*). The specified sodium chloride content of each vial was 0.54–0.66 mg.

Glucose Hydrate Analysis

Glucose hydrate was determined on the water-reconstituted final product using Statzyme glucose reagent (Worthington). The specifiation was 4.5–5.5 mg of glucose hydrate/vial.

Sodium Content

Sodium content was determined using the atomic absorption method (*14*).

Phosphate Content

Phosphate was determined on the saline-reconstituted final product using the phosphomolybdenum blue colorimetric assay (*15*) after deproteinization with 2*N* sulfuric acid.

Glycine Assay

Glycine content was determined by an amino acid analysis method using high-performance liquid chromatography (HPLC). The primary amino acids were allowed to react with orthophthaladldehyde and form fluorescent derivatives that were chromatographed on a C_{18} column (Waters Associates) (*16*).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE)

We used the SDS PAGE method described by Laemmli (*17*). Gels of 1.5 mm thickness were constituted with 10% polyacrylamide and stained with Coomassie blue. Molecular weight protein markers (Pharmacia) included phosphorylase b, 94,000; BSA, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 20,000; and α-lactalbumin, 14,000.

Isoelectric Focusing (IEF)

Isoelectric focusing was performed on Ampholine PAG plates (LKB, pH range 3.5–9.5) according to the manufacturer's instructions. Gels were stained with 0.03% Coomassie brilliant blue G-250 in 3.5% perchloric acid (*18*).

Native Gel Electrophoresis

Electrophoresis for native asparaginase was performed according to the method described by Reisfeld et al. (*19*), with some modifications in the running gel. The running gel consisted of 7.5% acrylamide, 0.2% bisacrylamide, 0.06*N* KOH, 0.375*M* acetic acid, 0.75% *N,N,N′,N′*-tetramethylethylenediamine, and 0.14% ammonium persulfate. Electrophoresis was performed in a minislab gel apparatus (Bio-Rad, 0.75 mm thickness). Basic fuchsin (Sigma) was used as a dye marker.

Amino Acid Sequencing

N-terminal sequencing was performed on a Beckman sequencer, model 809C, according to the methods described by Copeland et al. (*20*).

Peptide Mapping

The proteins were oxidized with performic acid (*21*) and digested with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Millipore) at 37°C, using a protein to trypsin ratio of 50:1 (w/w). The peptide mapping was performed with HPLC using a μ Bondapak C_{18} column (Waters). A linear gradient of 0–60% acetonitrile, containing 0.1% trifluoroacetic acid, over 40 min at a flow rate of 1 mL/min was used.

Endotoxin Assay

The concentration of endotoxins in solution was determined with a chromogenic limulus amebocyte lysate (LAL) assay, using a QCL-1000 kit (M.A. Bioproducts). An inhibition control was routinely used to check the validity of the assay.

Sterility Test

Sterility of the final product was determined by putting the contents into a sterile growth medium of trypticase soy agar (Difco) with 0.1% glucose and checking for microbial contamination over a period of 24–48 h at 37°C.

RESULTS

A summary of the recovery and purification is presented in Table 1. The overall purification factor achieved with ion-exchange and affinity chromatography was 46-fold. The yield from cell harvest through the acetone powder extraction step was 32%, whereas the yield through ion-exchange and affinity chromatography was 72%, giving an overall yield of 23%.

Because *Erwinia* L-asparaginase is a basic protein (*22*) and most bacterial proteins are acidic, a significant purification was achieved by initial cation-exchange chromatography. As shown in Fig. 1 (lanes 2 and 5), the material eluted from the cation-exchange column (starting material for the affinity column) contained only one extra protein contaminant compared to the standard. This extra protein contaminant was successfully removed by affinity chromatography. Figure 2 is a typical loading and elution profile from an affinity column. Affinity column wash material was concentrated by ultrafiltration and applied to SDS gel (Fig. 1, lane 4). The gel indicates that this protein is, in fact, the contaminant identified in the cation-exchange column eluent. The removal of this extra protein contaminant by affinity chromatography produced the material equivalent to the standard (Fig. 1, lanes 2 and 3).

The affinity column eluent pool in glycine buffer could be stored at −20°C after adjustment to neutral pH. This was a convenient holding point in the process.

Erwinia is a gram-negative microorganism (*23*), and, as such, the cell-free extract was expected to contain large quantities of endotoxins. The

TABLE 1
Recovery and Purification of L-Asparaginase from *Erwinia carotovora*

Step	Total[a] activity, IU × 10^{-6}	Total[b] protein, g	Specific activity, IU/mg	Endotoxin, ng/mL	Purification, -fold	Recovery, %
1. Whole broth	9.0	ND[c]	ND	ND	ND	100
2. Cell pellet	7.0	ND	ND	ND	ND	78
3. Acetone powder (1st)	5.8	ND	ND	ND	ND	64
4. Acetone powder (2nd)	4.8	ND	ND	ND	ND	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.

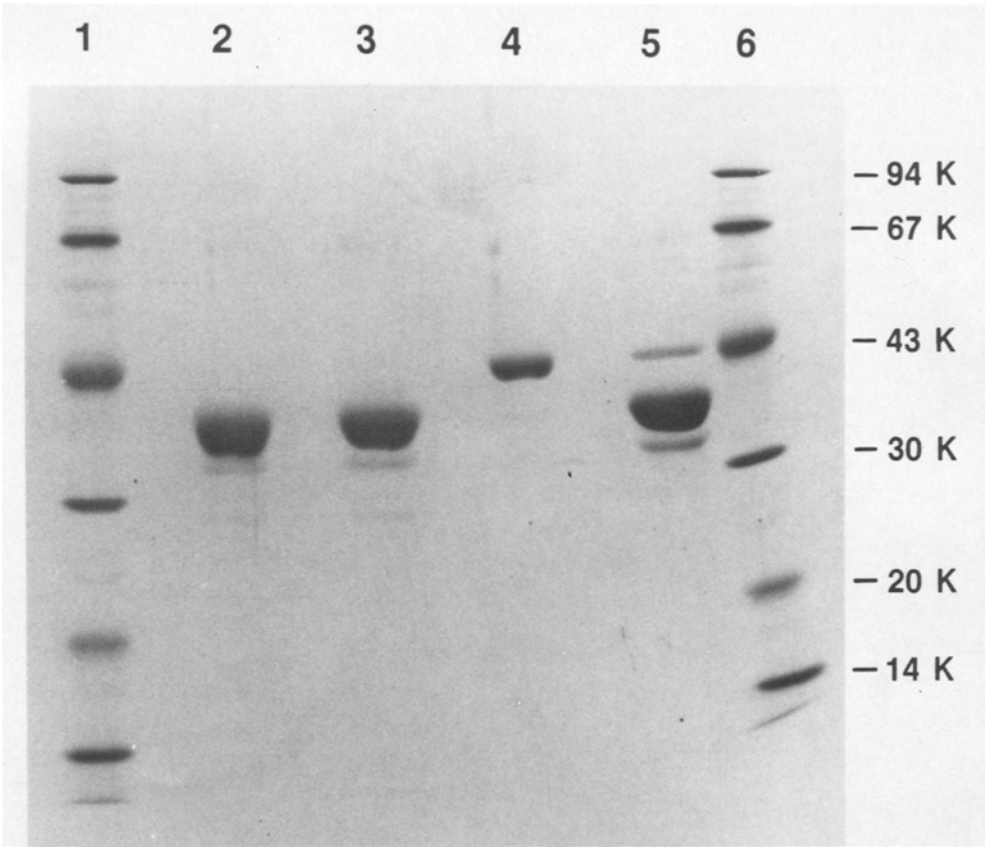

Fig. 1. SDS Gel of column fractions and standard. Lanes 1 and 6: 4 μg of each molecular weight standard; lane 2: standard (lot MRE 33), 10 μg; lane 3: affinity column elution pool, 10 μg; lane 4: affinity column wash, 5 μg; lane 5: CM column elution pool (affinity column starting material), 10 μg.

endotoxin level dropped from 11.5 μg/mL to 0.676 ng/mL following cation-exchange chromatography and further decreased to 0.45 ng/mL through affinity chromatography (Table 1). When depyrogenated water was used in all chromatographic operations, the enzyme-rich affinity column eluent usually contained endotoxins in the range of 0.1–0.5 ng/mL. However, endotoxins are known to form high-molecular-weight aggregates (*24*) and may then concentrate with the protein product.

Aluminum oxide gel is an effective endotoxin adsorbent for certain proteins (*25–27*). It has also been demonstrated to be effective for pyrogen removal from L-asparaginase (*7*). To determine optimum conditions for depyrogenation, concentration and 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 pH was varied. Aluminum oxide appeared to be equally effective for depyrogenation at pH 5, 6, and 7 at 1% (w/v) and 0.5% (Table 2). However, at pH 7 some protein loss occurred.

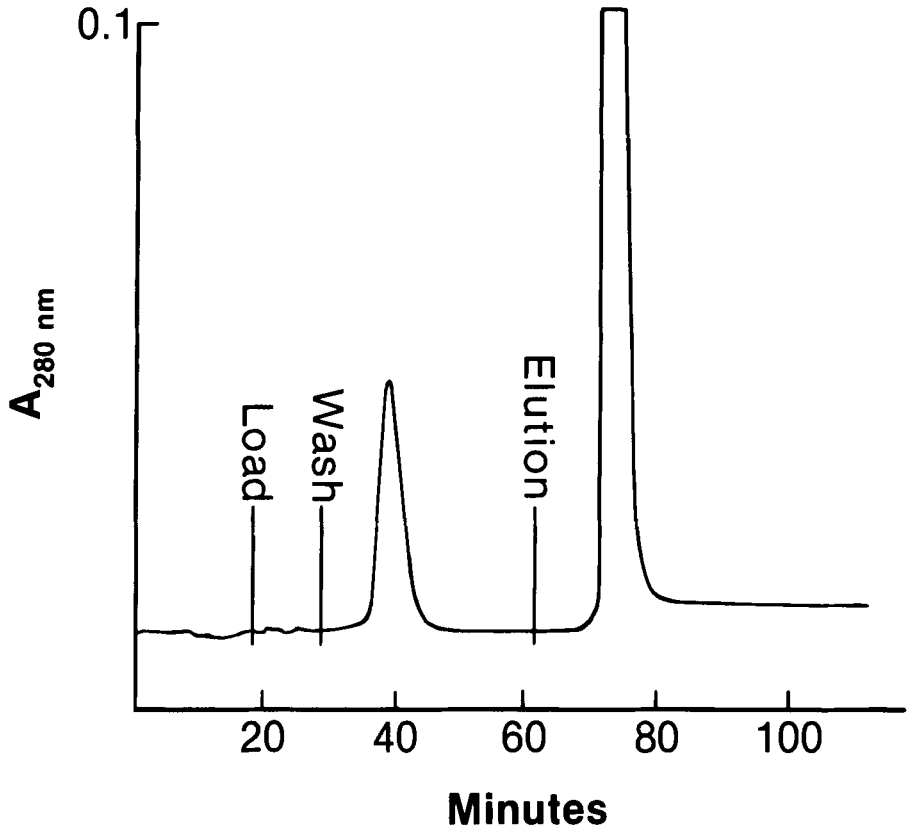

Fig. 2. Affinity column loading and elution profile. A total of 4600 IU of enzyme (CM column elution pool) was applied to an affinity column (1.6 × 7.5 cm). Column effluent was monitored at 280 nm for protein using an ISCO UA-5 absorbance monitor. The flow rate was set at 2 mL/min.

TABLE 2
Dose and pH Effect of Aluminum Oxide Depyrogenation

Aluminum oxide, % w/v	pH	Protein concentration[a] after treatment, mg/mL	Endotoxin level after treatment, ng/mL
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
0.5	6	1.3	<0.006
0.5	7	1.1	<0.006

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

Table 3 summarizes the final processing of lot XX04B. Aluminum oxide treatment resultd in 9% loss of enzyme as a result of the hold-up volume in the gel. We did not try to recovery this trapped enzyme. The yield from the affinity column pool through dialysis-concentration, depyrogenation, and formulation was 82%, giving an overall yield from fermentation to final formulated product of 19%.

This process yielded lots XX04A (72 vials) and XX04B (197 vials) in single-dose form. Table 4 and Figs. 3–7 present the results of final-product analysis. To ensure comparable analysis to the standard, we analyzed the final product and the standard together in most of the assays.

TABLE 3
L-Asparaginase Lot XX004B Final Processing

Step	Enzyme[a] activity, IU/mL	Total volume, mL	Total activity, IU × 10^{-6}	Recovery, %
1. Affinity column pool	2610	1000	2.61	100
2. Dialysis concentration	54,600	46	2.51	96
3. Aluminum oxide treatment	26,700	85	2.26	87
4. Dilution of enzyme before formulation	13,300	167	2.22	85
5. Formulation and sterile filtration	10,800	197	2.13	82

[a]Determined by Nessler assay.

TABLE 4
L-Asparaginase Final-Product Analysis

Assays	Lot XX04A	Lot XX04B	Standard[a]
Potency, IU/vial	11,400	10,400	10,245
Protein, mg/vial	17.2	15.4	16.4
Sodium chloride, mg/vial	0.55	0.56	ND[b]
Sodium, mg/vial	0.20	0.19	ND
Glucose hydrate, mg/vial	4.4	5.3	ND
Phosphate, μg/vial	<15	<15	ND
Glycine, μg/vial	<0.2	<0.2	ND
Endotoxin, ng/vial	0.007	0.007	<0.013
pH	6.35	6.10	ND
Specific activity, IU/mg	661	676	627
Uniformity, %	3.1	2.8	ND
Sterility	Pass	Pass	ND
Visual examination	Pass	Pass	Pass
Reconstitution	Pass	Pass	Pass
SDS gel	>90% purity	>90% purity	>90% purity
Native gel	>95% purity	>95% purity	>95% purity

[a]Data obtained from assays of different lots.
[b]Not determined.

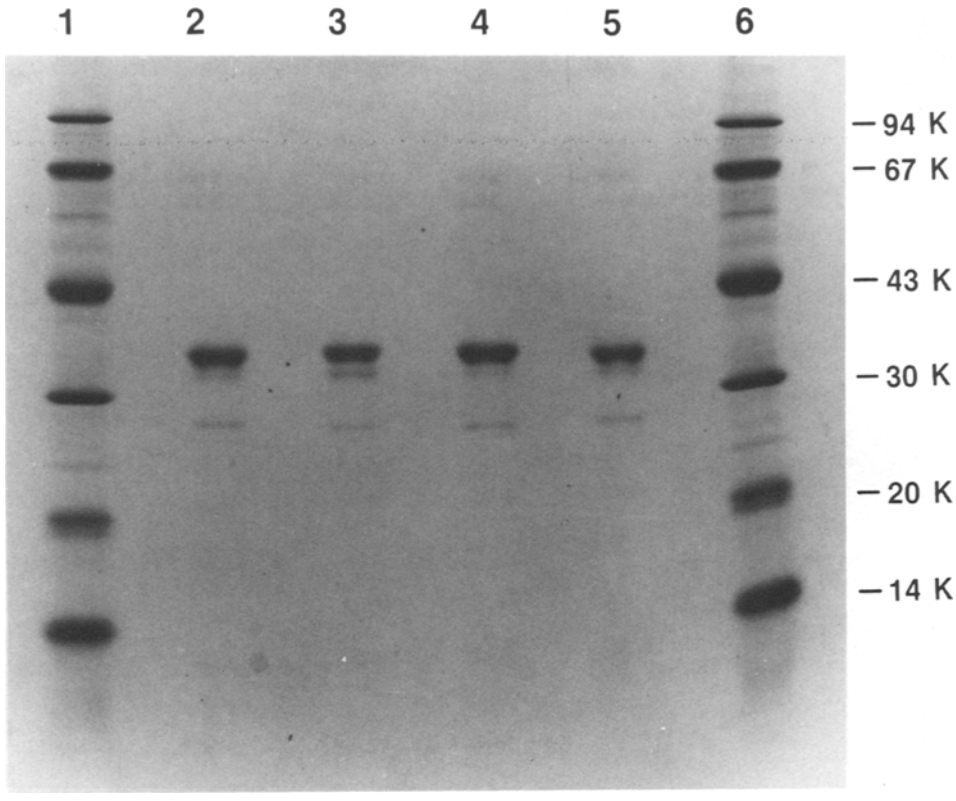

Fig. 3. SDS gel of final product. Lanes 1 and 6: 4 μg of each molecular weight standard: lanes 2 and 5: standard (lot MRE 33), 3 μg; lane 3: lot XX04A, 3 μg; lane 4: lot XX04B, 3 μg.

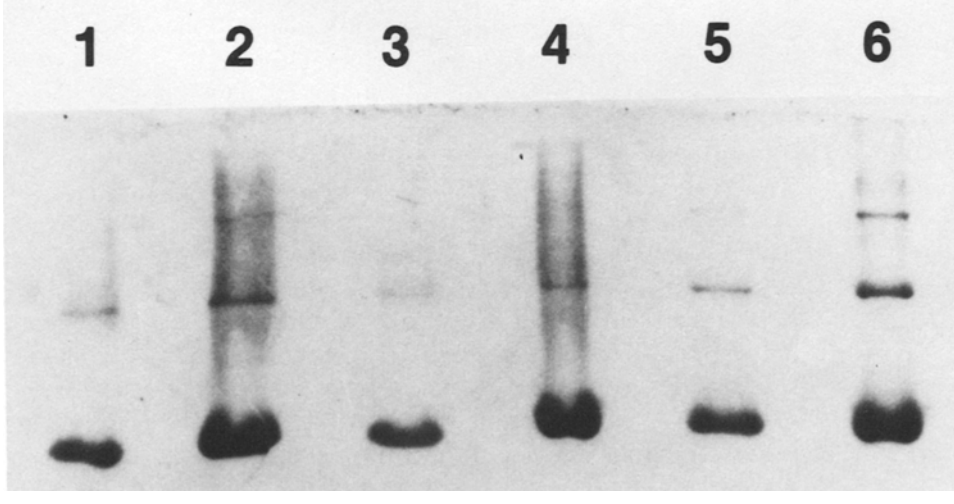

Fig. 4. Gel electrophoresis for native L-asparaginase. Lanes 1 and 2: lot XX04A, 4 and 20 μg, respectively; lanes 3 and 4: lot XX04B, 4 and 20 μg, respectively; lanes 5 and 6: standard (lot MRE 35), 4 and 20 μg, respectively.

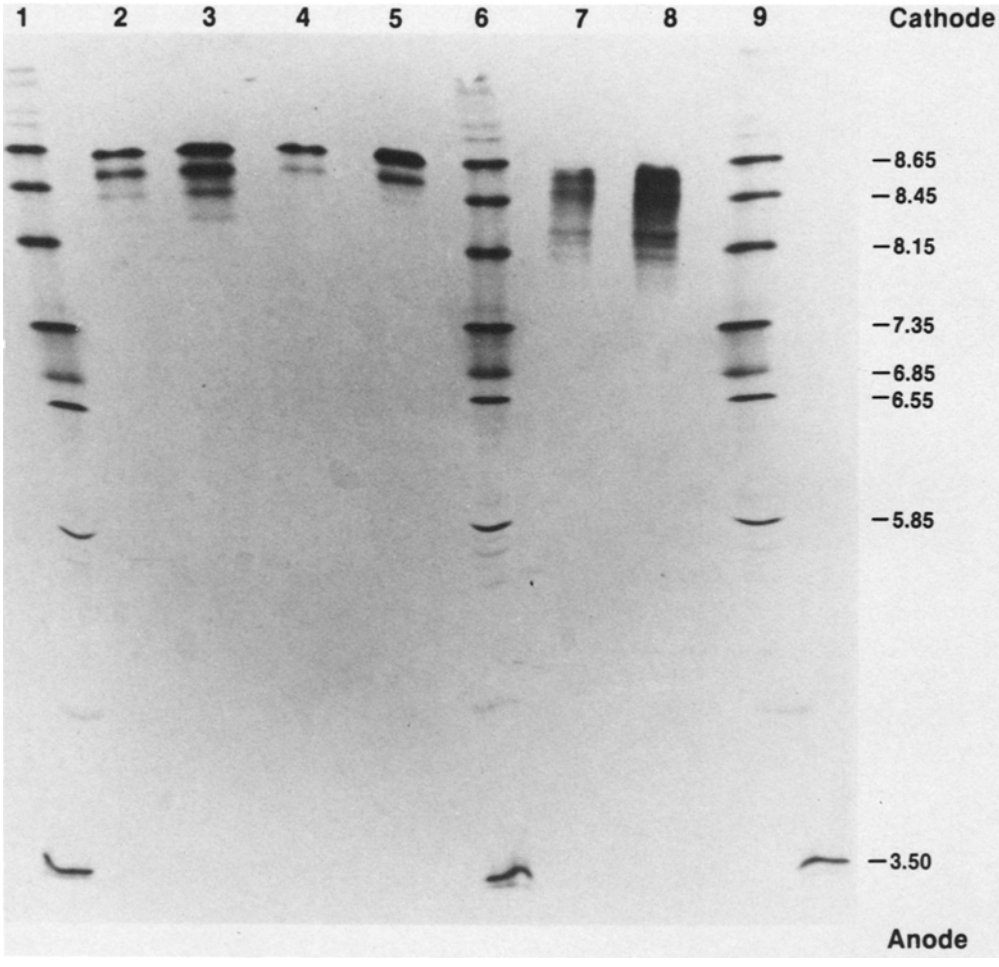

Fig. 5. IEF gel of L-asparaginase final product. Lanes 1, 6, and 9: isoelectric point calibration standards (Pharmacia); lanes 2 and 3: lot XX04A, 20 and 40 μg, respectively; lanes 4 and 5: lot XX04B, 20 and 40 μg, respectively; lanes 7 and 8: standard (lot MRE 35), 20 and 40 μg, respecitively.

	1				5					10					15					20						25					30
XX04A	A	D	K	L	P	N	I	V	I	L	A	T	G	G	T	I	A	G	X	A	A	(T)	G	X	E	X	A	(G)	Y	X	A
XX04B	A	D	K	L	P	N	I	V	I	L	A	T	G	G	T	I	A	G	X	A	A	(T)	G	V	Q	(G)	X	G	Y	X	A
Standard	A	D	K	L	P	N	I	V	I	L	A	T	G	G	T	I	A	G	X	A	A	(T)	G	V	Q	G	X	G	Y	X	A
				1				5					10					15					20					25			
E. coli[a]				L	P	N	I	T	I	L	A	T	G	G	T	I	A	G	G	G	D	S	A	T	K	S	N	Y	T	A	G

[a]Maita, T., and Matsuda, G. (1980), Hoppe-Seyler's Z. Physiol. Chem. 361, 105.

Fig. 6. *N*-terminal sequence of *Erwinia* L-asparaginase (standard and lots XX04A and XX04B) and *E. coli* L-asparaginase. The underlined amino acids contain sequence homologies. X indicates that the specific amino acid could not be identified. Parentheses indicate possible amino acids.

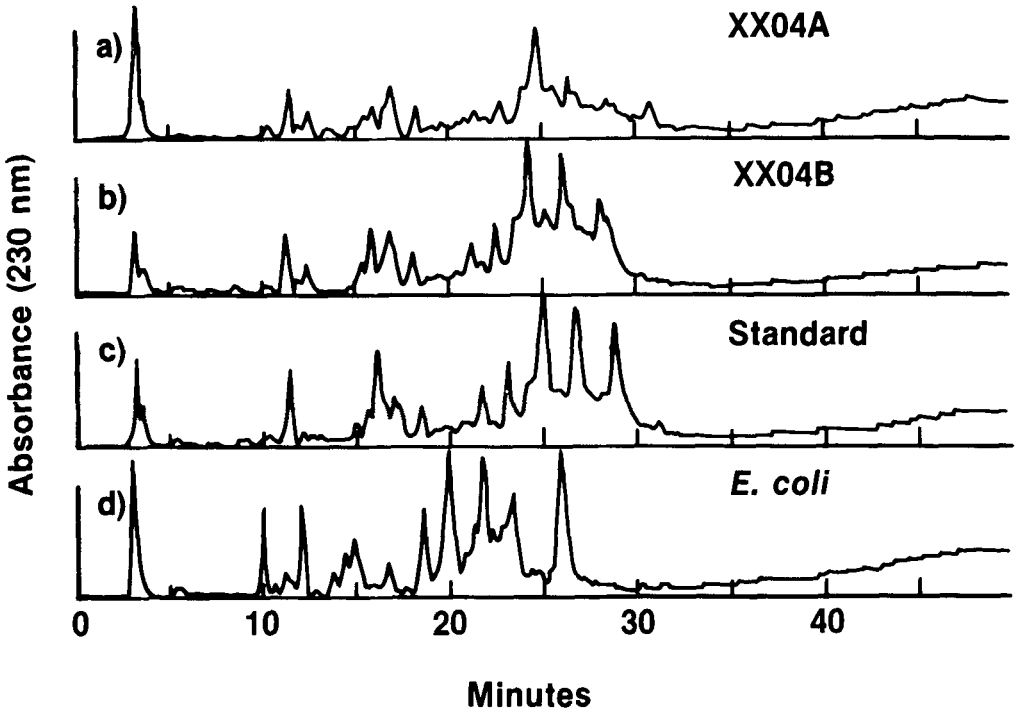

Fig. 7. Peptide mapping or *Erwinia* L-asparaginase (standard and lots XX04A and XX04B) and *E. coli* L-asparaginase.

These assays included evaluations of protein and endotoxin content, potency, specific activity, visual examination, reconstitution, SDS PAGE, native gel electrophoresis, IEF, amino acid analysis, N-terminal sequencing, peptide mapping, and immunological comparison. Several additional assays were performed on our final product only. These included analysis of the content of sodium chloride, sodium, glucose hyrate, phosphate, and glycine, as well as pH, uniformity of fill, and sterility. All results presented in Table 4 met the specifications except for glucose hydrate content in lot XX04A.

The SDS PAGE was performed to assess enzyme purity (Fig. 3). The purities of the standard and lots XX04A and XX04B were higher than 90%. The enzyme is a tetramer with identical subunits (22). Gel electrophoresis was performed for native, nondenatured L-asparaginase (Fig. 4). Our products were shown to be equivalent to the standard.

The IEF gel is presented in Fig. 5. The standard contained a series of unresolved bands. Lot XX04A had two major bands of near equal intensity and at least one minor band. Lot XX04B contained one major band and one minor band. The reason for this charge heterogeneity is not clear. One possibility is the deamidation of glutamine and asparagine residues during processing when a higher pH was used.

The N-terminal sequences of the standard and lots XX04A and XX04B were identical, except for a glutamine (Q) glutamic acid (E) differ-

ence in position 25 (Fig. 6). This deamidation is common during Edman degradation (*28*) and does not necessarily indicate a different sequence. Based on the sequence determination, we found that *E. coli* and *E. carotovora* L-asparaginases share some sequence homologies (Fig. 6). Figure 7 is the peptide map of lots XX04A and XX04B, the standard, and *E. coli* L-asparaginase (Merck, Sharp and Dohme). The standard and lots XX04A and XX04B were identical, whereas the *E. coli* enzyme was different, as we expected (*29*). The amino acid composition of the standard and lots XX04A and XX04B were identical (data not shown) and confirmed the composition reported (*22*). Immunological comparison (data not shown), using the Ouchterlony test with rabbit anisera against the standard, lots XX04A and XX04B, and *E. coli* L-asparaginase, indicated the immunological reactions of complete identity between the three *Erwinia* enzyme preparations (standard and lots XX04A and XX04B). The results also confirmed that the *Erwinia* and *E. coli* L-asparaginases were not immunologically related (*30*). The final product analysis indicated that lots XX04A and XX04B were equivalent to the standard and were essentially endotoxin free.

DISCUSSION

We have presented here a process to manufacture *Erwinia* L-asparaginase. We used continuous flow centrifugation to harvest cells. This method was satisfactory for 150 L of whole broth, and the process time was usually 3 h. Centrifugation was chosen for cell harvest based on equipment availability and the scale of operation. Alternative methods of cell harvest, such as cross-flow microfiltration, produced unsatisfactory results in our experience of working with other microorganisms, although Le et al. (*31*) claimed that the filtration method was cost efficient compared to the centrifugation method for *E. carotovora* harvesting.

In the process reported here, the cells were treated with acetone and the enzyme was recovered by borate extraction. The average step yield for acetone powder preparation was about 60% and for extraction about 80%. The yields were slightly lower than the other methods reported [toluene–urea lysis (*6*): 85%; alkali lysis (*32*): 60–100%]. In our experience, alkali lysis gave a very poor yield (6%), as reported by Grossowicz and Rasooly (*6*). The evaluations of toluene–urea treatment, osmotic shock, and mechanical fracture produced unsatisfactory results as well.

Quirk and Woodrow (*32*) compared membrane filtration and centrifugation for separating bacterial enzyme from cell debris. They reported that the centrifugation process gave a higher yield for L-asparaginase from *E. carotovora*; however, the supernatant had higher turbidity. Basket centrifugation using a perforated bowl at low g forces was not as efficient in removing cell debris as high *g* force centrifugation; it was used only because of its explosion-proof design, which was necessary to process organic solvent in acetone treatment.

In contrast to the multiple purification and dialysis steps used by other workers (*7*), the process reported here includes only two chromatographic steps and one dialysis step. The enzyme was eluted from the cation-exchange column with a change in the buffer pH rather than a change in ionic strength. The enzyme eluted from the column was very concentrated and contained low salt, thus avoiding a dialysis-concentration step between the ion-exchange and affinity chromatographic steps.

Two types of affinity coupling chemistry were examined. Our initial attempt at affinity gel preparation involved activation of Sepharose with cyanogen bromide (*33*). The four-step procedure involved activation with cyanogen bromide, coupling with ϵ-amino caproic acid to form a linker, activation of the linker with *N*-hydroxysuccinimide in the presence of *N,N'*-dicyclohexylcarbodiimide using dioxane as a solvent, and coupling with L-Asn (*34*). The epoxy-activation method offers numerous advantages: (1) Fewer toxic chemicals and no organic solvents are used, avoiding the problems of waste disposal and personal hazard; (2) the activation step simultaneously puts the linker onto the gel matrix; and (3) unlike the unstable isourea linkage formed with cyanogen bromide activation (*35*), epoxy-activation offers a relatively stable linkage (*12*).

The capacity evaluation showed that the CM-Sepharose Fast Flow beads could bind 4000 IU of enzyme/mL of packed gel, whereas the L-Asn Sepharose CL-4B could bind a minimun of 2000 IU of enzyme/mL of packed gel. Thus, both production columns were loaded below capacity. The column used for the cation-exchange step could handle only 1 bar of running pressure, so that the full flow rate of CM-Sepharose Fast Flow beads (180–300 cm/h) could not be used.

Gel media used in large-scale processes are susceptible to fouling from two sources particulate material and accumulation of adsorbed materials (*36*). Because we could not adequately remove the cell debris with the centrifugation and submicron filtration methods, the production scale column had to be protected. We used CDR, an inexpensive medium, as a precolumn packing material to protect the more valuable CM-Sepharose column. The CDR is a slightly hydrophobic, fibrous, celulous product, lightly substituted with a diethylaminoethyl (DEAE) functional group (*37*). The particulate matter and pigments from the cell-free extract were trapped by CDR, as indicated by the brown color of the precolumn after use. The specific activity of cell-free extract usually increased two- or threefold across the CDR column; the removal of undesirable protein and nucleic acids was apparently a result of the anion-exchange function of CDR.

Two asparaginase assays were used throughout the process. The Nessler assay was used for final product analysis and final processing. The ammonia probe assay was used exclusively in the purification process because of its speed. Two protein assay methods were used throughout. The Lowry protein assay was used to analyze the final product and

the standard. The Bio-Rad protein assay was a better assay for the in-process monitoring of protein because of its speed. Because *Erwinia* asparaginase has a very low aromatic content (*22*) and the Lowry protein assay measures the aromatic amino acids (*11*), the results obtained with the Lowry protein assay were generally only half as high as those obtained with the Bio-Rad assay. Thus, specific activity data varied according to the protein assay used. When the Lowry assay was used, the final product and the standard had a specific activity of 600–750 IU/mg. With the Bio-Rad assay, the final product and the standard had a specific activity of 300–370 IU/mg.

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