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L-Asparaginase from *Erwinia carotovora*

An Improved Recovery and Purification Process Using Affinity Chromatography

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ABSTRACT

A large-scale process was developed to purify L-asparaginase from submerged cultures of *Erwinia carotovora*. Cells from 880 L of fermentation broth were harvested and washed using a plate and frame type filter press. A cellular acetone powder was prepared from the washed cells by suspending the cells twice in acetone and the residual acetone was removed by washing the acetone powder in the filter press with 10 m*M* phosphate buffer (pH 7.0). The cellular acetone powder was extracted with 10 m*M* borate buffer at pH 9.5. The enzyme-rich borate extract was recovered by filtration and clarified by an in-line bag filter. The filtrate was adjusted to pH 7.5 and filtered through a 1-μm bag filter precoated with Celite and then through a 0.22-μm cartridge filter. The cell-free extract, containing 21×10^6 IU of enzyme and 448 g of total protein, was applied to an L-Asparagine Sepharose 6 Fast Flow affinity column (9 L) using a bag filter loaded with Cell Debris Remover as an in-line prefilter. The affinity gel was prepared by coupling L-Asn at pH 9.0 to epoxy-activated Sepharose 6 Fast Flow beads. A total of 14×10^6 IU of enzyme (35 g protein) was eluted at pH 9.0 in 10.5 L. The eluted enzyme was determined to be greater than 90% pure using sodium dodecyl sulfate polyacrylamide gel electrophoresis. The total process time from whole broth to affinity

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column elution was 68 h and the enzyme yield was 38%. This improved process for the 880 L fermentation broth produced a cell-free extract of high specific activity, shortened the process time, increased the column capacity, and yielded a product with high purity.

Index Entries: L-asparaginase; *Erwinia carotovora;* affinity chromatography; large-scale purification; downstream processing.

INTRODUCTION

L-Asparaginase (EC 3.5.1.1) catalyzes the hydrolysis of L-asparagine (L-Asn) to L-aspartic acid and ammonia. Interest in this enzyme arose 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, 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 (*5,6*), they offer an important alternative therapy if a patient becomes hypersensitive to one of the enzymes (*2*).

The fermentation and purification processes for L-asparaginase production have been thoroughly reviewed (*7*). L-Asparaginase from *E. carotovora* is an intracellular enzyme (*8*). One purification process involved alkali lysis, multiple ion-exchange adsorptions, ammonium sulfate precipitation, multiple dialysis-concentration steps, and ethanol crystallization (*9*). We have reported a simplified process to produce gram quantities of *Erwinia* L-asparaginase from 150 L of fermentation broth (*10*); this process involves preparation of a cellular acetone powder, ion-exchange chromatography, and affinity chromatography. We describe here an improved process for the purification of L-asparaginase from 880 L of fermentation broth.

MATERIALS AND METHODS

Asparaginase Assay

The ammonia probe assay (*10*) was used throughout the entire process; this assay uses an ammonia electrode to measure the ammonia liberated during the enzymatic reaction of L-asparaginase with L-Asn.

Protein Determination

Protein was determined using the Bio-Rad dye binding assay (*11*) with bovine γ-globulin as a standard.

Affinity Gel Preparation

The L-Asn Sepharose 6 Fast Flow was prepared using an adaptation of the Sundberg and Porath method (*12*). Sepharose 6 Fast Flow (Pharmacia, 10 kg) was activated by reaction with 10 L of 1,4-butanediol diglycidyl ether (Sigma) and 10 L of 0.6 *N* sodium hydroxide solution containing 20 g of sodium borohydride. The activation reaction was allowed to proceed at room temperature (RT) for 15 h in a 115 L glass lined pot still (Pfaudler). The activated gel was recovered by suction filtration, washed seven times with 400 L of reverse osmosis deionized (RODI) water each time, and coupled to L-Asn by reaction of the gel with L-Asn (5.7 kg) in 100 L of 0.5 *M* sodium carbonate buffer at pH 9.0. The coupling reaction was allowed to proceed at RT for 15 h with slow agitation in the same pot still. After the coupling step, the gel was washed seven times with 400 L of RODI 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

Fermentation broth (880 L) was mixed with 45 kg of filter aid [Filter-Cel (Johns-Manville Co.) and Pre Co Floc (Eagle Picher)] in a 3700 L refrigerated processing vessel. The cells were harvested by filtration through a horizontal 208 sq ft plate and frame filter press (Shriver Co.), which had been sequentially precoated with 11 kg of Filter-Cel followed by the same amount of Super-Cel (Johns-Manville Co.). The filter aids were employed to increase the efficiency of the filter. The action was to increase the porosity of the cake of retained solids upstream of the filter surface and so to maintain the filter permeability. Filter-Cel and Super-Cel are two types of celite diatomite, which is the skeletal remains of tiny aquatic plants called diatoms. Filter-Cel is the finest celite grade giving the highest clarity and lowest flowrate. Super-Cel is a coarser, faster flowrate filter aid. To operate filtration process more efficiently, filter aids were used as precoat and body feed. The precoat was built on the filter press by recirculating a fine filter aid slurry followed by a coarser filter aid slurry. The purpose of multilayer coating was to retain particles of different properties. The filter aids (body feed) were also added to the broth. Pre Co Floc is a tradename of cellulose fiber, when added to celite, will prevent filter medium from cracking. As filtration progressed, the filter aids were deposited on the precoat and a new filtering surface continuously formed. The filter aids entrapped suspended impurities but allowed clean liquid to pass through.

The wet biomass, recovered from the filter press, was mixed with 880 L of 10 m*M* potassium phosphate buffer, pH 7.0, in an agitated tank for 30 min and recovered by filtration through the same press. The washed

cells were mixed with 880 L of dry acetone at 10°C and agitated for 30 min. The slurry was recirculated through the filter press until a clear filtrate was obtained. The acetone treatment was repeated. After the second treatment, 600 L of 10 m*M* sodium phosphate buffer, pH 7.0, was circulated through the press to remove residual acetone. The cell biomass was then extracted with 1400 L of 10 m*M* sodium borate buffer, pH 9.5, for 30 min. The crude extract recovered from the precoated filter press was clarified by in-line passage through a 1-μm bag filter (GAF Corp.) packed with about 3 kg of Cell Debris Remover (CDR, Whatman). The CDR in the bag filter was replaced four times during the run. The enzyme-rich extract was then serially filtered through a Super-Cel precoated 1-μm bag filter and a 0.22-μm cartridge filter (Sartorius).

Affinity Chromatography

The L-Asn Sepharose 6 Fast Flow gel was packed in a T252 column (25.2×18 cm, 9 L, Pharmacia), and the packed column was equilibrated with 5 m*M* sodium phosphate buffer, pH 7.5. CDR was pre-equilibrated batchwise with 0.1 *M* sodium phosphate buffer (pH 7.5), basket centrifuged (Western States) to remove the buffer, re-equilibrated twice with 5 m*M* sodium phosphate buffer (pH 7.5), and loaded in a 1-μm bag filter to be used as a column prefilter. The cell-free extract (1400 L) was clarified through the in-line CDR bag filter and loaded in an upward direction onto the affinity column. A diaphram pump (American Lewa) was used to maintain flow rate of 80 L/h during the loading stage. After loading, the CDR packed bag filter was detached and the affinity column was extensively washed with 5 m*M* sodium phosphate buffer, pH 7.5, until the effluent was devoid of protein, as determined with the Bio-Rad protein microassay (*11*). The enzyme was eluted in a downward direction from the column with 50 L of 10 m*M* glycine buffer, pH 9.0, at a flowrate of 60 L/h. Ten-L fractions were collected and the majority of the eluted enzyme came out at the second fraction. This enzyme-rich fraction was adjusted to pH 7.0 with dilute HCl and kept at −20°C.

The affinity column was regenerated by sequential washing with 0.2 *M* sodium carbonate buffer (pH 9.5) containing 1 *M* NaCl and 0.1 *M* sodium acetate buffer (pH 4.0) containing 1 *M* NaCl.

Dialysis Concentration

The affinity column eluent (10.5 L) was thawed at 2–8°C, dialyzed to remove buffer salt with continuous feeding of neutralized depyrogenated water in a DC 10 L ultrafiltration system (Amicon) with a spiral cartridge (type S10 Y30, 10 ft², Amicon), and finally concentrated down to 2 L. The time spent in dialysis concentration was about 6 h.

Standard

Standard *Erwinia* L-asparaginase was provided by Matthew Suffness, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE)

We used the SDS PAGE method described by Laemmli (*13*). 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; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 20,000; and α-lactalbumin, 14,000.

Endotoxin Assay

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

RESULTS

A summary of the recovery and purification is presented in Table 1. From 880 L of fermentation broth containing 37×10^6 IU of L-asparaginase, 1400 L of cell-free extract was obtained that contained 21×10^6 IU of enzyme and 448 g of protein. This cell-free extract was adsorbed onto the affinity column and 14×10^6 IU (35 g protein) of purified enzyme was eluted in a total volume of 10.5 L. The yield was 57% through the acetone powder extraction step, 67% through affinity chromatography step, and 100% through the dialysis concentration step, giving an overall yield of 38%.

The affinity column eluent pool was better than 90% pure and was equivalent to the standard as well as the finished product from the 150 L process according to SDS PAGE (Fig. 1). The specific activity of the affinity column eluent from the present study (Table 1, 394 IU/mg) compared favorably to that from the 150 L process (*10*), which was reported to be 310 IU/mg. The overall purification factor achieved with the single step affinity chromatography was about eightfold.

The capacity evaluation showed that the L-Asn Sepharose 6 Fast Flow could bind 20,000 IU of enzyme per mL of packed gel. Compared to the L-Asn Sepharose CL-4B we used for the 150 L process (*10*), a tenfold increase in capacity was achieved.

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

Step	Volume L	Specific activity, IU/mg	Total activity, IU $\times 10^{-6}$	Total protein, g	Purification, fold	Recovery, %
Whole broth	880	ND[a]	37	ND	ND	100
Acetone powder extract	1400	47	21	448	1.0	57
L-Asn Sepharose 6 Fast Flow	10.5	394	14	35	8.4	38
Dialysis concentration	2	417	14	33.6	8.9	38

[a]Not determined.

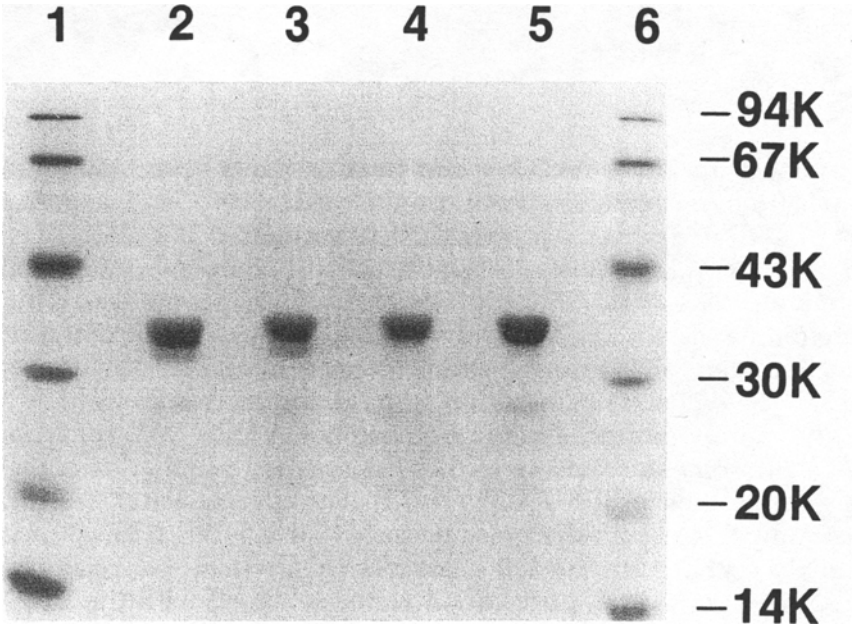

Fig. 1. SDS PAGE of purified L-asparaginase. Lanes 1 and 6: 4 μg of each molecular weight standard; lane 2: standard, 3 μg; lanes 3 and 4: the finished product of the 150 L process, lots XX04A and XX04B, respectively, 3 μg; lane 5: affinity column eluent of the 880 L process, 3 μg.

Table 2
Endotoxin Level in Affinity Column Fractions

Fraction	Volume, L	Endotoxin level, ng/mL	Enzyme activity, IU/mL
Wash fraction 2	35	2.25	0.18
Wash fraction 3	19	1.01	0.12
Wash fraction 4	9	0.66	0.11
Elution fraction 1	10	9.18	1.68
Elution fraction 2	10.5	1.34	1313
Elution fraction 3	10	1.16	6.48
Elution fraction 4	10	1.15	0.53
Wash buffer	—	0.045	—
Elution buffer	—	0.020	—

The affinity column can be eluted in several ways.

1. Using 5 m*M* sodium phosphate buffer, pH 7.5, containing at least 1 m*M* L-Asn;
2. Using 10 m*M* sodium phosphate buffer, pH 6.0, containing at least 0.3 *M* NaCl; or
3. Using 10 m*M* glycine buffer at pH 9-10.

The poor solubility of L-Asn made it very inconvenient for large-scale enzyme elution. NaCl elution yielded a broader peak and diluted enzyme. High pH elution with glycine buffer was chosen for the large-scale production, because the enzyme eluted from the column was very concentrated and contained only a small amount of a biogenic amino acid, glycine, thus an extensive dialysis concentration step was not necessary for final product processing.

Erwinia is a gram-negative microorganism (*14*), and as such the cell-free extract was expected to contain large quantities of endotoxins. These endotoxins were almost entirely removed by the affinity column wash (Table 2). However, some endotoxins appeared to bind to the affinity column nonspecifically and coeluted with the enzyme. The enzyme-rich fraction (elution fraction 2) still contained a significant level of endotoxin. The residual endotoxin in L-asparaginase can be removed by aluminum oxide treatment. Aluminum oxide is effective for L-asparaginase depyrogenation at pH 5 and 6, at 1 and 0.5% (*10*).

Table 3 presents a comparison of two processes, one for 150 L of fermentation broth (*10*) and the other for 880 L of fermentation broth. A total of 76 h was required to process 150 L of broth using the method reported earlier (*10*), whereas it took only 68 h to process 880 L of broth in the present study, a 6.5-fold increase in processing efficiency. For initial solid–liquid separation, Sharples centrifuge (M-16) operating at a flowrate

Table 3
Comparison of the 150 L Process and the 880 L Process

	Processing efficiency, L/h	Specific activity of cell-free extract, IU/mg	Yield, %	Purity by SDS gel, %
150 L process	2	6.7	23	>90
880 L process	13	47	38	>90

of 1 L/min did not have enough capacity to handle the sixfold increase in volume; therefore, a plate and frame type filtration process was used. To prepare cell-free extracts, basket centrifugation was used for the 150 L process and plate and frame type filtration was used for the 880 L process. This modification represents a significant improvement in processing efficiency owing to a 2.3-fold increase in filter surface area per volume of broth, which allowed a much greater throughput in volume and resulted in greater effluent stream clarification. The inclusion of CDR together with the depth filtration process in the filter press produced a cell-free extract of much higher specific activity than did the centrifugation process. The elimination of ion-exchange chromatography in the 880 L process significantly shortened the process time and the capacity of affinity gel cut down the process cost. In addition, the overall recovery of the 880 L process increased slightly (from 23 to 38%). As shown in Fig. 1, the L-asparaginase produced by the 150 L process (lanes 3 and 4) and the L-asparaginase produced by the 880 L process (lane 5) were both better than 90% pure.

DISCUSSION

There are several reasons why single-step affinity purification has not yet been widely used in large-scale operations. First, industrial-scale clarification of large volumes of bacterial homogenates is difficult to achieve (*15*). If the supernatant is not properly clarified, the column can easily be blocked and the flow reduced. Second, economic factors may create reluctance to use the affinity technique in large-scale operations (*16*). Finally, pumping large volumes of crude extract through a relatively small column can be both time consuming and impractical.

We have overcome most of these difficulties in that mechanical cell disruption methods such as homogenization or sonication were not used. Instead, the cells were permeabilized by acetone treatment. This resulted in a biomass that was relatively easy to filter. Acetone also removed lipids that could adhere, congeal and result in downstream column fouling. The depth filtration process using a filter press has an inherently high partic-

ulate removal efficiency. The crude extract recovered by filtration through the filter press was subsequently subjected to multiple filtration steps for further clarification. The additional filtration steps included using a CDR-packed bag filter, a Celite-coated 1-μm bag filter, a 0.22-μm cartridge filter, and a second CDR-packed bag filter (affinity column pre-filter). The purpose of the multiple filtrations was to remove particulate matter, nucleic acids and strongly adsorbed proteins in order to extend the life of the affinity column. The CDR-packed bag filter was replaced several times during the run to ensure the clarification of the cell-free extract.

The large-scale affinity gel preparation involved using epoxy activation followed by L-Asn coupling. The reagents were relatively inexpensive and no organic solvent was used.

In the current study, the affinity gel coupling procedure was modified slightly from that of the 150 L process (*10*). The coupling pH was raised from 8.5 to 9.0. The coupling reaction proceeded more efficiently at pH 9.0 where the amino group (pKa = 8.8) on the ligand L-Asn was predominantly unprotonated. This could be one of the reasons for the observed increase in capacity. Gel matrix differences, 4% agarose for Sepharose CL-4B and 6% agarose for Sepharose 6 Fast Flow, may contribute another reason for the increase in capacity.

By using Sepharose Fast Flow beads as an affinity column matrix, we were able to achieve a linear flowrate of 160 cm/h, which was equivalent to 80 L/h for the T252 column. Higher flowrates (300 L/h) may be achieved by using a larger diameter column (37×15 cm, Pharmacia) with 0.5 in (inner diameter) inlet and outlet tubings. Alternatively, the cell-free extract could be concentrated using ultrafiltration before loading it onto the affinity column.

The transition from the 150 L process using a basket centrifuge to the 880 L process using a plate and frame type filter press was not without difficulty. Residual acetone in the wet cellular acetone powder must be removed from the biomass prior to extraction. The basket centrifuge was inherently efficient for this operation; however, the horizontal design of the filter press did not allow total purge of the acetone in the biomass. In addition, maximum clarification and efficiency parameters had to be established for the filter press. These parameters are determined by the proportion and size classification of the filter aids used for body feed and filtration surface precoat. We chose to use a combination of a noncalcined fine grade Celite (Filter-Cel) and a calcined coarse grade Celite (Super-Cel) for optimal operation.

After acetone treatment, the bacterial cells become "leaky" and the asparaginase will extract, to 60% (*10*), in an aqueous solution. It was observed that asparaginase would bind to Filter-Cel at neutral pH. Because of this binding, a "post acetone treatment" wash of the biomass using 10 m*M* sodium phosphate buffer (pH 7.0) was incorporated, which allowed residual acetone to be purged from the filter press by circulation of the buffer through the press after the second acetone treatment.

During process development work, the process time incidentally extended to 4–5 d. We have, on these occasions, consistently experienced a drop in enzyme specific activity together with an increase of a 34,000 dalton contaminant protein. When the same batch of cell-free extract was purified the same way on a chromatography column, the 6-d and 1-d column process time yielded the 34,000 dalton contaminate at 50 and 5%, respectively. This contaminant appeared to behave chromatographically similar to the active asparaginase and thus coeluted with the active asparaginase on cation-exchange as well as affinity column. Since *Erwinia* L-asparaginase had an observed subunit molecular weight of 37,000, the 34,000 low molecular weight contaminant was probably a proteolytic product of L-asparaginase. Protease inhibitors were not added to the crude extract because the contamination of a therapeutic enzyme with toxic protease inhibitors had to be avoided. If the process time was kept within 2–3 d, the percentage of low molecular weight impurities was usually less than 10% and the specific activity was equivalent to or higher than the standard.

During the course of affinity purification, L-asparaginase bound very tightly to the column throughout the loading and wash conditions and was totally eluted with the application of 1 m*M* L-Asn, high salt or high pH. This observation seems to indicate that the ligand L-Asn was recognized but not hydrolyzed by the enzyme. The hydrolysis of ligand by the enzyme would lead to the release of enzyme from the affinity matrix under non-eluting conditions, and this was never observed.

The life of affinity column in large-scale operation was not tested. However, if CDR-precolumn was changed after each run and care was taken to keep the affinity column from fouling, the bench-scale column has been used for over 10 cycles with consistent performance.

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