Original Article

# Cloning, expression, purification and characterisation of *Erwinia carotovora* L-asparaginase in *Escherichia coli*

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## Abstract

**Background:** For the past 30 years, bacterial L-asparaginases have been used as therapeutic agents in the treatment of acute childhood lymphoblastic leukemia. It is found in a variety of organisms such as microbes, plants and mammals. Their intrinsic low-rate glutaminase activity, however, causes serious side-effects, including neurotoxicity, hepatitis, coagulopathy and other dysfunctions. *Erwinia carotovora* asparaginase shows decreased glutaminase activity, so it is believed to have fewer side-effects in leukemia therapy. Our aim was to clone, express, purify and characterize *E. carotovora* asparaginase.

**Materials and Methods:** L-asparaginase from *E. carotovora* NCYC 1526 (ErA) was cloned and expressed in *Escherichia coli* strain BL21 (DE3). The enzyme was purified to homogeneity by affinity chromatography. Various conditions were tested to maximize the production of recombinant asparaginase in *E. coli.*

**Results:** A new L. asparaginase from *E. carotovora* NCYC 1526 (ErA) was successfully cloned, expressed and purified in *E. coli* BL21 (DE3). The specific activity of the enzyme was 430 IU/mg.

**Conclusion:** The results of the present work form the basis for a new engineered form of ErA for future therapeutic use, which could be extended with crystallographic studies.

**Key Words:** Characterization, cloning, ErA, *Erwinia carotovora*, *Escherichia coli* BL21 (DE3), L-asparaginase, purification

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## INTRODUCTION

L-Asparaginases (EC 3.5.1.1) are the enzymes catalyzing the conversion of L-asparagine to L-Asn and ammonia and also in a smaller level the hydrolysis of L-Gln to L-Glu. Two types of bacterial L-asparaginase have been identified and named type I and type II.[1] Type I asparaginases are expressed in the cytoplasm and characterized by enzymatic activity for both L-Asn and L-Gln. Type II asparaginases are expressed under anaerobic conditions in the periplasmic space of the bacterial membranes with high specific activity against L-Asn. The interest in L-asparaginases arose from its antitumor activity. Especially, type II asparaginases, display antitumor activity and are used as chemotherapeutics in acute lymphoblastic leukemia (ALL).[2-4] The antineoplastic activity results from depletion of the circulating pools of L-Asn by L-asparaginase. Malignant cells can only slowly synthesise L-Asn and are dependent on an exogenous supply.[5] Administration of L-asparaginase

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will reduce the Asn blood levels, leading to inhibition and regression of tumors. For an effective treatment a noticeable reduction of L-Asn concentration in blood and tissue fluids is required (<10% of the normal level).[6]

The L-asparaginases of *Erwinia chrysanthemi* and *Escherichia coli* have been in use for many years as effective drugs in the treatment of ALL and leukemia lymphosarcoma,[7,8] but their L-glutaminase activity causes severe side-effects as a result of L-Gln deprivation and limits their use.[6]

Desired properties for therapeutic L-asparaginases include low L-glutaminase specificity, high L-asparaginase activity with a long ½-time in the bloodstream.[9] These criteria have directed the search for an optimal therapeutic asparaginase that started >30 years ago,[10] but still has not led to a noticeable success. As a result, many asparaginases with promising catalytic properties did not pass preclinical trials, due to their therapeutic inefficiency.[11] The interest in L-asparaginase from *Erwinia carotovora* arose from its decreased glutaminase activity and therefore it is expected to show fewer side-effects when used in the anti-cancer therapy.[12]

All asparaginases are homotetramers with 222 symmetry and a molecular mass in the range of 140-150 kDa with a highly conserved overall fold.[13-15] They are composed of four identical subunits denoted A-D.[8] Each monomer consists of about 330 amino acid residues that form 14 β-strands and 8 α-helices, arranged into two easily identifiable domains, the larger N-terminal domain and the smaller C-terminal domain, connected by a linker consisting of ~20 residues.

Despite the therapeutic potential of ErA, this enzyme is poorly characterised, compared to other bacterial L-asparaginases. Differences in the biological activity among available *E. coli*-asparaginase preparations and variation of the tolerated dose among trials, all suggestive of differences in the relative potency of the available asparaginase products.[16-19] Therefore, we aimed to clone the *E. carotovora* gene into *E. coli* BL21 (DE3), express, purify and characterize to find a potentially better L-asparaginase with lower side-effects.

## MATERIALS AND METHODS

All chemicals and materials were obtained from Sigma-Aldrich®, Merck®, Fluka® and Oxoid® unless otherwise stated. Nessler's reagent, d-Asn, β-alanine amide and glutamate dehydrogenase (GDH) were obtained from Fluka® (Taufkirchen, Germany). Cells were grown in Luria-Bertani (LB) medium containing 100 μg/ml ampicillin to a culture density of $OD_{600}$ 1.5-2.0 at 37°C and with continuous shaking at 150-180 rpm.

### Cloning of L-asparaginase gene from *E. carotovora* and expression in *E. coli*

A BLAST search for the *E. carotovora* genome sequence using as a quire the gene encoding for L-asparaginase from *E. chrysanthemi* yielded a single 1041 bp open reading frame (ORF) that was supposed to encode L-asparaginase. Polymerase chain reaction (PCR) was used to amplify the full-length ORF from genomic deoxyribonucleic acid (DNA) using the following oligo primers:

Forward:(5'-GC*CATATG*TTTAACGCATTATTCGTT GTTG-3) (NdeI)

Reverse:(5'-GC*GGATCC*CAAGCGATTAATAAGCGT GGAAG-3) (BamHI)

*E. carotovora* (NCYC 1526) which kindly provided by Dr. Korbekandi from his previous project, was grown at 30°C in LB medium containing 1% peptone, 0.5% yeast extract and 1% NaCl. After 18-20 h, cells were pelleted by centrifugation (2500 g, 15 min) and genomic DNA was isolated according to a standard procedure.[20]

The PCR reaction was carried out in a total volume of 50 μl contained 60 ng of each primer, 20 ng template genomic DNA, deoxyribonucleotide (dNTPs) containing 0.2 Mm of each dNTP, 5 μl 10× Pfu buffer and two units of Pfu DNA polymerase (Stratagene, USA). The PCR procedure was optimized to the best condition comprised 95°C for 4 min, followed by 30 cycles of 2.5 min at 95°C, 2 min at 50°C and 3 min at 72°C. A final extension time at 72°C for 10 min was performed after the 30 cycles. The resulting PCR product was purified and subjected to restriction enzyme digestion by BamHI and NdeI which has been ligated into pET22b vector in order to produce 6His-tagged fusion protein using standard T4 DNA ligase protocol.[20] This vector facilitated Western blot analysis and purification of the product with histidine tag.

The resulting construct was used to transform *E. coli* BL21 (DE3) competent cells using heat shock method.[20] *E. coli* cells, harboring constructed plasmids, were grown at 37°C in LB medium containing 100 μg/ml ampicillin. Several successful colonies were analyzed using miniprep and the purified plasmids were digested to linear the construct and analyzed on agarose gel using 1 kb marker (Bioline). The resulting construct was sequenced using ABI PRISM BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Corporation) along both strands to confirm the right construct. *E. coli* cells, harboring

plasmid, were grown at 37°C in LB medium containing 100 μg/ml ampicillin. The synthesis of the product was induced by the addition of different concentrations (0.1-2 mM) of isopropyl β-D-1-thiogalactopyranoside (IPTG) when the absorbance at 600 nm was 0.5.

### Purification of recombinant asparaginase

At 5 h after the induction, cells were harvested by centrifugation (8000 rpm, 4°C, 20 min), resuspended in sodium phosphate buffer (5 mM, pH 7.7) and subjected to French press (Aminco). We used French press to prepare cell-free extract, which is suitable for large scale purification compared with sonication. It is an effective and economical technique for cell-free extract preparation providing high recovery of the enzyme. Cell debris and precipitated proteins were removed by centrifugation (10,000 g, 20 min). Recombinant L-asparaginase attached to the histidine was purified in a two-step procedure.

As the first step in the purification scheme, the soluble fraction of the cell-free extract was used for purification of recombinant asparaginase by nickel affinity chromatography. A HisTrapHP (GE Health care) column (containing Ni Sepharose) was equilibrated with equilibration buffer A (50 mM Tris, 500 mM NaCl, 12.5 mM imidazole (Sigma®), 10% glycerol, adjusted to pH 8.5 with HCl. A total volume of 35 ml supernatant was loaded on to the column. The column was washed with 60 ml of buffer to wash off non-adsorbed protein. The bound protein was eluted by using elution buffer (Buffer B: 50 mM Tris, 500 mM NaCl, 250 mM imidazole, 10% glycerol, adjust to pH 8.5 with HCl by increasing imidazole concentration to 250 mM in a linear gradient. At this step L-asparaginase was purified from the contaminants of *E. coli* asparaginase. 1 mL fractions containing recombineant asparaginase were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), then pooled together and dialyzed against 50 mM Tris–HCl, pH 8.5. The protein solution was concentrated by Ultra-Filtration using Centricon MWCO 10 kDa (Amicon®, Germany).

As a next step in the purification procedure, an affinity chromatography was employed on concentrated purified product from step one. The purity of the final L-asparaginase preparation was evaluated by SDS-PAGE.

### Electrophoresis

SDS-PAGE was performed according to the method of Laemmli[21] on a slab gel containing 12.5% (w/v) polyacrylamide (running gel) and 2.5% (w/v) (stacking gel). Proteins separated by SDS-PAGE were transferred to polyvinyl difluoride membrane (Millipore) using the electro-blotting technique. Western blot analysis was performed using the enhanced chemiluminescence Western blot protocol (Amersham Pharmacia Biotech). Immediately after protein transfer, non-specific binding sites on membrane were blocked by skim milk or Bovine Serum Albumin in phosphate buffer saline (PBS). Membranes were then washed in PBS-Tween 20®washing buffer and incubated with antisera against the hexa-histidine tag (Qiagen). The membranes were then washed with PBS-Tween 20® to remove excess antibodies prior to incubation with anti-mouse IgG-horseradish peroxidase conjugate (Qiagen). After washing steps by PBS, the membranes were added with the detection solution of ECL. The signals were detected by exposing to Kodak X-OMAT™ film before being developed by film processor. Molecular size of the purified enzyme was measured using molecular marker. A sample from each purification step was subjected to SDS-PAGE analysis and the proteins were stained with Coomassi Brilliant Blue R-250.

### Assay of enzyme activity and protein concentration

Enzyme assays were performed at 37°C using a Hitachi U-2000 double beam ultraviolet-vis spectrophotometer carrying a thermostated cell holder (10 mm path length). Activities were measured by determining the rate of ammonia formation, by coupling with GDH according to literature.[22] The final assay volume of 1 ml contained 71 mM Tris-HCl buffer, pH 8.0; 1 mM Asn; 0.15 mM α-ketoglutaric acid; 0.15 mM nicotinamide adenine dinucleotide; four units GDH and a fraction showing ErA activity. Alternatively, the rate of ammonia formation was measured at 37°C using Nessler method.[23] L-Asparaginase activity was expressed in generally accepted international units (IU). 1 IU was determined as the amount which released 1 μmol of ammonia from the substrate in 1 min. Protein content of the purified samples was determined by measurement of $A_{280}$ (2 μl samples, in triplicates, by Nanodrop) using estimates of extinction coefficient and molecular mass calculated using ProtParam.[24] Reference buffers 1 and 2 were used for determination and $dH_2O$ was used as blank.

Reference 1 (58% B): 580 μl buffer B, 420 μl buffer A.

Reference 2 (74% B): 740 μl buffer B, 260 μl buffer A.

## RESULTS

### Cloning, expression and purification of ErA

A BLAST search for *E. carotovora* NCYC 1526 genome sequence in the Sanger Centre database (http://www.sanger.ac.uk/) using as a quire the gene encoding for lasparaginase from *E. chrysanthemi* yielded a single 1041 bp ORF which was supposed to

encode for L-asparaginase. To determine whether the putative *E. carotovora* protein was indeed a functional asparaginase, we cloned and expressed the full-length gene in *E. coli* and tested for its ability to catalyze asparagine hydrolysis. PCR was used to amplify the full-length gene from genomic DNA of *E. carotovora* NCYC 1526. The resulting PCR amplicon was cloned into the T7 expression vector plasmid pET22b [Figure 1]. This plasmid was used to transform the expression host *E. coli* BL21 (DE3). Cell-free extract from lysate preparation of the *E. coli* transformants showed a high asparaginase activity.

## Finding the optimal production condition

After construction of Hexa-His-L asparaginase several media cultures were employed for the optimum production (data not shown). Finally LB media was selected for the experiments. To investigate the optimal condition for protein production and purification, several conditions were examined in the small scale level using the culture induced with 0.1-2.0 mM IPTG on $OD_{600}$ = 0.5 [Figure 2].

## Large scale purification of the protein

His-tag on the recombinant asparaginase allowed quick and easy purification from the supernatant. The purity of L-asparaginase was assayed by SDS-PAGE (12% gels) stained with Coomassie Brilliant Blue R250 by the Laemmli method.[21]

After optimization of production in small scale [Figure 3], 500 ml of the culture was inoculated with an overnight culture in a 5 l flask and the pellet from this culture was collected and subjected to French press. The supernatant was used to load the column after removing debris and intact cells. After the purification process, samples from fractions 58-75 and 77-92 were selected as a base of absorbance pick provided by recorder [Figure 4] and subjected to SDS-PAGE [Figures 5a and b].

Following the confirmation of the favored eluted protein on stained gel, all samples from tubes 58 to 76 were pooled together as pool 1 containing 19 ml Samples from tubes 77 to 89 were pooled to make 13 ml content as pool 2 as well. The amount of protein in pool 1 and pool 2 were determined using the reference values. The amounts were 17.7 mg and 13 mg, respectively. The specific activity of the enzyme was 430 IU/mg. The content of pool 1 was subjected to concentrateion using Vivaspin 6 filter to minimize the volume. Following this step, the concentration of protein in pool 1 was increased from 17.7 mg/ml to 30 mg/ml.

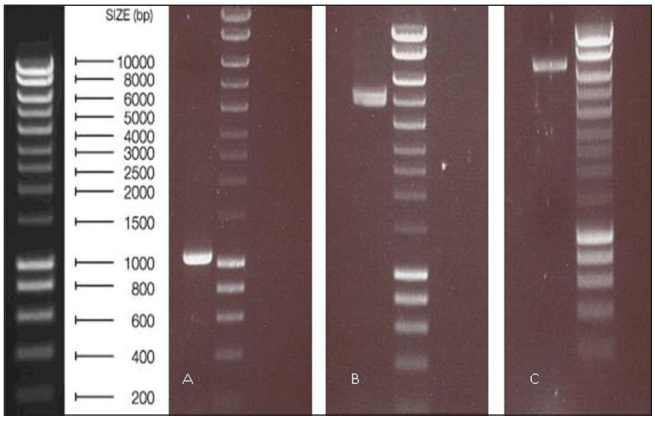


**Figure 1:** Electrophoresis in 0.75% agarose gel of restriction endonuclease digestion of polymerase chain reaction product (a; 1.05 kb), pET22b (b; 5.4 kb) using BamHI and NdeI and recombinant construct (c; 6.5 kb) using just BamHI. Marker Hyperlader I from Bioline

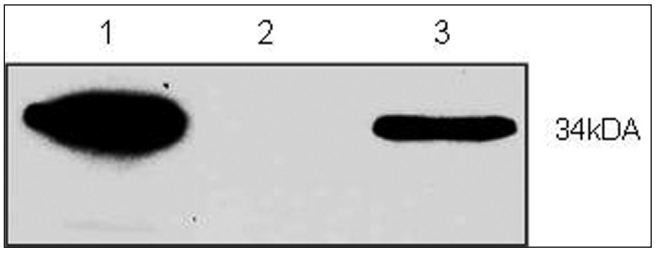


**Figure 3:** Western blot analysis of the samples for small scale purification, probed with His-tag antisera. Lane 1: Total lysate, Lane 2: Flow through sample from the column, Lane 3: The purified protein

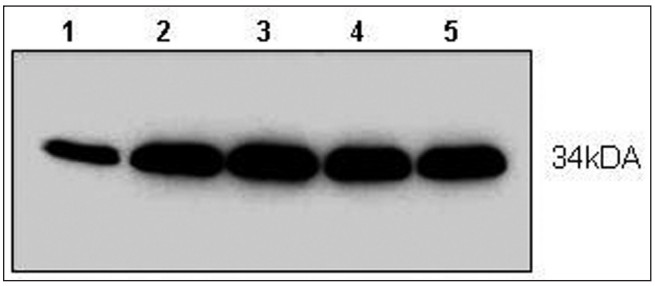


**Figure 2:** Western blot analysis of samples to find optimal induction conditions, probed with His-tag antisera. Lane 1, uninduced sample, Lane 2-5, induced with 0.1, 0.5, 1 and 2 mM isopropyl β-D-1-thiogalactopyranoside

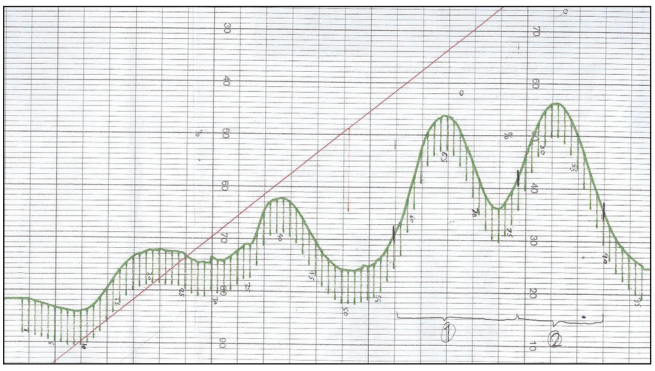

**Figure 4:** The change of absorbance in collected samples from the fraction collector

As the next step in the purification procedure, an affinity chromatography was employed on concentrated purified products from step one. The purity of the final ErA preparation was evaluated by SDS-PAGE from the eluted samples based on the printed graph [Figure 6] showing the presence of polypeptide chain in much higher concentration [Figure 7].

## DISCUSSION

The study by Gilbert *et al*.[25] and Liu *et al*.[26] have cloned and expressed *E. coli* and E. *chrysanthemi* L-asparaginase gene into *E. coli*. However, a major drawback of using microorganisms as a source of this drug was a number of adverse effects of the enzyme. Cytotoxicity measurements by Papageorgiou *et al*. revealed that *E. carotovora* asparaginase was 30 times less toxic than the *E. coli* enzyme against human leukemia cell lines. Moreover, they showed that *E. carotovora* asparaginase decreases thermodynamic stability when compared with the *E. coli* enzyme and is rapidly inactivated in the presence of urea. On the basis of these results, they proposed that *E. carotovora* asparaginase has limited potential as an antileukemic drug, despite its promising low glutaminase activity.[27] Thermodynamic analysis of *E. carotovora* asparaginase activity by Kotzia and Labrou showed that the activation energies are dependent on the substrate and the main determinant contributing to the substrate specificity was entropy changes.[28]

As a viable alternative to bacterial L-asparaginases, recently some scientists selected plant gene as a source to overcome the limitations of the bacterial enzyme. They successfully cloned the L-asparaginase gene of *Withania somnifera* and expressed it at a high level in *E. coli*.[29]

In the present study, T7 promoter was used along with IPTG, for the induction of the enzyme. The first part of this work dealt with determining the optimal condition for L-asparaginase expression. His-tag on the recombinant asparaginase allowed quick and easy purification of the recombinant protein. The protein was purified with the aid of affinity chromatography. The purified recombinant asparaginase appeared occasionally as a doublet in SDS-PAGE similar to the results of Khushoo *et al*.[30] The reason for the appearance of a double band has already been investigated by analyzing the redox state of the protein. The results suggested that the concentration of beta-mercaptoethanol present in the standard protein-loading dye was not sufficient to reduce all the protein molecules in the sample.[31]

The specific activity of the enzyme in our study was 430 IU/mg. The reported activities of wild-type

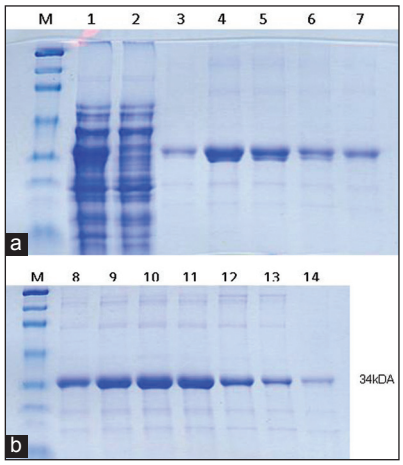


**Figure 5a and b:** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% gel) with the purified L-asparaginase to determine the presence of protein in the eluted samples. The gel was stained with Coomassie Brilliant Blue R-250. M: molecular size marker. Lane 1: sample of loaded crude supernatant to the column, Lane 2: Column effluent (Flow through), Lanes 3-7: Eluted fractions from tubes 58, 65, 69, 72 and 75. (a) Lanes 8-14: Samples of eluted fractions from tubes 77, 80, 82, 85, 88, 91 and 95 (b)

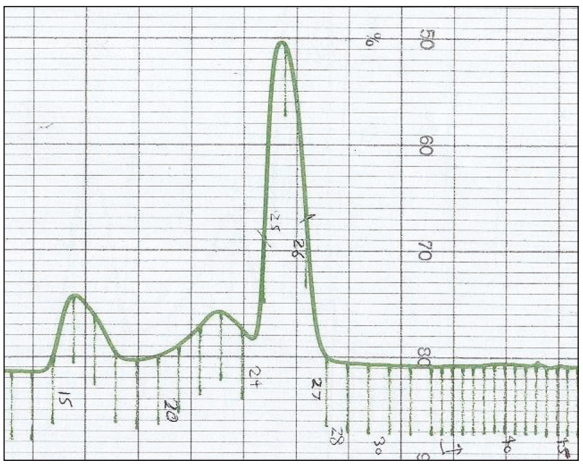


**Figure 6:** The change of absorbance in collected concentrated samples from fraction collector

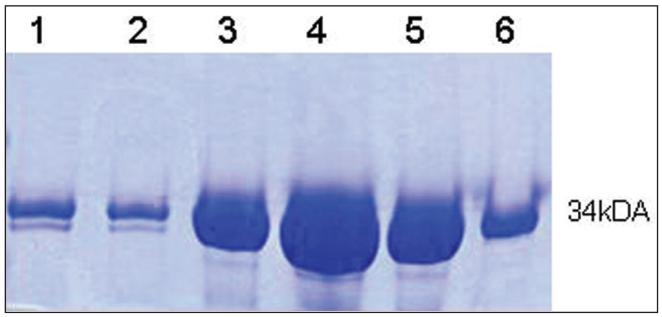


**Figure 7:** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% gel) with the purified L-asparaginase to determine the presence of protein in the eluted samples from the concentrated samples (Tubes 23, 24,25,26,27 and 28, respectively)

L-asparaginase from *E. carotovora* varied from 310 IU/mg[5] to 550 IU/mg.[32]

Current status of and recommendations for the use of *Erwinia* asparaginase form a cornerstone of ALL treatment protocols with three main preparations for use in treatment protocols: the native *E. coli* asparaginase, a pegylated form (PEG-asparaginase) and an alternative enzyme isolated from *E. chrysanthemi*, referred to as *Erwinia* asparaginase. Despite the availability of these agents, much debate remains regarding the optimal formulation and dose for the treatment of pediatric and adult ALL patients. Erwinia asparaginase is a valid second or third-line therapy, depending upon protocols, regulatory factors and availability.[27]

## CONCLUSION

In the present study, a new L. asparaginase from *E. carotovora* NCYC 1526 was cloned and expressed. The results of the present work forms the basis for a new engineered form of ErA for future therapeutic use, which could be extended with crystallographic studies.

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