Journal of Microbial & Biochemical Technology - Open Access
JMBT/Vol.2 Issue 1

Research Article | OPEN ACCESS Freely available online | doi:10.4172/1948-5948.1000017

Comparison between Two *Erwinia carotovora* L-Asparaginase II Constructions: cloning, Heterologous Expression, Purification, and Kinetic Characterization

Priscila Lamb Wink[1,2], Heique Marlis Bogdawa[3], Gaby Renard[3], Jocelei Maria Chies[3], Luiz Augusto Basso[1,2*] and Diógenes Santiago Santos[1,2*]

[1]Centro de Pesquisas em Biologia Molecular e Funcional (CPBMF), Instituto Nacional de Ciência e Tecnologia em Tuberculose (INCT-TB), Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Av. Ipiranga, 6681, Tecnopuc, Prédio 92A, Partenon, Porto Alegre, 90619-900, Brazil
[2]Programa de Pós-Graduação em Medicina e Ciências da Saúde - PUCRS. Av. Ipiranga, 6690, Partenon, Porto Alegre, 90610000, Brazil
[3]Quatro G Pesquisa e Desenvolvimento LTDA. Av. Ipiranga, 6681, Tecnopuc, Prédio 92A, Partenon, Porto Alegre, 90619900, Brazil

Abstract

L-Asparaginase II from *Erwinia carotovora* may represent an important alternative therapy in the treatment of acute childhood lymphoblastic leukaemia, despite its promising lower glutaminase activity than *Escherichia coli* and *Erwinia chrysanthemi* L-asparaginases II, currently used in treatment of this disease. Here we describe cloning, expression, purification and determination of steady-state kinetic parameters for *E. carotovora* L-asparaginase II: with (AspSP) and without the signal peptide (AspMP). AspMP was purified to homogeneity by a single-step protocol with 91% yield, and AspSP by a two-step protocol with 28% yield. In addition, both enzymes presented similar high specific activities: 208.1 and 237.6 U mg^{-1}, respectively. The K_m and k_{cat} values showed that AspMP has lower glutaminase activity than AspSP. Moreover AspMP is produced by a simpler purification protocol, and at higher yield. This process can be amenable to large scale production and be of interest to researchers and biopharmaceutical companies.

Keywords: L-asparaginase II; *Erwinia carotovora*; Heterologous expression; Cancer therapy; Therapeutic protein; Biosimilar

Abbreviations: ALL: Acute Lymphoblastic Leukemia; CVs: Column Volumes; IPTG: Isopropyl β-D-thiogalactopyranoside; AspMP: Construct f L-asparaginase II gene (*ErA*) from *Erwinia carotovora* (*subsp. atroseptica*) not containing its signal peptide (mature protein); AspSP: Construct of L-asparaginase II gene (*ErA*) from *Erwinia carotovora* (*subsp. atroseptica*) containing its signal peptide

Introduction

L-asparaginase enzymes (L-Asparagine amidohydrolase; EC 3.5.1.1) catalyze the hydrolysis of L-asparagine (L-Asn) to L-aspartate (L-Asp) and ammonia (NH_3), and to a lesser extent the hydrolysis of L-glutamine (L-Gln) to L-glutamate (L-Glu). Two types of bacterial L-asparaginases have been identified: type I and type II (Campbell et al., 1967). Type I L-asparaginases are expressed constitutively in the cytoplasm and catalyze the hydrolysis of both L-Asn and L-Gln, whereas type II L-asparaginases are expressed under anaerobic conditions in the periplasmic space of the bacterial membranes and display higher specificity for L-Asn hydrolysis (Campbell et al., 1967; Cedar and Schwartz, 1968). Type II L-asparaginases, in particular, present tumor inhibitory activity and have thus been extensively studied (Campbell et al., 1967; Cedar and Schwartz, 1968). Tumor-inhibitory L-asparaginases have also been isolated from a number of bacterial sources including *Proteus vulgaris*, *Acinetobacter glutaminasificans*, *Pseudomonas putida*, *Wolinella succinogenes*, *Helicobacter piloris*, and others. However, the enzymes from *Escherichia coli* (*E. coli*) and *Erwinia* sp. have been more frequently used in cancer therapy (Avramis and Panosyan, 2005; Broome, 1968; Oettgen et al., 1970) due to their higher substrate affinity (Schwartz et al., 1966) and favorable factors affecting the clearance rate of these enzymes from the system (Broome, 1965). *E. coli* and *Erwinia chrysanthemi* (*E. chrysanthemi*) are the main sources of L-asparaginases II, which have identical mechanisms of action but different pharmacokinetic properties (Duval et al., 2002). *E. coli* L-asparaginase induces more coagulation abnormalities but it was shown to be superior to *E. chrysanthemi* L-asparaginase for the treatment of childhood lymphoid malignancies (Duval et al., 2002). The main side effects are liver dysfunction, pancreatitis, diabetes, leucopoenia, neurological seizures, and coagulation abnormalities that may lead to intracranial thrombosis or hemorrhage (Duval et al., 2002; Oettgen et al., 1970). Another limiting factor of *E. coli* L-asparaginase II is the development of hypersensitivity, which ranges from mild allergic reactions to anaphylactic shock (Schwartz et al., 1966). Because L-asparaginases II from *E. coli* and *Erwinia* possess different immunological specificities they offer an important alternative therapy if a patient becomes hypersensitive to one of these enzymes (Lee et al., 1989).

The bacterial enzyme L-asparaginase II has been used in com-

***Corresponding authors:** Luiz Augusto Basso, Centro de Pesquisas em Biologia Molecular e Funcional (CPBMF), Instituto Nacional de Ciencia e Tecnologia em Tuberculose (INCT-TB), Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Av. Ipiranga, 6681, Tecnopuc, Prédio 92A, Partenon, Porto Alegre, 90619-900, Brazil, Tel/Fax: +55 51 33203629; E-mail: luiz.basso@pucrs.br

Diógenes Santiago Santos, Programa de Pós-Graduação em Medicina e Ciências da Saúde - PUCRS. Av. Ipiranga, 6690, Partenon, Porto Alegre, 90610000, Brazil, Tel: + 55 51 33203318; E-mail: diogenes@pucrs.br

Received January 19, 2010; **Accepted** February 25, 2010; **Published** February 25, 2010

Citation: Wink PL, Bogdawa HM, Renard G, Chies JM, Basso LA, et al. (2010) Comparison between Two Erwinia carotovora L-Asparaginase II Constructions: cloning, Heterologous Expression, Purification, and Kinetic Characterization. J Microbial Biochem Technol 2: 013-019. doi:10.4172/1948-5948.1000017

bination with other drugs in acute lymphoblastic leukemia (ALL) treatment for approximately 30 years (Avramis and Panosyan, 2005; Amylon et al., 1999; Ortega et al., 1977). The antileukemic effect of L-asparaginase II is believed to result from the depletion of circulating L-Asn (Kiriyama et al., 1989; Moola et al., 1994; Oettgen et al., 1967; Richards and Kilberg, 2006). As certain tumor cells have decreased or absent activity of asparagine synthase, they depend on externally supplied L-Asn for protein synthesis and the decreased supply of L-Asn in the blood results in cancer cell death (Müller and Boos, 1998). L-Asparaginase II is also used to treat Hodgkin's disease, acute myelocytic leukemia, lymphosarcoma, reticulosarcoma, and melanosarcoma (Duval et al., 2002).

The interest in *Erwinia carotovora* (*E. carotovora*) L-asparaginase II stems from its significantly lower glutaminase activity as compared to that exhibited by *E. coli* and *E. chrysanthemi* enzymes. This is particularly important, since the glutaminase activity of therapeutic preparations of L-asparaginases II has been implicated in causing side effects such as serious liver disorders, acute pancreatitis, hyperglycemia and immunosuppression (Hawkins et al., 2004; Krasotkina et al., 2004). Interestingly, no report using the sequence of *E. carotovora* L-asparaginase II mature protein and recombinant DNA technology for its heterologous expression aiming at commercial production are available. The commercially available L-asparaginases are produced by non-recombinant bacteria fermentations in which the production of large quantities is restricted by the extremely low efficiency of the process (Guo et al., 2000). In order to study the best conditions for the production of recombinant *E. carotovora* L-asparaginase II in *E. coli*, we compared the expression, purification and activity of two L-asparaginase constructions. These constructions were *E. carotovora* L-asparaginase II with and without its signal sequence. This process represents an important step towards large-scale production of recombinant *E. carotovora* L-asparaginase II in *E. coli* cells.

Material and Methods

Amplification, cloning, and expression of *E. carotovora* L-asparaginase II

Two PCR amplifications from genomic *E. carotovora* (*subsp. atroseptica*) DNA (a kind gift of Dr. Valmir Duarte – Departamento de Fitossanidade – Faculdade de Agronomia - UFRGS), were carried out using: forward primer 1 (with *Nde*I restriction site) 5′-gaa ttc cat atg ttt aac gca tta ttc gtt-3′ and reverse primer (with *Bam*HI restriction site) 5′-ggc gga tcc tta ata agc gtg gaa gta atc-3′ for the DNA sequence encoding L-asparaginase II (*ErA*) with its native signal sequence (AspSP) and forward primer 2 (with *Nde*I restriction site) 5′-gaa ttc cat atg gca gaa aat cta cct aac-3′ and reverse primer for the other sequence without its native signal sequence (AspMP). The PCR products (1041 and 1002 bp, respectively) were purified from agarose gel, cloned into the pCR-Blunt® vector (Invitrogen) and subcloned into the pET-30a(+) expression vector (Novagen) using the *Nde*I and *Bam*HI restriction enzymes (Boehringer Mannheim). In order to confirm the identity and absence of PCR-introduced mutations in the cloned genes, the *ErA* genes were sequenced by automatic sequencing. *E. coli* C41(DE3) and BL21(DE3) electrocompetent cells were transformed with the recombinant pET30a(+) containing the L-asparaginase II genes (AspSP and AspMP, respectively) and grown on LB agar plates containing 30 μg mL^{-1} of kanamycin. It should be pointed out that the protocol of recombinant protein expression described here represents just one, out of several experimental conditions used to optimize the assay. The soluble fractions were analyzed by 12 % SDS-PAGE (Laemmli, 1970).

Expression of AspSP in *E. coli*

To analyze the L-asparaginase II expression in the periplasmic space, a osmotic shock protocol was used (Ausubel et al., 1989), in which *E. coli* C41(DE3) electrocompetent cells were transformed with the recombinant pET30a(+) containing the AspSP gene. A single colony was inoculated in 50 mL of LB medium containing 30 μg mL^{-1} kanamycin and grown in shaker flasks at 180 rpm at 37ºC to an OD_{600} of 0.4-0.6. The cells were grown for an additional 6 h after induction with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). Cells were harvested by centrifugation at 20,800*g* for 5 minutes at 4°C. The cell pellet was resuspended in 40 mL of 30 mM Tris-HCl, 20% sucrose, pH 8. Then 0.8 mL 0.5 M EDTA, pH 8 was added and the sample stirred slowly at room temperature for 10 minutes. The cells were collected by centrifugation at 4ºC for 10 minutes at 10,000*g*. The pellet was resuspended in 400 mL of ice-cold 5 mM $MgSO_4$ and the cell suspension stirred slowly for 10 minutes on ice, and centrifuged at 10,000*g* for 10 minutes at 4ºC. The supernatant (periplasmic fraction) was concentrated to 4 mL and analyzed by 12% SDS-PAGE. The pellet (cytoplasmic space) was resuspended in 4 mL buffer and disrupted by sonication, centrifuged at 48,000*g* for 30 min at 4ºC, and the supernatant analyzed by 12% SDS-PAGE. To verify the expression of AspSP into the culture medium, 50 mL of LB medium were grown as described above and 6 h after induction with IPTG, the supernatant was obtained by centrifugation at 20,800*g* for 5 minutes at 4°C, concentrated to 4 mL, and analyzed by 12% SDS-PAGE.

Expression of AspMP in *E. coli*

BL21(DE3) electrocompetent cells were transformed with the recombinant pET30a(+) containing the AspMP gene. Cell culture and IPTG induction of recombinant protein were as for AspSP. One mL of cell culture was harvested by centrifugation at 18,000*g* for 1 min and the pellet ressuspended in 0.5 mL of 50 mM Tris/HCl pH 8 was disrupted by sonication. The samples were centrifuged at 18,000*g* for 20 min at 4ºC and the soluble fraction was analyzed by SDS-PAGE.

Recombinant protein purification

To purify the recombinant proteins, a single colony was inoculated in 50 mL of LB medium containing 30 μg mL^{-1} kanamycin and grown in shaker flasks at 180 rpm at 37ºC up to an OD_{600} of 1. Ten mL of this cell culture were used to inoculate 5 flasks containing 500 mL of LB medium. After the cultures reached an OD_{600} of 0.4 - 0.6, recombinant protein expression was induced with 1 mM of IPTG. The cells were grown for additional 6 h after induction and harvested by centrifugation at 15,900*g* for 30 min at 4ºC and stored at -20ºC. The cells were resuspended (1g cells/10 mL buffer) in 20 mM potassium phosphate buffer pH 5.5 (buffer A), disrupted by sonication on ice (10 pulses of 1 second, 60% amplitude) and centrifuged at 48,000*g* for 30 min at 4ºC. The supernatant was incubated with 1% (w/v) of streptomycin sulfate, in order to precipitate the

nucleic acids, at 4ºC under slow agitation for 30 min, centrifuged at 48,000*g* for 30 min, and the resulting supernatant dialyzed twice against 2 L of buffer A. The purification protocol for the two proteins was performed using the ÄKTA System (GE Healthcare) and performed at 4ºC. Samples from dialysis were clarified by centrifugation (48,000*g* for 30 min at 4ºC) and the supernatants were loaded on a HiPrep 16/10 SP XL cation exchange chromatography column previously equilibrated with buffer A. The non-adsorbed proteins were washed out with 5 column volumes (CVs) of buffer A, and the adsorbed proteins were eluted with a linear pH gradient, pH 5.5 to 8.5, of 20 mM potassium phosphate buffer using 20 CVs. The eluted fractions containing the purified recombinant AspMP enzyme were pooled and stored at -20ºC. On the other hand, for AspSP recombinant protein, the eluted fractions were concentrated and loaded on Sephacryl S-200 HR column previously equilibrated with 20 mM potassium phosphate buffer pH 7.5, and the eluted fractions containing the purified AspSP enzyme were pooled and stored at -20ºC. Chromatographic protein fractions and homogeneity of recombinant AspMP and AspSP proteins were evaluated by 12% SDS-PAGE stained with either Comassie Blue or Silver (Owl Silver Stain, Owl Separation Systems). Protein concentration was determined by the Bradford's method (Bradford et al., 1976) using the BIO-RAD protein assay kit and bovine serum albumin as standard.

N-terminal amino acid sequence analysis

N-terminal amino acid residues of both AspMP and AspSP homogeneous recombinant L-asparaginase II proteins were identified by the automated Edman degradation method on a PPSQ-23 protein peptide sequencer (Shimadzu Co. Japan).

Activity assays and determination of steady-state kinetic parameters:

The assay employed to evaluate L-asparaginase II activity of the fractions of the purification protocol steps was performed as described by Meister (Meister, 1955) with minor modifications. Purified *E. carotovora* L-asparaginase II (100 nM) was added to 0.05 M Tris/HCl buffer pH 8.0, containing 0.04 M L-Asn and the reaction mixture was incubated for 1 to 20 minutes at 37ºC. The rate of NH_3 formation in the reaction was determined using the Nessler reagent. One unit of L-asparaginase activity is defined as the amount of enzyme that catalyzes the formation of 1 µM of NH_3 from L-Asn per minute.

The assay employed to measure L-Asn and L-Gln hydrolysis catalyzed by homogeneous recombinant AspMP and AspSP proteins was the direct method described by Krasotkina et al. (2004), with minor modifications. The reaction was carried out in a 1.5 mL quartz cuvette containing 198.9 nM AspSP or AspMP for L-asparaginase activity measurements and 662.9 nM AspSP or AspMP for L-glutaminase activity measurements, and one of the substrates (L-Asn or L-Gln) at varying concentrations (from 1 to 20 mM) in 0.02 M potassium phosphate buffer at pH 7.0. The kinetic parameters k_{cat} and K_m were calculated by non-linear regression analysis fitting the experimental steady-state data to the Michaelis-Menten equation. Turnover numbers were calculated on the basis of one active site per 34.5 kDa of subunit molecular mass. SigmaPlot 9.0 was used for mathematical processing of experimental data.

Results

DNA sequence amplification and cloning

A 1041 bp PCR amplification fragment with 100% identity to the L-asparaginase II coding region from *E. carotovora* gene (Accession number AY560097) (AspSP) and a 1005 bp PCR amplification fragment consistent with *ErA* gene lacking the signal peptide region (AspMP) were detected on agarose gel (data not shown). The fragments were subcloned into pET30a(+) vector between the *Nde*I and *Bam*HI restriction sites. The correct construction of both plasmids was confirmed by nucleotide sequence analysis.

Recombinant protein expression

Heterologous expression of *E. carotovora* AspMP in *E. coli* BL21(DE3) host cells could be achieved in the presence and absence of IPTG induction as shown by a strong protein band of approximately 39 kDa as determined by SDS-PAGE analysis of the soluble fraction (Figure 1). After the osmotic shock, the expression of recombinant *E. carotovora* AspSP in *E. coli* C41(DE3) host cells was observed in the periplasmic space and in the culture medium at similar quantities, and in the cytoplasm at negligible level (Figure 2). The recombinant AspMP was observed only in the cytoplasm, as expected.

Recombinant protein purification

The amount of cells obtained from 2.5 L of LB medium was: 7g for AspSP and 3g for AspMP. The purification of AspSP was achieved by a two-step purification protocol. Protein elution of recombinant AspSP loaded on a Hiprep 16/10 SP XL cation exchange column employed pH gradient with the recombinant protein eluting at pH 7.0. The pooled fractions were loaded on a Sephacryl S-200 HR gel filtration column. The pooled protein fractions were analyzed by SDS-PAGE (Figure 3A). On the other hand, purification of AspMP could be achieved by a single-step protocol using Hiprep 16/10 SP XL cation exchange column and pH gradient elution profile (Figure 3B). Apparently no con-

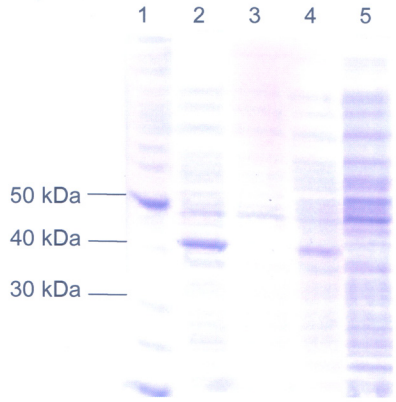

Figure 1: Analysis of recombinant AspMP expression by SDS-PAGE. SDS-PAGE (12 %) analysis showing the expression of AspMP in the soluble fraction of *E. coli* BL21(DE3) host cells. Lane 1: Molecular mass markers (Bench Mark™ Protein Ladder, Invitrogen). Lane 2: host cells containing the recombinant pET30a(+)-AspMP plasmid induced with 1 mM IPTG; lane 3: host cells containing the pET30a(+) plasmid lacking the coding sequence induced with 1 mM IPTG; lane 4: host cells containing the recombinant pET30a(+)-AspMP plasmid without induction and lane 5: host cells containing the pET30a(+) plasmid lacking the coding sequence without induction. The predicted molecular mass for L-asparaginase is 34.5 kDa. The gel was stained with Coomassie Blue.

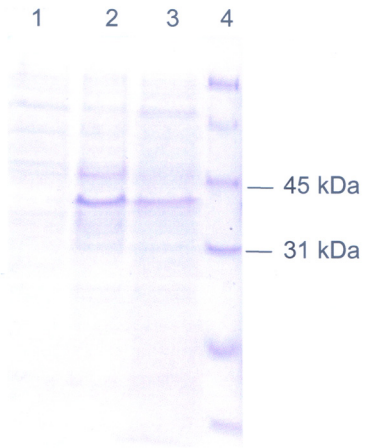

Figure 2: Analysis of recombinant AspSP expression by SDS-PAGE. SDS-PAGE (12 %) analysis showing the expression of AspSP in the periplasm and in the culture medium of *E. coli* strain C41(DE3) host cells 6 hours post-induction. Lane 1: the cytoplasm fraction showing negligible expression of AspSP (36.5 kDa); lane 2: AspSP expression in the periplasm fraction; lane 3: AspSP expression in the culture medium; and lane 4: Molecular mass markers (Low Range Protein Ladder, BIO-RAD). The predicted molecular mass for L-asparaginase II is 34.5 kDa. The gel was stained with Coomassie Blue.

taminants were detected with Coomassie Blue staining. In addition, SDS-PAGE analysis with silver staining attests the high degree of homogeneity for both proteins (Figure 3C). Approximately 0.63 mg of homogeneous recombinant AspSP and 2.2 mg of homogeneous recombinant AspMP were obtained from 1g of cells. The yield of recombinant active protein was 91% for AspMP and 28% for AspSP (Table 1). The specific activity of purified recombinant L-asparaginases II was calculated by measuring the rate of NH_3 formation, by the Nessler assay. Values of 208.1 and 237.6 U mg^{-1} were obtained for AspMP and AspSP, respectively (Table 1).

N-terminal amino acid sequencing

The first eleven N-terminal amino acid residues for the two recombinant L-asparaginases II were identified as AENLPNIVILA by the EDMAN degradation method. This result confirmed the identity of both homogeneous recombinant mature proteins as L-asparaginase II, and the removal of the N-terminal signal peptide from AspSP construct.

Enzyme kinetic analysis

The substrate specificity was assessed for L-Asn and L-Gln by steady-state kinetic analysis employing a direct assay that monitored the decrease in absorbance of the γ-amide bond upon carboxylic acid formation (Table 2). The K_m values for L-Asn were 0.16 mM for AspSP and 1.1 mM for AspMP; and the K_m values for L-Gln were determined as 2.6 mM for AspSP and 4.4 mM for AspMP. The catalytic constant values for L-asparaginase activity was 160 s^{-1} for both enzymes, and for L-glutaminase activity were 6.0 s^{-1} for AspSP and 7.1 s^{-1} for AspMP.

Discussion

To the best of our knowledge, the work here presented is the first report on cloning, expression in *E. coli*, and purification of the mature recombinant *E. carotovora* L-asparaginase II (AspMP) protein. Previous reports cloned the DNA sequence of *E. carotovora* L-asparaginase II containing the signal sequence (Krasotkina et al., 2004; Kotzia and Labrou, 2005). Our aim was to compare expression and activity of two constructs of *E. carotovora* L-asparaginase II (with and without signal peptide) to optimize the process for large scale production. As AspMP does not have a signal peptide sequence, its expression was restricted to the cytoplasm. Since bacterial L-asparaginases are extracellular, it has been proposed that a precursor should contain a signal peptide sequence to direct its transport across the periplasmic membrane (Kotzia and Labrou, 2005). Our results demonstrate that AspSP construct without the signal peptide was present in the periplasm as well as in the extracelular medium, and with the signal peptide at negligible levels in the cytoplasm (Figure 2). Secreted proteins can leak from the periplasmic space into the culture medium possibly due to an increased permeability of the cell membrane (Tong et al., 2000).

The N-terminal amino acid sequencing showed the removal of the N-terminal signal peptide from AspSP. We thus deemed

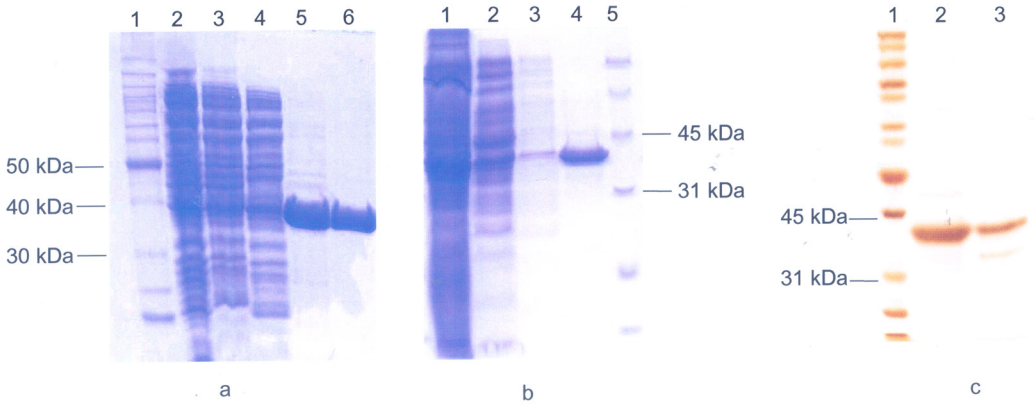

Figure 3: SDS-PAGE (12 %) analysis of purification protocol fractions containing L-asparaginase II. (A): AspSP Purification protocol fractions of SDS-PAGE stained with Coomassie Blue: Lane 1: Protein molecular mass markers (Bench Mark™ Protein Ladder, Invitrogen); lane 2: soluble fraction after cell disruption; lane 3: sample after 1% (w/v) of streptomycin sulfate precipitation; lane 4: sample after dialysis and loaded on cation exchange Hiprep SP XL column; lane 5: pooled fractions of cation exchange chromatography loaded on Sephacryl S-200; and lane 6: homogeneous AspSP (34.5 kDa) after elution from size exclusion chromatography. (B): AspMP Purification protocol fractions of SDS-PAGE stained with Coomassie Blue: Lane 1: soluble fraction after cell disruption; lane 2: sample after 1% (w/v) of streptomycin sulfate precipitation; lane 3: sample after dialysis loaded on cation exchange Hiprep SP XL column; lane 4: homogeneous L-asparaginase II (34.5 kDa) pool eluted from the cation exchange column; and lane 5: Protein molecular mass markers (Low Range Protein Ladder, BIO-RAD). (C): SDS-PAGE stained with Silver of purified AspMP and AspSP proteins: Lane 1: Protein molecular mass markers (Low Range Protein Ladder, BIO-RAD); lane 2: AspMP; and lane 3: AspSP.

Enzyme	Step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification (fold)	Yield (%)
AspSP	Crude extract	91.4	3738.3	40.9	1.00	100
	SP XL	3.2	143.4	44.8	1.1	3.8
	S200	4.4	1045.4	237.6	5.8	28
AspMP	Crude extract	49.5	1485	30.0	1.00	100
	SP XL	6.5	1352.6	208.1	6.9	91

The results presented are for a purification protocol from 2.5 g of *E. coli* host cells.

Table 1: Improved purification protocol for recombinant L-asparaginases II AspSP and AspMP from *E. carotovora*.

Enzyme	Substrates	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
AspSP	L-asparagine	0.16 ± 0.01	160 (± 1)	9.98 (± 0.62) x 10^5
	L-glutamine	2.6 ± 0.4	6.0 (± 0.3)	2.31 (± 0.37) x 10^3
AspMP	L-asparagine	1.1 ± 0.1	160 (± 4)	1.45 (± 0.14) x 10^5
	L-glutamine	4.4 ± 0.2	7.1 (± 0.1)	1.61 (± 0.08) x 10^3

[a]All initial velocities were determined in triplicate.

Table 2: Steady-state kinetic parameters of *E. carotovora* L-asparaginases II AspSP and AspMP[a].

more convenient to express the recombinant L-asparaginase protein in one compartment and avoid leakage to extracellular medium. Accordingly, we constructed L-asparaginase without the signal peptide sequence (AspMP). Amino acid sequencing (AENLPNIVILA) showed that the N-terminal methionine was removed from the AspMP. Thus, the N-terminal amino acid sequence of AspMP is identical to recombinant AspSP protein having the signal peptide removed. In addition, as the main sources of L-asparaginases II commercially available are produced by non-recombinant fermentation of *E. coli* and *E. chrysanthemi* and have thus their signal peptide sequence removed, the construct here described for AspMP may represents an attractive alternative for large scale production of *E. carotovora* L-asparaginases II. It should also be pointed out that the N-terminal amino acid sequencing showed that AspMP was separated from its *E. coli* counterpart, which has an important bearing on *E. carotovora* L-asparaginases II preparation since these two enzymes have distinct immunological specificities and provide alternative therapy if a patient develops hypersensitivity to either L-asparaginases II (Bascomb and Bettelheim, 1976).

The estimated molecular mass for the two L-asparaginases II without its signal peptide was 34.5 kDa, which is in contrast to the approximately 39 kDa value observed by SDS-PAGE analysis. Owing to its very alkaline character (estimated pI value of 9.04) the proteins may run differently (slower) on SDS-PAGE than would be expected based on their known molecular mass (Walker, 1996). There appears to be two minor components present in the AspMP preparation (Figure 3). As previously reported (Krasotkina et al., 2004), these low-molecular-mass contaminants were probably proteolytic products of L-asparaginase II, which accumulated during storage of the enzyme solutions. Incidentally, no cocktail of protease inhibitors were employed in the purification process because their presence in a therapeutic protein must be avoided. Notwithstanding, the AspMP preparation is clearly superior to AspSP one (Figure 3).

An additional favorable feature for using the L-asparaginase II construction without the signal peptide is that it can be purified by a single-chromatographic-step protocol as compared to two steps for AspSP. A two-step purification protocol for recombinant *E. carotovora* L-asparaginase II has been described by Kotzia and Labrou (2005). However, no active protein yield was reported by these authors. Recombinant *E. carotovora* L-asparaginase II has been purified by a one-step purification protocol with 72% yield of active protein (Krasotkina et al., 2004), however the construct was protected by patent. Notwithstanding, the yield of active recombinant AspMP described here was 91% (Table 1), which is superior than reported by others (Krasotkina et al., 2004). In addition, the yield of active recombinant AspSP employing the protocol here described was 28%, which strengthen the proposal of using the AspMP construct for further attempts at large scale production.

We found similar specific activities for AspSP and AspMP, 237.6 and 208.1 U mg^{-1}, respectively (Table 1) by the Nessler method. These values are lower as compared to Krasotkina et al. (2004), and cannot be compared to a measurement using the same method because this value was not provided (Kotzia and Labrou, 2005). On the other hand, when compared to *E. chrysanthemi* L-asparaginase (Kotzia and Labrou, 2007), these values are higher, and, surprisingly, our values were also higher than *E. coli* L-asparaginase. According to Duval et al. (2002), it would be expected that *E. coli* L-asparaginase would be more effective in depletion L-Asn than *Erwinia* L-asparaginase during the childhood lymphoid malignancies treatment. Notwithstanding, the Nessler method was only employed to accompany the degree of purification of recombinant *E. carotovora* L-asparaginase II. Despite the sensitivity of this assay, a more convenient direct continuous method was chosen to determine the steady-state parameters as described below.

The K_m value for L-Asn was approximately 7-fold larger for AspMP as compared to AspSP (Table 2), a result that is somewhat intriguing since they should be the same values for a protein obtained from different constructs and purification protocols. However, the k_{cat} values for hydrolysis of L-Asn are the same for both proteins (Table 2). The K_m value for L-Gln was approximately 2-fold larger for AspMP as compared to AspSP and similar k_{cat} values for hydrolysis of L-Gln (Table 2). The larger K_m value for L-Gln hydrolysis is a favorable feature of AspMP protein as compared to AspSP because a higher concentration of this substrate in the physiological milieu would be required to achieve maximum velocity. Moreover, the K_m value (4.4 mM) for L-Gln hydrolysis catalyzed by AspMP construct here described is larger than that found for L-asparaginases II from *E. carotovora* (Krasotkina et al., 2004), *E. coli* (Derst et al., 2000) and *E. chrysanthemi* (Moola et al., 1994), which were 3.4, 3.5 and 1.7 mM, respectively. L-Asparaginases with low K_m values for L-Asn and large values for L-Gln have been re-

ported to be preferable during the course of anti-cancer therapy (Hawkins et al., 2004), since the toxicity of the enzyme is partially attributable to the L-glutaminase activity (Howard and Carpenter, 1972). The k_{cat}/K_m ratio is an apparent second-order rate constant that determines the specificity for competing substrates (Fersht, 1999). Accordingly, the ratio of L-Asn k_{cat}/K_m value over L-Gln k_{cat}/K_m value indicates that the former is a better substrate for AspSP (432-fold increase) as compared to AspMP (90-fold increase). However, AspMP is produced in higher yield using a one-step protein purification protocol.

Conclusions

It has recently been reported that *E. carotovora* L-asparaginase II has decreased stability as compared to the *E. coli* enzyme and its cytotoxic efficiency was 30 times lower than that of *E. coli* periplasmic L-asparaginase (Papageorgiou et al., 2008). However, further efforts towards increasing *E. carotovora* L-asparaginase II enzyme stability (e.g., pegylation) are warranted due to its better therapeutic potential by combining the advantages of *E. coli* periplasmic L-asparaginase (low glutaminase activity) and *E. chrysanthemi* L-asparaginase (less severe immunorelated side-effects). Moreover, because L-asparaginases II from different sources possess different immunological specificities, *E. carotovora* L-asparaginase II may represent an important alternative therapy as a patient develops hypersensitivity to the other enzymes. In addition, it has more recently been shown that *Erwinia* asparaginases should be considered as alternative therapy for children with ALL who develop allergy to *E. coli*-derived asparaginase preparations (Vrooman et al., 2010). The experimental approach here described represents an efficient and high-yield method to obtain homogeneous recombinant *E. carotovora* L-asparaginase II with high specific activity and low glutaminase activity. This process can be amenable to large scale production and may be of interest to researchers and biopharmaceutical companies interested in developing biosimilars and improving their therapeutic properties, which offer a great opportunity to scientific, biotechnological, economical, and industrial growth.

Acknowledgements

This work was supported by National Institute of Science and Technology on Tuberculosis (Decit/SCTIE/MS-MCT-CNPq-FNDCT-CAPES) and Millennium Initiative Program (CNPq), Brazil, to DSS and LAB. DSS and LAB also acknowledge grants awarded by FINEP. DSS (304051/1975-06) and LAB (520182/99-5) are research career awardees from the National Council for Scientific and Technological Development of Brazil (CNPq). PLW acknowledges a scholarship awarded by CAPES. The authors also acknowledge Dr. Carlos Bloch Jr. for N-terminal amino acid sequencing.

References

1. Amylon MD, Shuster J, Pullen J, Berard C, Link MP, et al. (1999) Intensive high-dose asparaginase consolidation improves survival for pediatric patients with T cell acute lymphoblastic leukemia and advanced stage lymphoblastic lymphoma: a Pediatric Oncology Group study. Leukemia 13: 335-342. » CrossRef » PubMed » Google Scholar

2. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, et al. (1989) Current Protocols in Molecular Biology. John Wiley & Sons, New York. » CrossRef » PubMed » Google Scholar

3. Avramis VI, Panosyan EH (2005) Pharmacokinetic/pharmacodynamic relationships of asparaginase formulations: the past, the present and recommendations for the future. Clin Pharmacokinet 44: 367-393. » CrossRef » PubMed » Google Scholar

4. Bascomb S, Bettelheim KA (1976) Immunological relationships of bacterial L-asparaginases. J Gen Microbiol 92: 175-182. » CrossRef » PubMed » Google Scholar

5. Bradford MM, McRorie RA, Williams WL (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254. » CrossRef » PubMed » Google Scholar

6. Broome JD (1965) Antilymphoma activity of L-asparaginase in vivo: clearance rates of enzyme preparations from guinea pig serum and yeast in relation to their effect on tumor growth. J Natl Cancer Inst 35: 967-974. » CrossRef » PubMed » Google Scholar

7. Broome JD (1968) Studies on the mechanism of tumor inhibition by L-asparaginase. Effects of the enzyme on asparagine levels in the blood, normal tissues, and 6C3HED lymphomas of mice: differences in asparagine formation and utilization in asparaginase-sensitive and -resistant lymphoma cells. J Exp Med 127: 1055-1072. » CrossRef » PubMed » Google Scholar

8. Campbell HA, Mashburn LT, Boyse EA, Old LJ (1967) Two L-asparaginases from *Escherichia coli* B. Their separation, purification, and antitumor activity. Biochemistry 6: 721-730. » CrossRef » PubMed » Google Scholar

9. Cedar H, Schwartz JH (1968) Production of L-asparaginase II by *Escherichia coli*. J Bacteriol 96: 2043-2048. » CrossRef » PubMed » Google Scholar

10. Derst C, Henseling J, Röhm KH (2000) Engineering the substrate specificity of *Escherichia coli* asparaginase. II. Selective reduction of glutaminase activity by amino acid replacements at position 248. Protein Sci 9: 2009-2017. » CrossRef » PubMed » Google Scholar

11. Duval M, Suciu S, Ferster A, Rialland X, Nelken B, et al. (2002) Comparison of *Escherichia coli*-asparaginase with *Erwinia*-asparaginase in the treatment of childhood lymphoid malignancies: results of a randomized European Organisation for Research and Treatment of Cancer-Children's Leukemia Group phase 3 trial. Blood 99: 2734-2739. » CrossRef » PubMed » Google Scholar

12. Fersht A (1999) Structure and mechanism in protein science: a guide to enzyme catalysis and protein folding. WH Freeman and Company, NY, USA. » CrossRef » PubMed » Google Scholar

13. Guo L, Wang J, Yan X, Chen R, Qian S, et al. (2000) Characterization of L-asparaginase fused with a protective ScFv and the protection mechanism. Biochem Biophys Res Commun 27: 197-203. » CrossRef » PubMed » Google Scholar

14. Hawkins DS, Park JR, Thomson BG, Felgenhauer JL, Holcenberg JS, et al. (2004) Asparaginase pharmacokinetics after intensive polyethylene glycol-conjugated L-asparaginase therapy for children with relapsed acute lymphoblastic leukemia. Clin Cancer Res 10: 5335-5341. » CrossRef » PubMed » Google Scholar

15. Howard JB, Carpenter FH (1972) L-asparaginase from *Erwinia carotovora*. Substrate specificity and enzymatic properties. J Biol Chem 247: 1020-1030. » CrossRef » PubMed » Google Scholar

16. Kiriyama Y, Kubota M, Takimoto T, Kitoh T, Tanizawa A, et al. (1989) Biochemical characterization of U937 cells resistant to L-asparaginase: the role of asparagine synthetase. Leukemia 3: 294-297. » CrossRef » PubMed » Google Scholar

17. Kotzia GA, Labrou NE (2005) Cloning, expression and characterisation of *Erwinia carotovora* L-asparaginase. J Biotechnol 119: 309-323. » CrossRef » PubMed » Google Scholar

18. Kotzia GA, Labrou NE (2007) L-Asparaginase from Erwinia Chrysanthemi 3937: cloning, expression and characterization. J Biotechnol 127: 657-669. » CrossRef » PubMed » Google Scholar

19. Krasotkina J, Borisova AA, Gervaziev YV, Sokolov NN (2004) One-step purification and kinetic properties of the recombinant L-asparaginase from *Erwinia carotovora*. Biotechnol Appl Biochem 39: 215-221. » CrossRef » PubMed » Google Scholar

20. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685. » CrossRef » PubMed » Google Scholar

21. Lee SM, Wroble MH, Ross JT (1989) L-asparaginase from *Erwinia carotovora*. An improved recovery and purification process using affinity chromatography. Appl Biochem Biotechnol 22: 1-11. » CrossRef » PubMed » Google Scholar

22. Meister A (1955) Glutaminase, Asparaginase, and α-Keto Acid-ω-amidase. In: S. Colowick and N. Kaplan (ed.). Methods in enzymology, New York, vol. II, pp380-385. » CrossRef » PubMed » Google Scholar

23. Moola ZB, Scawen MD, Atkinson T, Nicholls DJ (1994) *Erwinia chrysanthemi* L-asparaginase: epitope mapping and production of antigenically modified enzymes. Biochem J 302: 921-927. » CrossRef » PubMed » Google Scholar

24. Müller HJ, Boos J (1998) Use of L-asparaginase in childhood ALL. Crit Rev Oncol Hematol. 28: 97-113. » CrossRef » PubMed » Google Scholar

25. Oettgen HF, Old LJ, Boyse EA, Campbell HA, Philips FS, et al. (1967) Inhibition of leukemias in man by L-asparaginase. Cancer Res 27: 2619-2631. » CrossRef » PubMed » Google Scholar

26. Oettgen HF, Stephenson PA, Schwartz MK, Leeper RD, Tallai L, et al. (1970) Toxicity of *E. coli* L-asparaginase in man. Cancer 25: 253-278. » CrossRef » PubMed » Google Scholar

27. Ortega JA, Nesbit ME Jr, Donaldson MH, Hittle RE, Weiner J, et al. (1977) L-Asparaginase, vincristine, and prednisone for induction of first remission in acute lymphocytic leukemia. Cancer Res 37: 535-540. » CrossRef » PubMed » Google Scholar

28. Papageorgiou AC, Posypanova GA, Anderson CS, Sokolov NN, Krasotkina J (2008) Structural and functional insights into *Erwinia carotovora* L-asparaginase. FEBS J 275: 4306-4316. » CrossRef » PubMed » Google Scholar

29. Richards NG, Kilberg MS (2006) Asparagine synthetase chemotherapy. Annu Review of Biochem 75: 629-654. » CrossRef » PubMed » Google Scholar

30. Schwartz JH, Reeves JH, Broome JD (1966) Two L-asparaginases from *E. coli* and their action against tumors. Proc Natl Acad Sci USA 56: 1516-1519. » CrossRef » PubMed » Google Scholar

31. Tong L, Lin Q, Wong WK, Ali A, Lim D, et al. (2000) Extracellular expression, purification, and characterization of a winter flounder antifreeze polypeptide from *Escherichia coli*. Protein Expr Purif 18: 175-181. » CrossRef » PubMed » Google Scholar

32. Vrooman LM, Supko JG, Neuberg DS, Asselin BL, Athale UH, et al. (2010) *Erwinia asparaginase* after allergy to *E. coli* asparaginase in children with acute lymphoblastic leukemia. Pediatr Blood Cancer 54: 199-205. » CrossRef » PubMed » Google Scholar

33. Walker JM (1996) SDS Polyacrylamide Gel Electrophoresis of Proteins. In: Human Press (ed) The Protein Protocols Handbook, Totowa, New Jersey, pp57-61. » CrossRef » PubMed » Google Scholar