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

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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 Erwinia 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 $V_{max}$ 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 fL-Asparaginase II gene (Er) from Erwinia carotovora (subsp. atroseptica) not containing its signal peptide (mature protein); AspSP: Construct of L-asparaginase II gene (Er) 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 (NH₃), 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, leukopenia, 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-
Combination 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 L-asparaginase II 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 Agricultura - UFRGS), were carried out using: forward primer 1 (with NdeI restriction site) 5’-gaa ttc cat atg ttt aac gca tta ttc gtt-3´ and reverse primer (with BamHI restriction site) 5’-ggc gga tcc tta aac gca tta ttc gtt-3´ and reverse primer (with BamHI restriction site) 5’-ggg gga tcc tta ata age tgg gaa gta ac-3´ for the DNA sequence encoding L-asparaginase II (ErA) with its native signal sequence (AspSP) and forward primer 2 (with NdeI restriction site) 5’-ggg gga cat atg gca gaa aat cta ctc ac-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 pET30a(+) expression vector (Novagen) using the NdeI and BamHI 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) electrocoment 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 37°C to an OD₆₀₀ 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 20,800g 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 4°C for 10 minutes at 10,000g. The pellet was resuspended in 400 mL of ice-cold 5 mM MgSO₄ and the cell suspension stirred slowly for 10 minutes on ice, and centrifuged at 10,000g for 10 minutes at 4°C. 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 48,000g for 30 min at 4°C, 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 20,800g for 5 minutes at 4°C, concentrated to 4 mL, and analyzed by 12% SDS-PAGE.

Expression of AspMP in E. coli

BL21(DE3) electrocoment 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 18,000g for 1 min and the pellet resuspended in 0.5 mL of 50 mM Tris/HCl pH 8 was disrupted by sonication. The samples were centrifuged at 18,000g for 20 min at 4°C 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 37°C up to an OD₆₀₀ 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 OD₆₀₀ 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 15,900g for 30 min at 4°C and stored at -20°C. 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 48,000g for 30 min at 4°C. The supernatant was incubated with 1% (w/v) of streptomycin sulfate, in order to precipitate the
The rate of NH₃ formation in the reaction mixture was incubated for 1 to 20 minutes at 37ºC. The supernatant was 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, 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 NH₃ 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 NH₃ 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 kcat and Km 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 NdeI and BamHI 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-

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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 was 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 NH₃ 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 AENLPNIVILYA 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-asparaginas 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 extracellular 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

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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.

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.
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) showing 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-asparaginase 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 Kᵦ 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 kᵦ values for hydrolysis of L-Asn are the same for both proteins (Table 2). The Kᵦ value for L-Gln was approximately 2-fold larger for AspMP as compared to AspSP and similar kᵦ values for hydrolysis of L-Gln (Table 2). The larger Kᵦ 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 Kᵦ 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 (Derd 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 Kᵦ values for L-Asn and large values for L-Gln have been re-

| Enzyme | Substrates | Kᵦ (mM) | kᵦ (s⁻¹) | kᵦ/Kᵦ (M⁻¹ s⁻¹) |
|--------|------------|---------|-----------|-------------------|
| AspSP  | L-asparagine | 0.16 ± 0.01 | 160 (+ 1) | 9.38 (± 0.62) x 10⁴ |
| AspMP  | L-asparagine | 1.1 ± 0.1 | 160 (+ 4) | 1.45 (± 0.14) x 10⁵ |
|        | L-glutamine  | 4.4 ± 0.2 | 7.1 (± 0.1) | 1.16 (± 0.08) x 10⁴ |

*All initial velocities were determined in triplicate.

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 | 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       |
|        | 200           | 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        |

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

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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-asparaginas. 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 quantitates 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 chEHDed 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-asparaginas 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, Farser R, Aialand 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 Leukaemia 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. http://www.ncbi.nlm.nih.gov/pubmed/10247603

13. Guo L, Wang J, Yan X, Chen R, Qian S, et al. (2000) Characterization of L-asparaginase II from different sources possess different immunological specificities. E. coli and E. carotovora L-asparaginase II may represent an important alternative therapy for children with ALL who develop allergy to E. coli-derived asparaginase preparations. J Biol Chem 274: 197-203. CrossRef PubMed Google Scholar

14. Hawkins DS, Park JR, Thomson BG, Felgenhauer JL, Holenberg 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: 3535-3541. 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 characterization 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. Krasokina J, Borisova AA, Gervaziyev 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. Lennmuki UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685. CrossRef PubMed Google Scholar

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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-α-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 LI, 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 lymphoblastic 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