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

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 pilori, 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 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 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 atc ggt gaa gta acc cta gtc ggg tgc tta ttc gta atc ggt gaa gta acc cta gtc ggg tgc tta ttc gta atc ggt gaa gta acc cta gtc ggg tgc tta ttc gta atc ggt gaa gta acc cta gtc ggg tgc tta ttc gta atc ggt gaa gta acc cta gtc ggg tgc tta ttc gta atc ggt gaa gta acc cta gtc ggg tgc tta ttc gta atc ggt gaa gta acc cta gtc ggg tgc tta ttc gta atc ggt gaa gta acc cta gtc ggg tgc tta ttc gta atc ggt gaa gta acc cta gtc ggg tgc tta ttc gta atc ggt gaa gta acc cta gtc ggg tgc tta ttc gta atc ggt gaa gta acc cta gtc g

Expression of AspSP in E. coli
To analyze the L-asparaginase II expression in the periplasmic space, a osmotic shock protocol was used (Ausbet 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 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 AspSP 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 18,000g 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 an 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 (lg 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 f
to 0.05 M Tris/HCl buffer pH 8.0, containing 0.04 M L-Asn and
Healthcare) and performed at 4ºC. Samples from dialysis were
the two proteins was performed using the ÄKTA System (GE
lyzed twice against 2 L of buffer A. The purification protocol for
parameters:
23 protein peptide sequencer (Shimadzu Co. Japan).
tified by the automated Edman degradation method on a PPSQ-
compositions 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
(Approval 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 ex-
pression 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 cyto-
plasm 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 AspMP loaded on a HiPrep 16/10 SP XL cation ex-
change column employed pH gradient with the recombinant
protein eluting at pHe 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-

N-terminal amino acid sequence analysis
N-terminal amino acid residues of both AspMP and AspSP
homogeneous recombinant L-asparaginase II proteins were iden-
tified by the automated Edman degradation method on a PPSQ-
23 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 37°C.
The rate of NH₃ formation in the reaction was determined using the
Nessler reagent. One unit of L-asparaginase activity is de-
defmed as the amount of enzyme that catalyzes the formation of 1
mM NH₃ from L-Asn per minute.

The assay employed to measure L-Asn and L-Gln hydrolysis
catalyzed by homogeneous recombinant AspMP and AspSP pro-
teins 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 AspMP 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-lin-
ear regression analysis fitting the experimental steady-state data to
the Michaelis-Menten equation. Turnover numbers were cal-
culated on the basis of one active site per 34.5 kDa of subunit
molecular mass. SigmaPlot 9.0 was used for mathematical pro-
cessing of experimental data.

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 nM IPTG; lane 3: host cells containing the pET30a(+) plasmid lacking the
coding sequence induced with 1 nM 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: Electrophoresis analysis of recombinant L-asparaginase II expressed in E. coli
in the presence (DE3) and absence (C41) of IPTG. Lane 1: molecular mass markers (Bench
Mark™ Protein Ladder, Invitrogen). Lane 2: host cells containing the recombinant pET30a
(+) L-asparaginase plasmid without induction; lane 3: host cells containing the recombinant
pET30a(+) L-asparaginase plasmid induced with 1 nM IPTG; lane 4: host cells containing the recombinant
pET30a(+) L-asparaginase plasmid without induction; lane 5: host cells containing the
pET30a(+) L-asparaginase 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.

N-terminal amino acid sequence analysis
N-terminal amino acid residues of both AspMP and AspSP
homogeneous recombinant L-asparaginase II proteins were iden-
tified by the automated Edman degradation method on a PPSQ-
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The assay employed to evaluate L-asparaginase II activity of
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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 37°C.
The rate of NH₃ formation in the reaction was determined using the
Nessler reagent. One unit of L-asparaginase activity is de-
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The assay employed to measure L-Asn and L-Gln hydrolysis
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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-lin-
ear regression analysis fitting the experimental steady-state data to
the Michaelis-Menten equation. Turnover numbers were cal-
culated on the basis of one active site per 34.5 kDa of subunit
molecular mass. SigmaPlot 9.0 was used for mathematical pro-
cessing of experimental data.
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: homogenous 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: homogenous 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 (AENLPVINIIVA) 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 (Bascob 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 8.8) 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 Km 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 kcat values for hydrolysis of L-Asn are the same for both proteins (Table 2). The Km value for L-Gln was approximately 2-fold larger for AspMP as compared to AspSP and similar kcat values for hydrolysis of L-Gln (Table 2). The larger Km 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 Km 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 Km values for L-Asn and large values for L-Gln have been re-

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

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U mg⁻¹)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AspSP</td>
<td>Crude extract</td>
<td>91.4</td>
<td>3738.3</td>
<td>40.9</td>
<td>1.00</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>SP XL</td>
<td>3.2</td>
<td>143.4</td>
<td>44.8</td>
<td>1.1</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>S200</td>
<td>4.4</td>
<td>1045.4</td>
<td>237.6</td>
<td>5.8</td>
<td>28</td>
</tr>
<tr>
<td>AspMP</td>
<td>Crude extract</td>
<td>49.5</td>
<td>1485</td>
<td>30.0</td>
<td>1.00</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>SP XL</td>
<td>6.5</td>
<td>1352.6</td>
<td>208.1</td>
<td>6.9</td>
<td>91</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrates</th>
<th>Km (mM)</th>
<th>kcat (s⁻¹)</th>
<th>kcat/Km (M⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AspSP</td>
<td>L-asparagine</td>
<td>0.16 ± 0.01</td>
<td>160 (± 1)</td>
<td>9.38 (± 0.62) x 10⁵</td>
</tr>
<tr>
<td></td>
<td>L-glutamine</td>
<td>2.6 ± 0.4</td>
<td>6.0 (± 0.3)</td>
<td>2.31 (± 0.37) x 10⁷</td>
</tr>
<tr>
<td>AspMP</td>
<td>L-asparagine</td>
<td>1.1 ± 0.1</td>
<td>160 (± 4)</td>
<td>1.45 (± 0.14) x 10⁷</td>
</tr>
<tr>
<td></td>
<td>L-glutamine</td>
<td>4.4 ± 0.2</td>
<td>7.1 (± 0.1)</td>
<td>1.61 (± 0.08) x 10⁷</td>
</tr>
</tbody>
</table>

*All initial velocities were determined in triplicate.*
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 \( k_{\text{cat}}/K_m \) ratio is an apparent second-order rate constant that determines the specificity for competing substrates (Fersht, 1999). Accordingly, the ratio of L-Asn \( k_{\text{cat}}/K_m \) value over L-Gln \( k_{\text{cat}}/K_m \) value indicates that the former is a better substrate for AspSP (432-fold increase) as compared to AspMP (90-fold increase). However, AspSM 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-FNDC1-CAPE) 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 (320182/99-5) 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.

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