SUSTAINABLE CHEMICAL PROCESSES

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Extracellular expression and affinity purification of L-asparaginase from *E. chrysanthemi* in *E. coli*

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Abstract

Background: L-asparaginases (E.C.3.5.1.1, L-ASNases) are successfully used as anti-neoplastic agents in the chemotherapy of acute lymphoblastic leukemia (ALL) and therefore are of high interest for the medical and scientific community. In the present study we report the recombinant, extracellular expression and affinity purification of L-asparaginase from *Erwinia chrysanthemi* 3937 (ErL-ASNase) in *E. coli*.

Results: Recombinant *E. coli* strains were screened for their ability to express and translocate ErL-ASNase to the culture medium. The strain *E. coli* Rosetta (DE3) exhibited the highest extracellular expression levels among all the strains tested and it was chosen for further optimization and the development of purification protocol. Affinity adsorbents with immobilized L-Asn, L-Asp and L-Glu were synthesized by solid-phase chemistry and evaluated for their ability to bind and purify ErL-ASNase directly from the culture medium. The affinity adsorbent with immobilized L-Asp (L-Asp-Sepharose CL-6B) showed the highest purifying ability for ErL-ASNase. Adsorption equilibrium studies revealed that the adsorption of ErL-ASNase follows Langmuir isotherm with K_D = 0.21 μM and maximum binding capacity 4.7 mg enzyme/g moist wet adsorbent. This affinity adsorbent was used for the development of one-step purification protocol. The proposed protocol is simple, fast, gentle and afforded ErL-ASNase with high purity and yield.

Conclusions: We show that the recombinant expression of ErL-ASNase in *E. coli* results in the secretion to the culture medium due to the presence of its natural peptide leader at the N-terminus. We developed an L-Asp-based affinity adsorbent which allowed the purification of the enzyme in one step, achieving high purity levels. This approach is advantageous over the other conventional tag-based purification methods, which require additional treatment steps for the cleavage and isolation of the affinity tags. Overall, the strategy employed for expression and purification of this protein drug uses green chemistry principles allowing the reduction of processing time and purification steps, making the approach more sustainable and attractive.

Keywords: L-asparaginase, Enzyme purification, Extracellular expression, Leukemia, Therapeutic enzymes

Introduction

L-asparaginase (E.C.3.5.1.1, L-ASNase) catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonia. The enzyme attracts attention due to its efficient use in the therapy of Acute Lymphoblastic Leukemia (ALL) [1-3]. L-ASNase is also used in combination with other drugs for the treatment of other type of malignancies such as non-Hogkins Lymhoma, chronic lymphoblastic leukemia, lymphosarcoma, melanosarcoma [4,5]. In the case of ALL the therapeutic function of L-ASNase is due to the fact that the asparagine synthetase of the leukemic lymphoblasts is downregulated resulting in deficiency of L-asparagine synthesis [6-8]. Therefore, the survival of those cells is dependent on the exogenous supply of L-asparagine from the blood. L-ASNase depletes the available L-Asn in the blood, causing inhibition of protein synthesis and apoptosis to the malignant cells [8,9]. It must be underlined that the normal cells are not affected since they can synthesize L-Asn.

L-ASNase is the only bacterial enzyme that has been approved by the FDA for human therapy [10]. The two approved preparations are from *E. coli* and *E. chrysanthemi* [11]. The *E. coli* ASNase is the first choice drug as it is capable of leading to remission very fast and causing relatively mild toxic effects [12]. Nevertheless, in cases where immunogenicity and allergic side effects

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arise due to *E. coli* L-ASNase, treatment is immediately switched to the Erwinia enzyme [13,14]. It has been reported, that the two enzymes exhibit totally different immunogenicity patterns in patients [15].

Affinity chromatography is considered to be one of the most refined and efficient techniques, which has been widely used in downstream processing with outstanding success in certain cases [16]. In this technique, the molecule of interest binds to another immobilized molecule, most often called the ligand. Ideally, the ligand has such structural and physicochemical properties that it is recognized specifically only by the target biomolecule [17]. As a result, the final purity depends on the specificity between the ligand and the biomolecule as well as, the strength of their interaction [18].

Periplasmic protein expression and potentially, extracellular secretion in the growth medium facilitates enormously the downstream processing protocol due to lower level of extracellular expressed proteins compared to intracellular ones [19-27]. As a result, higher protein purity can be achieved applying less number of chromatographic steps.

The long-term usage of L-asparaginase leads to life-threatening multiple toxic effects. Among its multiple toxic effects, L-asparaginase induces allergic reactions usually due to low purity enzyme preparations and the presence of unwanted contaminations by bacterial proteins. Therefore, efficient recombinant expression, coupled with simple, rapid and low cost purification protocol would assist the overall production scheme of such an important enzyme, whose purity is extremely critical due to its use for human treatment. The aim of the present work is to study the optimization of the extracellular expression of ErL-ASNase in *E. coli* by attaching its natural peptide leader at the N-terminus. The secreted recombinant L-ASNase was ultimately purified by applying an efficient one-step affinity chromatographic protocol.

Experimental

Materials

L-Asn, L-Asp, L-Glu, trichloroacetic acid, 1,4-butanediol diglycidyl ether and crystalline bovine serum albumin (fraction V) were obtained from Sigma-Aldrich (U.S.A.). Sepharose CL-6B were purchased from Pharmacia. Nessler's reagent was obtained from Fluka (Germany). Isopropyl-beta-D-thiogalactoside (IPTG) was from Genaxis (U.K.). Yeast extract, peptone, agar and glycerol were purchased from Scharlau (Spain).

Solid phase synthesis of the affinity adsorbents

Synthesis of the affinity adsorbents was carried using a two-step procedure as follows: i) Activation of agarose: Sepharose CL-6B beads (5 g) were thoroughly washed with double distilled water in a glass filter funnel and drained. The washed beads were suspended in 1M NaOH solution (5 mL) and shaken on a rotary shaker (140 rpm, 25°C, 120 min). The beads were washed with double distilled water to remove excess NaOH and 1,4-butanediol diglycidyl ether (5 mL) was added to the Sepharose CL-6B. The mixture was shaken at 140 rpm, 25°C for 12 h. After completion of the reaction the 1,4-butanediol diglycidyl ether-activated Sepharose CL-6B was washed with double distilled water.

ii) Coupling with the ligand (L-Asp, L-Glu, or L-Asn): Activated Sepharose CL-6B (5 g) was resuspended in 20 mL KH_2PO_4, pH 7.5, along with either L-Asp or L-Glu or L-Asn (1 g dissolved in 10 mL Na_2CO_3, pH 10). The mixture was shaken on a rotary shaker (210 rpm, 25°C) for 64 h. After completion of the reaction, the gel was filtered, washed with distilled water and stored in water at 4°C.

Optimization of extracellular expression of ErL-ASNase in *E. coli*

ErL-ASNase cloned in pCR T7/CT TOPO vector as described previously [28], was expressed in seven different *E. coli* strains (Table 1) in order to investigate the expression levels as well as potential secretion in the culture medium. A single colony of each *E. coli* strain harboring the recombinant plasmid [28] was used to inoculate LB medium containing 100 μg/mL ampicillin. Cultures were grown overnight at 37°C under vigorous rotation (240 rpm). Subsequently, either LB or 2xYT or TB main cultures supplemented with 100 μg/mL ampicillin, were inoculated with 1/100 of overnight cultures. Cell growth

Table 1 *E. coli* strains used in the present study

E. coli strains	Genotypes	Supplier
XL1-Blue	endA1 gyrA96(nal^R) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB$^+$ lacI^q Δ(lacZ)M15] hsdR17(r_K^- m_K^+)	Stratagene
TOP10	F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ$^-$	Invitrogen
UT 5600	F$^-$ ara-14 leuB6 secA6 lacY1 proC14 tsx-67 Δ(ompT-fepC)266 entA403 trpE38 rfbD1 rpsL109 xyl-5 mtl-1 thi-1	New England Biolabs
BL21(DE3)	F$^-$ ompT gal dcm lon hsdS_B(r_B^- m_B^-) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])	Novagen
BL21(DE3)Star	F$^-$ ompT gal dcm lon hsdS_B(r_B^- m_B^-) λ(DE3),(cm^R)	Invitrogen
Rosetta(DE3)	F$^-$ ompT hsdSB(r_B^- m_B^-) gal dcm (DE3) pRARE (CamR)	Novagen
BL21(DE3)pLysS	F$^-$ ompT gal dcm lon hsdS_B(r_B^- m_B^-) λ(DE3) pLysS(cm^R)	Novagen

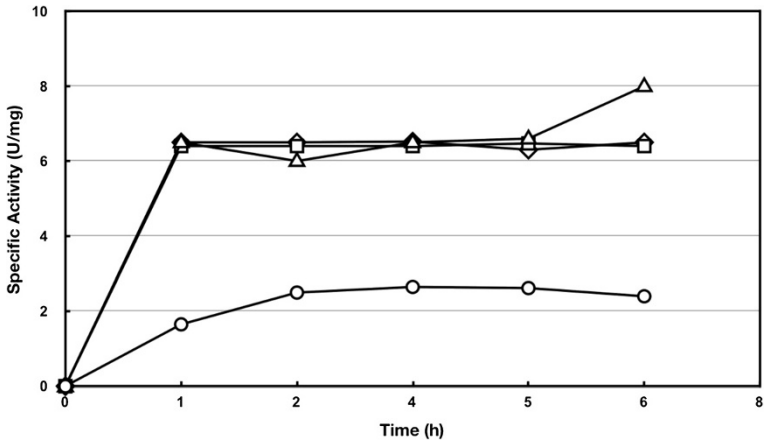

Figure 1 Evaluation of extracellular secretion of ErL-ASNase expressed by different *E. coli* strains (△: *E. coli* Rosetta(DE3); ◊: *E. coli* BL21 (DE3) Star; □: *E. coli* BL21(DE3); ○: *E. coli* TOP10). As time zero is considered the point at which IPTG is added. Cells were grown at 37°C as described in text. The standard deviation for the specific activities (U/mg), evaluated from three independent determinations was found to be less than 15%.

was monitored spectrophotometrically and when absorbance at 600 nm reached 0.5-0.7, expression was induced by the addition of IPTG to a final concentration of 1 mM. Cultures were incubated at 37°C or 18°C and at different time points cells were harvested by centrifugation at 10,000g (4°C) for 20 min, suspended in 50 mM KH_2PO_4 pH 7.5, sonicated (the cell suspensions were divided into 2-mL fractions, which were sonicated by applying short pulses of 15 s; the process was repeated three times), and centrifuged at 10,000g (4°C) for 20 min. The supernatant was collected and L-ASNase activity was determined in the intracellular extract as well as in the culture medium.

Enzymatic activity assay

Enzyme assays were performed at 37°C by measuring the amount of ammonia liberated upon reaction with Nessler's reagent. Activities were measured as described by Kotzia & Labrou [28]. One unit of L-ASNase activity is defined as the amount of enzyme that liberates 1 μmol of ammonia from L-Asn per min at 37°C. Protein concentrations were determined at 25°C by the method of Bradford [29] using bovine serum albumin (fraction V) as standard.

Screening of affinity adsorbents for ErL-ASNase binding

Columns packed with affinity adsorbents (2 mL) were washed with 50 mL double distilled H_2O and equilibrated with 30 mL of 20 mM KH_2PO_4, pH 7.5. Culture medium supernatant containing the secreted ErL-ASNase (approximately 80 U) was dialysed overnight against 20 mM KH_2PO_4 pH 7.5 and was loaded onto each column. Non-adsorbent proteins were washed off with 30 mL of equilibration buffer. The adsorbent's binding capacity was determined as the proportion (%) of L-ASNase (units) bound on the affinity adsorbent to the overall units loaded.

Adsorption equilibrium studies

In a total volume of 1 mL of 20 mM KH_2PO_4, pH 7.5, varying amounts of purified L-ASNase, previously dialysed in 20 mM KH_2PO_4 pH 7.5, were mixed with 3 mg of affinity adsorbent (L-Asp-Sepharose CL-6B). The suspensions were shaken for 120 min in order for the system to reach equilibrium. The suspension was then

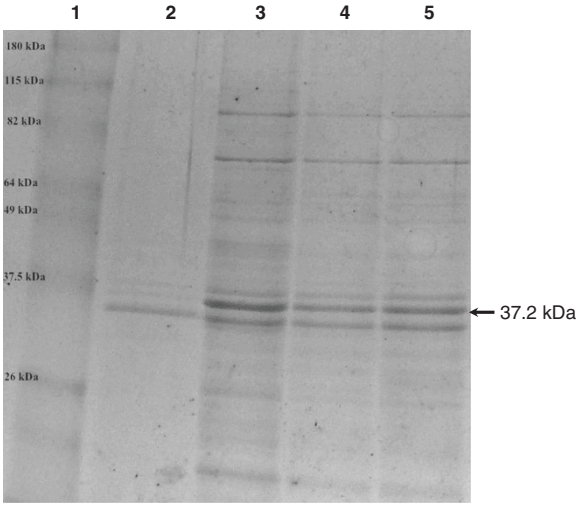

Figure 2 Electrophoretic profiles of the culture media of the *E. coli* strains that secreted the ErL-ASNase. Lane 1, protein marker; Lane 2, culture medium from *E. coli* TOP10 strain; Lane 3, culture medium from *E. coli* Rosetta(DE3); Lane 4, culture medium from *E. coli* BL 21(DE3) Star; Lane 5, culture medium from *E. coli* BL 21(DE3) cells.

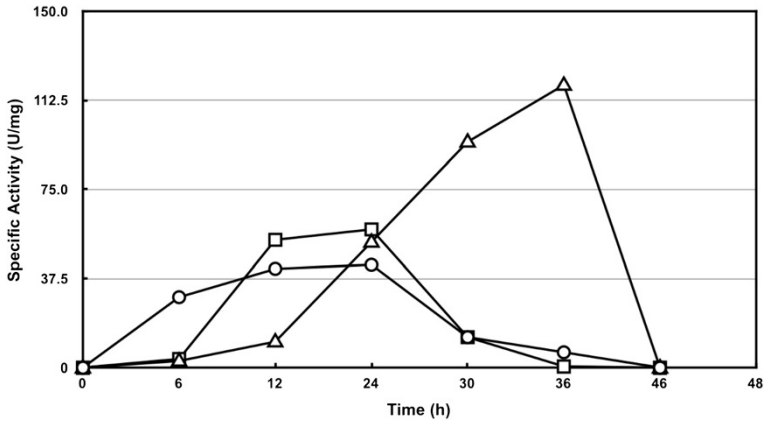

Figure 3 Supernatant specific activities of ErL-ASNase expressed in culture medium by *E. coli* Rosetta(DE3) strain using different culture media (△: TB; □: 2xYT; ○: LB) at 37°C. The standard deviation for the specific activities (U/mg), evaluated from three independent determinations was found to be less than 15%.

centrifuged (4000g, 2 min) and the amount of unbound protein in the supernatant was determined by the Bradford method [29]. Bound protein was calculated by subtracting the amount of unbound protein from the total amount of protein added. The data were analyzed using Igor Pro software platform (Wavemetrics Co.).

Purification of extracellular ErL-ASNase on immobilized L-Asp affinity adsorbent

Culture medium (10 mL) was dialyzed overnight at 4°C against binding buffer (20 mM KH_2PO_4, pH 7.5) containing different concentrations of NaCl (0.05-0.2 M) and adjusted to different pH values (7.5-8.6) and loaded onto L-Asp adsorbent, previously equilibrated with 30 mL of the binding buffer. The column was kept closed for 10 min for the system to reach equilibrium. Subsequently, the column was exhaustively washed either with binding buffer containing 2 M NaCl or with ice-cold ddH_2O. Finally, bound ErL-ASNase was eluted with 10 mL of 20 mM L-Asp dissolved in 20 mM potassium phosphate buffer, pH 7.5. Both flow-through and washing fractions were kept and the protein content was determined by Bradford and analyzed by SDS-PAGE and activity assays.

Electrophoresis

SDS polyacrylamide gel electrophoresis was performed according to the method of Laemmli [30] on a slab gel containing 12.5% (w/v) polyacrylamide (running gel) and 2.5% (w/v) stacking gel. The protein bands were stained with Coomassie Brilliant Blue R-250.

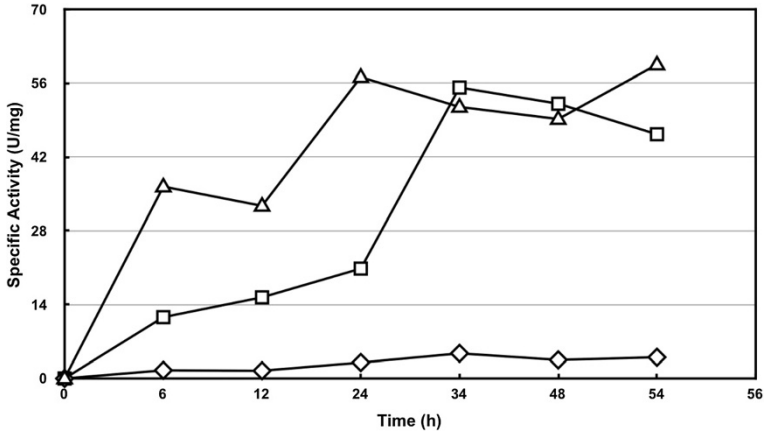

Figure 4 Supernatant specific activities of ErL-ASNase expressed in culture medium by *E. coli* Rosetta(DE3) strain using different culture media (◊: TB; □: 2xYT; △: LB) at 18°C. The standard deviation for the specific activities (U/mg), evaluated from three independent determinations was found to be less than 15%.

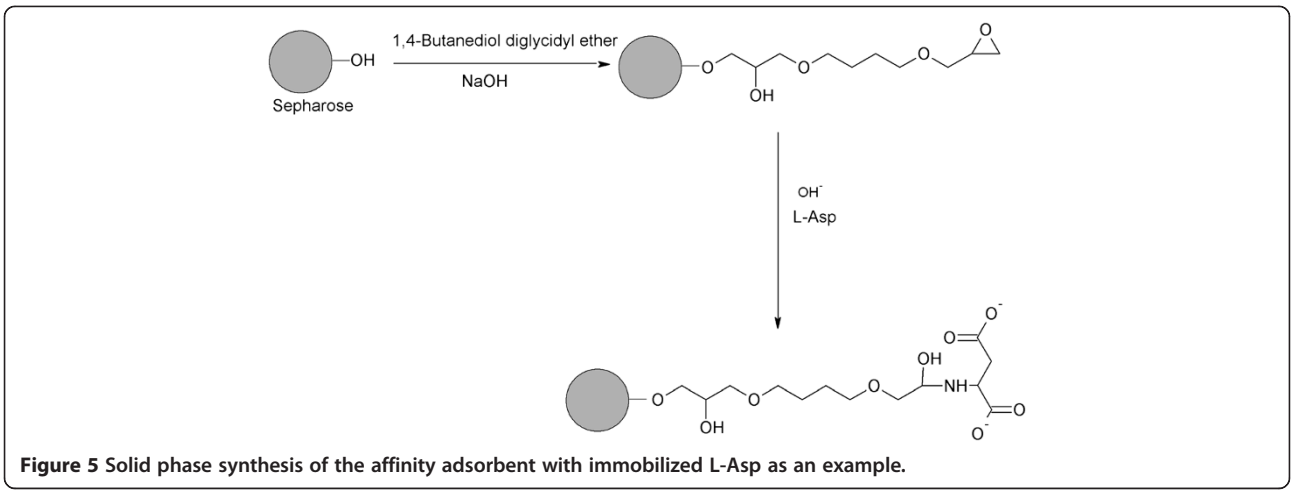

Figure 5 Solid phase synthesis of the affinity adsorbent with immobilized L-Asp as an example.

Results & discussion

Optimization of extracellular expression of ErL-ASNase in *E. coli*

In order to investigate and determine the optimum conditions for extracellular expression of L-ASNase, seven *E. coli* strains (Table 1) were evaluated for their ability to secrete the recombinant enzyme into the culture medium, following induction by IPTG [31]. The results (Figure 1) showed that four [*E. coli* TOP10, *E. coli* BL21(DE3), *E. coli* BL21(DE3) Star, *E. coli* Rosetta(DE3)] out of seven strains were able to secrete recombinant ErL-ASNase into the culture medium at high level even during the early period of induction i.e. the first hour. Figure 2 shows the electrophoretic profile of the proteins secreted in the culture medium. It is evident that, the predominant protein band corresponds to ErL-ASNase (~37.2 kDa). Among all tested strains, *E. coli* Rosetta(DE3) secreted the highest amounts of ErL-ASNase, whereas *E. coli* TOP10 secreted the lowest. The other *E. coli* strains (XL-1 Blue, WT 5600, BL21(DE3)pLysS) failed to secrete ErL-ASNase and therefore, *E. coli* Rosetta(DE3) was chosen for further study. Three different culture medium compositions (LB, 2XYT and TB) and two different temperatures (18°C and 37°C) were evaluated for their effect on the enzyme production and secretion, using the *E. coli* Rosetta(DE3) strain. As shown in Figure 3 the TB medium appears to provide the optimum conditions regarding the secretion of the enzyme, at 37°C. Surprisingly enough, unlike 37°C, TB was the worst at 18°C (Figure 4). Under these conditions approximately 17,800 UI/L of enzyme were secreted in the culture medium. On the other hand the intracellular expression level of ErL-ASNase was ~ 4,200 UI/L.

Synthesis and screening of affinity adsorbents for ErL-ASNase binding

The synthesis of affinity adsorbents with immobilized L-Asp, L-Asn and L-Glu was carried out on the solid phase (Sepharose CL-6B) (Figure 5) according to the well established protocols [32]. Briefly, Sepharose CL-6B was activated by a bis-oxirane (1,4-butanediol diglycidyl ether) under alkaline conditions and the activated Sepharose CL-6B was substituted by L-Asp, L-Asn and L-Glu at pH 10. In order to determine the most effective adsorbent, all adsorbents were evaluated for their ability to bind and purify ErL-ASNase from the extracellural medium. Dialyzed supernatant (80 U, in 20 mM KH_2PO_4, pH 7.5), was loaded on each adsorbent (2 mL). The columns were washed with equilibration buffer and bound ErL-ASNase was eluted with L-Asp (20 mM) dissolved in 20 mM KH_2PO_4, pH 7.5. It has been shown [33] that L-Asp is a strong competitive inhibitor (K_i ~ 80 μM) of L-ASNase. All affinity adsorbents were able to bind ErL-ASNase at pH 7.5 (Table 2). In particular, the adsorbent with immobilized L-Asp exhibited the highest binding capacity, followed closely by L-Glu, while L-Asn showed the lowest capacity. Taking into consideration the above observation, the adsorbent L-Asp-Sepharose CL-6B was selected for further study.

Adsorption equilibrium studies

In the present work, equilibrium adsorption studies were employed to characterize the interaction of ErL-ASNase with the L-Asp-Sepharose CL-6B affinity adsorbent. This approach gives a relationship between the concentration

Table 2 Binding (%) of ErL-ASNase to the affinity adsorbents

Adsorbent	Binding (%)
L-Asn-Sepharose CL 6B	49
L-Asp-Sepharose CL 6B	71
L-Glu-Sepharose CL 6B	59

Binding (%) was expressed as the proportion (%) of ErL-ASNase (U) bound on the affinity adsorbent to the overall units loaded. The standard deviation for the enzyme activities (U), evaluated from three independent determinations was found to be less than 10%.

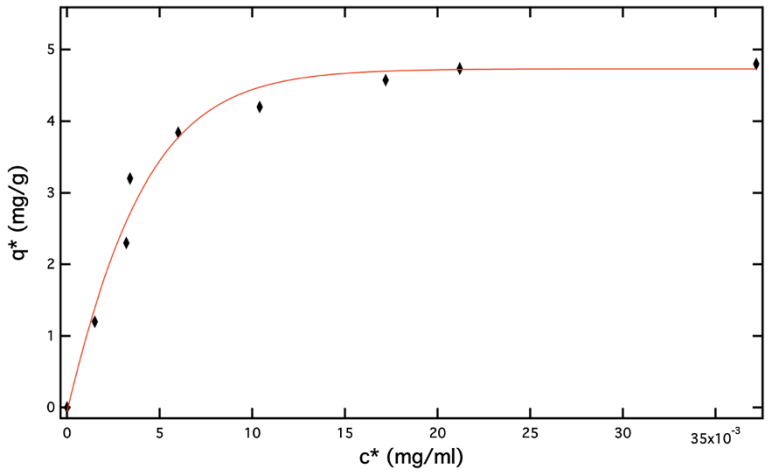

Figure 6 Equilibrium adsorption of ErL-ASNase on L-Asp-Sepharose CL-6B affinity adsorbent in batch system. The plot describes the equilibrium concentration of the enzyme in liquid-phase vs the equilibrium concentration in the solid-phase for the Langmuir isotherm. Non-linear regression curve fitting was performed using the Igor Pro software (Wavemetrics Co.).

of the enzyme in the solution and the amount of enzyme adsorbed to the adsorbent at equilibrium [17,32,34]. The theoretical model most often used to characterize affinity systems is the second-order reversible interaction, where the enzyme is bound to the ligand by a monovalent interaction (equation 1) that has a characteristic binding energy:

$$E + L \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E.L \qquad (1)$$

where E is the enzyme in solution, L is the ligand, and E.L is the enzyme-ligand complex. The parameters k_1 and k_2 are the forward and reverse rate constant, respectively. From equation 1 the Langmuir isotherm, described by equation 2, can be obtained [17,34]:

$$q* = q_{max} c* / (K_D + c*) \qquad (2)$$

where q^* is the adsorbate concentration at equilibrium (mg/g adsorbent), c^* is the equilibrium liquid phase concentration (mg/mL), q_{max} is the Langmuir isotherm maximum capacity (mg/g adsorbent), and K_D is the apparent dissociation constant.

The batch adsorption of ErL-ASNase on L-Asp-Sepharose CL-6B affinity adsorbent is shown in Figure 6. The line corresponds to Langmuir isotherm which gives the best fit for the experimental data. The calculated dissociation constant was estimated to be $K_D = 0.21$ μM. This value falls within the range expected for a highly selective affinity adsorbent [35-37]. The maximum binding capacity q_{max} was estimated 4.7 mg/g moist wet adsorbent.

Purification of ErL-ASNase on immobilized L-Asp affinity adsorbent

The challenge for the development of a purification protocol is to be considerably fast, gentle, low cost and efficient regarding yield and purity levels. Initial screening unveiled that the adsorbent L-Asp-Sepharose CL-6B displayed the higher capacity and purification ability for ErL-ASNase and consequently it was employed for the development of a purification protocol. It is noteworthy that bound ErL-ASNase on L-Asp adsorbent tolerated washing steps with even 2 M NaCl with negligible loss of protein. This is indicative of the strong binding of the enzyme on the affinity adsorbent. However, despite the fact that bound ErL-ASNase could resist against salt

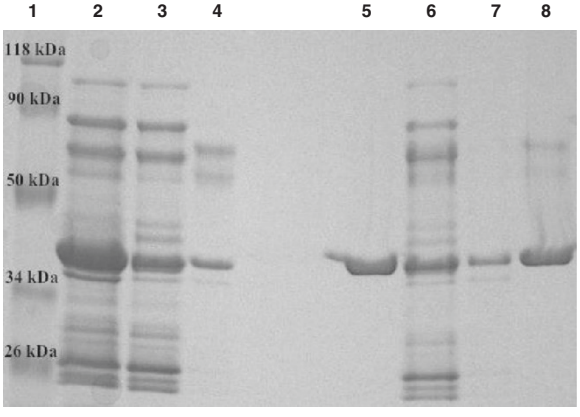

Figure 7 Electrophoretic profile of the purification protocol. Lane 1, protein marker; Lane 2, extracellular medium. Lane 3, flow through fraction; Lane 4, washing fraction, performed with ddH_2O; Lane 5, elution of ErL-ASNase after washing with ddH_2O; Lane 6, flow through fraction; Lane 7, washing fraction, performed with 2 M NaCl; Lane 8, elution of ErL-ASNase after washing with 2 M NaCl.

washing, up to 2 M NaCl, unfortunately this step resulted in only moderate removal of non-specific bound contaminating proteins (Figure 7, Lanes 4,7). Other agents such as glycerol or sucrose that destabilizes hydrogen bond interactions between molecules, failed to remove non-specific bound contaminating proteins (data not shown). Surprisingly enough we found that non-specific contaminating proteins can be totally removed after exhaustive washing with ddH_20. This finding agreed with the observation that salt concentration (0.1 M NaCl) present in the binding buffer, maximized the binding specificity of the enzyme on the adsorbent at pH 7.5 (data not shown). The total absence of salt (as NaCl) or salts at higher concentration (0.2 M NaCl) in this step resulted in a decrease in binding specificity. These findings led us to the development of single-step affinity purification procedure for ErL-ASNase. Dialyzed culture medium (20 mM KH_2PO_4, containing 0.1 M NaCl, pH 7.5), was applied directly on L-Asp-Sepharose CL6B adsorbent, previously equilibrated with the dialysis buffer. Washing step included exhaustive washing with ice-cold ddH_2O. Elution of ErL-ASNase was affected specifically by 20 mM L-Asp with a recovery >80%. The purity of the enzyme preparation was evaluated by SDS-PAGE (Figure 7, Lane 5) and shown to be homogeneous. The present method provides a simple and effective way for preparing in one-step highly purified recombinant ErL-ASNase.

Concluding remarks

Given the existing high interest in L-ASNase serving as a neoplastic agent and being a milestone for the treatment of ALL, we focused on the development of an efficient protocol for the expression and purification of this protein drug. The achievement of high purity levels in the case of pharmaceutical proteins is of great importance and plays a vital role in therapeutic applications [38,39]. Therefore, it is a challenging task to establish an efficient protocol not only for the recombinant expression of a therapeutic protein, but also for its purification, which must be simple, fast, mild and of low cost. Recently, L-ASNase from *E. coli* was extracellular expressed by fusing the gene coding for 6-His-tagged L-ASNase to the pelB leader sequence. In that work the recombinant 6-His-tagged protein was purified from the culture supernatant in a single step using Ni-NTA affinity chromatography [40,41]. However, this approach necessitates additional purification steps such as the cleavage and the removal of the 6-His-tag, which add to the overall cost of the purification process.

In the present work we showed that recombinant ErL-ASNase is efficiently expressed in *E. coli* Rosetta(DE3) and secreted into the culture medium. The expression levels were optimized by testing different *E. coli* strains, culture media, as well as temperature and incubation times. The secreted recombinant protein was purified directly from the culture medium, thereby simplifying considerably the downstream processing. The purification protocol was based on affinity adsorbent with immobilized L-Asp as a ligand, which exhibited high specificity and affinity for ErL-ASNase. The final purity of the enzyme (as judged by SDS-PAGE) justifies the potential for scaling up the whole process, taking also into account the low-cost materials used.

Abbreviations

ALL: Acute lymphoblastic leukaemia; L-ASNase: L-asparaginase; ErL-ASNase: L-asparaginase from *Erwinia chrysanthemi* 3937; FDA: Food and drug administration; GDH: Glutamate dehydrogenase; PAGE: Polyacryamide gel electrophoresis; SDS: Sodium dodecyl sulfate.

Competing interests

The authors declare that they have no competing interests.

Authors' contribution

CSK carried out the experimental work, analyzed the results and drafted the manuscript; NEL designed the experiments and drafted the manuscript. Both authors read and approved the final manuscript.

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