Research Article

Biotechnology Progress
DOI 10.1002/btpr.2410

Heterologous expression and purification of active L-asparaginase I of *Saccharomyces cerevisiae* in *Escherichia coli* host

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Received: Apr 05, 2016; Revised: Nov 04, 2016; Accepted: Nov 04, 2016

Abstract

L-asparaginase (ASNase) is a biopharmaceutical widely used to treat child leukemia. However, it presents some side effects, and in order to provide an alternative biopharmaceutical, in this work, the genes encoding ASNase from *Saccharomyces cerevisiae* (*Sc*_ASNaseI and *Sc*_ASNaseII) were cloned in the prokaryotic expression system *Escherichia coli.* In the 93 different expression conditions tested, the *Sc*_ASNaseII protein was always obtained as an insoluble and inactive form. However, the *Sc*_ASNaseI $(His)_6$-tagged recombinant protein was produced in large amounts in the soluble fraction of the protein extract. Affinity chromatography was performed on a Fast Protein Liquid Chromatography (FPLC) system using Ni^{2+}-charged, HiTrap Immobilized Metal ion Affinity Chromatography (IMAC) FF in order to purify active *Sc*_ASNaseI recombinant protein. The results suggest that the strategy for the expression and purification of this potential new biopharmaceutical protein with lower side effects was efficient since high amounts of soluble *Sc*_ASNaseI with high specific activity (110.1 ± 0.3 $IU.mg^{-1}$) were obtained. In addition, the use of FPLC-IMAC proved to be an efficient tool in the purification of this enzyme, since a good recovery ($40.50 \pm 0.01\%$) was achieved with a purification factor of 17-fold.

Keywords: biopharmaceutical, Fast Protein Liquid Chromatography (FPLC), protein purification, recombinant DNA, enzyme technology.

1. Introduction

The L-asparaginase (ASNase), L-asparagine amidohydrolase [EC 3.5.1.1] is an enzyme which catalyzes the hydrolysis of the amino group of L-asparagine, forming L-aspartic acid and ammonia. The discovery of this enzyme started with Kidd's[1] remarkable observation that Guinea pig serum had anti-tumor activity against several types of lymphoma in mice. Broome[2] elucidated this anti-leukemic activity attributing it to ASNase action. Nevertheless, only in 1967, the ASNase was isolated in enough quantity to prove its anti-tumoral activity in clinical trials[3]. Since then, the demand for the proper and efficient isolation and purification techniques for ASNase has increased, due to its pharmacological importance and proved application.

ASNase was shown to be very useful in treating acute lymphocytic leukemia (ALL)[4–6], acute myeloid leukemia[7] and lymphomas[8]. ALL is a blood and bone marrow-related cancer whose prevalence is higher in childhood[9,10]. Nowadays, ASNase based drugs are available in different preparations/formulations as described in Table 1[11–16]. However, there are some factors limiting its use as a chemotherapeutic agent, namely i) the daily dose demand (10-200 $IU.Kg^{-1}$ *per* day), for different periods of time, which may last for 21 days, and ii) the toxic side effects that can occur during therapy, *i.e.* hyperglycemia, decreased serum albumin, lipoproteins and fibrinogen, increased liver fat, some mild brain dysfunctions[6,11,17–20] and iii) the development of hypersensitivity to treatment. The hypersensitivity reactions ranging from a small allergy in the drug injection area, bronchospasm and even anaphylactic shock, occurs in 5 to 50% of the treated patients. Nowadays, the researchers are focused in the partial or total elimination of these problems, regarding some protein structural modification approaches, through genetic engineering, gene enhancement and/or by ASNase conjugation with biopolymers (*e.g.* polyethylene glycol (PEG), dextran, albumin and heparin)[5,11,21–23] or by changing the expression host systems[24].

In this work, the genes codifying ASNase were isolated from the *Saccharomyces cerevisiae* BY4741 (Invitrogen), a microorganism able to synthesize two distinct forms of ASNase: *Sc*_ASNaseI, an intracellular constitutive enzyme and *Sc*_ASNaseII, a periplasmic enzyme which is secreted when the microorganism is exposed to some stress conditions, in particular to nitrogen starvation. Both enzymes are poorly characterized; Ferrara et al.[25] obtained a better endogenous amount of *Sc*_ASNaseII using the double mutant strain *Δure2Δdal80.* However, the posterior yield of *Sc*_ASNaseII heterologous expression in *Pichia pastoris* was 7-fold higher than in the

mutant *S. cerevisiae*[26] and the protein has been successfully expressed and purified in its active form, using this host cell[27].

In this work, the intention was to analyze the expression of the *Sc*_ASNases, eukaryotic forms of the biopharmaceutical ASNase, by applying the well-characterized, simple, fast and cheap host heterologous *E. coli* expression system. In order to access this system capacity, a bioinformatics analysis was conducted to evaluate both enzymes requirements as for its post-translational modifications in the native host, followed by the expression of the enzymes and evaluation of their activities. Moreover, the Fast Protein Liquid Chromatography (FPLC) purification of *Sc*_ASNaseI was applied as a downstream methodology platform. Actually, the use of the FPLC purification in proteins has been previously explored[28–30], thus being considered a well-established technique in different biotechnological industries.

2. Materials and methods

2.1. *E. coli* expression vector construction

In order to amplify the *ASP1* and *ASP3* genes, the genomic DNA of *S. cerevisiae* extracted with PureLink® Genomic DNA Purification Kit (Invitrogen®, Carlsbad, CA, USA) was used. Also, the *ASP3* synthetic gene with optimized codons to express protein in *E. coli* was purchased from GenScript Corporation. Primers to amplify the target yeast genes were commissioned to the company *Exxtend* (Paulínea, SP, Brazil) (sequences described in Table 2). *Sc_ASNase* genes were cloned into expression vectors described in Table 3. Restriction enzymes *NdeI, BamHI* and *XhoI* were purchased from New England Biolabs (NEB, Ipswich, UK). T4 DNA ligase was obtained from Promega (Madison, WI, USA). The bacteria transformation was carried out by electroporation in a MicroPulser Electroporator (BioRad®, Hercules, CA, USA). Luria Bertani – L.B. (0.5% NaCl, 0.5% yeast extract, and 1% triptone) solid medium (2% agar added) with added carbenicillin (50 $\mu g.\mu L^{-1}$) was used to select transformants which contain pET15b or pET22b vectors or added of kanamycin (15 $\mu g.\mu L^{-1}$) to select transformants with pET28a+SUMO construction. The recovery of the plasmids from bacteria was performed using a QIAprep Spin Miniprep Kit (Qiagen®, Hilden, Germany), according to the manufacturer's instructions. The correct insert of *Sc_ASNase* genes into the pET vectors were confirmed by DNA sequencing, for all expression vectors described in Table 3.

2.2. Heterologous expression of *Sc*_ASNaseI

The *E. coli* BL21 (DE3) strain was grown in LB liquid media, supplemented with the appropriate antibiotics, at 37°C until OD_{600nm} = 0.6-0.7. When the bacteria were in the log-phase, 1 mM of isopropylthio-β-D-galactoside (IPTG) was added, and the solution was incubated for 3 h at 37°C to induce the transcription and, consequently, to allow the production of *Sc*_ASNaseI. The sample pre-treatment was conducted by a chemical lysis procedure; herein cell pellets from genetically transformed *E. coli* BL21 (DE3) were suspended in 10 mL of BugBuster Master Mix (Merck-Millipore™, Billerica, MA, USA) *per* g of cell, mixed gently for 20 min and centrifuged at 16,000 x *g* for 20 min at 4°C; the pellet was discarded and the supernatant was subjected to vacuum filter membrane (0.45 μm, Merck-Millipore); to the filtered sample, the final concentration of 20 mM imidazole was added.

The affinity chromatography was performed on a FPLC™ System (ÄKTA purifier, GE Healthcare) using a Ni^{2+}-charged, 5 mL HiTrap IMAC FF column (GE Healthcare). A linear gradient, running from 0-500 mM of imidazole at 5.0 $mL.min^{-1}$ was carried in order to be possible to identify the lowest imidazole concentration of the elution buffer required to extract the highest amount of the purified *Sc*_ASNaseI. This concentration range was further used in an imidazole gradient step, in which two-step concentrations of imidazole (160 and 272 mM) were applied. The initial sample volume applied was 5.0 mL. A binding buffer (20 mM sodium phosphate buffer at pH 8.0 and 500 mM NaCl) and an elution buffer (20 mM sodium phosphate buffer at pH 8.0, 500 mM NaCl and 500 mM imidazole) were used in the purification process. The samples were previously centrifuged 4,000 x *g* for 20 min at 4°C and desalted through an exclusion chromatography step in a PD-10 column (GE Healthcare Life Sciences) with Tris-HCl buffer (20 mM) at pH 8.6.

2.3. Screening of conditions for *Sc*_ASNaseII expression

The *E. coli* strains (Table A.1 at ESI†) were genetically transformed with the chosen construction containing the *ASP3* gene (Table 3). The screening of conditions for *Sc*_ASNaseII protein expression was carried out in 10 mL of LB medium plus the selective antibiotic medium, starting with a cell optical density of OD_{600nm} = 0.2, at 37°C and 180 rpm until a cell density of OD_{600nm} = 0.6-0.7. At this point, an IPTG solution was added at the described concentrations (0.01, 0.1, 0.5 and 1.0 mM) and incubated for 3 h at 37°C or overnight at 20°C to induce the production of *Sc*_ASNaseII using the conditions, vectors and gene combinations described in Table A.2. At the end of this procedure, the cells were pelleted, the supernatant discarded and 1.5 mL of BugBuster Master Mix (Merck-Millipore) was added, gently mixed for 20 min and centrifuged at 16,000 x *g* for 20 min. The pellet containing the cellular debris and inclusion bodies (containing insoluble proteins), was separated from the supernatant (with the soluble proteins) and both were analyzed by SDS-PAGE 12%, under reducing conditions, in accordance with the Laemmli' method[31]. The electrophoresis was performed at 120 V using a vertical BioRad® system. The protein markers of different molecular weights were acquired from BioRad®. The volume of the samples added to the gel was 10 µL with a protein concentration ranging from 0.01 to 3 mg/mL. The gels were silver stained for the protein bands detection, using the Silver Staining Kit (Amersham Pharmacia Biotech®) and following the manufacturer's instructions.

2.4. Nessler activity assay and purification parameters calculation

The determination of the enzymatic activity was performed by the Nessler activity assay (expressed in $IU.mL^{-1}$)[32]. The sample volume of 0.1 mL was incubated at 37°C for 30 min in Tris-HCl buffer (50 mM, pH 8.6) with L-asparagine (9 mM). Then, the reaction was stopped with the addition of trichloroacetic acid (TCA). The Nessler reagent (Merck) was mixed by inversion for 1 min and then the reactions were measured in a microplate reader at λ_{436nm} (Spectramax Plus384, Molecular Devices). A standard curve was prepared using ammonium sulfate. In order to remove the possible interferences of quantification, two blanks composed by Tris buffer + L-asparagine + TCA were made for each sample. Moreover, at least three samples of each enzymatic activity measurement were prepared, being the results described, the average of the three replicas.

The total protein concentration was measured through absorbance assay, measuring the absorbance at λ_{280nm}, by UV-Vis spectroscopy. A calibration curve with bovine serum albumin (BSA) (lyophilized powder, ≥ 96 wt%, Sigma-Aldrich, St. Louis, MO, USA) was used to determine the protein concentration in each sample.

2.5. Computational analysis of recombinant *Sc*_ASNases

The possible N-glycosylation sites were predicted using NetNGlyc 1.0 program[33]. The presence and location of signal peptide cleavage sites in the amino acid sequence was determined using the SignalP 4.1 program[34]. The method incorporates a prediction of cleavage sites and a signal peptide/non-signal peptide prediction based on a combination of several artificial neural networks and hidden Markov models.

3. Results and discussion

3.1. Bioinformatics analysis of *Sc*_ASNases

A computational analysis of the *Sc*_ASNases using bioinformatics tools was carried out. This analysis included the prediction of glycosylation sites using sequence information (NetNGlyc 1.0) and the presence, prediction and location of signal peptide cleavage sites in the ASNase amino acid sequences (SignalP 4.1).

The bioinformatics results obtained predicted that the *Sc*_ASNaseI could be expressed in its active form in *E. coli*, despite its prokaryotic expression nature. These data

(NetNGlyc 1.0 and SignalP 4.1) resulted in three possible glycosylation sites (Fig. 1A), even in the absence of a signal peptide (Fig. 2A). This observation suggests that the glycosylation, if present in its native host, is not essential for the enzyme activity, which is supported by the high value of the enzyme specific activity (*SA*) found for the purified *Sc*_ASNaseI (see below). This is an important result, since *E. coli* is a robust expression system, largely adopted by biopharmaceutical companies due its high titers outcomes.

In contrast, *Sc*_ASNaseII presents 3 possible glycosylation sites (Fig. 1B) and a signal peptide to direct this protein to the endoplasmic reticulum (Fig. 2B); therefore, it is highly probable that this protein undergoes glycosylation. In fact, endogenously produced *Sc*_ASNaseII was observed with a high molecular weight due to hypermannosylation[35]. Moreover, Castro-Girão et al.[27] have described that heterologous produced *Sc*_ASNaseII produced by *P. pastoris* undergoes glycosylation, since after the purification process, two bands were observed by electrophoresis, which were then turned into one, just after treatment of *Sc*_ASNaseII with PNGase F (a deglycosylation protein). In addition, as previously discussed, these authors observed the specific activity of *Sc*_ASNaseII produced by *P. pastoris* in the soluble fraction. Summing up, glycosylation seems to play an essential role for this enzyme activity and a prokaryotic system might be inappropriate to produce *Sc*_ASNaseII.

3.2. Expression and purification of *Sc*_ASNaseI

Recombinant protein expression in industrial scale demands careful evaluation of all process production steps. The choice of an appropriate host lies as a major step to this goal, moreover in the production of biopharmaceuticals. The proper protein folding, activity, purity and yield are dependent on the host ability to translate, fold and keep high amounts of the desirable protein in its soluble form. Sajitha et al.[24] successfully expressed the ASNase from *E. coli* into the eukaryotic host *P. pastoris*. The authors obtained a soluble and active ASNase in the supernatant and speculate that this protein might undergo eukaryotic glycosylation conferring more stability to the protein and fewer side effects.

In an attempt to obtain a cost effective, fast and efficient *Sc*_ASNase expression system, the gene *ASP1* was cloned into pET15b between *NdeI* and *BamHI* restriction sites, which results in a *Sc*_ASNaseI N-terminal His-tag fusion protein. The native protein is predicted to present a molecular weight of 41,385.6 Da, but the recombinant protein

with His-tag and thrombin recognition site, presents a molecular weight of 43,139.0 Da. As depicted in Fig. 3, the prokaryotic *E. coli* cell was able to express high amounts of soluble *Sc*_ASNaseI. However, the protein obtained in this first test presented a considerable number of contaminant proteins, especially in the fraction of higher *Sc*_ASNaseI concentration (Fig. 3, lane 6).

Taking into account that the administration of this biopharmaceutical (ASNase) will be potentially carried by its direct injection into the human blood, the high purity of ASNase is certainly a crucial demand for it to be considered as a promising therapeutic alternative. In this sense, an FPLC purification was performed considering two main tasks; the first one using a linear gradient of imidazole in order to identify the lowest imidazole concentration able to elute the highest amount of *Sc*_ASNaseI, and the second one using the optimized imidazole concentration to develop a purification protocol in which the same buffer was used to perform a two-step purification procedure. Both approaches were applied and from the general results (Table 4), it was concluded that, while fractions 4-5 contain the expression of host contaminant proteins resulting in a very low specific *Sc*_ASNaseI activity (0.02 ± 0.01 IU.mg^{-1}), the purified fractions 20-31 exhibited the highest specific activity (6.61 ± 0.08 IU.mg^{-1}). Considering the results obtained, the imidazole concentrations chosen for the stepwise purification were 160 and 272 mM, which correspond to fractions 23 and 30, respectively. Table 5 summarizes the results obtained, correlating the fractions collected with the respective *Sc*_ASNaseI activity results, along with the protein concentration, enzymatic activity (*EA*), specific activity (*SA*), purification factor (*PF*), and recovery (%*Rec*) parameters.

From the set of results obtained (Fig. 4 and Table 5), we conclude that the most pure fraction exhibited a *SA* of 110.1 IU.mg^{-1} comparable to the commercially available ASNase formulations, like Erwinaze® (from *E. chrysanthemi*) and Elspar® (from recombinant *E. coli*) containing 225 IU.mg^{-1} (information displayed in the Product Datasheet - Active Asparaginase full-length protein ab73439). In what concerns the application of the purification step (FPLC purification), even higher specific activities would be reached, these similar to the values described for the commercial ASNase. Moreover, not only the enzyme activity is maintained but also the yields of purification of ASNase obtained were considerable (enzyme yield *circa* 1,001 IU.g cell^{-1}). It should be highlighted that *Sc*_ASNaseI was remarkably purified at 15% of total cell dry weight, which is a sizable piece of total cellular protein.

Taking into account the results obtained and the demands of future applications, further optimization studies should be performed to the FPLC purification protocol, namely evaluating the effect of the fraction volume, pump pressure, initial volume injected, elution and binding buffer formulations in order to further improve the purifying yields. Other authors have reported some attempts to purify *Sc*_ASNaseI in the same line of work, namely Dunlop and collaborators.[35] These authors used several steps of purification to obtain the endogenous *Sc*_ASNaseI from *S. cerevisiae*; being the best specific activity found around 5 $IU.mg^{-1}$, which is almost 20-fold lower than the obtained in the present work. In addition, the same authors commented on the difficulty to separate both isoforms endogenously expressed *Sc*_ASNaseI from *Sc*_ASNaseII[35], a difficulty surpassed in this work by the use of an heterologous strategy and just one chromatographic purification step, which allowed the purification of *Sc*_ASNaseI resulting in a much higher specific activity than that obtained from endogenous expression.

For a method/process to be of industrial relevance, the polishing of the target macromolecule being produced and purified is a crucial step. In this case, and considering the potential application of the biopharmaceutical in the ALL treatment, the elimination of the $(His)_6$-tag from the biopharmaceutical[36] can be achieved through the use of the aminopeptidase dipeptidyl peptidase I (DPPI), either alone or in combination with glutamine cyclotransferase (GCT) and pyroglutamyl aminopeptidase (PGAP). In both cases, the $(His)_6$-tag is cleaved off by DPPI, which catalyses a stepwise excision of a wide range of dipeptides from the N-terminus of a peptide chain[37]. Other major concern considering the potential use of the biopharmaceutical in the ALL treatment is related to metal leaching from the column during purification[38]. Although additional costs may be inserted for validation, new technologies have been developed in a lab scale in this context, in particular the treatment of the final sample with EDTA (GE Healthcare's excel[39] and Roche's cOmplete[40]). Albeit, large-scale application of this system still needs to be fully evaluated.

3.3. Expression and purification of *Sc*_ASNaseII

Initially, the same expression and purification procedures described for *Sc*_ASNaseI were applied to express *Sc*_ASNaseII. Fig. 5 depicts the high level of *Sc*_ASNaseII expression in the *E. coli* host cells. However, the protein was completely concentrated in the insoluble fraction of the cell lysate (Fig. 5, lane 2). From the results of the lower

growth temperature test, the conclusion was that this test was not improving the insoluble fraction presence as suggested elsewhere[41]. In this context, several growth conditions including temperature of induction, concentration of (the inductor) IPTG and the time of induction were tested, since the optimization of these parameters is being recognized as a good strategy to overexpress soluble recombinant proteins in bacterial cells (as reviewed by Papaneophytou and Kontopidis[42]). None of these conditions were able to produce soluble *Sc*_ASNaseII in the *E. coli* host cell. It was hypothesized that the native gene from the amino-acids 1 to 362, *i.e.* with the yeast periplasmic signal, should enhance the solubility of the protein; however, this expression also resulted in an insoluble protein. Then, several genetic attempts to obtain soluble *Sc*_ASNaseII were carried out, namely (i) the synthetic *ASP3* gene was expressed with optimized codons to the *E. coli* host, (ii) by changing the expression vector modifying the position of His-tag from the N- to C-terminal, and (iii) constructing a vector that expresses *Sc*_ASNaseII in fusion with the SUMO protein (a highly soluble protein). Moreover, eight different types of *E. coli* strains were also tested (Table A.1 in ESI†). In conclusion, 93 different expression conditions (described in Table A.2 in ESI†) were tested without any success in the production of soluble *Sc*_ASNaseII. The only condition that resulted in low amounts of soluble *Sc*_ASNaseII (described briefly in Fig. 6) produced a protein with a low specific activity (3.8 $IU.mg^{-1}$ of protein) after the Immobilized Metal ion Affinity Chromatography (IMAC) purification step. These results are in accordance with the bioinformatics analysis, since *Sc*_ASNaseII was predicted to be glycosylated in the native host. The absence of glycosylation, caused by *E. coli* lack of glycosylation machinery, seems to be a key factor in the *Sc*_ASNaseII activity. Dunlop et al.[35] also purified *Sc*_ASNaseII endogenously produced in *S. cerevisiae;* the authors obtained a maximum *SA* of 60 $IU.mg^{-1}$ of protein. However, this protein was characterized as hypermannosylated, which means that endogenous obtained *Sc*_ASNaseII is highly immunogenic and cannot be used as a biopharmaceutical from this source, because human immune system cells possess a receptor for mannose[43]. In addition, *Sc*_ASNaseII has been described as possessing 204 $IU.mg^{-1}$ of protein, when expressed in *P. pastoris* host, a 3-fold higher activity than the endogenous source[27]. From the main results, it is suggested that, in contrast with *Sc*_ASNaseI, which is soluble and active when expressed in *E. coli* host cells, the *Sc*_ASNaseII cannot be properly produced in this host cell, independent of the growth conditions used.

4. Conclusions

The optimized two-step FPLC purification method allowed the successful purification of *Sc*_ASNaseI from *S. cerevisiae* expressed in *E. coli* BL21 (DE3). Moreover, the bioinformatics analysis together with the enzyme activity results obtained suggested that N-glycosylation is not essential for *Sc*_ASNaseI activity. Antitumor activity and biochemical characterization of *Sc*_ASNaseI are being now conducted to evaluate the potential of this enzyme in acute lymphocytic leukemia treatment. By contrast, the results suggest that *Sc*_ASNaseII requires glycosylation, and therefore a eukaryotic expression host system to produce soluble and active enzyme.

Acknowledgements

This research was supported by grants from the Coordination for Higher Level Graduate Improvements (CAPES/Brazil), National Council for Scientific and Technological Development (CNPq/Brazil) and State of São Paulo Research Foundation (FAPESP/Brazil, process numbers 2013/08617-7 and 2015/07749-2). This work was developed in the scope of the project CICECO-Aveiro Institute of Materials (Ref. FCT UID/CTM/50011/2013), financed by national funds through the FCT/MEC. The authors also thank FCT for the doctoral SFRH/BD/102915/2014 and post-doctoral grants SFRH/BPD/79263/2011 of J.H.P.M. Santos and S.P.M. Ventura, respectively. JS doctoral grant and when applicable co-financed by FEDER under the PT2020 Partnership Agreement. Moreover, S.P.M. Ventura acknowledges the FCT/MEC (Portugal) for a contract under Investigador FCT 2015 contract number IF/00402/2015.

Abbreviations

ALL: acute lymphocytic leukemia

ASNase: L-asparaginase

BSA: bovine serum albumin

FPLC: fast protein liquid chromatography

IMAC FF: immobilized metal ion affinity chromatography fast flow

IPTG: isopropylthio-β-$_D$-galactoside

IU: International unit

L.B.: Luria Bertani

OD: optical density

PEG: polyethylene glycol

PNGase F: peptide-N-Glycosidase F

*Sc*_ASNaseI and *Sc*_ASNaseII: genes encoding ASNase from *Saccharomyces cerevisiae* were cloned in the prokaryotic expression system *Escherichia coli*

TCA: trichloroacetic acid

Authors' contributions

JS, IC, ML and MP constructed vectors and expressed ASNase, carried out enzyme activity assay and purification process, and wrote the manuscript drafting, JM participated in data analysis and carried out the computational analysis of recombinant enzyme, JC, PJ and SV participated in the design of the study analysed data, and wrote the manuscript, AL and GM conceived the study and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Conflict of interest

I confirm that authors have read BioMed Central's guidance on competing interests and declare none of the authors have any competing interests.

Ethics Statement

The manuscript does not contain any data collected from humans, animals or plants.

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Table 1 – Commercially approved ASNase[11–16].

Drug Name	Production Source	Type	Manufacteur
Elspar®	*Escherichia coli*	Native enzyme	Lundbeck Inc
Kidrolase®	*Escherichia coli*	Native enzyme	EUSA Pharma S.A.
Oncaspar® (PEG-asparaginase)	*Escherichia coli*	PEGylated native enzyme	Sigma Tau Pharmaceuticals
Graspa®	*Escherichia coli*	Native enzyme encapsulated in erythrocytes	ERYTECH Pharma
Leunase®	*Escherichia coli*	Modified *E.coli* HAP strain	Kyowa Hakko Kirin Co. Ltd.
Spectrila®	*Escherichia coli*	Recombinant Enzyme	Medac Gesellschaft fuer klinische Spezialpraeparate mbH
Erwinase®	*Erwinia chrysanthemi*	Native enzyme	EUSA Pharma
Crisantaspase®	*Erwinia chrysanthemi*	Native enzyme	Ohara Pharmaceutical

Table 2. Sequence of primers used to carry out the constructions of the *Sc*_ASNases overexpression vectors.

Primer name	***Sequence (5'→3')***	***Restriction enzyme site***
ASP1-fw	GGGAATTCCATATGAAAAGCGATTCAGTTGAAATCA	*NdeI*
ASP1-rv	CGCGGATCCTCACCACCATAGAC	*BamHI*
ASP3-1-fw	GGGAATTCCATATGAGATCTTTAAATACCC	*NdeI*
ASP3-26-fw	CGGGATCCGAAGAGAAGAATTCTTC	*BamHI*
ASP3-32-fw	GGGAATTCCATATGTTGCCATCAATCAAAATTTTTGG	*NdeI*
ASP3-26opt-fw	GGGAATTCCATATGGAAGAAAAAAATAGCTC	*NdeI*
ASP3-BamHI-rv	CGGGATCCTTAACCACCGTAGACG	*BamHI*
ASP3-XhoI –rv	CCGCTCGAGACCACCGTAG	*XhoI*
ASP3-opt-rv	CATTGGATCCTCAACCGCC	*BamHI*

Table 3. Description of the primers combination, type of DNA templates and expression vectors used in this work.

Forward primer	*Reverse Primer*	*DNA template*	*Vector*	*Final construction*
ASP1-fw	ASP1-rv	*S. cerevisiae* genomic DNA	pET15b	ASP1+pET15b
ASP3-1-fw	ASP3-BamHI-rv	*S. cerevisiae* genomic DNA	pET15b	ASP3_1-362+pET15b[A]
ASP3-32-fw	ASP3-BamHI-rv	*S. cerevisiae* genomic DNA	pET15b	ASP3_32-362+pET15b[B]
ASP3-26-fw	ASP3-XhoI -rv	*S. cerevisiae* genomic DNA	pET22b	ASP3_26-362+pET22b[C]
ASP3-26opt-fw	ASP3-opt-rv	*ASP3* optimized gene	pET15b	ASP3opt_26-362_pET15b[D]
ASP3-26-fw	ASP3-XhoI -rv	*S. cerevisiae* genomic DNA	pET28b+SUMO	ASP3_26-362+ pET28b+SUMO[E]

[A] Complete *Sc*_ASNaseII fused with N-terminal His_6-tag and expressed in cytoplasm in *E. coli.*

[B] *Sc*_ASNaseII without the first 32 amino acids – sequence of protein that aligns with bacterial *Ec*_ASNaseII- fused with N-terminal His_6-tag and expressed in cytoplasm in *E. coli.*

[C] Mature *Sc*_ASNaseII without the 26 first amino acids involved in the intermembrane signalling secretion in yeast *S. cerevisiae.* Protein fused with C-terminal His_6-tag and expressed in periplasmic space in *E. coli.*

[D] Mature *Sc*_ASNaseII without the 26 first amino acids involved in the intermembrane signalling secretion in yeast *S. cerevisiae.* The gene was synthesised with optimal codons to express in *E. coli;* expressed with N-terminal His_6-tag and in cytoplasm.

[E] Mature *Sc*_ASNaseII without the 26 first amino acids involved in the intermembrane signalling secretion in yeast *S. cerevisiae* fused with N-terminal high solubilizing protein SUMO, expressed in cytoplasm.

Table 4. FPLC purification with an imidazole linear gradient (0-500 mM) step at 5.0 $mL.min^{-1}$. Fractions eluted with *Sc*_ASNaseI activity are shown, along with the protein concentration ($mg.mL^{-1}$), enzymatic activity ($IU.mL^{-1}$) and specific activity ($IU.mg^{-1}$) values.

Sample	***Protein concentration*** **($mg.mL^{-1}$)**	***Enzymatic activity*** **($IU.mL^{-1}$)**	***Specific activity*** **($IU.mg^{-1}$)**
Fractions 4-5	3.52 ± 0.03	0.06 ± 0.01	0.02 ± 0.01
Fractions 14-18	0.05 ± 0.01	0.03 ± 0.01	0.49 ± 0.01
Fractions 20-31	0.02 ± 0.01	0.11 ± 0.01	6.61 ± 0.08

Table 5. FPLC purification with the two-step concentration methodology of the initial imidazole concentration (160 and 272 mM), applied at 5.0 mL.min^{-1}. Fractions eluted with *Sc*_ASNaseI activity are shown, along with protein concentration (mg.mL^{-1}), enzymatic activity (IU.mL^{-1}), specific activity (IU.mg^{-1}), purification factor (fold) and recovery (%) parameters.

Sample	*Protein concentration* (mg.mL^{-1})	*Enzymatic activity* (IU.mL^{-1})	*Specific activity* (IU.mg^{-1})	*Purification factor (fold)*	*Recovery Activity (%)*
Cell extract	0.38 ± 0.01	2.50 ± 0.01	6.52 ± 0.01	1.00	100.00
Stepwise FPLC purification (Fractions 38-42)	0.02 ± 0.01	2.017 ± 0.01	110.06 ± 0.34	16.88	40.50

Figure Captions

Figure 1. Prediction results of N-glycosylation in the *Sc*_ASNaseI (A) and *Sc*_ASNaseII (B), using the NetNGlyc 1.0 software. The sequence position represents the protein amino-acids position. The graph depicts the possible sites of N-glycosylation in the *Sc*_ASNases sequence (- blue lines), represented by the blue bars that rise vertically and pass the program threshold (- red line).

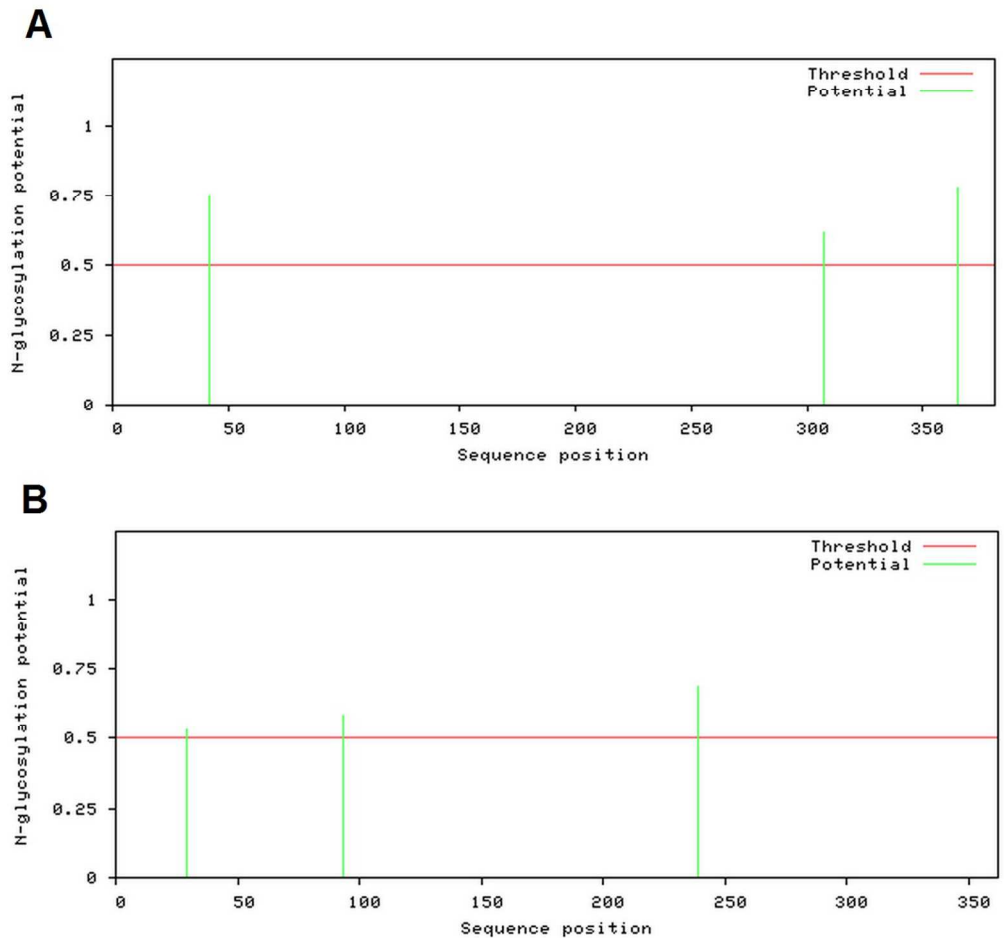
Figure 2. Presence and location predictions of signal peptide and signal peptide cleavage sites in the *Sc*_ASNaseI (A) and *Sc*_ASNaseII (B) sequences by the SignalP-4.1 software. C-score represents the raw cleavage site score generated by the software. S-score: signal peptide score generated by the software. Y-score: combined cleavage site score generated by the software. If a signal peptide cleavage site is predicted, a C-score above the threshold (dashed purple line) would be present for some of the amino-acids. In our specific case, a signal peptide cleavage site and signal peptide itself is not predicted.

Figure 3. SDS-PAGE stained with Comassie-Blue representing the first attempt of *Sc*_ASNaseI purification by IMAC. In the left are indicated the molecular protein weights of the highlight bands of the BenchMark protein ladder (lane 1). Lanes 2-6: sequential eluted *Sc*_ASNaseI from a nickel column purification.

Figure 4. Polyacrylamide gel electrophoresis combined with silver staining, showing the purified samples from the two-step FPLC purification. The brown box stresses the (His)6-tagged ASNase bands (~45.0 kDa). The different lanes represented the eluted fractions (5, 6 and 7: washout unbound proteins; 26-40: purified *Sc*_ASNase I and 48-57: non-purified *Sc*_ASNase I) and PM means Unstained Protein *Molecular weight* marker.

Figure 5. SDS-PAGE stained with Comassie-blue representing the expression of *Sc*_ASNaseII in the insoluble fraction of the *E. coli* cell lysate. - Lane 1: BenchMark™ Protein Ladder; Lane 2: insoluble fraction pelleted after clarification of the cell lysate by centrifugation. Lane 3: supernatant of cell lysate, representing the protein soluble fraction.

Figure 6. SDS-PAGE stained with Comassie-blue representing the expression of *Sc*_ASNaseII in the soluble fraction of the *E. coli* cell lysate and the specific activity of this protein. The construction pET15b+ASP3_32-362 was expressed in the bacterial host strain CodonPlus (DE3) (Merck™); cells were pre inoculated in LB media overnight at a 37ºC and 250 rpm. The inoculum was initially diluted to OD_{600nm} 0.2 in fresh culture media, grown at the same conditions until OD_{600nm} 0.4 when 0.1mM of IPTG was added and culture temperature was changed to 12ºC. This *Sc*_ASNaseII induction was performed for 24 hours. In this expression vector construction, the protein starts at the amino-acid residue number 32, without the predicted yeast periplasmic signal. Fractions of nickel affinity purification. Lane 1: Total supernatant after cell lysis; Lane 2: BenchMark™ Protein Ladder; Lanes 3 and 4: Fractions from column elution with 500 mM of imidazole.



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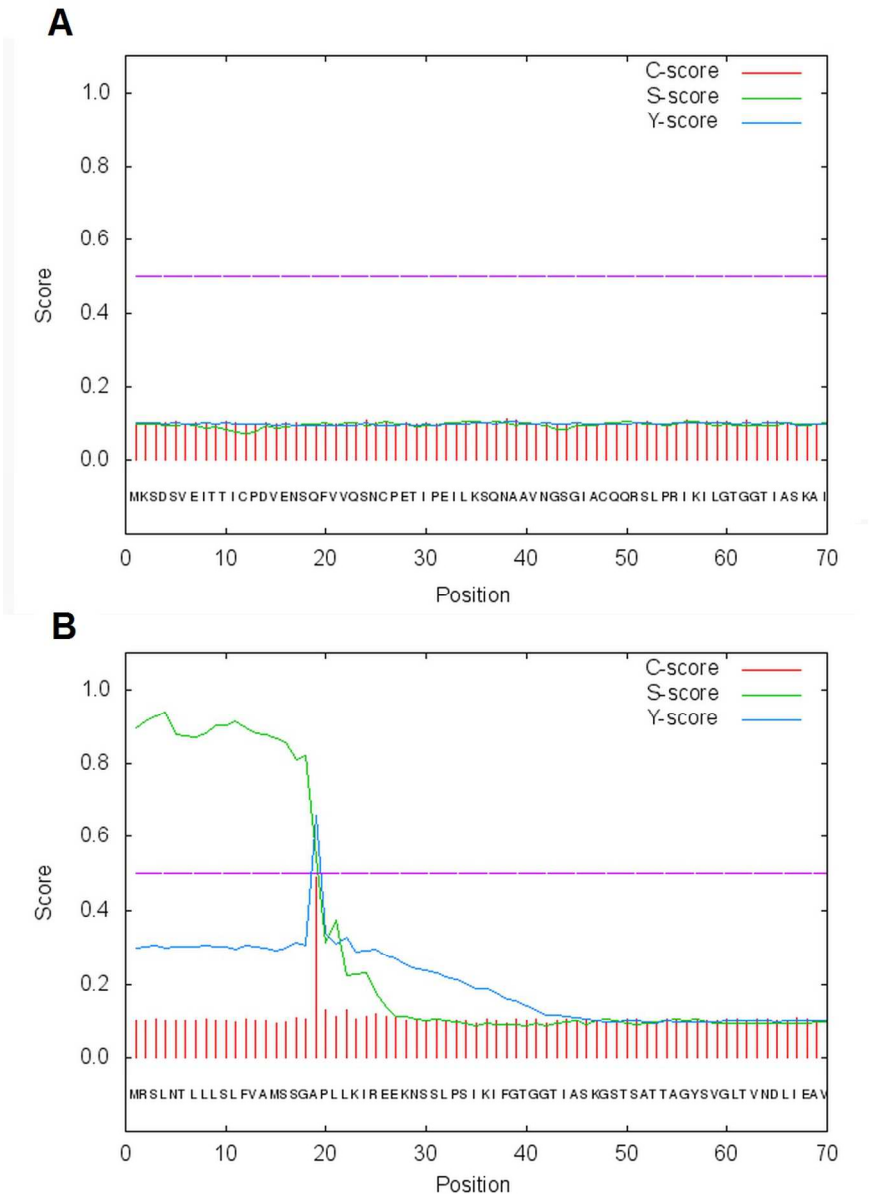

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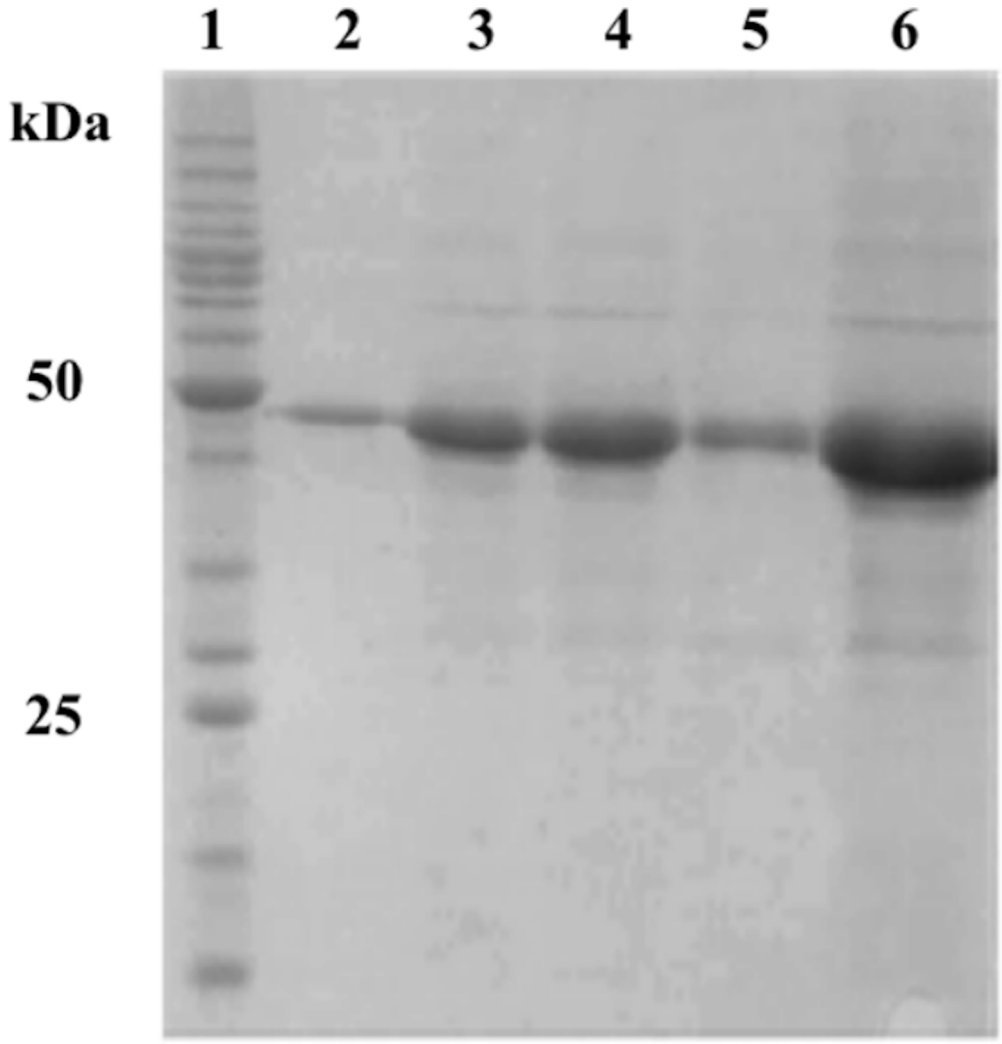

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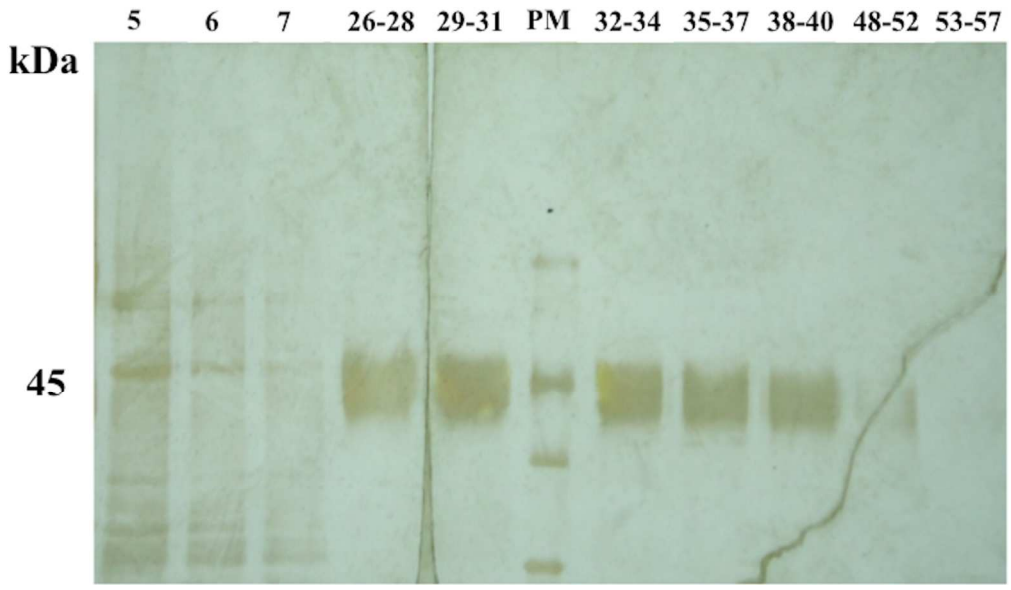

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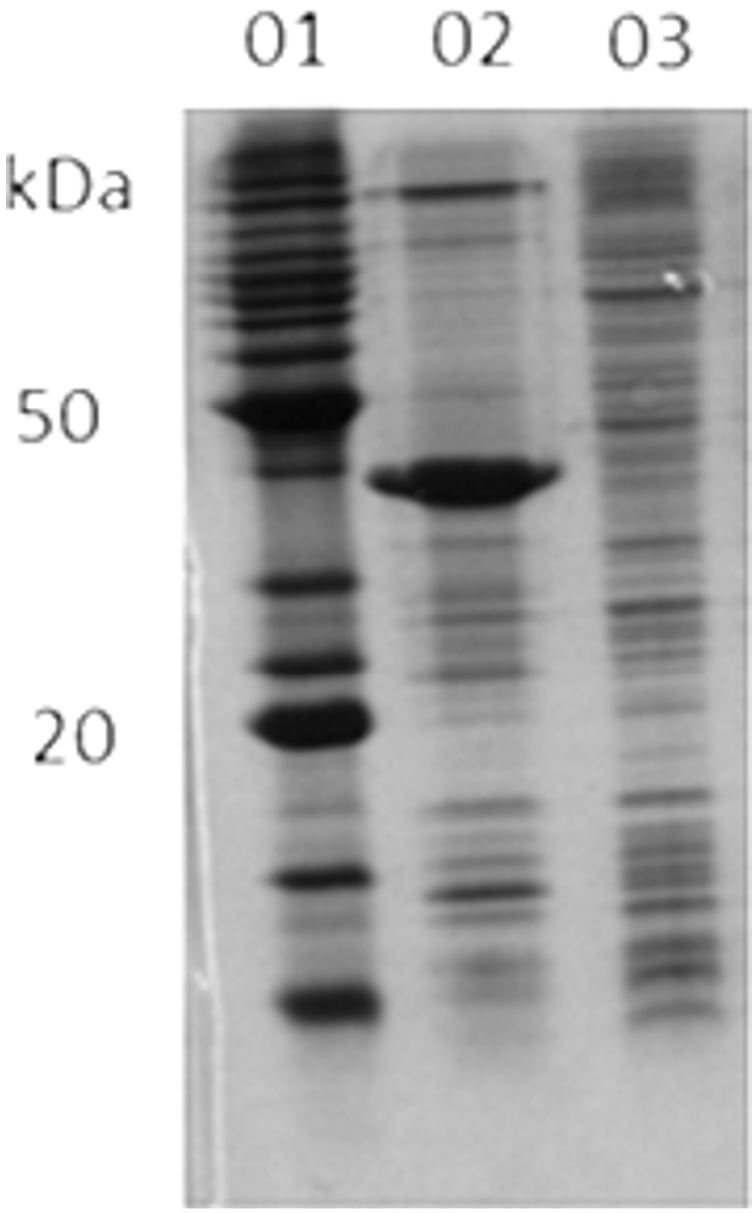

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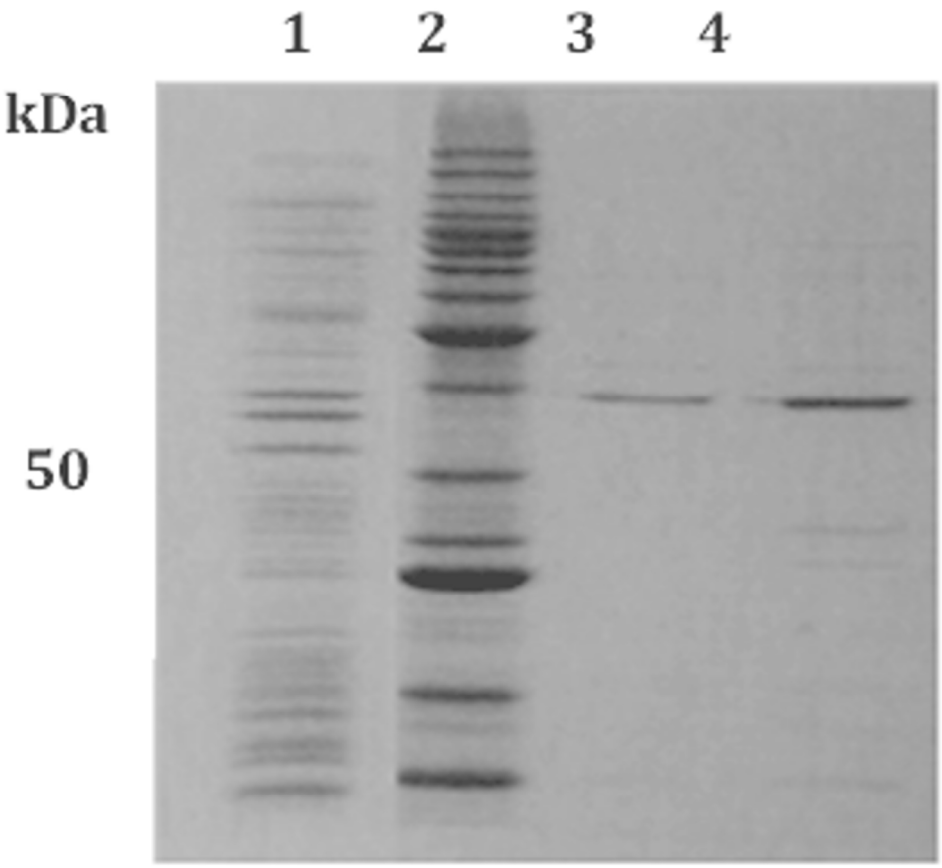

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