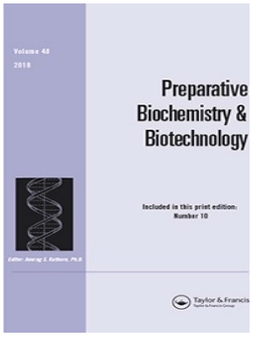

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L-Asparaginase from *E. chrysanthemi* expressed in glycoswitch®: effect of His-Tag fusion on the extracellular expression

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ABSTRACT

L-Asparaginase (L-ASNase) is an important enzyme used to treat acute lymphoblastic leukemia, recombinantly produced in a prokaryotic expression system. Exploration of alternatives production systems like as extracellular expression in microorganisms generally recognized as safe (such as *Pichia pastoris* Glycoswitch®) could be advantageous, in particular, if this system is able to produce homogeneous glycosylation. Here, we evaluated extracellular expression into Glycoswitch® using two different strains constructions containing the *asnB* gene coding for *Erwinia chrysanthemi* L-ASNase (with and without His-tag), in order to find the best system for producing the extracellular and biologically active protein. When the His-tag was absent, both cell expression and protein secretion processes were considerably improved. Three-dimensional modeling of the protein suggests that additional structures (His-tag) could adversely affect native conformation and folding from L-ASNase and therefore the expression and cell secretion of this enzyme.

KEYWORDS

L-Asparaginase; extracellular expression; Glycoswitch; His-tag

Introduction

L-Asparaginase (L-ASNase) is an important enzyme used as a biopharmaceutical product to treat acute lymphoblastic leukemia (ALL) because it catalyzes the conversion of serum asparagine (Asn) to aspartic acid and ammonia, causing the death of leukemic cells by starvation due to lack of Asn for protein synthesis.[1] The commercial versions of the L-ASNase approved by the US Food and Drug Administration (FDA) to treat ALL are derived from bacteria (*E. coli*: Kidrolase®, EUSA Pharma Inc.; Oncaspar®, Enzon Pharmaceutical Inc.; and *E. chrysanthemi*: Erwinase®, JZP-416®; EUSA Pharma Inc.) or produced as a recombinant in *E. coli* expression systems.[2] This prokaryotic system is widely used due to its rapid cell growth, high protein yield, low-cost production medium, and its genome has been well studied.[3] However, bacteria tend to accumulate lipopolysaccharide in their outer membrane (which is immunogenic in humans)[4] and methods to produce extracellular proteins have still not been entirely described,[5] resulting in either cytoplasmic or periplasmic production, which requires cell lysis and further recombinant protein purification. In addition, a high expression rate can produce protein in insoluble inclusion bodies, thereby requiring extra steps to fold recombinant active proteins.

Other research groups have tried to use a eukaryotic expression system such as *Pichia pastoris* in order to express L-ASNase,[6–9] since it is generally recognized as safe (GRAS) by the FDA,[10] presents the possibility of post-translational modifications added to the proteins and it can produce them extracellularly, which considerably improves the downstream process to get L-ASNase. However, for the high recovery rate and to facilitate purifications steps of the recombinant protein, researchers usually use His-tag in the C- or N-terminus from the protein; but it does not always work properly and the likelihood of altering the native conformation or protein functionality could be considerable.[11–13]

Currently, there are several strains of *P. pastoris* available called Glycoswitch®, which can produce both homogeneous and humanized glycosylation in the recombinant proteins,[14] which could be advantageous in covering some immunogenic epitopes of the bacterial proteins such as L-ASNase, representing an optimal system for the production of biopharmaceuticals applied to human health.

Here, we report on the construction of two different expression vectors aiming to express L-ASNase coded by the bacterial gene *asnB* from *E. chrysanthemi* and to determine the effect of the His-tag on the extracellular production of

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L-ASNase in the Glycoswitch® *P. pastoris* strain, as a potentially easier approach to produce biopharmaceutical products derived from bacteria and applied to human health.

Experimental

Construction of recombinant strains

The Glycoswitch® SuperMan$_5$ (his$^-$) strain from *P. pastoris* and expression vector pJAG-s1 containing α mating factor (αMF) from *S. cerevisiae* (used for extracellular secretion of recombinant proteins) was acquired from Biogrammatics Inc. The synthetic gene *asnB* (UniprotKB – P06608) (sequence in Supplemental data 1) was purchased from GenScript, and further used as a template to be amplified in two different ways: (1) adding the His-tag in the C-terminus (forward 5'ggcGGTCTCgGGCTGAACGCTGGTTTAAGTC C3' and reverse5'ggcGGTCTCcATTAATGATGGTGGTGA TGGTGTGGACCCTGGAACAAAACTTCAAGGTAGGTG-TGGAAGTATTCTTGG3'), and (2) excluding the His-tag in the reverse primer (5'ggcGGTCTCcATTAGTAGGTGTG GAAGTATTCTTGG3'). The underlined sequences in the primers are the restriction sites of the *BsaI* enzyme (Biolabs Inc.). PCR was performed using the following conditions: denaturation at 95 °C/30 s, annealing at 69 and 65 °C/30 s for the insert including His-tag and excluding His-tag, respectively; and extension at 72 °C/7 min.

After the amplification, both the gene *asnB* and the plasmid pJAG-s1 were digested by the *BsaI* (BioLabs® Inc.) restriction enzyme. The ligation reaction between the gene and the plasmid was performed by the T4 DNA ligase (Promega), and the resulting plasmids (pJAG_*asnB*_His and pJAG_*asnB*) were transformed by electroporation in DH5α cells.

Colony PCR was performed by taking DH5α clones as templates and using either the primers for the respective genes mentioned earlier or primers specific for the vector: pJAG-s1_fw 5'CAACGGTTTGTTGTTCATTAACAC3' and pJAG-s1_rv 5' CTCGTACGAGAAGAAACAAAATGAC3'. Correct clones were confirmed using agarose gel electrophoresis (1%) based on the size of the expected amplifications.

Recombinant plasmids were isolated from DH5α cells using the QIAprep® Spin Miniprep (QIAGEN) plasmid DNA extraction kit and linearized using *PmeI* (Biolabs Inc.) restriction enzyme, which favors homologous recombination into the *AOX1* locus. Linearized plasmids were used to transform Glycoswitch® SuperMan$_5$(his$^-$) by electroporation. A Glycoswitch® strain was transformed with an empty plasmid being used as a negative control. Electroporation was performed using a MicroPulser™ electroporator with 1 mm cuvettes (BIORAD). After the electroporation, YPD (yeast extract, 10.0 g L^{-1}, peptone, 20.0 g L^{-1}, and dextrose, 20.0 g L^{-1}) medium was added and cells were then incubated for 3 h at 37 °C. The culture was then spread onto YPD-agar plates containing 1 mg ml^{-1} of Geneticin G418 antibiotic and finally incubated at 30 °C until the colonies were grown.

The colony PCR was performed using transformed Glycoswitch® clones as templates, previously treated with Zymolyase at 5 U µl^{-1} concentration (Zymo Research) for 10 min at 37 °C. Specific primers for either the gene or the plasmid were used as previously described. Correct clones (Glycoswitch®—pJAG-s1, negative control; Glycoswitch®—pJAG-s1_*asnB*_His and Glycoswitch®—pJAG-s1_*asnB*) were analyzed using agarose electrophoresis gel (1%) based on the expected size of the DNA amplifications.

Expression of the recombinant protein

Briefly, cells were cultivated in pH 6.0 BMGY (buffered glycerol complex medium: yeast extract, 10.0 g L^{-1}; peptone, 20.0 g L^{-1}; yeast nitrogen base, 3.4 g L^{-1}; ammonium sulfate, 10.0 g L^{-1}; glycerol, 10 g L^{-1}) (500 ml in 2000 ml Erlenmeyer baffled flask) at 30 °C and 250 rpm in Excella E24 Incubator Shaker Series (New Brunswick Scientific) until OD_{600} of approximately 12. The cultures were centrifuged at 2147 × *g* and suspended in pH 6.0 BMMY (buffered methanol-complex medium: yeast extract, 10.0 g L^{-1}; peptone, 20.0 g L^{-1}; yeast nitrogen base, 3.4 g L^{-1}; ammonium sulfate, 10.0 g L^{-1}; methanol instead 10 g L^{-1} glycerol) and returned to the shaker at the same conditions described above, using methanol pulses (10 ml L^{-1}) added each 24 h to induce the recombinant protein production. A control strain (Glycoswitch®—pJAG-s1) was also cultured in the same conditions. Following the induction for 72 h, both the culture supernatants and cells were separated by centrifugation at 10,956 × *g* for 10 min at 4 °C and stored at 4 and −20 °C, respectively until further analysis.

Intracellular content (intracellular lysate) from the different constructions was obtained by the mixture of 5 g of cell pellet in lysis buffer (50 mM Tris—HCl pH 7.5; 1 mM ethylenediamine tetra acetic acid (EDTA); 1 mM phenylmethanesulfonyl fluoride (PMSF); 50 ml L^{-1} glycerol) and (1:2) glass beads 0.5 mm. The mixture was vortex-mixed for 30 s and immediately refrigerated at 4 °C for 1 min, repeating the procedure eight times. Afterwards, the mixture was centrifuged at 10,956 × g at 4 °C for 10 min and the supernatant was submitted to analysis to determine enzymatic activity.

Enzymatic activity was determined in intracellular content (intracellular activity), whole cell suspensions (periplasmic activity) and culture supernatants (extracellular activity) by formation of (AHA),[15] a product of the reaction between aspartic acid (hydrolysis product resultant from the reaction catalyzed by the enzyme L-ASNase) and hydroxylamine + $FeCl_3$, in which the presence of iron (III) chloride could be monitored through a spectrophotometer at 500 nm, enabling the quantification of AHA produced per minute.

One hundred microliters of intracellular content or culture supernatants were mixed with 100 mM of Asn as a substrate, 1000 mM of hydroxylamine pH 7.0 and 50 mM of Tris-HCl pH 8.6; then the samples were incubated at 37 °C for 10 min. The reaction was stopped with the addition of a solution containing iron (III) chloride ($FeCl_3$) (100.0 g L^{-1}), trichloroacetic acid (TCA) (50.0 g L^{-1}) and hydrochloric

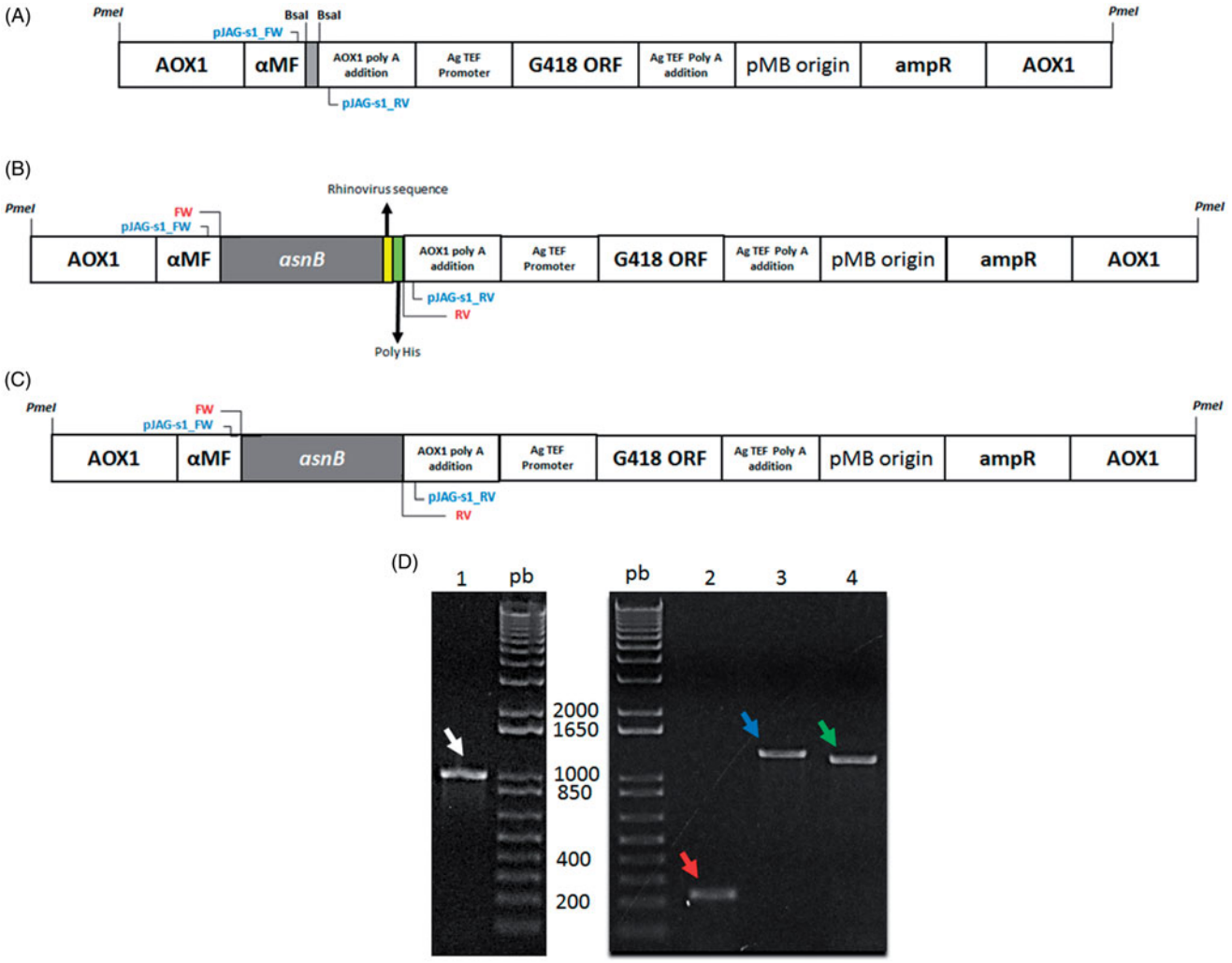

Figure 1. Diagram showing linearized expression vectors (A, B, and C) and confirmation of recombinant strain obtained through amplification of *asnB* gene in the genomic DNA of yeast (D). Empty pJAG-s1 (A), showing the *BsaI* restriction enzyme site where the gene of interest was further inserted in fusion with His-tag (pJAG-s1_*asnB*_His, B), and the same construct without His-tag (pJAG-s1_*asnB*, C). Both primer annealing sites in vector and in gene are shown in blue and red, respectively. (Linearization was done by the *PmeI* restriction enzyme, which recognizes the *AOX1* locus in the vector). Agarose gel (1%) from PCR resulting from *asnB* gene of 989 pb (D, white arrow) and Glycoswitch® strain construction (D, lane 2, 3 and 4). Amplicon from Glycoswitch®—pJAG-s1 empty vector strain (negative control) of 233 pb (red arrow), Amplicon from Glycoswitch®—pJAG-s1_asn*B*_His strain of 1234 pb (blue arrow) and from Glycoswitch®—pJAG-s1_asn*B* strain of 1192 pb (green arrow) using the vector primers.

acid (HCl) (660 mM), further analyzed at 500 nm. In order to evaluate periplasmic protein activity, 100 µl of cell suspension were taken and centrifuged at 2147 × *g*; the pellet was rinsed two times with ultrapure water (Direct—Q® 8 UV, Millipore) and finally resuspended in 100 µL of ultrapure water. Enzymatic activity was measured from this 100 µL of whole cell suspension, using the same conditions described above. AHA (0–3,5 mM) (Sigma–Aldrich) was used to build the calibration curve to determine the enzymatic activity, expressed in U L^{-1}, where 1 U corresponds to the amount of enzyme required to produce 1 µmol of AHA per minute.

In silico *analysis*

Modeling of our expected recombinant proteins from vectors pJAG-s1_*asnB*_His and pJAG-s1_*asnB* was carried out using the Modeler 9.17 software (University of California, USA). A basic modeling was performed since the amino acid sequences to be modeled have a considerable similarity to the amino acid sequence used as a template for this modeling (mature L-ASNase from *E. chrysanthemi*, PDB: 5F52). Archives generated from this modeling step were edited using the Pymol software (www.pymol.org).

Results and discussion

Construction of the recombinant strains

In order to express extracellular L-ASNase from *E. chrysanthemi* in Glycoswitch®, the coding sequence of *asnB* was cloned in two ways: (1) in fusion with a tag containing 6 histidine residues linked to a rhinovirus protease recognition-site sequence at the C-terminal portion of the protein in order to facilitate downstream process, and (2) coding the *asnB* without His (like native mature protein). Figure 1 describes the design of the three expression vectors as well as their linearization with the *PmeI* restriction enzyme to finally obtain the cassettes to introduce in the Glycoswitch® strain.

Table 1. Enzymatic activity values obtained using culture supernatants (extracellular), whole cell suspensions (periplasmic) and intracellular lysate (cytoplasmic) from different recombinant strains, with AHA methodology.[15]

Strains	Enzymatic activity	Culture supernatants	Whole cell suspensions	Intracellular lysate
Glycoswitch – pJAG-s1 (Negative control)	[]μmolβ-Hidro	0	0	0
	U L^{-1}	–	–	–
Glycoswitch – pJAG-s1_*asnB*_His	[]μmolβ-Hidro	0.09 ± 0.02	0.51 ± 0.02	0.29 ± 0.06
	U L^{-1}	30.69 ± 5.19	168.88 ± 27.48	96.6 ± 5.5
Glycoswitch – pJAG-s1_*asnB*	[]μmolβ-Hidro	1.37 ± 0.03	0	0.08 ± 0.01
	U L^{-1}	456.66 ± 8.82	–	26.66 ± 2.31

Absence of the His-Tag enhances the extracellular expression of L-ASNase in glycoswitch®

To determine both the presence and location of the active recombinant protein, enzymatic activity was assessed in the culture supernatants (if the enzyme was extracellular), intracellular content (if the enzyme was intracellular—cytoplasmic) and in whole cell suspensions (if the enzyme was directed to the yeast periplasm) from all constructions (Table 1).

The total activity of the L-ASNase in fusion with the His-tag was 296.17 U L^{-1}. This expression was mostly both periplasmic (168.88 U L^{-1}) and intracellular (96.6 U L^{-1}) compared to the native protein (without His-tag). Similar results have been reported by Lubinaeau et al.[16] which intended to express extracellularly the human fucosyltransferase in *P. pastoris*, but the protein was accumulated mostly in the periplasmic space. It is important to highlight that fucosyltransferase is originally located at Golgi transmembrane, and both cytoplasmic, as well as transmembrane domains, were deleted in the construction of the expression strain. This modification favored the protein export until to the periplasmic space but did not result in its extracellular secretion. Similar results were also described for the Levansucrase protein (EC 2.4.1.10) which is naturally secreted to extracellular space by bacterium *Acetobacter diazotrophicus*; when this protein was expressed in *P. pastoris*, the highest percentage of the protein was accumulated in the periplasmic space.[17] In our case, when the His-tag was absent, both the cell expression and the secretion process improved considerably; the extracellular expression increasing 6.7 times (456.66 U L^{-1}), with low periplasmic accumulation and very low intracellular activity (26.66 U L^{-1}). This could be evidence of a possible harmful effect of His-tag on the expression and secretion process. It could be similar to results reported by Nguyen et al.[6] who expressed L-ASNase of *E. chrysanthemi* into *P. pastoris* (SMD1168 and X33) adding His-tag in the C-terminus with low extracellular L-ASNase production (See SDS-page gel in Nguyen *et al.*); but it is unknown if this result was influenced for the His-tag or the glycosylation process in the L-ASNase.

Considering the production of the L-ASNase without His-tag in the yeast secretory pathway, the transcript of the *asnB* gene is translated by ribosomes associated with the endoplasmic reticulum (ER), the recently translated signal sequence (α-matting factor) is cleaved and the protein is modified with sugars and exported to secretion vesicles as far as the Golgi apparatus (GA). After this process, the protein is secreted toward the extracellular supernatant as a mature and active protein (Figure 2(A)). Notwithstanding, in our production of the L-ASNase in fusion with His-tag, this additional structure (His-tag) is translated in each monomer, what could negatively affect the tetrameric conformation and possibly affect a proper protein folding and also lead to most of the proteins being accumulated for degradation through ER-associated degradation (ERAD) (Figure 2(B)).[18] Some of the remaining recombinant protein could be folded and exported to the yeast periplasm and/or for the extracellular supernatant. This hypothesis could explain the activities reported in Table 1. Unlike this work, Sajitha et al.[7] were able to express *asnB* from *E. coli* with a considerably higher activity compared to those reported in this study, where their construction included His-tag. Such differences may be due to the fact that (1) the proteins (L-ASNase of *E. coli* and L-ASNase of *E. chrysanthemi*) have only 47% of identity, so although both enzymes are bacterial asparaginases, they are very different; (2) different strains were used to express recombinant proteins. In fact, the PichiaPink™ strain used by Sajitha et al. presents a double knockout gene (*PEP4* and *PRB1*) responsible for encoding proteases A and B, respectively, thus avoiding protein degradation at the extracellular supernatant. The Glycoswitch® strains used in this study do not contain the same silenced genes but counterbalance them by having a powerful humanized glycosylation architecture within the cells, which is not in the PichiaPink™ strains.

In silico analysis shows his-tag going inside the L-ASNase

The Modeler 9.19 software was used to build recombinant L-ASNase tridimensional structures. Four models were considered for this purpose: (1) *H. pylori* (PDB: 2WLT_A);[19] (2) *E. coli* (PDB: 3ECA_A);[20] (3) *E. carotovora* (PDB: 1ZCF_A);[21] and (4) *D. chrysanthemi* (PDB: 5F52_A),[22] presenting 47, 48, 79, and 100% of amino acid sequence similarity, respectively, compared to the recombinant L-ASNase synthetized in this work. The L-ASNase amino acid sequence containing the highest percentage of similarity was used as a model by the software (*D. chrysanthemi*). The resulting 3D structures are represented in Figure 3. The Modeler program makes it possible to choose the best model from among several generated through parameters such as Discrete Optimized Protein Energy (DOPE), Statistically Optimized Atomic Potentials (SOAP) and the GA341 score. DOPE scores for each amino acid residue belonging to each predicted model are presented graphically in Supplemental data 2.

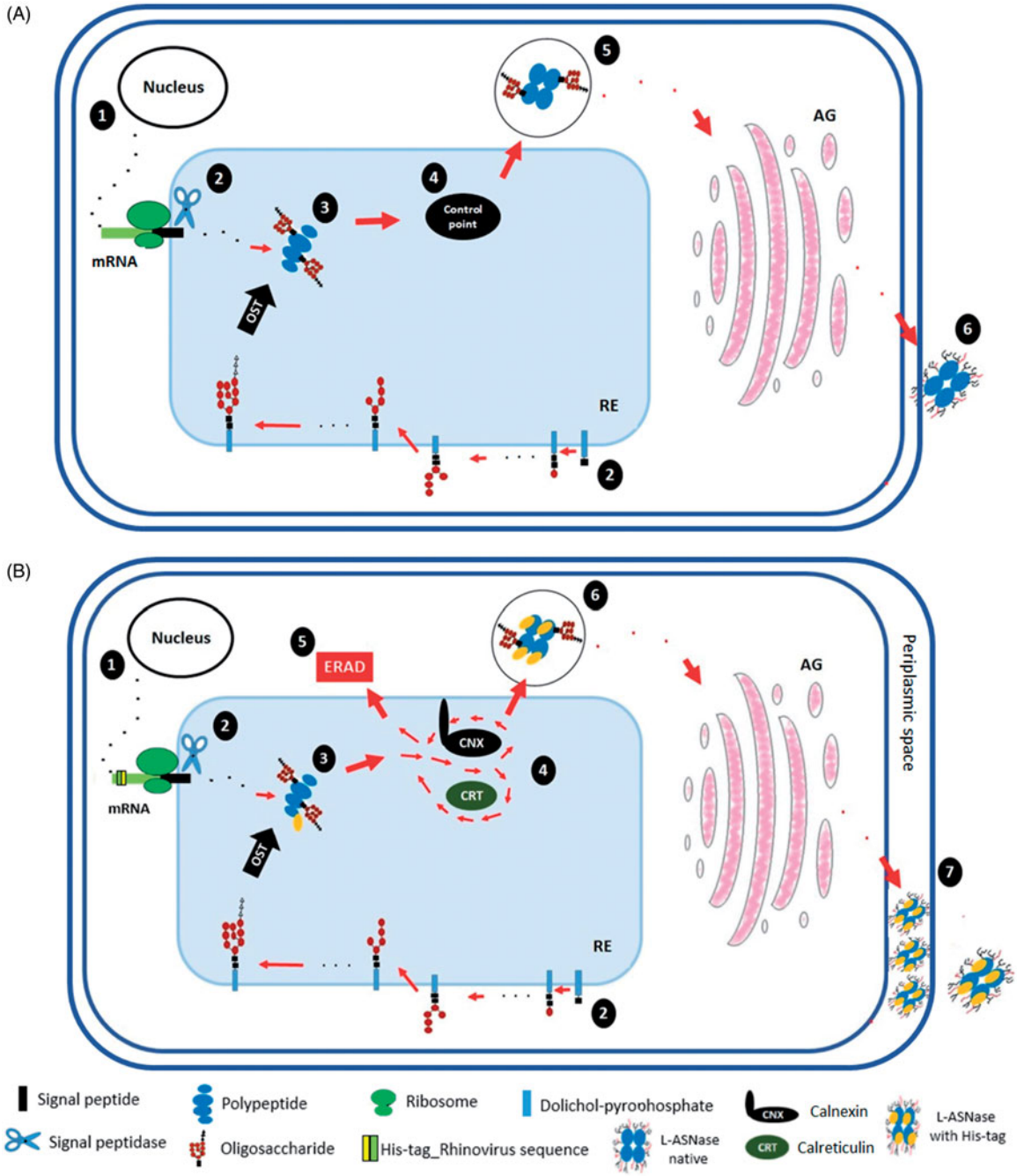

Figure 2. Representation of a possible recombinant L-ASNase production by Glycoswitch®—pJAG-s1_*asnB* strain (A) and Glycoswitch®—pJAG-s1_*asnB*_His (B). A) mRNA of L-ASNase is recognized by ribosomes which translate the signal sequence and then transport it to the endoplasmic reticulum (ER) (1); newly synthetized signal peptide is recognized and cleaved by a signal peptidase located inside the ER where the recombinant protein synthesis finishes (2); translation process is restarted and occurs at the same time, transferring an oligosaccharide to an oligosaccharyltransferase (OST) (3); proteins then undergo folding (4) and are transferred to vesicles where they will be transported to the Golgi apparatus (5), ending their modification to finally be secreted to the extracellular supernatant as functional proteins (6). B) In the case of Glycoswitch®—pJAG-s1_*asnB*_His, mRNA is synthetized containing the His-tag_Rhinovirus sequence (1), where signal peptidase cleaves the signal sequence (2), resulting in a recombinant protein containing His-tag (3), and processed mainly by CNX and CRT (4). Folding impairment and degradation by ERAD could further affect the total protein activity (5). A small amount of proteins would be able to pass through the cell regulation processes and could reach the Golgi apparatus (6), completing their modification and being secreted to the periplasmic space and the extracellular supernatant (7).

According to the model in Figure 3, the 6 histidine residues (green in Figure 3(B)), as well as the rhinovirus sequence (orange in Figure 3(B)) (used as a site for proteases to remove the histidine residues after protein purification), were localized inside the tridimensional protein monomer, which could adversely affect its native conformation and folding, blocking its passage to the extracellular medium by failing cell quality control mechanisms, represented by calnexin and calreticulin, and promoting its degradation via ERAD.[18] Similar findings have been observed by Chant et al.,[11] where conformational changes appeared when they used a 6 His-tag in the HZFB AreA protein (transcription factor) compared to the ZFB AreaA protein (without 6 His-tag); however, significant functional changes were not found in this protein. This is in contrast to the results reported by Fonda et al.,[12] where they

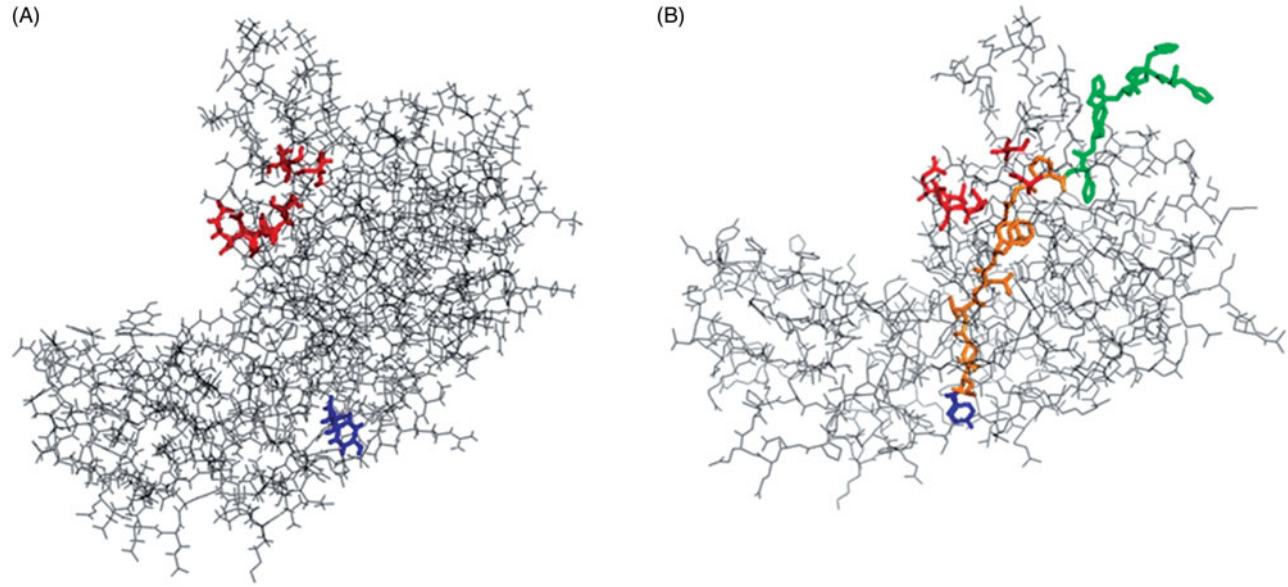

Figure 3. Modeling of recombinant L-ASNase from *E. chrysanthemi*. L-ASNase monomer was used as a control (PDB: 5F52_A) (A); L-ASNase containing His-tag (green) and rhinovirus sequence (orange) (B). The modeling suggests that addition of both structures (His-tag and rhinovirus sequence) to L-ASNase can be harmful, due to its proximity to the active site amino acids highlighted in red (Thr15, Ser62, Glu63, Thr95, Asp96, Ala120). C-terminal amino acids are shown in blue.

produced TNF-alpha recombinant with 7 His-tag attached to the N-terminal portion (His7-(ΔN6)TNF), which resulted in significant reduction of biological activity compared to wild type. In relation to the tridimensional model of His7-(ΔN6)TNF, the 7 histidine was not inside structure itself as in our study. Goel et al.[13] expressed scFV (monoclonal antibody) in *P. pastoris*, fused to 6 His-tag either C-(scFv-His6) or N-terminus (His6-scFv) of the polypeptide. They found the lowest antigen binding (20–25%) in the scFv-His6 construction because 6 His-tag partially covered the antigen binding site of the protein. In our case, the 6 His-tag does not cover the active site from L-ASNase (red in Figure 3(A,B)); instead, the 6 His-tag goes next to the active site and penetrates the monomeric structure, which can be detrimental to correct protein folding or oligomerization.

The results of this study suggest that additional structures like His-tag sequence in the L-ASNase protein of *D. chrysanthemi* had a negative impact on its extracellular production.

Disclosure statement

The authors declare no competing financial interests.

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