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Glycosylation of L-asparaginase from *E. coli* through yeast expression and site-directed mutagenesis

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HIGHLIGHTS

- WT L-ASNase expressed in yeast was successfully glycosylated, secreted and purified.
- The yeast strain was able to glycosylate all glycosylation sites of WT L-ASNase.
- Mutations at L-ASNase glycosylation sites influenced its biological properties.
- Mutated L-ASNase showed a significant increase in biological activity relative to WT.
- Glycosylation increases L-ASNase stability and masks immunogenic epitopes.

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ABSTRACT

L-Asparaginase (L-ASNase) is a key component in the treatment of acute lymphoblastic leukemia (ALL), but several clinical disadvantages, such as immunogenicity and rapid clearance, are still present. We evaluated the possibility to synthesize a new L-ASNase from *Escherichia coli* with human-like glycosylation and study the glycosylation effect on the biochemical properties of the enzyme. Six L-ASNase mutants were also created in which L-ASNase glycosylation sites were removed through site-directed mutagenesis. The WT L-ASNase was successfully expressed, secreted and glycosylated by an engineered *P. pastoris* strain and presented predominantly $Man_5GlcNAc_2$ glycans on its structure, which were then able to decrease L-ASNase immunogenicity *in vitro*. The purified glycosylated L-ASNase has shown a 30-fold decrease in specific enzymatic activity compared to the non-glycosylated proteoform, but a triple mutant L-ASNase (3M) was able to restore L-ASNase biological activity to significant levels. 3M accumulated in the yeast periplasmic space and there presented a 28-fold increase in enzymatic activity when compared to the fully glycosylated proteoform. Both WT and 3M L-ASNases presented increased stability in human serum compared to non-glycosylated L-ASNase. This study demonstrates the important effects of glycosylation on L-ASNase properties and opens up new possibilities to use glycosylated L-ASNases for the treatment of ALL.

1. Introduction

L-Asparaginase (L-ASNase) is an enzyme used for the treatment of acute lymphoblastic leukemia (ALL). This therapeutical product catalyzes the conversion of amino acid L-asparagine into aspartate and ammonia, a reaction through which L-ASNase exerts its antineoplastic activity. The amino acid L-asparagine is essential for the growth of both normal and leukemic cells, but the latter is exclusively dependent on an exogenous supply of L-asparagine. Therefore, when L-ASNase is administered into the bloodstream and serum asparagine is depleted, leukemic cells became unable to utilize this amino acid, leading to starvation and eventually death of the malignant lymphoid cells [1].

For the treatment of ALL, there are three types of L-ASNases commercially available. Two of these enzymes (either native or pegylated form) are derived from *Escherichia coli,* whereas a third type is originated from *Erwinia chrysanthemi*. While *E.coli*-derived preparations are used as first-line for the treatment of ALL, L-ASNases derived from *E. chrysanthemi* are mainly administrated as second-line in cases of

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hypersensitivity to the first type [2]. In addition, the PEGylated asparaginase (Oncaspar®) from *E. coli* has been approved by the U.S. Food and Drug Administration (FDA) as first-line of treatment for ALL in 2006 [3]. Compared to its native form, PEGylated L-ASNase has shown decreased immunogenicity and prolonged half-life, thanks to the covalent conjugation of polyethylene glycol chains to the enzyme [4].

Despite the known advantages of using PEGylation to enhance pharmacokinetic profiles of therapeutical products, this approach still presents several drawbacks. For instance, the formation of antibodies against PEG molecules (anti-PEG) represents one of the limitations of using PEGylated therapeutic proteins clinically [5]. In the case of PEGylated L-ASNase, presence of anti-PEG has been shown to cause rapid clearance of the enzyme which may negatively affect the therapy [6]. Another limitation is the non-homogeneous conjugation of PEG on drugs. Variations in both the number as well as in the architecture of the attached PEG molecules may adversely affect the biological activity of many therapeutic biomolecules [5,7]. Many adverse events have also been reported in patients treated with PEGylated L-ASNase. Most serious toxicities included thrombosis, pancreatitis as well as bleeding [8].

Glycosylation of recombinant proteins can serve as a potential alternative to PEGylation. Glycosylated bioproducts are typically produced in mammalian cell lines, which are able to correctly perform complex post-translation modifications to the final bioproduct, including glycosylation. In fact, bioproducts manufactured in mammalian cell lines represented 79 % of all biopharmaceutical approvals between 2015 and 2018 [9]. The list of approved glycosylated products includes a variety of agents such as antibodies, blood factors, colony-stimulating factors, hormones, among other naturally occurring glycosylated therapeutic proteins [9]. However, studies on glycosylation of prokaryotic proteins intended for clinical applications, as in the case of L-ASNase, have not been extensively reported yet.

The production of recombinant glycosylated proteins could enhance *in vivo* efficacy by optimizing biological stability and minimizing renal and hepatic clearance, hence improving both drug pharmacokinetic and pharmacodynamic properties [10]. Increasing the number of carbohydrate chains on therapeutic proteins, for instance, has already been shown to produce bioproducts with enhanced *in vivo* activity and prolonged half-life. Recombinant human erythropoietin, for example, has been engineered bearing new N-glycosylation consensus sequences in strategic regions of the protein backbone, resulting in an analog with substantially greater *in vivo* activity and longer duration of action [11,12]. Using a similar approach, Ceaglio et. al. have introduced four N-glycosylation sites on recombinant human alpha interferon through site-directed mutagenesis and obtained at the end hyperglycosylated variants with a 25-fold increase in serum half-life [13].

The biomanufacturing of such glycosylated bioproducts entails the use of different host cells, mainly Chinese hamster ovary (CHO) cell-based systems [10]. The reason for its popularization lies in its capacity to express recombinant proteins with a humanized glycosylation pattern, allowing the final biopharmaceutical product to properly exerts its biological activities [14]. Notwithstanding the current popularity of these expression systems, production of recombinant proteins in mammalian cells has important disadvantages such as low protein secretion, easy culture contamination by viruses, high lot variability as well as expensive industrial processes [15].

Alternatively, yeast cells can be used to express recombinant glycosylated bioproducts given their high productivity and low fermentation costs. Although yeast glycosylation pattern is significantly different from the humanized profile existent in mammalian cells, recent advances in engineering glycosylation pathways in yeast has opened up new cost-effective alternatives to produce human-like glycosylated proteins. Engineering of the N-glycosylation pathway in yeast *Pichia pastoris* has enabled the expression of recombinant proteins with mammalian-type glycosylation. The strategy mainly consists in the disruption of the endogenous glycosyltransferase gene (*OCH1*), which is responsible for adding α-1,6-mannose residues to the growing glycan chain and hence producing hyper-mannosylated structures; the introduction of other important enzymes present in higher-eukaryote glycosylation pathways are conjointly implemented [16]. By using this engineered host, recombinant proteins with human-like glycosylation can then be produced. Advantages include more uniform glycosylation pattern, high-expression of recombinant proteins, prevention of aberrantly glycosylated products and increased homogeneity of the final bioproduct [17].

Here we report the expression of a novel recombinant L-ASNase from *Escherichia coli* in an engineered *Pichia pastoris* host able to perform humanized glycosylation. In addition, we confirmed that the organism was able to glycosylate and secrete the recombinant prokaryotic protein outer of the cell, thus allowing the production of an alternative L-ASNase with improved biological properties. The effect of glycosylation on the biological properties of the enzyme as well as on their cellular trafficking through the yeast periplasm to the extracellular medium was also assessed.

2. Material and methods

2.1. Host strains and culture medium

Both *E. coli* DH5α and *E.coli* BL21 cells were grown in lysogenic broth - LB medium (1 % tryptone, 0.5 % yeast extract and 0.5 % NaCl) while both *Pichia pastoris* GS115 (Invitrogen™, USA) and *Pichia pastoris* SuperMan5 (his-) (BioGrammatics, Inc., USA) strains were grown in YPD medium (1 % yeast extract, 2 % peptone and 2 % glucose). When solid media was used, agar was added at a concentration of 2 %. Transformant bacteria were selected using carbenicillin (50 μg/mL) and transformant yeasts were selected using geneticin G418 (800 mg/mL).

For recombinant protein expression, *Pichia pastoris* strains were initially inoculated in BMGY medium (0.75 % glycerol, 1 % yeast extract, 2 % peptone, 0.34 % YNB, 1 % ammonium sulphate and 100 mM potassium phosphate buffer, pH 6.0) and then shifted to BMMY medium (0.5 % methanol, 1 % yeast extract, 2 % peptone, 0.34 % YNB, 1 % ammonium sulphate and 100 mM potassium phosphate buffer, pH 6.0). Methanol was added to the culture medium every 12 h to a final concentration of 0.5 %. For recombinant protein expression in *E. coli* BL21 strain, cells were grown in 25 ml of LB medium at 37 °C and then transferred to 200 ml of LB medium up to an OD_{600nm} of 0.2. At OD_{600nm} 0.7, cells were induced with 1 mM IPTG for 22 h at 37 °C.

2.2. Vector engineering and cells transformation

For construction of the yeast expression vector, optimized L-ASNase II encoding gene (*ansB)* was previously synthesized by GenScript Optimum Gene™ and cloned into a pUC57 plasmid. *ansB* was amplified from the plasmid *via* PCR using primers 1 and 2 (Exxtend Biotecnologia São Paulo, Brazil) (Supplementary Table), corresponding to amino acids 22–348. Primer sequences included *BsaI* recognition sites and allowed removal of original periplasmic signal coding sequence from the *ansB* gene.

pJAG-s1 plasmid from BioGrammatics, Inc. was used as an integrative vector. The amplified *ansB* gene was cloned into the pJAG-s1 plasmid in frame with the α-mating factor (α-MF) signal sequence. Both the amplified gene and the pJAG-s1 vector were pre-digested with the *BsaI* restriction enzyme and then joined with T4 DNA ligase. *ansB* gene was sequenced to confirm the absence of mutations using primers 3 and 4 (Exxtend Biotecnologia São Paulo, Brazil) (Supplementary Table) as well as BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems™, USA). Primers were designed to anneal at flanking regions of the ligation sites present within the pJAG-s1 plasmid. The ligation product was transformed in *E.coli* DH5α cells by electroporation.

Confirmed expression vectors were linearized with *PmeI* restriction enzyme and transformed in yeast strains by electroporation. Yeast genomic DNA was extracted using Purelink™ Genomic DNA Mini Kit

(Invitrogen™) and chromosomal integration was confirmed *via* PCR, using primers 3 and 4 (Exxtend Biotecnologia São Paulo, Brazil) (Supplementary Table).

A pET15b bacterial vector containing the *ansB* gene (including the bacterial signal sequence) was used for expression of L-ASNase in the *E.coli* BL21 strain.

2.3. Analysis of yeast protein expression

Samples of culture supernatants were initially acetone-precipitated (80 % acetone). Precipitates were kept at −20 °C for 30 min and then centrifuged at 16,000*g* for 10 min at room temperature. The supernatant was discarded and the remaining acetone was evaporated at room temperature for 30 min. Pellet containing precipitated proteins were resuspended in 50 mM Tris-HCl, pH 8.6. The resulting samples were mixed with loading dye, heated to 95 °C for 5 min and then loaded on a 12 % reductant SDS-PAGE gel for analysis of protein expression and glycosylation.

2.4. Purification of recombinant L-ASNase expressed in P. pastoris

Culture supernatant was collected after 48 h post-shift to methanol medium. The medium broth was centrifuged at 10,000 *g* at 4 °C for 3 min and the pellet discarded. The extracellular medium was concentrated 10X and buffer exchanged to 50 mM Tris-HCl, pH 8.6 with a 10 kDa Pellicon XL tangential flow membrane coupled with a *Labscale™ TFF System* device.

The sample was used for purification by anion exchange chromatography. HiTrap DEAE Sepharose FF column (GE Healthcare Life Sciences) of 5 ml was equilibrated with 50 ml of equilibration buffer (50 mM Tris-HCl, pH 8.6). Two hundred milliliters of the sample were then loaded onto the column at a flow rate of 3 ml/min. After, the anionic column was washed with 25 ml of equilibration buffer and recombinant L-ASNase was eluted with 20 ml of elution buffer (50 mM Tris-HCl plus 75 mM NaCl, pH 8.6) at a flow rate of 1 ml/min by FPLC (*fast protein liquid chromatography)* using an *AKTA Purifier (GE Life Sciences)*. Fractions of 1 ml were collected and submitted to SDS-PAGE and enzymatic assays for qualitative analysis. Fractions contained purified recombinant L-ASNase were mixed and used for quantitative enzymatic assays.

2.5. Purification of recombinant L-ASNase expressed in E. coli BL21

Culture supernatant was collected after 22 h of IPTG induction. The medium broth was centrifuged at 3220*g* at 4 °C for 20 min and the supernatant discarded. The pellet was then suspended with 40 ml of hyperosmotic buffer (100 mM Tris-HCl, pH 8.0; 500 mM sucrose and 0.5 mM EDTA) and incubated on ice for 5 min. Cells were harvested again by centrifugation at 3,220*g* at 4 °C for 20 min. Pellet was resuspended in 3 ml of 0,5 mM PMSF solution and incubated on ice for 5 min, causing bacterial external membrane lysis. Suspension was finally centrifuged at 3220*g* at 4 °C for 40 min and the supernatant was collected.

The sample was used for purification by anion exchange chromatography. HiTrap DEAE Sepharose FF column (GE Healthcare Life Sciences) of 5 ml was equilibrated with 50 ml of equilibration buffer (50 mM Tris-HCl, pH 7.4) and the sample was then loaded onto the column at a flow rate of 3 ml/min. After, the anionic column was washed with 25 ml of equilibration buffer and recombinant L-ASNase was collected after salt gradient elution using NaCl at a flow rate of 1 ml/min by FPLC (*fast protein liquid chromatography)* using an *AKTA Purifier (GE Life Sciences)*. Fractions of 0.5 ml were collected and submitted to SDS-PAGE and enzymatic assays.

2.6. Glycosylation analysis

Proteins were deglycosylated by treatment with PNGase F from *Elizabethkingia meningoseptica* (Sigma Aldrich, USA). Proteins samples were mixed with 5 % SDS solution and 1 M DTT and then heated to 95 °C for 5 min. 500 mM sodium phosphate buffer pH 7.5, 10 % Triton X-100 and PNGase F were added to the mixture and resulting samples were incubated for 3 h at 37 °C in order to be N-deglycosylated. These samples were analyzed by SDS-PAGE and mass spectrometry.

2.7. Mass spectrometry analysis

For the mass spectrometry analysis, protein samples were firstly isolated from Coomassie-stained acrylamide gel and later submitted to in-gel trypsin-digestion (Trypsin Gold – Promega, USA). Sample desalting was performed using a Zip-Tip C18 cartridge column (Merck – Millipore, USA). The final desalted peptide mixture was then submitted to Easy-nLC -LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). Raw data were analyzed by Byonic software (Protein Metrics) and intact glycopeptides were identified with PEP2D FDR less than 0.001.

2.8. Protein concentration and enzymatic activity

Protein concentration was measured by the Bradford dye method and results were quantified spectrophotometrically at $\lambda = 595$ nm. Different concentrations of BSA samples were used as standards to construct a calibration curve, in which absorbance values of the samples were interpolated.

L-ASNase enzymatic activity was quantified either by L-aspartyl-β-hydroxamic acid (AHA) method, which measures the total amount of L-aspartic acid β-hydroxamate released in the reaction medium or by Nessler's reagent, which measures the total amount of ammonia released. One International Unit (U) of L-ASNase was defined as the amount of enzyme capable of hydrolyzing 1 μmol of amino acid L-asparagine into aspartate and ammonia per minute at 37 °C. For the AHA method, samples were incubated at 37 °C for 30 min with 10 mM L-Asparagine, 100 mM hydroxylamine, 100 mM NaOH and 15 mM Tris-HCl, pH 8.6. The reaction was stopped with 50 μl of a solution containing 5 % TCA, 10 % $FeCl_3$ and 0.66 M HCl and results measured spectrophotometrically at $\lambda = 500$ nm. Known concentrations of L-aspartic acid β-hydroxamate were used as a standard to construct a calibration curve. For the Nessler's method, samples were incubated at 37 °C for 10 min with 8.6 mM L-Asparagine and 19.5 mM Tris-HCl, pH 8.6. The reaction was stopped with 10 ul of a solution containing 5 % TCA and diluted 4.5-fold in water. 25 ul of Nessler's reagent was added and the results were measured spectrophotometrically at $\lambda = 436$ nm. Known concentrations of ammonium sulphate were used as a standard to construct the calibration curve.

2.9. Site-directed mutagenesis

All mutants were obtained using a QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, USA). For each mutation, a pair of specific primers were synthesized (Exxtend Biotecnologia São Paulo, Brazil) (Supplementary Table). Either the *pJAG_ansB* or the pET15b expression vector was used as a template and the products of each PCR reaction were later treated with *DpnI* for 1 h at 37 °C. Amplified vectors were then transformed in XL1-Blue Supercompetent Cells (Agilent Technologies) and resulting colonies were randomly screened for the presence of the desired plasmid. Finally, codon substitution was confirmed by vector sequencing as described previously.

2.10. Yeast cell lysis analysis

Cell pellets were centrifuged at 16,000 *g* for 5 min and the

supernatant discarded. Then, wet cell pellets were resuspended in 400 µl of lysis buffer (50 mM Tris-HCl at pH 8.6, 1 mM EDTA, 1 mM PMSF, and 5 % Glycerol) and 200 mg of 425 − 600 µm acid-washed glass beads (Sigma). Suspensions were finally vortexed for 30 s and then kept on ice for 1 min; this step was repeated 8 times until the release of the intracellular content.

2.11. Enzyme extraction from Periplasm

Protocol from Ferrara et al. was used as a reference and adapted for this work [18]. Cell pellets were centrifuged at 16,000 *g* for 5 min and the supernatant discarded. Wet cell pellets were then submitted to 5 cycles of freezing and thawing (22 h and 2 h, respectively). After the last cycle, pellets were resuspended in 50 mM Tris-HCl pH 8.6 and suspension was centrifuged at 16,000 *g* for 10 min. Pellet was discarded and the supernatant was used for further analysis.

2.12. Western-blotting

Samples from different preparations were loaded into 12 % SDS-PAGE gel. Following separation, proteins from the gel were transferred to PVDF membrane through horizontal electrophoresis at 95 V for 50 min in transfer buffer (25 mM Tris, 192 mM Glycine and 2 % Methanol). After electrophoretic transfer, the membrane was incubated for 3 h in blocking solution (5 % non-fat milk and TBS-T buffer) and then probed with anti-L-asparaginase polyclonal rabbit antibody (1:2000) (Rheabiotech - Prod. Des. Com. Prod. Biotecnologia Ltda, Campinas – SP, Brazil). The membrane was washed three times with TBS-T buffer (20 mM Tris-HCl, 500 mM NaCl and 0.05 % Tween® 20, pH 7.5), with 5 min of incubation for each time. Anti-rabbit alkaline-phosphate conjugated secondary antibody solution was then applied to the membrane for 1 h. The membrane was washed again three times and revelation was performed using NBT BCIP substrate solution (100 mM Tris HCl, 100 mM NaCl, 5 mM $MgCl_2$, 0.4 mM NBT and 0.4 mM BCIP, pH 9.5) until chromogenic detection of bands. Uncropped immunoblots images in Supplementary Fig. 1.

2.13. Enzymatic stability in human serum

Purified WT L-ASNase expressed in *E.coli* BL21 or *P. pastoris* SuperMan5 (his$^-$) and non-purified 3M L-ASNase from *P. pastoris* SuperMan5 (his$^-$) periplasm were incubated at 37 °C (water bath) with 10 % human serum (Sigma) diluted in Tris-HCl buffer 50 mM, pH 8.8, for 168 h (7 days). Enzymatic activity was measured using Nessler's reagent as described above and results were normalized to 100 % at time zero (before serum incubation).

2.14. Enzyme-linked immunosorbent assay (ELISA)

Recognition of L-ASNases by anti-L-ASNase from *E. coli* antibody was quantified by ELISA. ELISA plate (Bio-Rad) was coated overnight at 4 °C with 10 ng of pure WT L-ASNase expressed in *E.coli* BL21 and with different amounts of pure WT L-ASNase expressed in *P. pastoris* SuperMan5 (his-) (from 5 to 2000 ng of protein). The plate was washed four times with washing solution (WS) (PBS, 0.05 % Tween-20) and blocked with 1 % (w/v) BSA in PBS buffer overnight at 4 °C. The supernatant was discarded, washed with WS four times and probed with anti-L-ASNase polyclonal rabbit antibody (Rheabiotec) diluted 10,000-fold in solution containing PBS buffer, 10 % bovine serum and 0.2 % Tween-20. The plate was then incubated at 37 °C with gentle shaking for 90 min. The remaining solution was discarded and the plate was washed four times with WS and probed with secondary antibody (anti-rabbit conjugated with alkaline phosphatase - KPL) diluted 3,000-fold in PBS, 10 % BSA and 0.2 % Tween-20. Plate was incubated at 37 °C with gentle shaking for 90 min. Wells were once again washed two times with WS and two times with PBS only. The substrate (1 % (w/v) o-phenylenediamine, 0.003 % (v/v) H_2O_2 in 50 mM citrate-phosphate buffer, pH 5.5) was added and incubated at 37 °C for 10 min and the reaction was stopped with 2 M H_2SO_4. The absorbance of the solution was measured at 492 nm using a SpectraMax Microplate Reader (Molecular Devices).

3. Results

3.1. Cloning of the ansB gene in the yeast strains

The L-ASNase encoding gene from *Escherichia coli* (named *ansB)* was used in this work. Optimized *ansB* gene sequence was inserted in-frame with the α-MF signal sequence present in expression vector, resulting in the *pJAG_ansB* plasmid (Supplementary Fig. 2). Expression vector construct was analyzed by colony PCR; resulting PCR electrophoresis bands from *E. coli* DH5α transformed cells matched the expected DNA length for the *ansB* gene insertion (Supplementary Fig. 3), confirming the construction of the *pJAG_ansB* plasmid. Further plasmid isolation and sequencing confirmed the absence of mutations within the *ansB* gene sequence.

Vectors were successfully integrated into *Pichia pastoris* chromosomes *via* homologous recombination at the *AOX1* locus. Both the engineered *Pichia pastoris* SuperMan5 (his$^-$) strain, as well as the GS115 (positive control), were transformed with the *pJAG_ansB* plasmid; PCR analysis from purified genomic DNAs confirmed integration of the *ansB* gene into the yeast genome (Supplementary Fig. 4). Since *Pichia pastoris* GS115 have not been engineered to express proteins with human-like glycan structures, a fair comparison regarding the glycosylation pattern between the two strains could later be established (see "3.2 Expression of Recombinant L-ASNase").

3.2. Expression of recombinant L-ASNase

At 48 h post shift to BMMY medium, we wanted to confirm if the yeast cells were able to secrete recombinant glycosylated L-ASNases to the extracellular medium. For that purporse, concentrated protein samples from the culture supernatant were treated with PNGase F, an enzyme capable of removing N-glycans residues from glycoproteins), allowing us to evaluate the presence of N-glycosylation modifications in each expressed protein.

We confirmed the expression of our recombinant and glycosylated protein in both yeast *Pichia pastoris* strains after treatment with PNGase F (Fig. 1). Bands of the length corresponding to deglycosylated L-ASNase (35 kDa) were visualized in the gels (yellow arrows). Interestingly, the existence of *two* similar L-ASNase protein band sizes instead of one single band after PNGase F treatment may indicate the presence of extra post-translation modifications in our recombinant protein rather than just N-glycosylation.

Treatment with PNGase F confirmed variability in glycosylation pattern between the engineered SuperMan5(his$^-$) and GS115 strains. Smearing at GS115-produced proteins demonstrated the presence of hyperglycosylation on this strain, since treatment with PNGase F with the consequent removal of N-glycans has not only revealed recombinant L-ASNase bands at around 35 kDa, but also diminished the smeared band intensity. Finally, bands at ~40 kDa on SuperMan5 (his-)-produced proteins not treated with PNGase F (red arrow) confirmed the presence of recombinant L-ASNase exhibiting a more homogenous and human-like glycosylation pattern. Expression profile of strains not transformed with any plasmid DNA was also analysed and indicated no presence of L-ASNase (both GS115 and SuperMan5 (his-) negative controls).

3.3. Mass spectrometry analysis

N-Glycosylation sites, as well as site-specific oligosaccharide composition in the recombinant L-ASNase, were analyzed by mass

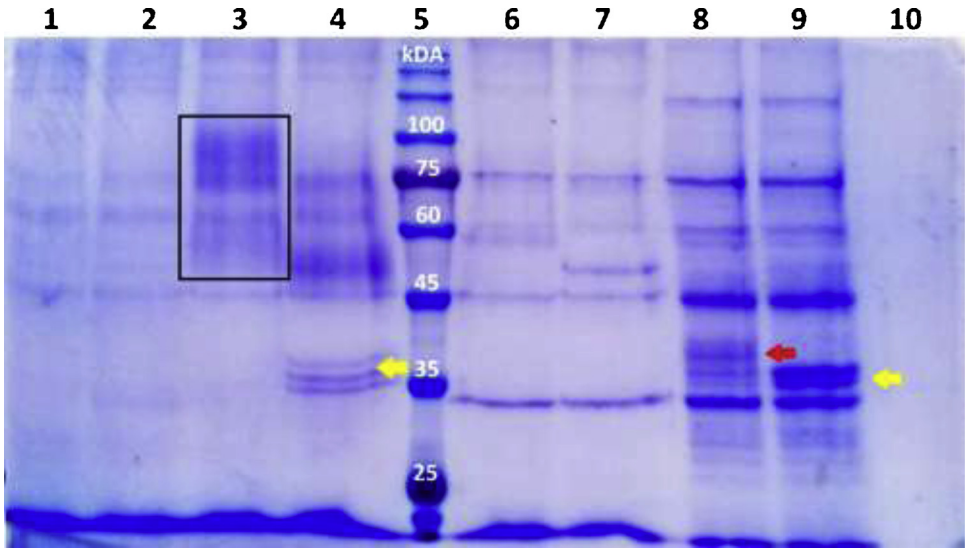

Fig. 1. SDS PAGE Analysis of concentrated proteins from the supernatant. Lane 1 – GS115 control; Lane 2 – GS115 control treated with PNGase F; Lane 3 - GS115-producing L-ASNase; Lane 4 - GS115-producing L-ASNase treated with PNGase F; Lane 5 – Protein Ladder; Lane 6 – SuperMan5 (his-) control; Lane 7 - SuperMan5 (his-) control treated with PNGase F; Lane 8 - SuperMan5 (his-)-producing L-ASNase; Lane 9 - SuperMan5 (his-)-producing L-ASNase treated with PNGase F; Lane 10 – PNGase reaction negative control (all components except concentrated proteins from the supernatant). Red arrow indicates glycosylated L-ASNase produced in SuperMan5 (his-) cells and yellow arrows indicate deglycosylated L-ASNase. Black box indicates smeared bands likely containing hyperglycosylated L-ASNase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

spectrometry. N-glycosylation is a post-translation modification that results in the attachment of a glycan onto specific asparagine residues from a given protein. In eukaryotes, this asparagine residue is present within the *N-X-S/T sequon*, a conserved acceptor protein sequence where N is asparagine, X is any amino acid except proline, and S/T is either serine or threonine [19].

Regarding the glycan composition, N-linked glycosylation in yeasts is of high-mannose type. This pattern can reduce protein serum half-life and increase its immunogenicity [20]. In *Pichia pastoris,* the number of mannose residues ranges from 8 to 14 mannose residues at the outer oligosaccharide chain of secreted proteins [21]. In the engineered SuperMan5(his$^-$) strain, however, inactivation of the *OCH1* gene results in glycans structures that are not hypermannosylated [16].

Based on its amino acid sequence, five potential N-glycosylation sites were identified in L-ASNase: N3, N24, N151, N164 and N222 [22]. To confirm if all residues were in fact glycosylated, three L-ASNase samples were submitted to mass spectrometry: (1) native L-ASNase expressed in *Escherichia coli*, (2) glycosylated L-ASNase expressed in *Pichia pastoris* SuperMan5(his-) and (3) L-ASNase expressed in *Pichia pastoris* SuperMan5(his$^-$) deglycosylated by PNGase F.

As expected, no glycans were found in samples containing either native L-ASNase expressed in *Escherichia coli* or deglycosylated L-ASNase expressed in *Pichia pastoris* SuperMan5(his$^-$) (Table 1). In addition, attachment of oligosaccharides containing few mannose residues (no more than ten) was confirmed in all five potential glycosylation sites present in samples containing the glycosylated L-ASNase (Table 1).

Most of the found attached glycans contained five mannose residues ($Man_5GlcNAc_2$) (Fig. 2), followed by six and four mannose residues. Therefore, the resulting profile matched the glycosylation profile expected for protein expression in the *Pichia pastoris* SuperMan5(his$^-$) strain. In addition, asparagine residue 151 (N151) was the preferred glycosylation site on the protein, where 32.7 % of all identified glycans were attached at this residue (Fig. 2)

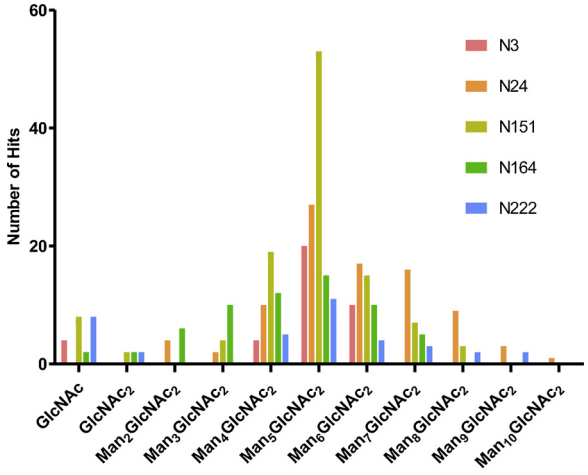

Fig. 2. Glycan profiling of recombinant L-ASNase expressed in the *Pichia pastoris* SuperMan5(his$^-$) strain. GlnNAc, N-Acetylglucosamine; Man, mannos. For better interpretation of the figure, the reader is referred to the web version of this article.

3.4. Purification and evaluation of enzymatic activity of recombinant L-ASNase

The secreted recombinant L-ASNase was purified from the crude extract by tangential ultrafiltration followed by anion exchange chromatography. Enzymatic activity was observed in concentrated samples from the original supernatant as well as in the retentate obtained from the ultrafiltration step. L-ASNase activity could also be assessed in

Table 1
Glycan profiling in different samples containing L-ASNase by mass spectrometry. Number in parenthesis indicates asparagine residue positions in which oligosaccharides were covalently bound.

Peptide Sample	Glycan Structure
(1) Native L-ASNase expressed in *Escherichia coli*	None found
(2) Glycosylated L-ASNase expressed in *Pichia pastoris* SuperMan5(his$^-$)	$Man_{4\text{-}6}GlcNAc_2$ (N3) $Man_{2\text{-}10}GlcNAc_2$ (N24) $Man_{3\text{-}8}GlcNAc_2$ (N151) $Man_{2\text{-}7}GlcNAc_2$ (N164) $Man_{4\text{-}9}GlcNAc_2$ (N222)
(3) Deglycosylated L-ASNase expressed in *Pichia pastoris* SuperMan5(his$^-$)	None found

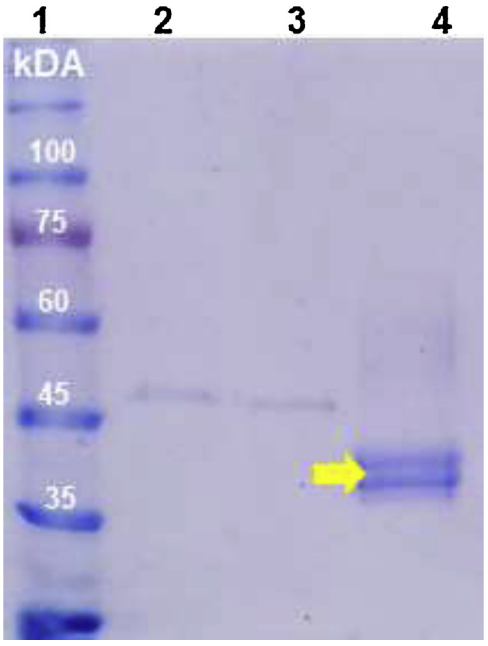

Fig. 3. SDS PAGE Analysis of purified samples. Lane 1 – Protein Ladder; Lane 2 – Extracellular medium; Lane 3 - Ultrafiltrate; Lane 4 – Eluted fractions collected from anion exchange chromatography in 50 mM Tris-HCl buffer and 75 mM NaCl. Yellow arrow indicates glycosylated L-ASNase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

samples collected from the anionic chromatographic step. In the end, we were able to obtain a purified glycosylated L-ASNase using only one chromatographic step (Fig. 3).

The L-ASNase enzymatic activity was then determined from the purified protein using the AHA method [23]. In this work, the specific activity of two types of L-ASNase was quantified: human-like glycosylated L-ASNase expressed in *P. pastoris* SuperMan5 (his$^-$) as well as native non-glycosylated L-ASNase expressed in *E. coli* BL21(DE3) (Fig. 4). Enzymatic activity was measured by the initial velocity of L-asparagine hydrolysis (μmols of L-aspartic acid β-hydroxamate produced per minute or enzyme units (U)) as a function of the amount of protein in milligrams. Our recombinant L-ASNase presented specific activity of approximately 2.98 ± 0.12 U/mg which represents 3.35 % of the enzymatic activity of non-glycosylated L-ASNase (88.94 ± 1.82 U/mg). So, glycosylation in the five Asn residues as observed by mass spectrometry analysis impaired the L-ASNase activity.

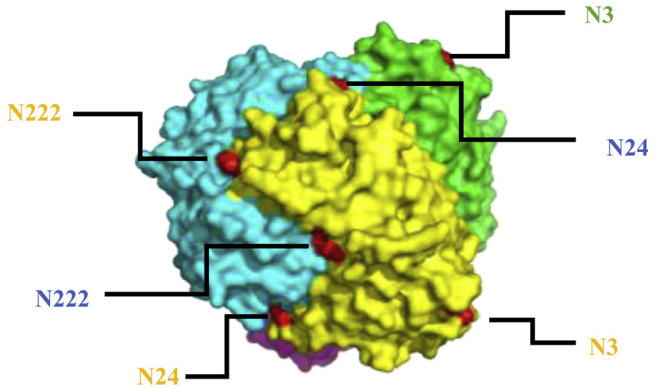

Fig. 5. Structure of L-ASNase from *E.coli*. Each chain is represented by a different color: chain A in green, chain B in light blue, chain C in purple and chain D in yellow. Red surfaces indicate exposed asparagine residues located within the N-glycosylation consensus sequence. Asparagine residues N151 and N164 are not shown because these residues are not exposed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Recovery of L-ASNase enzymatic activity by site-directed mutagenesis

L-ASNase from *E. coli* is a tetrameric enzyme composed of two intimate pairs of identical subunits (Fig. 5). The formation of this homotetramer is required for L-ASNase biological activity [24]. Since L-ASNase can only act as a homotetramer, we postulated that glycosylation of critical residues located at the interface of these subunits could significantly impair protein stability and that consequent removal of some of the N-glycosylation consensus sequences could restore the native L-ASNase enzymatic activity by preventing the addition of glycan moieties at these critical regions (Supplementary Fig. 5).

Therefore, each of the asparagine residues from the five N-glycosylation consensus sequences of the enzyme was individually mutated to glutamine, resulting in five L-ASNase variants (N3Q, N24Q, N151Q, N164Q and N222Q). Enzymatic activity of intracellular, extracellular and periplasmic content of *P. pastoris* SuperMan5 (his-) strain harboring each individual mutation, indicated no substantial increase in L-ASNase enzymatic activity (Fig. 6A, C and E). We then decided to remove one additional glycosylation site by creating a novel double mutant enzyme (N151Q and N164Q). Sites N151 and N164 were chosen since they are critical residues located at the interface between two L-asparaginase monomers (Fig. 5) and would likely be involved in protein oligomerization. However, no increase in activity was observed (Results are not shown).

Finally, a triple mutated variant (3M) was created. For this purpose, the amino acids residues N24, N151 and N164 were mutated to glutamine. Residue N24 was chosen because it is one well known to be

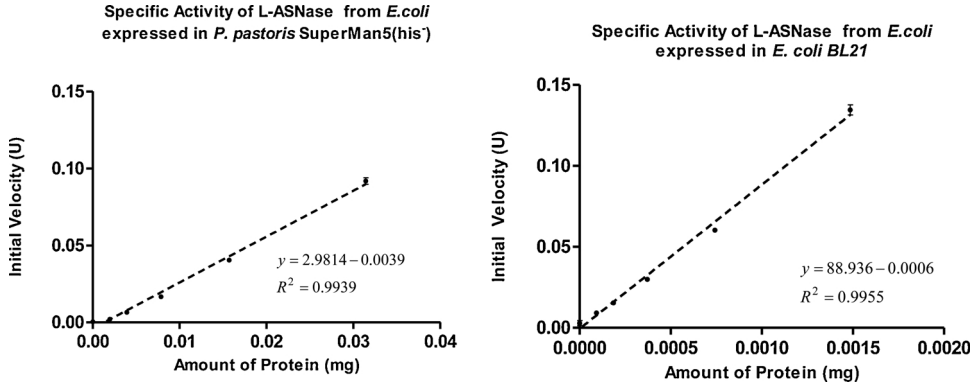

Fig. 4. Enzymatic activity of recombinant L-ASNase expressed in *P. pastoris* SuperMan5(his-) and recombinant L-ASNase expressed in *E. coli* BL21.

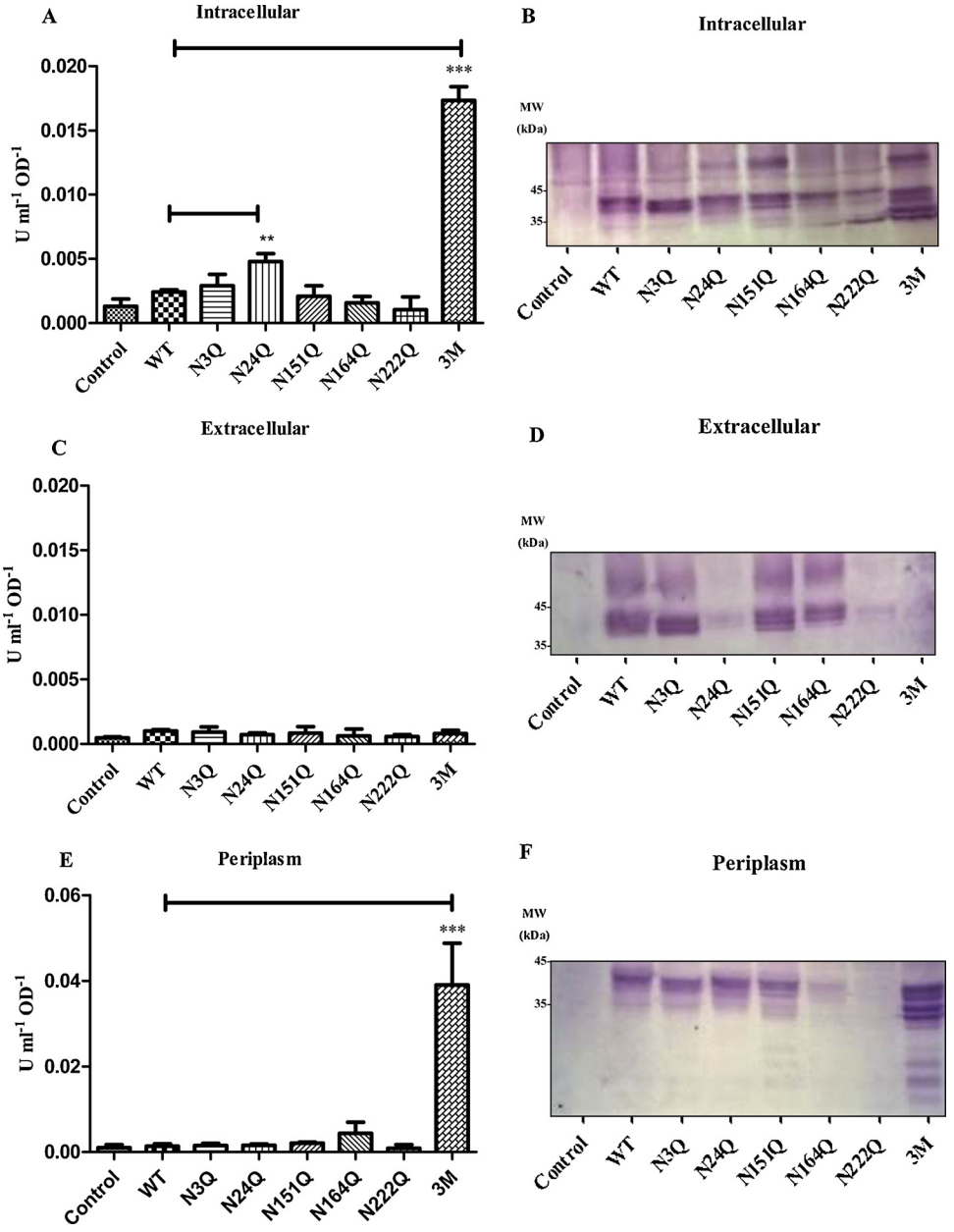

Fig. 6. Analysis of the influence of the removal of N-glycosylation consensus sequence on the biological activity and secretion of L-ASNase mutants. **A** Intracellular enzymatic activity of recombinant L-ASNase variants. **B** Western-blotting analysis of intracellular expression of recombinant L-ASNase variants. **C** Extracellular enzymatic activity of recombinant L-ASNase variants **D** Western-blotting analysis of extracellular expression of recombinant L-ASNase variants. **E** Periplasmic enzymatic activity of recombinant L-ASNase variants. **F** Western-blotting analysis of periplasmic presence of recombinant L-ASNase variants. U ml^{-1} OD^{-1} represents the amount of L-ASNase able to produce 1 μmol L-aspartic acid β-hydroxamate per minute per ml per OD of reaction. (**, $P < 0.01$; ***, $P < 0.001$, One way ANOVA with Dunnett post test compared with WT L-ASNase; n = 3 replicates; error bars represent standard deviation).

involved in L-ASNase stabilization and enzymatic activity [40,41], whereas N3 and N222 are residues located on the protein surface and would likely no interfere with L-ASNase enzymatic activity. All mutants were produced by site-directed mutagenesis and integrated in *P. pastoris* SuperMan5(his-) genome strain. Enzymatic assay of intracellular content of *P. pastoris* strains harboring the 3M gene variant revealed a substantial increase in its enzymatic activity compared to all remaining proteoforms (Fig. 6A). Enzymatic activity of 0.017 U ml^{-1} OD^{-1} was obtained with the 3M variant, which corresponds to a 7-fold increase relative to the wild-type (WT) form. The increase in enzymatic activity was solely caused by the novel glycosylation profile present on the enzyme and not by the mutations themselves, since recombinant non-glycosylated 3M variant expressed in *E.coli* BL21 has shown a significant decrease of 25 % in specific enzymatic activity when compared to the WT proteoform (Supplementary Fig. 6). The N24Q proteoform has also exhibited an increase in enzymatic activity (2-fold higher than the WT), whereas the remaining mutants (N3Q, N151Q, N164Q and N222Q) did not show significant changes in biological activity. Similar results were obtained when specific activity of each single mutant variant was quantified after purification (Supplementary Fig. 7).

Immunoblots of intracellular contents showed synthesis of all glycosylated L-ASNase mutants at varying expression levels. Small variations in size were observed for proteoforms N24Q and 3M compared to WT. In fact, removal of glycosylation site N24 resulted in a decrease in size whereas the removal of the three asparagine residues in the 3M L-ASNase variant resulted in both decrease in protein size and formation of multiple glycoforms (Fig. 6B).

Mutations on amino acids residues N24, N151, N164 had a detrimental effect on the secretion of 3M. Differently from the other mutants, the triple mutant variant was the only proteoform not detected on the extracellular medium (Fig. 6D). Besides, no enzymatic activity was detected in the extracellular medium (Fig. 6C). Although both the WT and the single mutant variants (N3Q, N24Q, N151Q, N164Q and N222Q) were successfully secreted from the cell, their low enzymatic activity impaired us to detect them by our standard enzymatic assays.

3.6. Triple mutant L-ASNase is transported to and retained in the periplasm

Next, we sought to investigate whether the 3M variant would still be able to reach the yeast periplasm through the aid of the *S. cerevisiae* α-MF cloned in-frame with the respective gene. We proceeded to the extraction of periplasmic L-ASNase by repeated freeze-thaw cycles of yeast cells. Finally, the biological activity of the different extracted L-ASNase was quantified by our standard enzymatic assay (Fig. 6E).

Compared to the intracellular content, similar results were obtained for periplasmic extracts. L-ASNase was expressed and transported to the periplasm at varying concentrations and glycan profiles (Fig. 6F). The 3M L-ASNase gene variant was the only proteoform with detectable enzymatic activity: 0.039 U ml^{-1} OD^{-1}. The value represents an increase of 28-fold of periplasmic enzymatic activity compared to the WT variant (Fig. 6E).

3.7. Stability of glycosylated L-ASNases in human serum

Glycosylation plays a very important role in the stability of proteins, which is especially interesting for proteins intended for clinical purposes. Many approved biopharmaceuticals have successfully been stabilized by glycosylation [25], which prevents, for example, proteolytic degradation [26], aggregation [27] as well as thermal denaturation [28].

To test whether glycosylated L-ASNases are biologically more stable than aglycosylated L-ASNase, we decided to measure changes in L-ASNase enzymatic activity after incubation with human serum (Fig. 7). This *in vitro* assay would mimic the physiological conditions encountered by the protein upon clinical administration and would provide some evidence of its biological stability.

After 168 h of incubation (*i.e.* 7 days), recombinant WT L-ASNase expressed in BL21 has shown a significant 34 % drop in enzymatic activity compared to the initial measurements at time zero. By contrast, both WT and 3M glycosylated L-ASNases have not shown a decrease in enzymatic activity after 168 h of incubation. Glycosylated WT L-ASNase presented an increase of approximately 75 % in enzymatic activity whereas the 3M variant has shown an increase of approximately 18 % relative to the initial measurements at time zero.

3.8. Recognition of glycosylated L-ASNase by anti-L-ASNase antibody

Clinical administration of L-ASNase from *E. coli* has been associated with severe toxicities. Hypersensitivity, for example, is the most common reason for asparaginase therapy discontinuation, in which

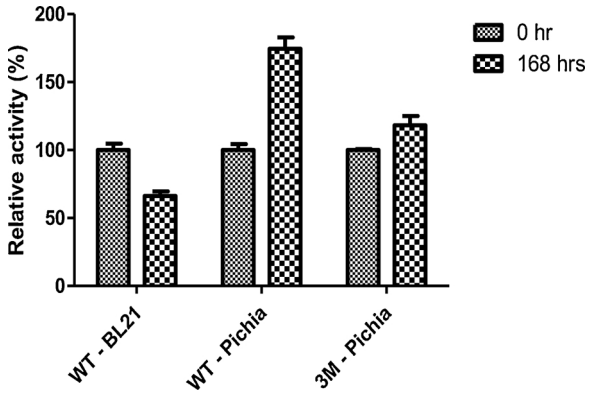

Fig. 7. *In vitro* stability analysis of L-ASNase on human serum. Samples were diluted in human serum and incubated at 37 °C. Enzymatic activity of each sample was measured at times 0 and 168 h. Values are given in relative enzymatic activity (%). Each protein enzymatic activity (U/ml) was normalized to 100 % at time zero. (n = 3 replicates for WT BL21 and n = 2 for WT and 3M *Pichia*; error bars represent standard deviation).

Fig. 8. Analysis of L-ASNase recognition by anti-L-ASNase antibodies through ELISA. 10 ng of aglycosylated L-ASNase expressed in *E.coli* BL21 (WT – BL21) and different concentrations of glycosylated L-ASNase expressed in *P. pastoris* SuperMan5 (his-) (WT – *Pichia*) were incubated with antibody anti-L-ASNase for 90 min (n = 3 replicates; error bars represent standard deviation).

patients are further required to switch from *E.coli* to *E. chrysanthemi* preparations [29].

The L-ASNase antigenicity is intrinsically related to the bacterial origin of the drug. L-ASNase epitopes are capable of activating the immune system and increasing the titer of antibodies against the molecule. This increase in antibody production is then followed by hypersensitivity reactions and a decrease in L-ASNase enzymatic activity [30]. We postulated that glycosylation could mask L-ASNase epitopes and, in order to confirm this hypothesis, we incubated WT L-ASNase (both glycosylated and aglycosylated) with anti-L-ASNase antibody (Fig. 8).

Different concentrations of glycosylated L-ASNase were incubated with anti-L-ASNase antibody on ELISA plates. After incubation, we observed that 100 ng of glycosylated L-ASNase were necessary to capture the same amount of anti-L-ASNase antibody than 10 ng of L-ASNase produced in *E.coli* BL21. In fact, a significant mean absorbance decrease of 85 % was observed for the glycosylated L-ASNase relative to aglycosylated L-ASNase when the same amount of both proteins (10 ng) was incubated with the same antibody. This result suggests that incorporation of glycans onto L-ASNase was capable of masking important protein epitopes involved in antibody recognition.

4. Discussion

Although a considerable number of biopharmaceutical products in the market are glycosylated, the possibility of expressing clinically-relevant glycosylated prokaryotic proteins has not yet been thoroughly investigated. In this work, we demonstrated the heterologous expression of several variants of L-ASNase in a glycoengineered *Pichia pastoris* strain. The WT L-ASNase proteoform was efficiently expressed, human-like glycosylated and secreted by the *Pichia pastoris* SuperMan5 (his-) strain, whereas a triple mutant L-ASNase variant showed increased biological activity, which demonstrates their potential to be used as novel biopharmaceutical products for the treatment of ALL. Expression of L-ASNase in *Pichia pastoris* has already been briefly described by Sajitha et al. [31], but information assessing either the L-ASNase specific activity or its glycosylation profile was not present.

Furthermore, recombinant L-ASNase glycosylation profile was successfully characterized. A noticeable glycosylation profile difference between both *Pichia pastoris* strains utilized in this work was confirmed. L-ASNase expressed in SuperMan5 (his^-) strain was homogenously glycosylated and contained predominantly $Man_5GlcNAc_2$ glycan residues in all its five potential glycosylation sites. On the other hand, L-ASNase expressed in GS115 strain presented high glycosylation heterogeneity as evidenced by smearing on SDS-PAGE analysis. Expression of other clinical relevant proteins by glycoengineered *Pichia pastoris* has also displayed a similar glycan profiling. Murine GM-CSF (mGM-CSF) has been successfully expressed containing $Man_5GlcNAc_2$ glycan

residues. The glycosylation has maintained mGM-CSF as much biologically active as the ones produced in *E. coli* [32]. Likewise, production of a functional monoclonal antibody in a glycoengineered yeast with human-like glycosylation demonstrated similar protein motility on SDS-PAGE, comparable size exclusion chromatogram profiles and same binding affinity when compared to antibodies produced in CHO cells [33].

Nonetheless, glycosylation has significantly impaired the enzymatic activity of the recombinant L-ASNase. The specific activity of the purified biopharmaceutical was found to be 3 U/mg, almost 30-fold lower than the value observed for its native counterpart (89 U/mg). Commercial L-ASNases have reported even higher specific activities when measured by Nessler's reagent. According to Merck & CO., Inc., ELSPAR® L-ASNase presents at least 225 U/mg protein per vial of the final biopharmaceutical formulation [34] whereas Oncaspar®, the PE-Gylated form, has a specific activity of at least 85 U/mg [35].

The effect of N-glycosylation on protein properties has been extensively studied. Besides contributing to protein folding, secretion, binding and substrate affinity, glycosylation plays a fundamental role in enzymatic activity [36]. Studies have shown that removal of individual N-glycans from protein structure can result in either reduction of its enzymatic activity or a significant increase in catalytic activity, depending on the protein being studied [37]. Removal of glycosylation sites through site-directed mutagenesis from human protein C (HPC), for instance, resulted in 2–3 fold increase in anticoagulant activity [38], which demonstrates that glycosylation, in this case, negatively affected protein function, in agreement with the results observed here for L-ASNase. Additionally, the presence of oligosaccharide chains on RNase B was responsible for diminishing the activity of the enzyme by preventing the oligomerization process to occur. This hindrance, however, was not observed in RNase A, the non-glycosylated variant of RNase B [39]. An oligomeric state analysis of recombinant L-ASNase by size exclusion chromatography (Supplementary Fig. 5) indicated the presence of both the active tetramer as well as the protein in its monomeric form, demonstrating the influence of N-glycosylation on protein oligomerization.

In fact, L-ASNase contains two asparagine residues from its glycosylation consensus sequences that are not exposed on the surface of the protein (N151 and N164). This feature could affect protein oligomerization and, consequently, could negatively affect L-ASNase biological activity (Fig. 5). Therefore, we believe that removal of glycans at these sites, as well as N24, an important residue involved in L-ASNase stabilization and enzymatic activity [40,41], was responsible for allowing the protein to restore its natural quaternary structure, resulting in an increase of L-ASNase biological activity to significant levels. When measured intracellularly, L-ASNase enzymatic activity was 7-fold higher than WT form and such increment was not observed in any of the single mutant variants. Since glycosylated WT L-ASNase has shown about 3.35 % of enzymatic activity relative to aglycosylated L-ASNase expressed in *E. coli* BL21, and that intracellular 3M L-ASNase has increased its enzymatic activity by 7-fold relative to WT, it is possible to conclude that the 3M variant presented about 24 % of the activity of WT L-ASNase expressed in *E. coli* (if we assume that WT and 3M L-ASNase concentrations from non-purified lysates were equal). It is noteworthy to mention that PEGylation of L-ASNase results in similar loss of activity [35].

On the other hand, removal of the number of glycans in the L-ASNase structure affected its ability to be secreted to the extracellular medium. L-ASNase enzymatic activity was only possible to be detected in the intracellular compartment of the cell, primarily at the periplasmic space. Through repetitive freeze-thaw cycles, we were able to extract and analyze the different L-ASNase variants that accumulated into the yeast's periplasmic space, following an adapted protocol from Ferrara et. al. This research group used a similar protocol to extract L-ASNase from *Saccharomyces cerevisiae* produced by *P. pastoris* yeast cells and obtained yields of 85 % when using six freeze-thaw cycles followed by potassium phosphate extraction [18]. We then showed that transport of the 3M L-ASNase mutant to the periplasm was carried out by the *S. cerevisiae* αMF signal sequence, although lack of sufficient glycan residues onto its structure prevented complete secretion to the extracellular medium. Nonetheless, mutation of L-ASNase N-glycosylation sites was still capable of modulating L-ASNase enzymatic activity and served as a means to understand and control L-ASNase biochemical properties. Looking forward, we expect that randomized combinations of glycosylation sites on L-ASNase could create mutant variants with enhanced extracellular activity.

Despite the decreases in enzymatic activity from both WT and 3M glycosylated L-ASNases (3 % and 24 % relative to aglycosylated L-ASNase enzymatic activity, respectively), both enzymes have shown a significant increase in stability after incubation with human serum. The enzymes were not only able to preserve their enzymatic activity after 168 h of incubation, but also to present higher values when compared to the enzymatic activity measured at time zero. The increase in enzymatic activity could be linked to the presence of osmolytes in the serum, which has already been shown to increase the stability and the specific activity of a L-ASNase from *E. chrysanthemi* [42]. Furthermore, we believe that the addition of several glycan moieties to the enzymes was responsible for this increase in stability and that this could have a positive impact on the pharmacokinetic profile of the drug. Chemical instability, such as proteolytic degradation, oxidation and chemical crosslinking, as well as physical instability, such as precipitation, thermal denaturation and aggregation, could easily be prevented by glycosylation, as this have already been evidenced in other proteins [25]. Glycosylated granulocyte-colony stimulating factor (G-CSF), for instance, was found to be less susceptible to elastase degradation than non-glycosylated G-CSF expressed in *E. coli*. In that case, the susceptibility to proteolytic degradation was correlated to a decrease in biological activity [43].

Finally, antigenicity of glycosylated WT L-ASNase appeared to be lower compared to aglycosylated L-ASNase, as this could be explained by masking of critical epitopes due to glycosylation. In PEGylation, for example, the same phenomena occurs: attachment of low-molecular-weight monomethoxypoly(ethylene glycol)(mPEG) to insulin has substantially decreased the protein's immunogenicity through sterically masking of antigenic determinants, which in turn, could increase the protein's plasma half-life [44]. Similar results were obtained using our ELISA method, in which a 10-fold increase in the amount of glycosylated L-ASNase relative to aglycosylated L-ASNase was necessary to be recognized by the same antibody titer.

5. Conclusion

In conclusion, the results indicated that the glycoengineered yeast strain is capable of expressing and secreting an active L-ASNase with human-like glycosylation. Even though glycosylation has had a detrimental effect on the L-ASNase enzymatic activity, the modulation of glycosylation sites through site-directed mutagenesis was capable of restoring L-ASNase enzymatic activity to significant values. In addition, both WT and 3M L-ASNases presented increased stability in human serum whereas WT L-ASNase has shown lower *in vitro* immunogenicity compared to aglycosylated L-ASNase. Expression of our prokaryotic protein in an engineered yeast strain as well as removal of glycosylated sites through site-directed mutagenesis allowed us to comprehend the different N-glycosylation effects on biological activity and secretion of L-ASNase, which could then be used as a strategy to design potential alternative L-ASNases to treat ALL.

Author contributions section

Contributions: GM and GP designed the research. GML and BE designed and performed experiments, from the construction of the expression vector to obtainment of P. pastoris transformed strains. GML

purified proteins, evaluated activity and produced / analyzed L-ASNase mutants. GM, BE and VF designed and performed LC/MS experiments and analyzed the data. HPB performed ELISA and serum stability assays. GM, AP and GP provided financial support and coordinated the research group. All authors contributed to write, review and edit the manuscript.

Declaration of Competing Interest

The authors declare no competing financial interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.bej.2020.107516.

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