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Characterization of a recombinant N-acetylgalactosamine-6-sulfate sulfatase produced in *E. coli* for enzyme replacement therapy of Morquio A disease

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ABSTRACT

Mucopolysaccharidosis IVA (MPS IVA) is a lysosomal storage disease caused by the deficiency of N-acetylgalactosamine-6-sulfate sulfatase (GALNS) enzyme. Currently, specific therapies are not available for MPS IVA patients. In this study, a biologically active recombinant GALNS enzyme (rGALNS) produced in *Escherichia coli* was purified through a two-step chromatography process. The effect of temperature and pH on purified rGALNS stability was evaluated, as well as the stability in human serum. Finally, the uptake of rGALNS by HEK 293 cells and MPS IVA fibroblasts was evaluated. The use of a semi-continuous process allowed the production of an active extracellular rGALNS, which was used for protein purification. The purified rGALNS showed a specific activity of 0.29 U mg^{-1} and a production yield of 0.78 mg L^{-1}. The rGALNS presented an optimal pH of 5.5 and was stable for 8 days at 4 °C. In human serum it was stable for up to 6 h. rGALNS was not taken up by the cultured cells, suggesting that N-linked oligosaccharides are not necessary for the production of an active enzyme or enzyme stability but for the cell uptake of protein. This study shows the first characterization of rGALNS produced by *E. coli*, and provides important information about purification, stability, and glycosylations effect for this type of enzymes.

1. Introduction

Mucopolysaccharidosis IV A (MPS IV A, Morquio A disease, OMIM #253000) is an autosomal recessive disease caused by the deficiency of the lysosomal enzyme N-acetyl-galactosamine-6-sulfate sulfatase (GALNS, EC 3.1.6.4) [1]. Deficiency of GALNS prevents the normal degradation of keratan- and chondroitin-6-sulfate causing their lysosomal accumulation [1,2]. Clinical features of MPS IV A patients include marked short stature, hypoplasia of the odontoid process, pectus carinatum, kyphoscoliosis, genu valgum, laxity of joints, and corneal clouding, without central nervous system impairment [1,3]. Life span is variable and depends on the spectrum of disease severity, but most patients die between the second and third decade of life [1,3,4].

At present, there is not specific treatment for this disease, and it is limited to supportive measures and surgical interventions to alleviate some manifestations of the disease [1,3]. Although bone marrow transplantation is the best treatment option for some mucopolysaccharidosis, it has a limited benefit in MPS IV A patients besides the risk of fatal complications [5]. Gene therapy, widely explored in animal models for almost all mucopolysaccharidosis types [6], has shown to be a feasible alternative for the treatment of Morquio A disease [7–9], albeit it is still in early stages.

Currently, the main treatment option for lysosomal storage disorders is the enzyme replacement therapy (ERT). Gaucher, Fabry, Hurler (MPS I), Hunter (MPS II), Maroteaux–Lamy (MPS VI), and Pompe diseases have approved ERT, and it has shown an improvement of some systemic manifestations of diseases [10]. In the case of ERT for MPS IVA, preclinical trials of ERT using recombinant enzymes have shown significant decrease of KS in blood and tissues [11,12] or in cultured cells [13].

Human GALNS is a homodimeric protein of 522 amino acids, composed of 60 kDa monomers processed to peptides with of 40 and 15 kDa [14,15]. Native GALNS has been purified from liver [16] and placenta [17], and produced in recombinant Chinese hamster

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ovary (CHO) cells [13,15,18]. Regardless of the source, GALNS is stable at 4 °C with an optimum pH between 3.5 and 5.5, and an isoelectric point between 5.5 and 6.5 [15,18,19].

We have reported the production of active lysosomal enzymes in *Pichia pastoris* and *Escherichia coli* [20–23]. Recently, we reported the production of rGALNS in *E. coli* BL21, suggesting that glycosylations are not necessary for the production of the active enzyme, and that bacterial formylglycine-generating enzyme can activate human sulfatases [23]. In addition, specific activity was similar to that reported for rGALNS produced in CHO cells [23]. In this study, we report the purification of rGALNS largely secreted by *E. coli* BL21. The effect of temperature and pH on rGALNS stability was evaluated, as well as the enzyme uptake by HEK 293 cells and MPS IVA fibroblasts. The results obtained show the first evidence for the purification of human rGALNS produced in *E. coli*, providing important information for these proteins toward the development of an ERT.

2. Materials and methods

2.1. Microorganism and culture conditions

The rGALNS enzyme was produced by the genetically modified strain *E. coli* BL21/pGEX-GALNS, cultured in a modified minimal growth medium with 20 g L^{-1} glucose previously described [23]. The cultures were conducted in a Bioflo 110 reactor (New Brunswick Scientific, Co., Inc., USA) with a working volume of 3 L, and agitated with a marine stirrer, under semi-continuous conditions. The semi-continuous process had two stages: (i) a 12 h batch culture conducted under previously described conditions [23], followed by (ii) a 24 h of a continuous medium replacement stage, under the same agitation, temperature, and aeration conditions of the batch stage. The medium replacement was done using a Pellicon XL Filter Module Durapore 0.45 μm 50 cm^2 (Millipore, Billerica, MA, USA), which allowed recirculating the biomass while removing the exhausted medium. Total culture volume was kept constant by using the same rates of medium removal and feed. After the semi-continuous stage was finished, rGALNS expression was induced with 1.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) during 6 h at 200 rpm and 18 °C.

2.2. Crude protein extract and protein purification

Under the described culture conditions the enzyme activity was detected both intra- and extra-cellularly. However, culture medium after induction was selected for rGALNS purification because of the ease to carry out the downstream process compared to the use of the cell lysate. Culture medium was filtered sequentially through paper Whatman No. 1, and 0.45 and 0.22 μm polyether sulphone membranes (Pall Corp, Port Washington, NY, USA). Permeate pH was adjusted to 5.5 with acetic acid, and ultra-filtered through a 30 kDa cut-off membrane (Millipore, Billerica, MA, USA) up to reach a final volume of 20 mL. Finally, the retentate was dialyzed overnight at 4 °C against 25 mM sodium acetate buffer (pH 5.5) with constant stirring. The dialyzed retentate (hereafter named crude protein extract) was stored at 4 °C until used for purification.

The rGALNS was purified from the crude protein extract by anion exchange chromatography using a Source Q-Sepharose™ Fast Flow column (Amersham Biosciences, Piscataway, NJ, USA) equilibrated with 25 mM Tris–HCl, pH 7.0, and eluted with a linear gradient of 0–0.5 M NaCl in 25 mM Tris–HCl, pH 7.0. The fractions with GALNS activity were pooled and applied to a Sephacryl™ S-200 High Resolution column (Amersham Biosciences) equilibrated with 25 mM Tris–HCl pH 7.0/10 mM NaCl. The column was eluted at 0.5 mL min^{-1} using the equilibration buffer. Finally, fractions with the highest GALNS activity were pooled and lyophilized at 0.28 mbar and −80 °C for 5 h in a Freezone 12.0 lyophilizer (Labconco, Kansas City, MO, USA). The freeze-dried sample was reconstituted in 1 mL of 25 mM phosphate buffer pH 5.5, and stored at −20 °C until use. Protein purification was monitored by SDS–PAGE under reducing conditions and GALNS enzyme activity. The production yield was calculated taking in to account the initial (800 mL) and final volumes (1.5 mL), and the final protein concentration (0.42 mg mL^{-1}).

2.3. GALNS enzyme activity

GALNS activity was assayed by using 4-methylumbelliferyl-β-D-galactopyranoside-6-sulfate (Toronto Chemicals Research, North York, ON, Canada) as substrate [24]. One unit (U) was defined as the amount of enzyme hydrolyzing 1 nmol of substrate per hour, and specific GALNS activity was expressed as U mg^{-1} of total protein determined by Bradford assay (BioRad, Hercules, CA, USA).

2.4. rGALNS characterization

2.4.1. Temperature effect

To evaluate the effect of temperature on rGALNS stability independent assays were conducted. In each trial, 20 μL of purified enzyme were incubated with 20 μL of 50 mM sodium acetate pH 5.5 at 4, 37, and 45 °C for 1, 2, 4, 6, 12, 96, 120, and 192 h. After incubation, enzyme activity and protein measurement were carried out. Each assay was done in triplicate.

2.4.2. pH effect

To evaluate the effect of pH on rGALNS stability independent assays were conducted. For each trial, 20 μL of the purified enzyme were incubated with 20 μL of 50 mM sodium citrate pH 3.0, or 50 mM sodium acetate pH 4.0, 5.0, 5.5, or 6.0, or 50 mM Tris–HCl pH 7.0 or 8.0, respectively. Samples were incubated at 37 °C for 1 h. After incubation enzyme activity and protein measurement were carried out. Each assay was done in triplicate.

2.4.3. Stability in human serum

To assess the stability of the rGALNS in human serum 20 μL of the purified enzyme were incubated for 6 h at 37 °C with 20 μL of pooled human serum obtained from healthy donors. After incubation enzyme activity and protein quantitation were carried out as previously described. Each assay was done in triplicate.

2.5. In vitro evaluation of recombinant enzyme GALNS

The ability of cells to take up the rGALNS was assessed in individual cultures of HEK 293 (ATCC CRL1573) and MPS IVA human skin fibroblasts. Cells were cultivated in Dulbecco's modified medium (DMEM, Gibco, Carlsbad, CA), supplemented with fetal bovine serum 15%, penicillin 100 U mL^{-1} and streptomycin 100 U mL^{-1}, at 37 °C in a CO_2 incubator. Twenty-four hours before the experiment, 1×10^5 cells per well were seeded in 12-well plates, and the culture medium was replaced with fresh medium 2 h before the experiment. The purified enzyme was added to 500 μL of culture medium at a final concentration of 10, 50 or 100 nM. After 5 h of incubation at 37 °C in a CO_2 incubator, culture medium was removed, and the cells were washed three times with cold PBS 1× (composition per liter: 8 g NaCl, 0.2 g KCl, 1.44 g NaH_2PO_4, 0.24 g KH_2PO_4, pH 7.2), trypsinized, and resuspended in lysis buffer (25 mM sodium acetate, 1% Triton X-100 1%, 1 mM β-glycerolphosphate, pH 5.5). Cells were lysed by three freeze/thaw cycles, and clarified by centrifugation at 3000 × *g* and 4 °C for 20 min. The enzyme activity was determined in the cell lysate and the culture medium as described above.

2.6. Computational analysis

Prediction of N-glycosylation sites was done by using the NetNGlyc 1.0 Server at Expasy Proteomics Server (http://www.expasy.ch/tools/). The sequence of the human GALNS enzyme available at GenBank (Accession Number NP_000503.1) was used for prediction. The GALNS subunits were established according to a previous report [14].

3. Results and discussion

3.1. Production of rGALNS in E. coli BL21

Previously, we reported the production of an active rGALNS enzyme produced in *E. coli* BL21, under batch culture conditions [23]. To increase the enzyme production, it was implemented a semi-continuous culture strategy. From three independent cultures it was obtained an intracellular rGALNS activity of 0.05 ± 0.01 U mg^{-1}, which was about 5-fold higher than that obtained under batch culture conditions. However, unlike previously reported [23], extracellular rGALNS activity was detected, which ranged from 2.95 ± 0.488 to 6.18 ± 1.16 U mg^{-1}. The highest activities were observed after 4 h of induction. The rGALNS activity was not detected in control cultures using a transformed *E. coli* BL21 strain with the plasmid without GALNS cDNA. Since the extracellular enzyme activity was higher than the intracellular activity and the facility to conduct the protein purification from culture medium rather than from cell lysate, rGALNS was purified from the culture medium obtained after 4 h of induction.

E. coli is one of the most used microorganism for producing recombinant proteins, and traditionally it has been considered as a non-secretory protein system [25]. However, several reports have shown that secretion of recombinant proteins may occur in commonly used laboratory strains through non-specific mechanisms

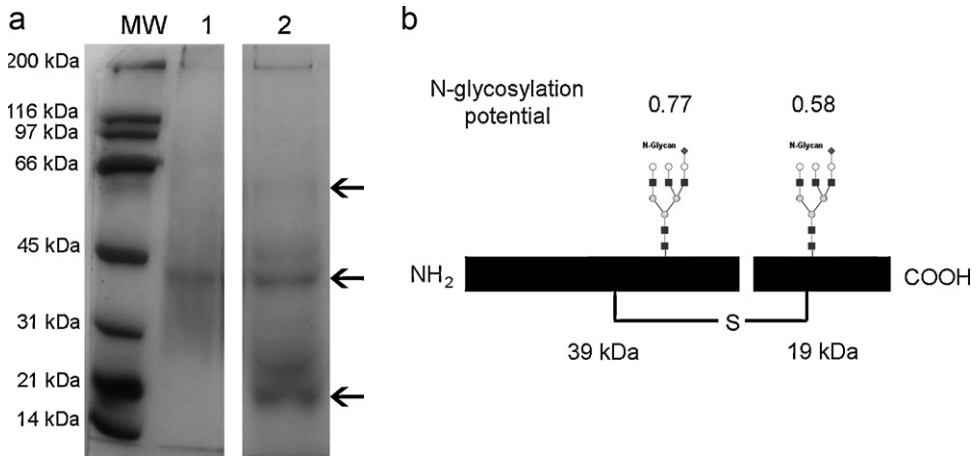

Fig. 1. SDS–PAGE analysis and prediction of N-linked oligosaccharides sites. (a) The crude protein extract (line 1) and the purified rGALNS (line 2) were subjected to SDS–PAGE under reducing conditions following by silver staining. MW = molecular weight. (b) The prediction of N-glycosylation sites was carried out by using NetNGlyc 1.0. According to NetNGlyc 1.0 Server, N-glycosylation potential is the averaged output of nine neural networks, and a potential crossing the default threshold of 0.5, represents a predicted glycosylated site. Molecular weights of subunits correspond to those reported for a GALNS produced in mammal cells.

[26]. Although the mechanisms of secretion have not been completely understood, it seems that both the secretion and the level of secretion could depend on the strain and the cultivation methods [26]. In addition, a similar result was previously reported for a recombinant iduronate-2-sulfate sulfatase produced in *E. coli* [27]. Although it cannot be excluded that extracellular rGALNS found in culture medium was lysis-dependent, the obtained results suggest that there was an effect of culture conditions since extracellular rGALNS was not found in batch cultures [23]. The presence of the native signal peptide, which was not removed in the GALNS cDNA used for plasmid construction, could be also involved in the secretion mechanism of the rGALNS through one of the *E. coli* secretion systems [26]. Currently, some of these conditions are under evaluation in order to understand the processes involved in rGALNS secretion.

3.2. rGALNS purification

A preliminary result showed that no significant loss of enzyme activity (<5%) was observed for the crude protein extract in pH above 5.5 (data not shown), which was the reported pH of maximum stability for a rGALNS enzyme produced in CHO [18]. For this reason, the first stages of the purification process were carried out at pH 5.5, while the anionic chromatography purification was carried out at pH 7.0. After this process, it was obtained a purified rGALNS with a specific enzyme activity of 0.29 U mg^{-1}, which is 500,000-fold lower than reported by Tomatsu et al. [18], but 10-fold lower than reported by Dvorak-Ewell et al. [13]. However, this specific enzyme activity was higher than the previously reported with the same microorganism from soluble or inclusion bodies fractions [23].

The rGALNS production yield was 0.78 mg L^{-1} which is lower than that reported for rGALNS produced in CHO cells [15,18]. These results agree with those that report the extracellular production of recombinant human epidermal growth factor and human proinsulin by *E. coli* in which production yields of 0.8 mg L^{-1} and 1.2 mg L^{-1} were observed, respectively [28,29]. However, the obtained rGALNS production yield is higher than reported for recombinant growth hormone (0.012 mg L^{-1}) [30] and recombinant human proinsulin (0.028 mg L^{-1}) [31] produced extracellulary by *E. coli*.

A rGALNS recovery yield of 1.3% was obtained, with most of the protein loss observed during the anion-exchange chromatography step. This result could be associated with the pH used for the anion-exchange chromatography, as it is discussed below. A final 1.7-fold purification was observed, suggesting that most of the protein presented in the crude protein extract was the rGALNS. This result is in accordance with that of the SDS–PAGE (Fig. 1a), in which the presence of mainly ~37 and 19 kDa bands were observed in the crude protein extract. The SDS–PAGE analysis of the purified rGALNS showed the presence of three species of polypeptides of ~53, 37, and 19 kDa bands which agrees with reports of GALNS enzymes purified from liver [16], placenta [17], and recombinant CHO cells [13,18].

The difference in the molecular weight of this *E. coli* rGALNS and those obtained from mammalian cells might be related to the absence of N-glycosylated proteins produced by *E. coli*, as previously discussed [23]. In mammals, GALNS is post-translationally modified through cysteine-to-formylglycine modification at the active site and addition of N-linked oligosaccharides [32]. This enzyme has two potential N-glycosylation sites at N204 and N423, which are located in the 39 and 19 kDa subunits, respectively (Fig. 1b). However, N204 seems to be the most probable site of N-glycosylation, with a potential of 0.77 vs 0.58 for the N423 site (threshold = 0.5). These results are in accordance with those observed on the SDS–PAGE (Fig. 1a), and in a previous Western-Blot assay [23]. The results showed a reduction in the molecular weight of the major subunit, in comparison with that observed for rGALNS produced in CHO cells, while the minor subunit maintained a molecular weight of ~19 kDa. These might suggest that only one of these sites appears to be utilized, as previously reported [15,17].

3.3. rGALNS characterization

The activity of the purified rGALNS was analyzed after enzyme incubation at different pHs and temperatures, and in human serum (Fig. 2). The purified rGALNS showed a maximum activity at pH 5.5, with a sharp activity reduction at pHs either above or below 5.5 (Fig. 2a). The highest activity reduction was observed at pHs < 5.0, in which it was below 30% of the levels observed at pH 5.5, while at pH > 5.5 the enzyme activity levels were about 50%. Temperature stability showed that at 37 °C no significant loss of enzyme activity was observed during the first 12 h, after which a marked reduction was observed (Fig. 2b). A residual activity of 42% was observed after 192 h of incubation at 37 °C. At 4 °C it was not observed a lost in the enzyme activity during the 192 h of the assay. Meanwhile, at 45 °C the enzyme activity remained constant during the first 6 h after which a significant reduction was observed, obtaining a

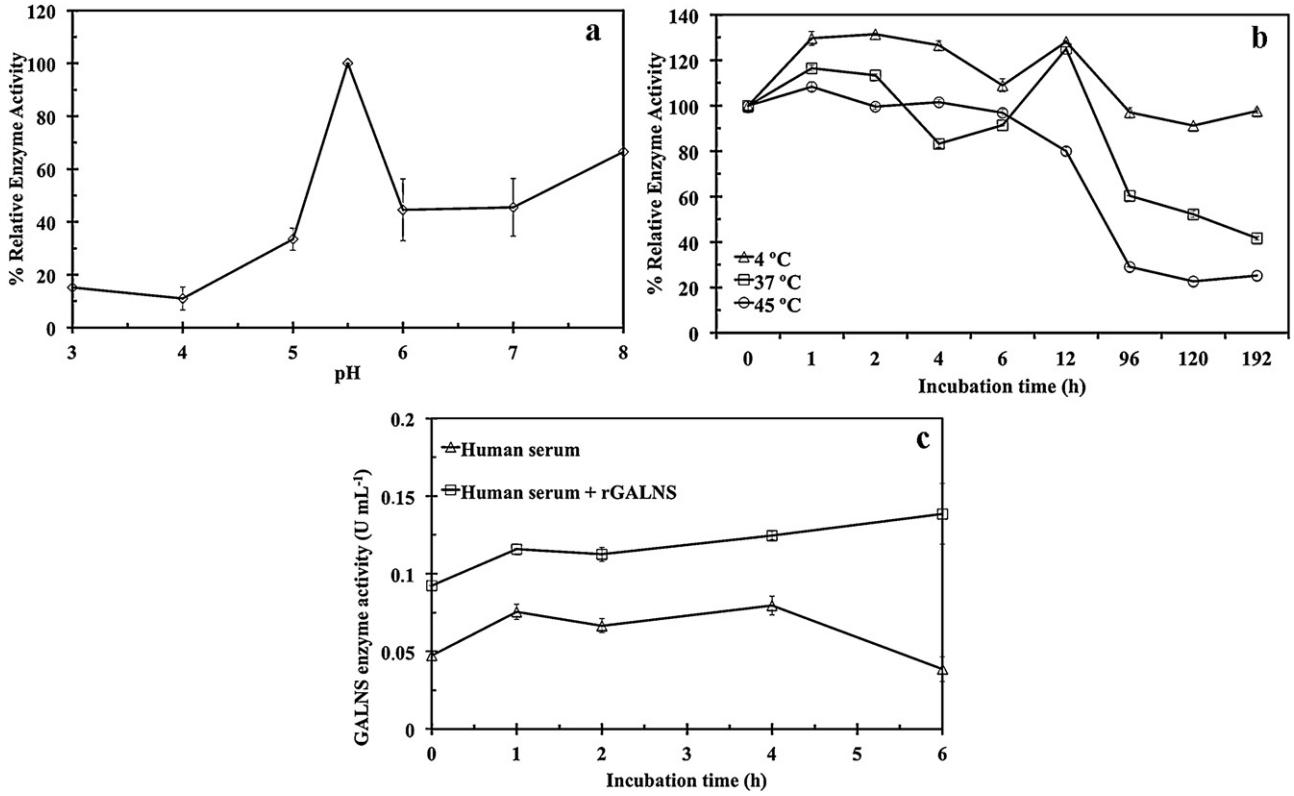

Fig. 2. Characterization of purified rGALNS. Effect of pH (a), temperature (b), and incubation in human serum (c) on enzyme activity for the purified rGALNS. The results are shown as percentage of relative enzyme activity against the activity at pH 5.5 (a) or at initial time (0 h) (b). Each assay was done in triplicate, and the results are presented as the mean ± SD.

residual activity of 25% at the end of 192 h of incubation. These results agree with those reported for rGALNS produced in CHO cells, which showed to be stable for over 24 days at 4 °C and pH 5.5, while at pH 6.5 and 7.5 the enzyme activity rapidly decreased regardless of the temperature, with a 20% lost during the first day [18]. In addition, the marked loss of enzyme activity at pH 7.0 could explain the low recovery percentage of the purified enzyme during the two-step chromatography purification.

Dvorak-Ewell et al. [13] reported that the rGALNS enzyme produced in CHO cells had an in vitro half life time of ~200 h in human serum. For this reason, it was evaluated the stability of the purified rGALNS in a pool of human serum from healthy donors. At initial time, the enzyme activity on spiked human serum was 2.0-fold higher than the levels observed in non-spiked human serum (Fig. 2c). The enzyme activity levels on spiked human serum remained relatively constant during the 6 h of evaluation, and above the levels observed in non-spiked human serum, suggesting that the rGALNS produced in *E. coli* could be stable at physiological conditions (Fig. 2c). In fact, it was observed that after 6 h of incubation, GALNS activity in non-spiked human serum showed a 48% reduction, while in the spiked human serum the activity remained constant. Tomatsu et al. [12,18] reported for a rGALNS produced by CHO cells that after an intravenous infusion into a MPS IVA knockout mouse the enzyme was cleared from circulation within the first 15–20 min post-infusion, and the enzyme was not detected after 60 min of post-infusion. Since the rGALNS produced was stable during 6 h, it is expected that this enzyme could have a half-life time that allowed its *in vivo* cell uptake. However, future studies should consider the pharmacokinetic evaluation of this enzyme.

3.4. Cell uptake of rGALNS

ERT for lysosomal storage diseases is based on the ability of the infused enzymes to be taken up by the cells, and targeted to the endosome/lysosome system. In mammals, these enzymes are post-translationally modified through the addition of N-linked oligosaccharides with terminal mannose-6-phosphate, which are recognized by M6P receptors to mediate the cell uptake [32]. Although in minor proportion, other cell membrane receptors could be used to mediate the M6P-independent cell uptake of lysosomal enzymes [32]. Previous reports of rGALNS produced in CHO cells have shown that the enzyme is largely taken up by fibroblasts and chondrocytes via M6P receptors [13,18]. To evaluate the cell uptake of the rGALNS produced in *E. coli*, the purified enzyme was added to cultures of HEK293 cells or MPS IV A skin fibroblasts. The concentration of rGALNS added to the cultures was selected based on the report of Dvorak-Ewell et al. [13] who used 1 and 10 nM of a rGALNS produced in CHO cells. Since the activity of the *E. coli* rGALNS was about 10-fold lower than that of Dvorak-Ewell et al. [13], it was decided to use 10, 50 and 100 nM of rGALNS. In cell lysates from HEK293 cells and MPS IV A fibroblasts, it was not observed an increment in GALNS enzyme activity levels above those levels observed in lysates from control cells (Fig. 3). Nevertheless, the enzyme activity in the culture medium was increased above the levels observed for the control cells. These results show that the rGALNS produced in *E. coli* cannot be taken up by cultured cells, suggesting that N-linked oligosaccharides are necessary for cell uptake but not for enzyme stability or the production of an active enzyme. In this sense, further work should be focused in

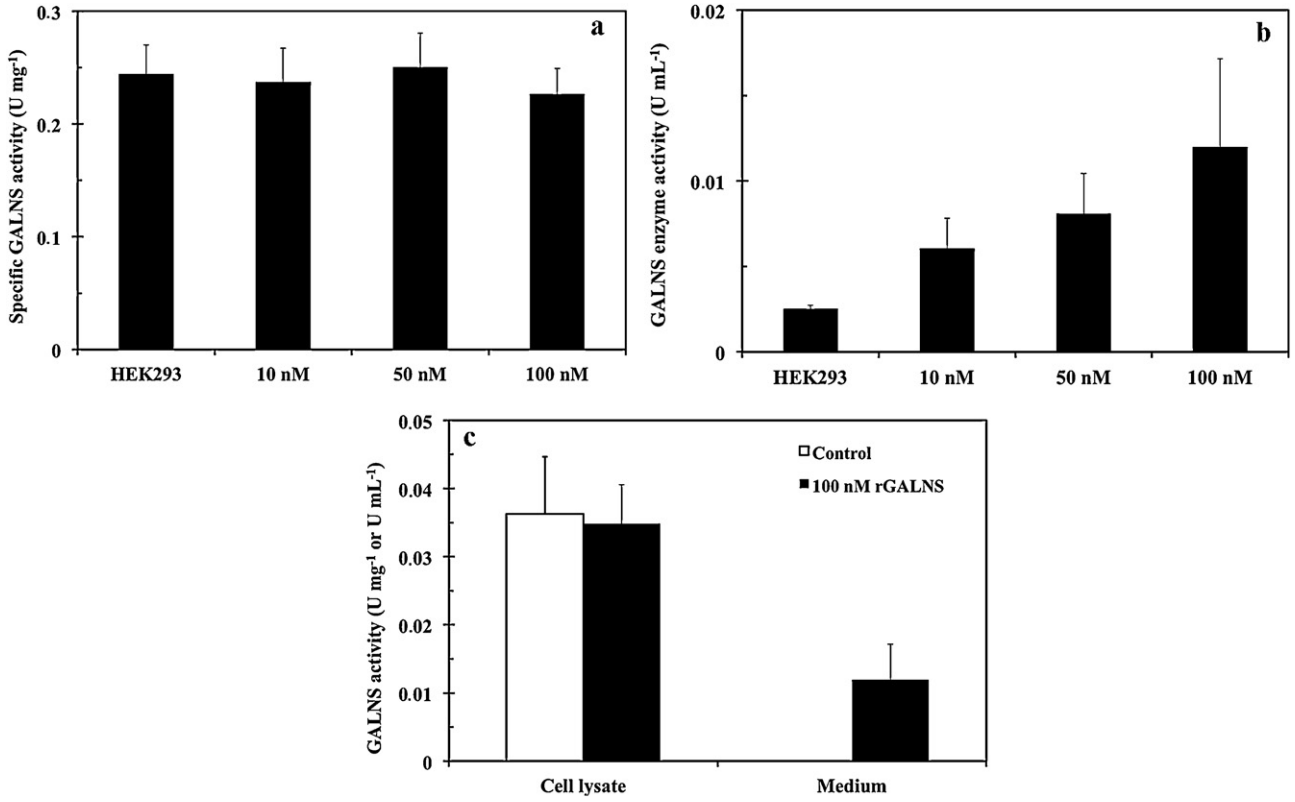

Fig. 3. Cell uptake of rGALNS. The cell uptake of the purified rGALNS was evaluated in HEK293 cells [cell lysate (a) and culture medium (b)] and MPS IVA skin fibroblasts (c). HEK293 and MPS IVA fibroblasts activities, both in cell lysate and culture media, are the enzyme activities before the addition of the rGALNS. Enzyme activity levels in cell lysate of MPS IVA fibroblasts correspond to background levels, which are >1% of normal levels. Enzyme activity was not observed in culture medium from MPS IVA fibroblasts. Each assay was done in triplicate, and the results are presented as the mean ± SD.

the evaluation of different strategies to produce an enzyme with specific N-linked oligosaccharides.

Several strategies could be explored to modify an *E. coli*-produced protein [25]. Wacker et al. [33] reported the presence of an N-glycosylation system in *Campylobacter jejuni*, which mediated the N-glycosylation of a recombinant protein when the system was overexpressed in *E. coli*. Although this study opened the possibility to produce N-glycosylated proteins in *E. coli*, the structure of the oligosaccharides consisted of a glycan composed of one amidohexose, five N-acetylhexosamines, and one trideoxydiacetamidohexose, which is completely different to that observed in eukaryotes [34]. To produce proteins with N-linked oligosaccharides similar to those observed in mammals, Schwarz et al. [35] developed a three-step method: (1) an *E. coli* overexpressing the N-glycosylation system of *C. jejuni* is used to produce a recombinant N-glycosylated protein, (2) the recombinant protein is purified and treated with exo-α-N-acetylgalactosaminidase to remove the attached glycan and produce a protein with the key GlcNAc-Asn linkage found in eukaryotic N-glycoproteins, and (3) the trimmed protein is used as substrate for an enzymatic sugar chain elongation, which mediated the modification of the protein with a natural high mannose – type glycan or a bi-antennary complex – type N-glycan. Another set of strategies available for protein modification are based on 'tag and modify' approaches [36]. These approaches involve the site-specific introduction of a tag into a protein, and then its chemo- and even region-selective modification with naturally occurring glycans. These strategies allow greater flexibility in the choice of the protein, carbohydrate, and glycosylation site. Another strategy could involve the use of polyethylene glycol (PEG) conjugates, which could allow the targeting of the enzyme, and to avoid the immune system [37].

It could be also necessary to include a tag that improve the targeting of the enzyme to bone since the main clinical obstacles for Morquio A patients are related to the bone deformity and dysplasia [3], and native GALNS produced in CHO cells has shown a limited delivery to the growth plate even in high doses [12]. Bisphosphonates, tetracyclines, polymers, and peptides, which are negatively charged, have been used for bone-targeted drug delivery, mainly through affinity to hydroxyapatite (HA) [38]. The fusion of a tissue-non-specific alkaline phosphatase enzyme with a peptide of acidic amino acid (AAA) residues allowed higher affinity of the modified enzyme for HA, substantially prolonged clearance from the bone and bone marrow [39], and a pathological improvement of bone mineralization on a hypophosphatasia mouse model infused with the modified enzyme [40]. In addition, the infusion of an AAA-ß-glucuronidase in mucopolysaccharidosis VII mice also showed that this enzyme was delivered to bone and bone marrow 4–5 times more efficiently than the unmodified enzyme [41]. Recently, it was showed that the addition of a N-terminal AAA peptide to a rGALNS enzyme, produced in CHO cells, markedly prolonged the enzyme clearance from blood after intravenous infusion into a MPS IVA murine model, and allowed a longer retention in bone and a substantial clearance of the storage materials in bone, bone marrow, and heart valves [11].

4. Conclusions

Several reports have shown the possibility of producing recombinant lysosomal sulfatases in *E. coli*. However, until now there were no reports about their purification and characterization, limiting the application of these enzymes for a possible ERT. In this work, a rGALNS produced in *E. coli* BL21 was purified by a

two-step chromatography process and characterized. We have shown for the first time that a purified rGALNS produced in *E. coli* has similar stability characteristics to those reported for GALNS enzymes produced in mammal cells. However, the enzyme produced in *E. coli* was not taken up by culture cells. These results suggest that the N-linked oligosaccharides presented in GALNS are necessary for enzyme uptake but not for enzyme stability or the production of an active enzyme.

Conflict of interest

The authors declare no conflict of interest

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