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ORIGINAL PAPER

Enzyme replacement therapy for Morquio A: an active recombinant *N*-acetylgalactosamine-6-sulfate sulfatase produced in *Escherichia coli* BL21

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Abstract Mucopolysaccharidosis IVA (MPS IVA) is an autosomal recessive disorder caused by *N*-acetylgalactosamine-6-sulfate sulfatase (GALNS) deficiency. Currently no effective therapies exist for MPS IVA. In this work, production of a recombinant GALNS enzyme (rGALNS) in *Escherichia coli* BL21 strain was studied. At shake scale, the effect of glucose concentration on microorganism growth, and microorganism culture and induction times on rGALNS production were evaluated. At bench scale, the effect of aeration and agitation on microorganism growth, and culture and induction times were evaluated. The highest enzyme activity levels at shake scale were observed in 12 h culture after 2–4 h induction. At bench scale the highest enzyme activity levels were observed after 2 h induction. rGALNS amounts in inclusion bodies fraction were up to 17-fold higher than those observed in the soluble fraction. However, the highest levels of active enzyme were found in the soluble fraction. Western blot analysis showed the presence of a 50-kDa band, in both soluble and inclusion bodies fractions. These results show for the first time the feasibility and potential of production of active rGALNS in a prokaryotic system for development of enzyme replacement therapy for MPS IVA disease.

Keywords *N*-acetylgalactosamine-6-sulfate sulfatase · Morquio A disease · *Escherichia coli* BL21 · Recombinant enzyme

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Introduction

Mucopolysaccharidosis IVA (MPS IVA, Morquio A disease; OMIM# 253000) is an autosomal recessive disorder caused by *N*-acetylgalactosamine-6-sulfate sulfatase (GALNS, EC 3.1.6.4) deficiency, leading to lysosomal accumulation of the glycosaminoglycans (GAGs) keratan sulfate (KS) and chondroitin-6-sulfate, mainly in cornea and bone [30]. Clinical manifestations vary from severe to attenuated forms, characterized by systemic skeletal dysplasia, laxity of joints, hearing loss, corneal clouding, and heart valvular disease [29]. Currently no effective therapies exist for MPS IVA, and only supportive measures and surgical interventions are used to treat some manifestations of the disease [29]. Bone marrow transplantation improves many aspects of the somatic manifestations, although it has limited impact on cardiac, eye, and skeletal abnormalities, in addition to the risk of fatal complications [55].

GALNS complementary DNA (cDNA) has been cloned and encodes a 522-amino-acid protein [48]. In mammals, posttranslational modifications are carried out by traffic from the endoplasmic reticulum (ER) to Golgi, where signal

peptide is removed and oligosaccharide chains are linked to the protein to produce a 120-kDa homodimer with monomers of 60 kDa. These homodimers are processed into polypeptides with final molecular weight of 40 and 15 kDa [6, 50]. The most important posttranslational GALNS modification, common to other sulfatases, is active-site activation by the formylglycine-generating enzyme (FGE) [11]. In mammals, FGE is an ER enzyme that catalyzes formation of a formylglycine (FGly) residue from a cysteine, necessary for sulfate removal from GAGs [11]; mutations in the catalytic-site domain of GALNS lead to null activity and severe forms of the disease [51]. However, FGE is an enzyme that is highly conserved from pro- to eukaryotes, with the exception of yeasts, in which a homologous enzyme has not been identified [23].

MPS IVA is a candidate disease feasible for enzyme replacement therapy (ERT), particularly due to the lack of central nervous system involvement [29]. Treatment of lysosomal storage diseases (LSDs) patients with ERT has shown significant clinical benefits by improvement of somatic manifestations and better quality of life [38]. ERT for MPS IVA is under development using a recombinant enzyme produced in Chinese hamster ovary (CHO) cells [50, 52]. Preclinical trials of ERT have shown significant decrease of KS in blood and tissues [52], and clinical trials are being formulated using a recombinant enzyme produced in CHO cells.

During the last two decades, production of recombinant proteins for human application has been carried out using mammalian cells, mainly CHO, baby hamster kidney, and the murine myeloma lines SP2/0 and NS0, due to their capability to express glycosylated proteins [2, 9, 27]. However, several human recombinant proteins [e.g., insulin, glucagon, β-type natriuretic peptide, tumor necrosis factor-alpha (TNF-α), and platelet-derived growth factor (PDGF)] are still being produced in bacteria such as *E. coli* or yeasts such as *P. pastoris* and *S. cerevisiae*, due to their rapid growth, high yield, and easy manipulation [3, 17, 44]. Nevertheless, to date, no studies of recombinant GALNS production have been reported using bacteria or yeasts.

In this work, we evaluated production of recombinant GALNS (rGALNS) enzyme in *E. coli* BL21. GALNS cDNA was subcloned in pGEX-3X plasmid, and its expression was induced using isopropyl-β-D-thiogalactopyranoside (IPTG). In a first stage, the effect of glucose concentration, microorganism culture, and IPTG induction times was evaluated at shake scale. In a second stage, production was carried out at bench scale, where parameters such as aeration, agitation, and IPTG induction time were investigated. The results presented in this work show for the first time the feasibility and potential of rGALNS production in *E. coli* BL21 for development of ERT for Morquio A disease.

Materials and methods

E. coli BL21/pGEX-GALNS microorganism production

Human GALNS cDNA (GenBank accession no. NM_000512.4) was obtained by *Eco*RI (Invitrogen, Carlsbad, CA, USA) digestion of pCXN-GALNS plasmid. The *Eco*RI 1.7-kb fragment was inserted into the *Eco*RI site of pGEX-3X (GE Healthcare, Piscataway, NJ, USA) to produce pGEX-3X-GALNS plasmid (6.6 kb). Ligation product was transformed in *E. coli* XL-Gold strain (Stratagene, La Jolla, CA, USA), and ampicillin-resistant clones were evaluated by correct-sense insertion of GALNS cDNA. Finally, *E. coli* BL21(D3) competent cells were transformed with pGEX-3X-GALNS plasmid to produce the BL21/pGEX-GALNS strain. All procedures were carried out using standard molecular biology methods [1].

Microorganism cultures at shake scale

Escherichia coli BL21/pGEX-GALNS microorganism was cultured in minimal growth medium (MGM) as previously described [15], with modifications [composition per liter: 13.23 g K_2HPO_4, 2.65 g KH_2PO_4, 2.04 g NaCl, 4.10 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.026 g $FeCl_3$, 0.01 g thiamine, 0.1 g ampicillin, and 2.86 ml trace-elements solution (0.022 g $AlCl_3$, 0.160 g $CoCl_6{\cdot}6H_2O$, 1.42 g $MnCl_2{\cdot}4H_2O$, 0.01 g $NiCl_2{\cdot}6H_2O$, 0.870 g $ZnSO_4{\cdot}7H_2O$, 1.44 g $CaCl_2{\cdot}2H_2O$, 0.023 g $Na_2MoO_4{\cdot}2H_2O$, 2.178 g $CuSO_4{\cdot}5H_2O$, and 0.010 g H_3BO_3); pH 7.2]. To evaluate the effect of glucose concentration on microorganism growth, 100 ml MGM supplemented with 15–40 g l^{-1} glucose was inoculated with 1.0 ml overnight-cultured microorganism in 100 ml Luria–Bertoni medium (LB, composition per liter: 10 g tryptone, 5 g yeast extract, and 5 g NaCl; pH 7.2–7.3). The culture was carried out in 500-ml shake flasks at 200 rpm and 37°C during 12 h. As a control, *E. coli* BL21(D3) strain without pGEX-3X-GALNS plasmid was cultured as described for *E. coli* BL21/pGEX-GALNS without ampicillin supplementation. Samples of 1.0 ml were removed every hour for optical density (OD) measurement at 620 nm and glucose analysis using a glucose assay kit (BioSystems Reagents & Instruments, Spain). Each assay was done in triplicate.

Effect of microorganism culture time on GALNS production was determined in individual BL21/pGEX-GALNS cultures carried out under the described growth conditions using 20 g l^{-1} glucose concentration. Cultures were stopped after 3, 6, 12 or 24 h of growth and cooled to 19 ± 1°C. At each culture time (3, 6, 12 or 24 h), GALNS production was induced with 1 mM IPTG (Invitrogen), with culturing at 200 rpm and 19 ± 1°C. Aliquots of 5 ml were removed 2, 4, and 6 h after IPTG induction. Samples

were centrifuged at 4,000 rpm and 4°C during 15 min, and biomass was washed three times with 1× phosphate-buffered saline (PBS) buffer (composition per liter: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4; pH 7.4). The washed biomass was stored at −20°C for further analysis. Each assay was done in duplicate.

Microorganism culture at bench scale

The effect of agitation and aeration rate on microorganism growth rate was evaluated by using a BL21(D3) strain without pGEX-3X-GALNS plasmid in a Bioflo 110 reactor (New Brunswick Scientific, Co. Inc. USA), with a 3-l working volume and a marine stirrer. Pre-inocula was prepared in a 1-l shake flask containing 0.3 l MGM supplemented with 20 g l^{-1} glucose concentration (MGM-glucose), which was inoculated with 3 ml overnight culture of BL21(D3) in 100 ml LB medium inoculated with a single microorganism colony. Pre-inocula was cultured at 200 rpm, 12 h, and 37°C and was used to inoculate 2.7 l MGM-glucose medium, which was cultured at pH 7.2 and 37°C during 24 h. Biomass and glucose were monitored as described above. This assay was done in duplicate.

GALNS production using BL21/pGEX-GALNS was carried out using the best culture conditions (time, and aeration and agitation rates) observed with the BL21(D3) strain. The BL21/pGEX-GALNS pre-inocula was prepared as described for BL21(D3) strain using MGM-glucose medium supplemented with 0.1 g l^{-1} ampicillin. After 12 h growth, the culture was cooled to 19 ± 1°C, and GALNS expression was induced using 1 mM IPTG during 6 h. Aliquots of 50 ml were removed after 2, 4, and 6 h induction and washed with 1× PBS buffer as described above. rGALNS production was analyzed in four independent cultures.

Crude protein extracts

Aliquots from the shake-scale cultures were resuspended in 1 ml lysis buffer [25 mM Tris, 1 mM phenylmethylsulfonyl fluoride, 1 mM ethylenediamine tetraacetic acid (EDTA), 2% β-mercaptoethanol, 5% glycerol, and 1% Triton X-100; pH 7.2], while aliquots from bench-scale cultures were resuspended in 5 ml lysis buffer. Samples were sonicated during 1 min at 4°C and 25% amplitude (Vibra-Cell, Sonics & Materials Inc., Newtown, CT, USA) and centrifuged at 3,500 rpm and 4°C during 15 min [46]. Preliminary studies showed that, under these conditions, the highest cell disruption and protein concentration were reached with a limited effect on protein degradation (data not shown). Supernatant (soluble fraction) was stored at −80°C for further analysis. Pellets from the sonicated 3-l culture samples were washed three times with 0.85% NaCl and solubilized with 1 ml solubilization buffer (25 mM Tris, 2% β-mercaptoethanol, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 8 M urea; pH 7.0). Solubilized samples were dialyzed overnight at 4°C against 25 mM potassium acetate buffer (pH 7.0). After dialysis, samples were centrifuged at 13,000 rpm and 4°C during 10 min to obtain the solubilized and refolded inclusion bodies fraction (hereafter named inclusion bodies fraction).

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 [54]. One unit (U) was defined as the amount of enzyme catalyzing 1 nmol substrate per hour, and specific GALNS activity was expressed as U mg^{-1} protein as determined by Bradford assay or as U mg^{-1} rGALNS as determined by enzyme-linked immunosorbent assay (ELISA). Enzyme activity was assayed in the soluble and inclusion bodies fractions, and in the growth media.

Western blotting

Crude protein extracts from soluble and inclusion bodies fractions were analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) under reducing conditions and electrotransferred to a nitrocellulose membrane (Hybond C-Extra; Amersham Bioscience, Piscataway, NJ, USA) previously soaked in transfer buffer (composition per liter: 3.03 g Tris, 14.04 g glycine, 1 g SDS, and 200 ml methanol; pH 8.3). The membrane was blocked with 5% nonfat dry milk, 0.2% Tween solution in 1× PBS buffer and incubated with a monoclonal mouse anti-human GALNS antibody [49] at 1:5,000 dilution, followed by incubation with rabbit anti-mouse immunoglobulin G (IgG) coupled with peroxidase (Sigma–Aldrich, St. Louis, MO, USA). Peroxidase activity was visualized using diaminobenzidine substrate (Sigma–Aldrich).

Enzyme-linked immunosorbent assay

An indirect ELISA technique was used for GALNS quantification in soluble and inclusion bodies fractions samples. Ninety-six-well polystyrene microplates (Nunc Maxisorp®) were coated with 10 μg ml^{-1} soluble or inclusion bodies fractions in 1× PBS and incubated overnight at 37°C in a wet chamber. The plate was blocked with solution 1 (5% nonfat dry milk, 0.05% Tween 20 in 1× PBS) during 2 h at 37°C. After two washes with 0.05% Tween 20 in 1× PBS, a polyclonal rabbit anti-GALNS IgG antibody, previously produced against a mix of highly immunogenic human GALNS

peptides (Sosa A, Espejo J, Lizaraso L, and Barrera A; unpublished data), was added at 1:600 dilution in solution 2 (2.5% nonfat dry milk, 0.025% Tween 20 in 1× PBS). The plate was incubated for 1 h at 37°C in a wet chamber and washed four times with 0.05% Tween 20 in 1× PBS. Anti-rabbit IgG coupled with peroxidase (Sigma–Aldrich) was applied to the wells at 1:2,000 dilution in solution 2, and the experiment was developed with 3,3′,5,5′-tetramethylbenzidine (KPL, Inc., Maryland, USA). The enzymatic reaction was stopped with 1 N HCl solution, and the plate was read at 450 nm using an Anthos 2020 microplate reader (Biochrom, Cambridge, UK). A calibration curve between 0 and 1 μg ml^{-1} was created using a recombinant human GALNS enzyme produced in CHO cells [50].

Statistical analysis

Differences between groups were tested for statistical significance using Student's *t*-test. *P* value lower than 0.05 was considered significant. All analyses were performed using Statgraphics Plus® software. All results are presented as mean ± standard deviation (SD).

Results and discussion

The aim of this study is to establish for the first time the feasibility and potential of rGALNS production in *E. coli* BL21 by evaluating: (1) the effect of glucose concentration and presence of pGEX-3X-GALNS plasmid on specific microorganism growth rate at shake scale, (2) the effect of parameters such as aeration and agitation on specific microorganism growth rate at bench scale, and (3) the effect of microorganism culture and IPTG induction times on rGALNS enzyme activity levels at shake and bench scales. Enzyme activity was assayed in the soluble and the solubilized and refolded inclusion bodies fractions.

Effect of presence of glucose or plasmid at shake scale

The effect of glucose concentration on specific growth of *E. coli* BL21 and *E. coli* BL21/pGEX-GALNS was evaluated in a range from 15 to 40 g l^{-1} at shake level to establish any significant difference due to replication of the plasmid pGEX-3X-GALNS in the strain. As previously reported [32], no significant effect on growth rates was observed for either strain at the assayed glucose concentrations, with mean growth rate of 0.500 ± 0.013 and 0.470 ± 0.019 h^{-1} for *E. coli* BL21 and *E. coli* BL21/pGEX-GALNS, respectively. Nevertheless, the growth rate of the recombinant strain was lower and statistically different ($P < 0.05$) for all glucose concentrations compared with that observed for the plasmid-free strain. This growth rate reduction could be explained by plasmid replication and synthesis of plasmid gene products, which require additional energy and material from the host cell [13, 47].

Most *E. coli* strains, such as JM109, secrete acetate even under very low glucose concentrations (0.5 g l^{-1}) in fed-batch cultures [42]. Nevertheless, *E. coli* strain BL21 has been reported to be less sensitive to high glucose concentrations, which allows its batch culture at glucose concentrations up to 40 g l^{-1} [33]. In addition, glucose concentration does not affect its growth rate and produces low acetate concentrations due to its active glyoxylate shunt pathway and acetyl-coenzyme A (CoA) synthetase [32–34, 53]. Since no significant effect of glucose concentration on growth rate was observed for either *E. coli* BL21 or *E. coli* BL21/pGEX-GALNS, and due to the fact that *E. coli* batch culture at high glucose concentration has been reported between 20 and 40 g l^{-1} [26, 42, 53], glucose concentration of 20 g l^{-1} was selected for batch experiments.

Effect of aeration and agitation at bench scale

Effect of aeration and agitation on growth rate was evaluated at bench scale for both strains. At the evaluated conditions, both strains showed the highest specific growth rate at 400 rpm and 2.5 standard temperature and pressure liters per minute (STPLPM), being 0.474 ± 0.008 and 0.293 ± 0.007 h^{-1} for *E. coli* BL21 and *E. coli* BL21/pGEX-GALNS, respectively. The lowest growth rate was obtained at 200 rpm and 1.0 STPLPM, being 0.421 ± 0.006 and 0.265 ± 0.004 h^{-1} for *E. coli* BL21 and *E. coli* BL21/pGEX-GALNS, respectively. However, no significant effect on growth rate was observed for either strain at 1.0 or 2.5 STPLPM aeration rates ($P > 0.05$). Consumption of glucose, dissolved oxygen, and optical density for both strains showed that the plasmid-bearing strain presented higher oxygen and glucose uptakes than did the plasmid-free strain (Fig. 1).

Although no results with the same culture medium have been reported, our results are well correlated with the growth rates reported by Phue et al. [32] and Wang et al. [58] for *E. coli* BL21 in 5-l (0.46 h^{-1}) and 3-l (0.55 h^{-1}) cultures, respectively, at 37°C and controlled pH 7.0. However, the *E. coli* BL21/pGEX-GALNS strain showed a lower growth rate than that reported for a BL21 strain carrying a low-copy-number plasmid (pGEX-3X) but comparable to that from a strain carrying a high-copy-number plasmid (0.29 h^{-1}) [58]. This difference could be associated with the plasmid (ColE1- versus pGEX-derived plasmids) or the gene (none versus GALNS) used in each case.

Culture at low growth rates has been related to high-quality and high-yield fermentations due to reduction of growth-rate–dependent plasmid instability [25, 41], which could have an important impact on recombinant protein production yields. For those reasons, aeration and agitation

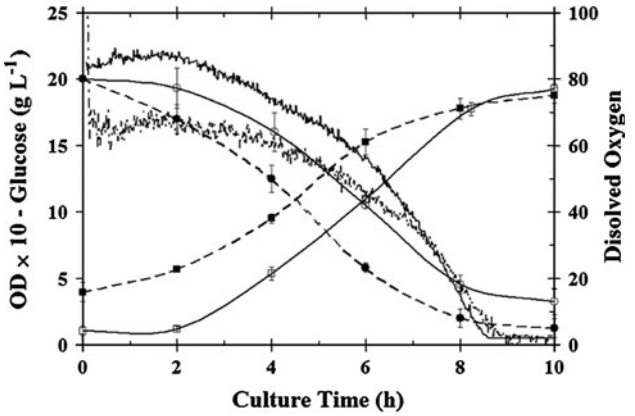

Fig. 1 Profiles of optical density (*open squares*), glucose concentration (*open circles*), and dissolved oxygen (*solid lines*) for *E. coli* BL21 (*open symbols*, *solid lines*) and *E. coli* BL21/pGEX-GALNS (*closed symbols*, *dashed lines*) at bench scale. Both microorganisms were cultured at 2.7 l, 200 rpm, 2.5 STPLPM, controlled pH 7.2, and 37°C

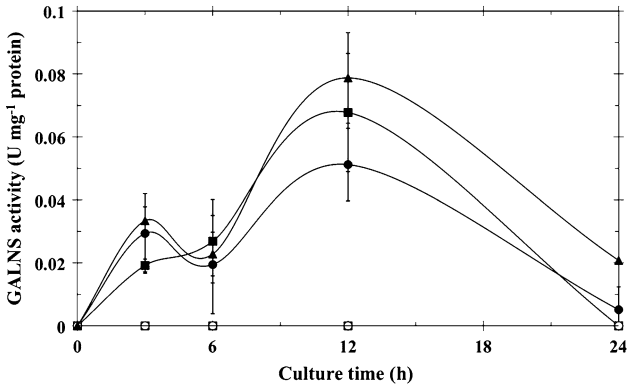

Fig. 2 Effect of microorganism culture and induction time on specific GALNS activity. GALNS expression was induced using 1 mM IPTG after 3, 6, 12, and 24 h of microorganism culture at shake scale. For each microorganism culture time, GALNS activity, in the soluble fraction, was monitored after 2 h (*filled squares*), 4 h (*filled triangles*) or 6 h (*filled circles*) IPTG induction. No GALNS activity was detected in *E. coli* BL21 (*open squares*) or in *E. coli* BL21/pGEX-GALNS without IPTG induction (*open circles*). GALNS activity in growth media was not detected

rates of 2.5 STPLPM and 200 rpm were selected as the conditions for rGALNS enzyme production at bench scale. Culture at 200 rpm and 1.0 STPLPM was not selected, because no statistical difference in specific growth rate was observed with respect to the culture at 2.5 STPLPM. In addition, high aeration rate has been associated with higher recombinant protein production [5, 8].

Production of rGALNS at shake and bench scales

The effect of microorganism culture and IPTG induction time on specific GALNS activity was investigated at shake level. The highest specific GALNS activity in soluble fraction was obtained after 12 h microorganism culture (Fig. 2), which corresponds to the beginning of the stationary stage (data not shown). Additionally, the best duration for induction by IPTG was found to be between 2 and 4 h, with GALNS activity of 0.067 and 0.078 U mg^{-1}, respectively. GALNS activity was not observed in either plasmid-free *E. coli* BL21 strain, or in *E. coli* BL21/pGEX-GALNS strain without IPTG induction (Fig. 2). From these results, the early stationary stage was selected as the best induction time.

At bench scale, the specific GALNS activity in the soluble and inclusion bodies fractions showed a similar profile in four independent cultures (Fig. 3a). Unlike shake-scale results, the highest GALNS activity in soluble fraction was observed after 2 h induction, being similar to the highest enzyme activity levels observed at shake scale (4 h induction). GALNS activity levels in inclusion bodies fraction ranged between 41% and 143% of the levels observed in the soluble fraction (Fig. 3a). However, no statistical difference was observed among the fractions at any induction time ($P > 0.05$).

The results of the ELISA assay showed that rGALNS amounts in inclusion bodies fraction were between 5.4- and 17.8-fold higher than those levels observed in soluble fraction (Fig. 3b). rGALNS in soluble and inclusion bodies fractions represented 2% and 24% of total protein, respectively, corresponding to 1.8 and 4.29 mg l^{-1} recombinant protein in each fraction. Protein aggregation (inclusion bodies) is a common issue found during production of biotherapeutics in *E. coli* [12]. Proteins in these inclusion bodies need elaborate solubilization, refolding, and purification procedures to recover their full functionality [31, 43, 56]. Although many commercial recombinant proteins are purified from inclusion bodies, loss of bioactivity of the expressed protein could be a major bottleneck [17, 39]. Due to the lability of GALNS enzyme [50], we tried to limit inclusion bodies formation by reducing the temperature during the induction stage [24, 39]. However, ELISA quantification results showed that the highest rGALNS amounts were obtained in the inclusion bodies fraction, corresponding to up to 71% of total rGALNS (Fig. 3b).

GALNS activity levels were normalized by using the results of the ELISA assay (Fig. 3c). This specific activity could be interpreted as a measure of the amount of active enzyme per microgram of recombinant protein (U μg^{-1} rGALNS). The highest levels of active enzyme were obtained in the soluble fraction, being approximately 15-fold higher than those observed in the inclusion bodies fraction. These results show that, although a larger amount of rGALNS was obtained in the inclusion bodies, only a limited fraction of it was active. This difference between enzyme amount and active enzyme levels could be associated with the solubilization and refolding process of

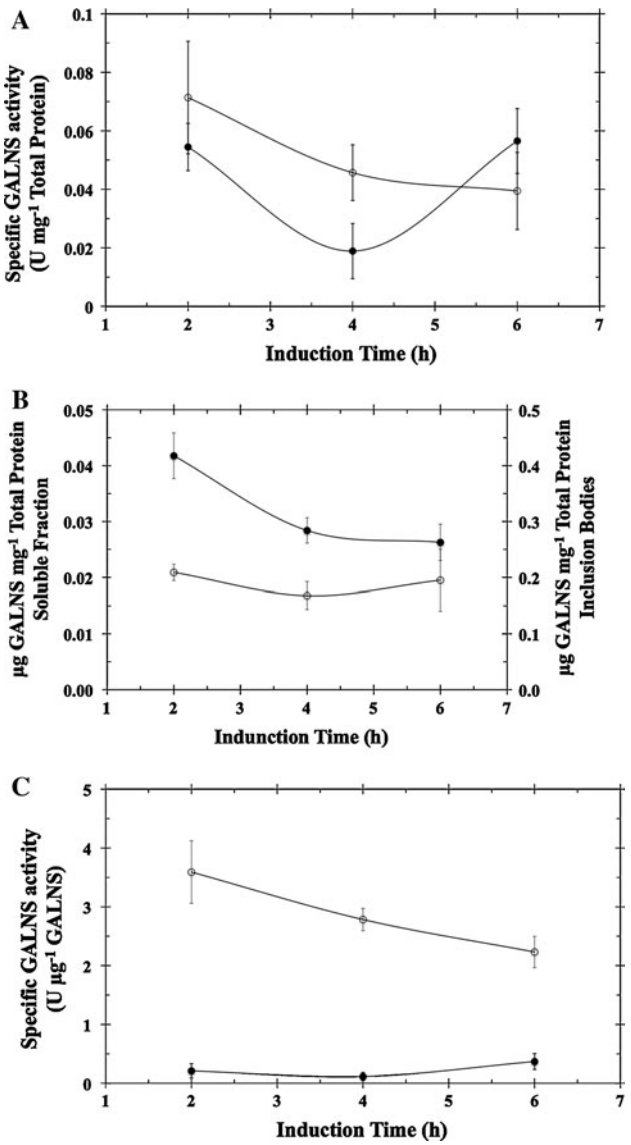

Fig. 3 Effect of induction duration on specific GALNS activity (**a**) and rGALNS amount (**b**) at bench scale. Analysis was done in both soluble (*open circles*) and inclusion bodies (*filled circles*) fractions. GALNS activity in growth media was not detected at any culture time. Specific GALNS activity was corrected using ELISA assay results (**c**) in soluble (*open circles*) and inclusion bodies (*filled circles*) fractions. Culture conditions for *E. coli* BL21/pGEX-GALNS: 12 h, 200 rpm, controlled pH 7.2, and 37°C. Induction conditions: 200 rpm, controlled pH 7.2, and 19 ± 1°C. Results are reported as the average of four independent cultures

inclusion bodies [31, 43, 56], or with the GALNS enzyme activation mechanism, which has been shown to be a limiting step in the enzyme maturation pathway requiring co-expression of sulfatase modifying factor 1 (SUMF1) to achieve complete enzyme activation [11].

Western blot analysis showed the presence of a ~50-kDa protein band in both soluble (Fig. 4a) and inclusion bodies (Fig. 4b) fractions. An additional 120-kDa band was observed in cell soluble fraction samples, which may correspond to soluble covalent dimers of GALNS [12]. As observed in the enzyme activity and ELISA assays, band intensity was higher in inclusion bodies than in soluble fraction. No band was recognized in samples from non-induced BL21/pGEX-GALNS and plasmid-free (BL21) strains, while rGALNS produced in CHO cells showed an expected 57-kDa band [50]. These results differ from those of a recombinant iduronate-2-sulfate sulfatase (IDS) produced in *Pichia pastoris* [10, 22] and *E. coli* JM109 [21, 36], for which a large number of bands were recognized by an anti-IDS monoclonal antibody. The use of *E. coli* BL21 strain, which lacks the major protease, significantly reducing endoproteolytic cleavage of damaged and recombinant proteins [45], could have a significant effect on the stability and processing of the produced rGALNS enzyme.

Like other lysosomal sulfatases, GALNS is characterized by two main posttranslational modifications: (1) conversion of cysteine to FGly at the enzyme active site [11], and (2) N-glycosylations that are carried out in the ER and Golgi apparatus [20]. All sulfatases studied to date contain at their catalytic site a FGly residue that is essential for enzyme activity [40]. Formation of FGly residue occurs by oxidation of a conserved cysteine residue, which is located at position 79 in the GALNS sequence [51], by the action of FGE [11]. FGE is encoded by sulfatase modifying factor 1 (SUMF1), which has been highly conserved during evolution and defines a new protein family conserved in pro- and eukaryotes [23]. In *E. coli*, as well as in other prokaryotes, cysteine- and serine-sulfatase types have been identified, which are activated by the enzymes FGE and AtsB, respectively [23]. The results presented herein show that prokaryotic FGE can activate human sulfatases, reflecting high conservation of this mechanism and the possibility to produce active sulfatases in prokaryotic systems. These results agree with findings of active recombinant human IDS enzyme production in *E. coli* JM109 [21, 36].

The second main modification, common to other lysosomal enzymes, is N-glycosylation of the protein, which adds 2–3 kDa per carbohydrate chain to the enzyme weight [20]. GALNS have two potential sites for N-glycosylation, indicating that about 4–6 kDa of the total GALNS weight should correspond to the carbohydrate chains. Western blotting analysis of rGALNS enzyme produced in *E. coli* BL21 showed that it differs by about 6 kDa from recombinant enzyme produced in CHO cells, which could be associated with the absence of those posttranslational modifications in *E. coli*. In mammals, N-glycosylation works as a recognition signal for mannose- and mannose-6-phosphate (M6P) receptors that mediate enzyme targeting to lysosomes and enzyme uptake by the cell [35]. Absence

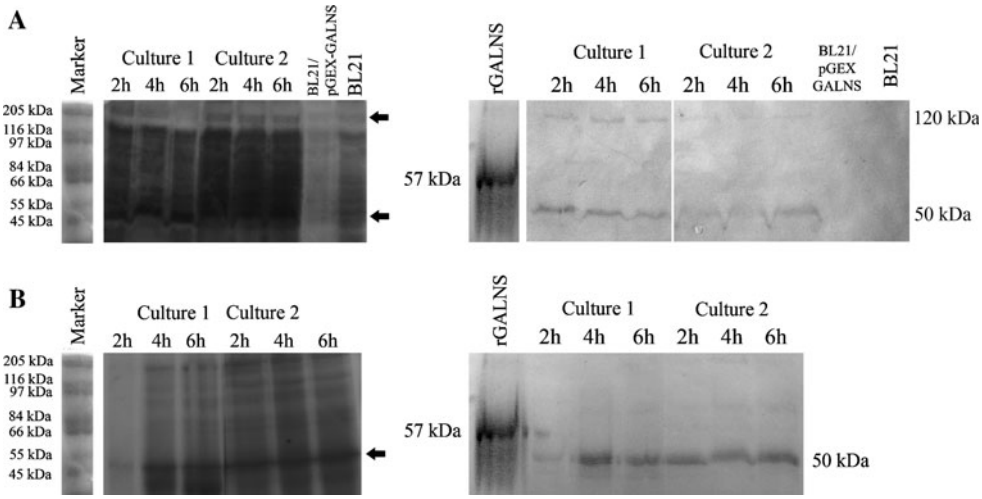

Fig. 4 SDS-PAGE and Western blot analysis of soluble (**a**) and inclusion bodies (**b**) fractions. *E. coli* BL21/pGEX-GALNS strain was cultured in 3 l, and GALNS expression was induced with IPTG after 12 h culture. Samples were analyzed after 2, 4, and 6 h induction. A plasmid-free *E. coli* BL21 strain (BL21) and a plasmid-bearing strain without IPTG induction (BL21/pGEX-GALNS) were used as negative controls. rGALNS produced in CHO cells (rGALNS) was used as a positive control

of N-glycosylation may affect activity, folding, stability, and oligomerization of the enzyme [35]. In Morquio A disease, mutations in N-glycosylation sites of GALNS enzyme result in reduction of its activity, which leads to mild or attenuated phenotypes, due to possible sorting of the enzyme, which results in its degradation [28]. Production of an active rGALNS enzyme without N-glycosylations in *E. coli* BL21 could be associated with the absence of an ER–Golgi–endosomal–lysosomal system, avoiding degradation of misfolded enzymes or sorting of the enzyme to a degradation cell compartment. These results suggest that N-glycosylation does not seem to be a requirement for GALNS enzyme activity and agree with recent reports that establish a variable effect of glycosylation on protein stability, folding, trafficking, and activity, among others (reviewed in [57]). However, further analysis should be carried out to evaluate the effect of absence of glycosylation on other enzyme properties.

Recombinant GALNS production has traditionally been carried out in CHO cells, with reported activities in crude extract of 3.06×10^{-3} U mg^{-1} [6] and 129 U mg^{-1} [50]. Whereas comparison with the former study is not possible due to difference in substrate type (radiometric versus fluorometric), the activity levels presented herein are about 100- to 2,500-fold lower than those reported by Tomatsu et al. [50] but similar to those reported in a recent communication of rGALNS production in CHO cells [16]. It is important to note that rGALNS enzyme obtained by Tomatsu's group was produced as a secreted protein in a protein-free medium [50], which facilitates its purification and increases its specific activity. As expected, GALNS activity was not found in the growth media under any of the evaluated culture conditions. Signal sequences of DsbA or MalE, two well-studied periplasmic proteins of *E. coli*, could be used to direct the recombinant protein to the periplasm, from where it is finally exported to the media, improving production and reducing the downstream steps required to obtain purified enzyme [37]. In addition, purification could be carried out by using affinity chromatography, as rGALNS is expressed as a fusion protein with glutathione *S*-transferase (present in the pGEX-3X plasmid).

Although active GALNS enzyme was produced, we could expect that, both in vitro and in vivo, this recombinant enzyme will not be taken up by the cell via mannose or M6P receptors. However, mannose- and M6P-independent pathways for transport of lysosomal enzymes have been described [7, 14], suggesting the possibility of using rGALNS enzyme produced in *E. coli* BL21 for development of ERT for Morquio A patients. Recently, use of a chemically modified β-glucuronidase, in which mannose- and M6P-receptor uptake was eliminated, showed the possibility and advantages of using carbohydrate-deficient or modified enzymes for treatment of lysosomal storage diseases [19]. In addition, this recombinant enzyme could be chemically or enzymatically modified to add specific glycosylation moieties [4] to favor its uptake via mannose or M6P receptors, or by poly(ethyleneglycol) conjugation (PEGylation), which significantly increases circulating half-life time and reduces immunogenicity and antigenicity [18].

Conclusions

In summary, we have produced active rGALNS enzyme in *E. coli* BL21 strain at shake and bench scales, with production yield of up to 4.29 mg l^{-1}. Although the specific activity levels are lower than those observed using mammalian cells, this recombinant enzyme could be used in development of ERT for Morquio A disease and shows the possibility to produce other human sulfatases due to the capacity of the bacterial FGE to activate those proteins. The results presented herein also show the advantage of combining an enzyme activity assay with GALNS quantification by

ELISA to obtain a specific performance of rGALNS production. Further work should focus on evaluation of high-density cell cultures to increase enzyme production, enzyme purification, and in vitro and in vivo evaluation of this recombinant enzyme.

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References

1. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1999) Short protocols in molecular biology. Wiley, Hoboken
2. Baker K, Rendall M, Hills A, Hoare M, Freedman R, James D (2001) Metabolic control of recombinant protein N-Glycan processing in NS0 and CHO cells. Biotechnol Bioeng 73:188–202
3. Baneyx F (1999) Recombinant protein expression in *Escherichia coli*. Curr Opin Biotechnol 10:411–421
4. Bernardes GJ, Castagner B, Seeberger PH (2009) Combined approaches to the synthesis and study of glycoproteins. ACS Chem Biol 4:703–713
5. Bhattacharya SK, Dubey AK (1997) Effects of dissolved oxygen and oxygen mass transfer on overexpression of target gene in recombinant *E. coli*. Enzyme Microb Technol 20:355–360
6. Bielicki J, Fuller M, Guo X, Morri C, Hopwood J, Anson D (1995) Expression, purification and characterization of recombinant human N-acetylgalactosamine-6-sulphatase. Biochem J 311:333–339
7. Braulke T, Bonifacino JS (2009) Sorting of lysosomal proteins. Biochim Biophys Acta 1793:605–614
8. Çalik P, Yilgör P, Ayhan P, Demir AS (2004) Oxygen transfer effects on recombinant benzaldehyde lyase production. Chem Eng Sci 59:5075–5083
9. Chu L, Robinson D (2001) Industrial choices for protein production by large-scale cell culture. Curr Opin Biotechnol 12:180–187
10. Córdoba-Ruiz HA, Poutou-Piñales RA, Echeverri-Peña OY, Algecira-Enciso NA, Landázuri P, Sáenz H, Barrera-Avellaneda LA (2009) Laboratory scale production of the human recombinant iduronate 2-sulfate sulfatase-like from Pichia pastoris. Afr J Biotechnol 8:1786–1792
11. Cosma M, Pepe P, Annunziata I, Newbold R, Grompe M, Parenti G, Ballabio A (2003) The multiple sulfatase deficiency gene encodes an essential and limiting factor for the activity of sulfatases. Cell 113:445–456
12. Cromwell ME, Hilario E, Jacobson F (2006) Protein aggregation and bioprocessing. AAPS J 8:E572–E579
13. Diaz-Ricci J, Hernandez M (2000) Plasmid effects on *Escherichia coli* metabolism. Crit Rev Biotechnol 20:79–108
14. Dittmer F, Ulbrich EJ, Hafner A, Schmahl W, Meister T, Pohlmann R, von Figura K (1999) Alternative mechanisms for trafficking of lysosomal enzymes in mannose 6-phosphate receptor-deficient mice are cell type-specific. J Cell Sci 112:1591–1597
15. Durany O, de Mas C, López-Santín J (2005) Fed-batch production of recombinant fuculose-1-phosphate aldolase in *E. coli*. Process Biochem 40:707–716
16. Dvorak-Ewell M, Wendt D, Shroff S, Koppaka V, Christianson T, Vellard M (2009) Functional evaluation of recombinant human N-acetylgalactosamine-6-sulfate sulfatase as enzyme replacement therapy. Mol Genet Metab 96:S22
17. Graumann K, Premstaller A (2006) Manufacturing of recombinant therapeutic proteins in microbiol systems. Biotechnol J 1:164–186
18. Greenwald RB, Choe YH, McGuire J, Conover CD (2003) Effective drug delivery by PEGylated drug conjugates. Adv Drug Deliv Rev 55:217–250
19. Grubb JH, Vogler C, Levy B, Galvin N, Tan Y, Sly WS (2008) Chemically modified b-glucuronidase crosses blood–brain barrier and clears neuronal storage in murine mucopolysaccharidosis VII. Proc Natl Acad Sci U S A 105:2616–2621
20. Kornfeld S (1987) Trafficking of lysosomal enzymes. FASEB J 1:462–468
21. Landázuri P, Gunturiz M, Gómez L, Poutou-Piñales RA, Torres A, Echeverri-Peña OY, Sáenz H, Delgado J, Barrera-Avellaneda LA (2003) Expresión transiente de la Iduronato 2 sulfato sulfatasa humana recombinante funcionalmente activa en *Escherichia coli*. Rev Asoc Col Ciencias Biol 15:33–42
22. Landázuri P, Poutou-Piñales RA, Acero-Godoy J, Córdoba-Ruiz H, Echeverri-Peña OY, Sáenz H, Delgado J, Barrera-Avellaneda LA (2009) Cloning and shake flask expression of hrIDS-like in *Pichia pastoris*. Afr J Biotechnol 8:2871–2877
23. Landgrebe J, Dierks T, Schamidt B, von Figura K (2003) The human SUMF1 gene, required for posttranslational sulfatase modification, defines a new gene family which is conserved from pro- to eukaryotes. Gene 316:47–56
24. Li R-Y, Cheng C-Y (2009) Investigation of inclusion body formation in recombinant *Escherichia coli* with a bioimaging system. J Biosci Bioeng 107:512–515
25. Lin-Chao S, Bremer H (1986) Effect of the bacterial growth rate on replication control of plasmid pBR322 in *Escherichia coli*. Mol Gen Genet 203:143–149
26. Luli G, Strohl W (1990) Comparison of growth, acetate production, and acetate inhibition of *Escherichia coli* strains in batch and fed-batch fermentations. Appl Environ Microbiol 56:1004–1011
27. Meuwly F, Ruffieux P, Kadouri A, von Stockar U (2007) Packed-bed bioreactors for mammalian cell culture: bioprocess and biomedical applications. Biotechnol Adv 25:45–56
28. Montaño AM, Sukegawa K, Kato Z, Carrozzo R, Di Natale P, Christensen E, Orii KO, Orii T, Kondo N, Tomatsu S (2007) Effect of 'attenuated' mutations in mucopolysaccharidosis IVA on molecular phenotypes of N-acetylgalactosamine-6-sulfate sulfatase. J Inherit Metab Dis 30:758–767
29. Montaño AM, Tomatsu S, Gottesman G, Smith M, Orii T (2007) International Morquio A registry: clinical manifestation and natural course of Morquio A disease. J Inherit Metab Dis 30:165–174
30. Neufeld E, Muenzer J (2001) The mucopolysaccharidosis. In: Scriver C, Beaudet A, Sly W, Valle D (eds) The metabolic and molecular bases of inherited diseases. McGraw-Hill, New York, pp 3421–3452
31. Patra AK, Mukhopadhyay R, Mukhija R, Krishnan A, Garg LC, Panda AK (2000) Optimization of inclusion body solubilization and renaturation of recombinant human growth hormone from *Escherichia coli*. Protein Exp Purif 18:182–192
32. Phue J-N, Lee SJ, Trinh L, Shiloach J (2008) Modified *Escherichia coli* B (BL21), a superior producer of plasmid DNA compared with *Escherichia coli* K (DH5a). Biotechnol Bioeng 101:831–836
33. Phue J-N, Noronha SB, Hattacharyya R, Wolfe AJ, Shiloach J (2005) Glucose metabolism at high density growth of *E. coli* B and *E. coli* K: differencs in metabolic pathways are responsible for efficient glucose utilization in *E. coli* B as determined by microarrays and northern blot analyses. Biotechnol Bioeng 90:805–820
34. Phue J-N, Shiloach J (2004) Transcription levels of key metabolic genes are the cause for different glucose utilization pathways in *E. coli* B (BL21) and *E. coli* K (JM109). J Biotechnol 109:21–30

35. Pohl S, Marschner K, Storch S, Braulke T (2009) Glycosylation- and phosphorylation-dependent intracellular transport of lysosomal hydrolases. Biol Chem 390:521–527
36. Poutou-Piñales RA, Vanegas A, Landázuri P, Sáenz H, Lareo L, Echeverri O, Barrera LA (2010) Human sulfatase transiently and functionally active expressed in *E. coli* K12. Electron J Biotechnol 13. doi:10.2225/vol13-issue3-fulltext-8
37. Puertas JM, Betton JM (2009) Engineering an efficient secretion of leech carboxypeptidase inhibitor in *Escherichia coli*. Microb Cell Fact 8:57
38. Rohrbach M, Clarke J (2007) Treatment of lysosomal storage disorders: progress with enzyme replacement therapy. Drugs 67:2697–2716
39. Sahdev S, Khattar SK, Saini KS (2008) Production of active eukaryotic proteins through bacterial expression systems: a review of the existing biotechnology strategies. Mol Cell Biochem 307:249–264
40. Sardiello M, Annunziata I, Roma G, Ballabio A (2005) Sulfatases and sulfatase modifying factors: an exclusive and promiscuous relationship. Hum Mol Genet 14:3203–3217
41. Shene C, Andrews B, Asenjo J (2003) Study of recombinant micro-organism populations characterized by their plasmid content per cell using a segregated model. Bioprocess Biosyst 25:333–340
42. Shiloach J, Kaufman J, Guillard A, Fass R (1996) Effect of glucose supply strategy on acetate accumulation, growth, and recombinant protein production by Escherichia coli BL21 (lambdaDE3) and *Escherichia coli* JM 109. Biotechnol Bioeng 49:421–428
43. Singh SM, Panda AK (2005) Solubilization and refolding of bacterial inclusion body proteins. J Biosci Bioeng 99:303–310
44. Swartz J (2001) Advances in *Escherichia coli* production of therapeutic proteins. Curr Opin Biotechnol 12:195–201
45. Terpe K (2006) Overview of bacterial expression systems for heterologous protein production: from molecular and biochemical fundamentals to commercial systems. Appl Microbiol Biotechnol 72:211–222
46. Thomas JG, Baneyx F (1996) Protein misfolding and inclusion body formation in recombinant *Escherichia coli* cells overexpressing Heat-shock proteins. J Biol Chem 271:11141–11147
47. Togna A, Shuler M, Wilson D (1993) Effects of plasmid copy number and runaway plasmid replication on overproduction and excretion of beta-lactamase from *Escherichia coli*. Biotechnol Prog 9:31–39
48. Tomatsu S, Fukuda M, Masue K, Sukegawa T, Fukao A, Yamagishi T, Hori H, Iwati T, Ogawa Y (1991) Morquio disease: isolation, characterization and expression of full-length cDNA for human N-acetylgalactosamine-6-sulfate sulfatase. Biochem Biophys Res Commun 181:677–683
49. Tomatsu S, Gutierrez M, Nishioka T, Yamada M, Tosaka Y, Grubb J, Montano A, Vieira M, Trandafirescu G, Pena O, Yamaguchi S, Orii K, Orii T, Noguchi A, Laybauer L (2005) Development of MPS IVA mouse (Galnstm(hC79S.mC76S)slu) tolerant to human N-acetylgalactosamine-6-sulfate sulfatase. Hum Mol Genet 14:3321–3335
50. Tomatsu S, Montaño A, Gutiérrez M, Grubb J, Oikawa H, Dung V, Ohashi A, Nishioka T, Yamada M, Tosaka Y, Trandafirescu G, Orii T (2007) Characterization and pharmacokinetic study of recombinant human N-acetylgalactosamine-6-sulfate sulfatase. Mol Genet Metab 91:69–78
51. Tomatsu S, Montaño A, Nishioka T, Gutierrez M, Peña O, Trandafirescu G, Lopez P, Yamaguchi S, Noguchi A, Orri T (2005) Mutation and polymorphism spectrum of the GALNS gene in mucopolysaccharidosis IVA (Morquio A). Hum Mutat 26:500–512
52. Tomatsu S, Montaño A, Ohashi A, Oikawa H, Oguma T, Dung V, Nishioka T, Orii T, Sly W (2007) Enzyme replacement therapy in a murine model of Morquio A syndrome. Hum Mol Genet 17:815–824
53. van de Walle M, Shiloach J (1998) Proposed mechanism of acetate accumulation in two recombinant *Escherichia coli* strains during high density fermentation. Biotechnol Bioeng 57:71–78
54. van Diggelen O, Zhao H, Kleijer W, Janse H, Poorthuis B, van Pelt J, Kamerling J, Galjaard H (1990) A fluorometric enzyme assay for the diagnosis of Morquio type A. Clin Chem Acta 187:131–140
55. Vellodi A, Young EP, Cooper A, Wraith JE, Winchester B, Meaney C, Ramaswami U, Will A (1997) Bone marrow transplantation for mucopolysaccharidosis type I: experience of two British centres. Arch Dis Child 76:92–99
56. Villaverde A, Carrió M (2003) Protein aggregation in recombinant bacteria: biological role of inclusion bodies. Biotechnol Lett 25:1385–1395
57. Walsh G, Jefferis R (2006) Post-translational modifications in the context of therapeutic proteins. Nat Biotechnol 24:1241–1252
58. Wang Z, Xiang L, Shao J, Węgrzyn A, Węgrzyn G (2006) Effects of the presence of ColE1 plasmid DNA in *Escherichia coli* on the host cell metabolism. Microb Cell Fact 5:34. doi:10.1186/1475-2859-1185-1134