# High Yield Process for the Production of Active Human α-Galactosidase a in CHO-K1 Cells through Lentivirus Transgenesis

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*Fabry disease is an X-linked recessive disorder caused by a deficiency in lysosomal α-Galactosidase A. Currently, two enzyme replacement therapies (ERT) are available. However, access to orphan drugs continues to be limited by their high price. Selection of adequate high-expression systems still constitutes a challenge for alleviating the cost of treatments. Several strategies have been implemented, with varying success, trying to optimize the production process of recombinant human α-Galactosidase A (rhαGAL) in Chinese hamster ovary (CHO-K1) cells. Herein, we describe for the first time the application of a strategy based on third-generation lentiviral particles (LP) transduction of suspension CHO-K1 cells to obtain high-producing rhαGAL clones (3.5 to 59.4 pg cell$^{-1}$ d$^{-1}$). After two purification steps, the active enzyme was recovered (2.4 × 10$^6$ U mg$^{-1}$) with 98% purity and 60% overall yield. Michaelis-Menten analysis demonstrated that rhαGAL was capable of hydrolyzing the synthetic substrate 4MU-α-Gal at a comparable rate to Fabrazyme®, the current CHO-derived ERT available for Fabry disease. In addition, rhαGAL presented the same mannose-6-phosphate (M6P) content, about 40% higher acid sialic amount and 33% reduced content of the immunogenic type of sialic acid (Neu5Gc) than the corresponding ones for Fabrazyme®. In comparison with other rhαGAL production processes reported to date, our approach achieves the highest rhαGAL productivity preserving adequate activity and glycosylation pattern. Even more, considering the improved glycosylation characteristics of rhαGAL, which might provide advantages regarding pharmacokinetics, our enzyme could be postulated as a promising alternative for therapeutic use in Fabry disease.* © 2017 American Institute of Chemical Engineers *Biotechnol. Prog.*, 33:1334–1345, 2017
*Keywords: rhαGAL, Fabry disease, lentiviral particles (LP), suspension CHO-K1 and glycosylation*

## Introduction

The lysosomal enzyme α-Galactosidase A (EC 3.2.1.22) catalyzes the removal of α-linked galactose from oligosaccharides, glycoproteins and glycolipids during the catabolism

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of macromolecules.[1] Human α-galactosidase A is a homodimeric glycoprotein (~101 kDa) with three N-linked oligosaccharide chains present on each subunit. Enzyme deficiency results in a systemic lysosomal accumulation of neutral glycosphingolipids, predominantly globotriaosylceramide (Gb3), leading to Fabry disease. The enzymatic defect, caused by an X-linked recessive gene, is characterized by a multiple organ pathology that most seriously affects the kidneys, heart, and the cerebrovascular system.[2,3]

Currently, two distinct recombinant protein replacement drugs are used for the treatment of Fabry patients: agalsidase beta (Fabrazyme®, Genzyme-Sanofi), and agalsidase alfa (Replagal®; Shire Human Genetic Therapies). Studies have shown that the two recombinant enzymes exhibit identical biochemical properties and are comparable with each other in terms of amino acid composition, specific activity, stability and uptake by cultured fibroblasts, with only minor differences in glycosylation composition and mannose-6-phosphate receptor-mediated cellular uptake.[4,5] Both agalsidase alfa and agalsidase beta are formulations of recombinant human α-Galactosidase A, but they are produced differently and different endpoints and dosing regimens were used in the clinical trials of these drugs. Agalsidase alfa is produced in a human fibroblast cell line and is administered by intravenous infusion at 0.2 mg $kg^{-1}$ every week; whereas agalsidase beta is produced in CHO-K1 cells and is administered by intravenous infusions at a dose of 1.0 mg $kg^{-1}$.[3] However, owing to the lack of therapeutic alternatives and given the small patient size, the annual treatment price is high, starting at ~€30,000 for infants to over €400,000 per patient per year, as dosage depends on the patients' weight.[2] In addition, it should be noted that the treatment for Fabry's patients is for life.[6]

Considering this background, the need for high levels of protein expression has prompted the search for new strategies aiming to reduce the final product cost.[7] Indeed, stable cell line generation and high protein expression levels constitute the most time-consuming and laborious procedures in the entire production process. Mammalian cells have been extensively used as platforms for protein production because of the characteristics of the post-translational modifications which they perform,[8] with CHO cells as the workhorse for mammalian protein production. The wide spread success of the CHO platform can be attributed to its unparalleled adaptability allowing for growth of these cells at high densities in suspension cultures, ease of adaptation to serum free conditions and the addition of human-like glycosylation.[9,10] However, the adaptability of the CHO cell line also has its drawbacks, as they are not optimal for the production of all recombinant proteins due to poor expression or other issues such as aggregation, proteolysis, or incorrect modification.[11] Regarding this, it is important to underline that several strategies have been implemented, with varying success, trying to optimize the production process of rhαGAL in CHO cells. Examples include the amplification of the rhαGAL transgene using dihydrofolate reductase/methotrexate selection[12] and an optimized transient gene expression protocol.[6] However, these procedures usually result in a low protein yield or a less active enzyme with different glycosylation profile.

In this study, we have replaced the amplification strategy based in dihydrofolate reductase/methotrexate selection by lentiviral transduction of suspension cells, obtaining productivities up to 59 pg $cell^{-1}$ $d^{-1}$. Here we report the production, purification and characterization or rhαGAL produced in CHO-K1 cells and we compared the results with one of the currently available formulations: agalsidase beta. To our knowledge, this is the first report that describes the use of lentiviral vectors to deliver the transgene of rhαGAL and drive its expression in manufacturing cell lines, improving the global production process. Furthermore, our enzyme exhibited a considerably higher sialic acid content than algasidase beta, which might result in a potentially improved half-life and stability, constituting an attractive therapeutic alternative for Fabry disease.

## Materials and Methods

### α-Galactosidase A

Recombinant agalsidase beta was used for all studies (Fabrazyme®, Genzyme), lot number E 2033 H 14. The vial was reconstituted according to the package instructions to give a nominal protein concentration of 5 mg $mL^{-1}$.

### Antibodies

Rabbit polyclonal sera anti-rhαGAL and biotinylated rabbit anti-rhαGAL polyclonal antibody used in this work were produced and characterized in our laboratory by immunization of rabbits with Fabrazyme® molecule, using standard protocols. Amdex streptavidin-horseradish peroxidase conjugate was purchased from GE Healthcare (UK).

### Plasmid construction and lentivirus production

The rhαGAL coding sequence was cloned in the self-inactivating (SIN) lentiviral vector construct (pEF-1α),[13] generating the pLV-rhαGAL transfer vector where transgene expression was driven by the human elongation factor-1α promoter.

Lentiviral particles (LP) were produced by simultaneous co-transfection of Human embryonic kidney 293 (HEK293) cells with four plasmids using the lipid reagent Lipofectamine 2000 (Invitrogen™, USA). These plasmids were the packaging construct (pMDLg/pRRE), the VSV-G expressing construct (pMDG), the Rev-expressing construct (pRSV-Rev),[14,15] and the transfer vector pLV-rhαGAL. The supernatant containing LP was harvested after 48 h, clarified by low speed centrifugation and frozen at −70°C prior to use. The titer was calculated with a HIV-1 p24 ELISA kit (QuickTiter™ Lentivirus Titer Kit, Cell Biolabs Inc., USA) following manufacturer's recommendations.

### Cell culture, transduction, and clonal isolation

Suspension adapted-CHO-K1 cells were maintained in serum-free medium at 37°C with 5% $CO_2$. Repeated transductions at a multiplicity of infection (MOI) of ~35 LP per cell were performed by incubating ~$1 \times 10^5$ cells per tube in CellSTAR® CellReactor™ tubes (Greiner Bio-One, Germany) with LP in a final volume of 1 mL of serum-free medium. At 48 h post-transduction, cell culture medium was replaced with fresh medium and transduced cells were allowed to recover by culturing overnight at 37°C with 5% $CO_2$. Transduced cells were cultured for another round of transduction as outlined above; transductions were repeated two more times, rendering TD1 to TD3 cell lines.

Clonal isolation was performed by limiting dilution in 96-well plates and visual examination of wells for single cell. After 2 weeks, single-cell clones were expanded sequentially from 96-well plate to 48-well, 24-well, 12-well, and CellSTAR® CellReactor™ tubes for further testing. Each cell population (TD1 to TD3) and cloned cell population were analyzed for rhαGAL production and enzyme activity as described below.

### *Measurement of rhαGAL activity and productivity*

To determine the specific rhαGAL productivity of each cell line and clone, cells were seeded at $7 \times 10^5$ cell $mL^{-1}$ in CellSTAR® CellReactor™ tubes and after 24 h the rhαGAL level and cell number were determined by ELISA and cell counting, respectively.

Enzyme activity was assessed by the hydrolysis of the artificial substrate 4-methylumbelliferyl α-D-galactoside (4MU-α-Gal, Sigma-Aldrich, USA), as explained below.

*ELISA Assay for rhαGAL Detection.* Briefly, 96-well micro-plate (Greiner Bio-One) were coated with 100 ng per well of rabbit polyclonal sera anti-rhαGAL in 50 mM carbonate/bicarbonate buffer (pH 9.6) and incubated for 1 h at 37°C and overnight at 4°C. After blocking 1 h at 37°C with 1% (w/v) BSA in phosphate-buffered saline (PBS), plates were incubated with 1:2 serial dilutions of rhαGAL reference (Fabrazyme®) or samples (culture supernatants, harvests, different purification fractions) for 1 h at 37°C. Then, plates were incubated with an appropriate dilution of biotinylated rabbit anti-rhαGAL polyclonal antibody for 1 h. Finally, Amdex streptavidin-horseradish peroxidase conjugate (GE Healthcare, UK) diluted 1:10.000 was added to the wells. After 1 h, plates were incubated with a chromogenic substrate solution (0.5 mg $mL^{-1}$ o-phenylenediamine (Sigma–Aldrich), 0.5 μl $mL^{-1}$ $H_2O_2$ 30 vol., 50 mM citrate-phosphate buffer, pH 5.3). The reaction was stopped by adding 50 μl of 2 N $H_2SO_4$, and the absorbance was measured at 492 nm in a microtiter plate reader (LabSystems Multiskan®, Thermo Fisher Scientific, USA). Between every step, plates were washed with PBS, 0.05% (v/v) Tween 20 (PBS-T). Dilutions were prepared in PBS-T containing 0.1% (w/v) BSA.

*Determination of rhαGAL Enzymatic Activity.* 4MU-α-Gal assay was performed by preparing a reaction mixture (60 μl) containing 0.1 M sodium citrate buffer (pH 4.6), 5 mM 4MU-α-Gal, and enzyme at 37°C for 45 min, followed by termination of the enzyme reaction by addition of 0.24 mL of 0.1 M glycine-NaOH buffer (pH 10.7). The released 4-methylumbelliferone was determined by fluorescence measurement (excitation 365 nm, emission 460 nm) against a 4MU standard curve in a microplate fluorometer (Fluoroskan Ascent®, Thermo Fisher Scientific). One unit of enzyme activity was defined as the amount of enzyme that releases one nmol of 4-methylumbelliferone per hour (U).

### *Measurement of transgene copy number*

Transgene copy number was determined by real-time quantitative PCR (qPCR) of genomic DNA isolated from transduced cells using the following primers: forward primer, 5′-CGCTGCTTTAATGCCTTTGT-3′ and reverse primer, 5′-GGGCCACAACTCCTCATAAA-3′. Transgene copy numbers were determined against a standard curve generated using genomic DNA from a cell line harboring two copies per cell of transgene. This cell line has been previously obtained in our laboratory and its copy number has been confirmed by genome sequencing. Briefly, PCR reactions for unknown samples were performed in 20 μL of total volume containing SYBR Green PCR master mix (Thermo Fisher Scientific), 10 μM forward and reverse primers, suitable dilution equivalent to $1.0 \times 10^4$ copies of unknown sample genomic DNA and control genomic DNA. PCR reactions to generate the standard curve were also performed in 20 μL of total volume containing SYBR Green PCR master mix, 10 μM forward and reverse primers and standard genomic DNA ranging from $1 \times 10^7$ to $1 \times 10^3$ copies generated by serial dilution. qPCR was performed using the following conditions: one cycle at 95°C for 15 min followed by 40 amplification cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 20 s.

### *q-PCR-based assay for detection of LP derived contaminants*

To analyze gene expression of LP components (Gag-Pol cassette and VSV-G) in packaging/producer cells, qPCR of genomic DNA isolated from transduced cells was developed. qPCR reactions were performed as explained above, using specific primers for each protein: Gag forward primer, 5′-GAGCCACCCCACAAGATTTA-3′ and reverse primer, 5′-TATCCCATTCTGCAGCTTCC-3′; Pol forward primer, 5′-TTGACTCAGATTGGCTGCAC-3′ and reverse primer, 5′-TTTAACTTTTGGGCCATCCA-3′ and VSV-G forward primer, 5′- AATGCCCAAGAGTCACAAGG-3′ and reverse primer, 5′-CATACCAGCGGAAATCACAA-3′. The reactions were performed using one and ten copies of each vector as standards and a suitable dilution equivalent to $1.0 \times 10^4$ copies of unknown sample genomic DNA. As a positive control, a reaction was performed using the primers described for the measurement of transgene copy number.

### *rhαGAL production*

Suspension rhαGAL producer CHO-K1 cells were grown in erlenmeyer flasks (Corning, USA) at 37°C with 5% $CO_2$, agitated at 140 rpm in a shaking incubator cabinet (CERTOMAT®CT plus, Sartorius, Germany). Thereafter, cells were cultured in one-liter bioreactor in perfusion mode (Biostat Q Plus, Sartorius). During the culture, pH was set at ~7.0, $pO_2$ at 40% air-saturation and agitation rate at 150 rpm. The system temperature was varied between 37°C and 31°C. Media exchange and perfusion rate were regulated according to the growth profile. Samples were collected daily to determine cell density, cell viability, lactate and glucose levels.

*Cell Counting and Metabolite Analysis.* Cells were stained with trypan blue and counted using a Neubauer haemocytometer.

Glucose and lactate concentrations were measured using the Reflectoquant® system (Merck, EE.UU). In this system test strips (with immobilized specific enzymes for glucose and lactate) are incubated with the test sample and the developed color is determined reflectometrically. Measuring ranges are 1–100 mg $L^{-1}$ and 3–60 mg $L^{-1}$ for glucose and lactic acid, respectively. Ammonium concentration was determined using a commercial kit (SB, Argentina) based on the Berthelot reaction.

### *rhαGAL purification*

Clarified supernatants were obtained by filtration with 0.45 μm membrane filters (Sartobran P, Sartorius). Conditioned medium containing ~$3.8 \times 10^4$ U $mg^{-1}$ of rhαGAL was loaded onto a weak ionic exchange (IEX) resin (DEAE Sepharose Fast Flow, GE Healthcare) packed in a 1.6 × 2.48 cm XK 16/20 column equilibrated with 10 mM phosphate buffer, pH 6.0, connected to an ÄKTAexplorer 100 Air (GE Healthcare). rhαGAL was eluted using an isocratic 0.18 M NaCl gradient in the same buffer. Fractions containing the enzyme were pooled and stored at −20°C until the next purification step. Then, the sample was loaded onto a hydrophobic (HIC) resin (Butyl Sepharose 4 Fast Flow, GE Healthcare) packed in a 1.6 × 1.49 cm XK 16/20 column equilibrated in a 2 M NaCl, 10 mM phosphate buffer, pH 6.0. The enzyme was recovered using an isocratic gradient, decreasing the salt concentration (0.75 M NaCl, 10 mM phosphate buffer, pH 7.5). Eluted protein was dialyzed and concentrated against storage buffer (PBS). Finally, purified rhαGAL was aliquoted and stored at −70°C.

### *Physicochemical characterization*

*Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).* The purity of rhαGAL was assessed using 12% (w/v) polyacrylamide resolving gels and 5% (w/v) stacking gels and compared to the commercial enzyme (Fabrazyme®). Separation was performed at 200 V for 65 min. Gels were stained with Coomassie blue and destained with a solution containing 15% (v/v) methanol and 10% (v/v) acetic acid.

*Isoelectric Focusing (IEF).* To separate the rhαGAL isoforms, a 1-mm thick 8% (w/v) polyacrylamide gel containing 8 M urea was prepared. The pH range was established using 75% (w/v) 3–5 ampholytes and 25% (w/v) 5–7 ampholytes (GE Healthcare). The gel was prefocused at 30 W, 250 V and 50 mA for 30 min. Then, 10 μg of the samples were applied over a sample application strip placed at 1 cm from cathode. Electrophoresis was performed at 30 W, 250 V, and 50 mA during 90 min. Finally, the gel was stained using a Coomasie blue colloidal solution.

*Intrinsic Fluorescent Spectroscopy.* Spectrofluorimetric measurements were performed using a Perkin-Elmer LS-55 luminescence spectrometer equipped with a Xenon discharge lamp, Monk-Gillieson type monochromators and a gated photomultiplier connected to an AMD Sempron PC using Windows Xp. The acquisition of data was performed using the FL Winlab v4.00.03 software supplied by Perkin Elmer. In all cases quartz cells with planar walls of 700 μL were used.

A 5 nm slit width was used in excitation and 7 nm in emission monochromators, a 650 V detector voltage and a scan speed of 600 nm $min^{-1}$ were set. Emission spectra were recorded from 290 to 500 nm in 1 nm steps with excitation at 278 nm.

### *Biochemical characterization*

*Specific Activity.* Specific enzymatic activities of purified rhαGAL and Fabrazyme® were measured as described above.

*In vitro Stability in Plasma.* Fresh frozen plasma was prepared in our laboratory from peripheral blood of a single donor. Immediately after collection, the whole blood treated with heparin was centrifuged at 4000 rpm during 5 min at 20°C. Then, to eliminate platelets, the plasma was centrifuged at 6400 rpm during 5 min at 4°C, complement-inactivated 30 min at 56°C and stored at −20°C until use. The sample was obtained with the donor's informed consent.

Afterwards, rhαGAL and Fabrazyme® were spiked into human plasma to a final concentration of 5 ng $mL^{-1}$, and incubated at 37°C for 0, 15, 30, 45, and 60 min. Following plasma exposure, the samples were analyzed for residual enzymatic activity (compared to $t = 0$), using 4MU-α-Gal as an artificial substrate for rhαGAL, as explained above.

*Michaelis–Menten Kinetics.* The enzyme kinetic parameters were measured using 4MU-α-Gal assay. The reaction was initiated by the addition of rhαGAL or Fabrazyme® to a final concentration of 5 ng $mL^{-1}$. Measurements were generated from three individual experiments of eight data points each. The kinetic parameters were calculated employing Lineweaver-Burk (double-reciprocal) plot.

### *Glycosylation analysis*

*Monosaccharide Composition.* Type and amount of monosaccharides present in purified rhαGAL and Fabrazyme® glycans were determined by acid hydrolysis of the samples followed by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using a DIONEX ICS-5000 system equipped with a CarboPac™ PA20 column (Thermo Fisher Scientific Dionex). Briefly, 30 μg of each glycoprotein were hydrolyzed with 2 M trifluoroacetic acid (TFA) (for neutral sugars detection including Galactose (Gal), Mannose (Man) and Fucose (Fuc) detection) during 3 h at 100°C. Then, reaction tubes were cooled down at room temperature, the reaction mixture was dried using Concentrator Plus (Eppendorf, Germany) and dried samples were resuspended in high purity water. Elution was accomplished with 200 mM sodium hydroxide over 15 min. Monosaccharide mix standard solutions (CM-Mono-Mix-10, Ludger, UK) were treated like sample solutions and used for the identification and quantification of peaks deriving from glycoproteins samples.

*Sialic Acid and Mannose-6-phosphate Quantification.* Thirty micrograms of purified rhαGAL and Fabrazyme® were hydrolyzed under acid conditions either with 0.1 M HCl at 80°C for 1 h or 6.75 M TFA at 100°C for 1.5 h to release the sialic acid or M6P respectively. Elution was accomplished with a linear gradient, employing eluent A (100 mM NaOH) and eluent B (500 mM NaAc in NaOH) over 24 min. N-acetylneuraminic (Neu5Ac) and N-glycolylneuraminic (Neu5Gc) acid standards (Calbiochem, France) and M6P standard (Sigma-Aldrich) were treated like sample solutions and used for the identification and quantification of peaks deriving from glycoproteins samples.

*N-deglycosylation and Fluorescent Labeling with 2-aminobenzamide (2-AB).* Twenty micrograms of purified rhαGAL and Fabrazyme® were mixed with 15 U of PNGase F (New England Biolabs, UK) in a total volume of 20 μl and incubated at 37°C overnight. Samples were analyzed using SDS-PAGE followed by Coomasie blue staining to verify the complete digestion of both samples.

N-glycans released after the enzymatic digestion were incubated with 5 μl of 0.76 M 2-AB fluorophore labeling solution (Sigma-Aldrich) during 2 h at 65°C. To remove 2-AB excess, ascending paper chromatography in acetonitrile

using Whatmann 3 MM paper strips was performed. Afterwards, glycans were eluted from the dried paper with water. Samples were filtered using 0.45 μm PDVF filters (Millex, Millipore, France), evaporated and finally resuspended in 20 μl of water for evaluation.

Aliquots of the 2-AB labeled samples were treated with 2% (v/v) TFA at 80°C for 1 h (to remove sialic acids) or with 5 U of Alkaline Phosphatase (ALP, Alkaline Phosphatase, calf intestinal, Promega, USA) in a total volume of 50 μL and incubated at 37°C ON (to hydrolize phosphate groups).

*Weak Anion Exchange Chromatography (WAX) for Charged Labeled N-glycans Analysis.* To analyze the relative amount of neutral, mono-, bi-, tri- and tetrasialylated and mono-phosphorylates structures of both enzymes, weak anion exchange (WAX) chromatography was performed. The 2-AB labeled samples were injected in an ASAHIPAK ES-502N7C column (100 × 7.5 mm; SHODEX, Japan) connected to a HPLC system equipped with a fluorescence detector module (excitation 330 nm, emission 420 nm). A fetuin N-glycan standard (Sigma-Aldrich) was used to identify the different sialic acid structures and a phosphorylated oligmannosidic standard (TheraProteins, Portugal) was employed to identify high-mannose mono-phosphorylated structures.

### *Statistical analysis*

Statistical analysis was performed using two-tailed student́s t-test using GraphPad Prism for Windows, version 5.01. Differences between treatments were considered significant when $P < 0.05$.

## Results

### *Cell culture, transduction, and clonal isolation*

Suspension CHO-K1 cells were serially transduced (three times) with third-generation LP containing the coding sequence of rhαGAL. Performing repeated transductions is a procedure whose aim is to increase the copy number of the transgene and, consequently, the expression of the protein of interest. For example, Spencer et al.[16] reported the generation of bioengineered recombinant coagulation Factor VIII producer BHK cell lines by serially lentiviral vector transductions; overall, fVIII production rate and proviral copy number were observed to increase with additional transduction events. In effect, both rhαGAL productivity and specific enzyme activity increased following each transduction, with productivity values ranging from 8 pg $cell^{-1}.d^{-1}$ for TD1 cell population to 15 pg $cell^{-1}$ $d^{-1}$ for TD3 cell line. Enzymatic activity increased from $3.5 \times 10^3$ U $mg^{-1}$ for TD1 cell line to $1.1 \times 10^4$ U $mg^{-1}$ for TD3 cell line. Thus, the cell population, which underwent three rounds of transduction events, was chosen to be cloned by limit dilution method. Thereafter, 15 clones were analyzed for rhαGAL production and specific enzyme activity, obtaining expression levels ranging from 3.5 to 59.4 pg $cell^{-1}$ $d^{-1}$ and specific enzyme activities from $3.0 \times 10^3$ to $2.4 \times 10^4$ U $mg^{-1}$. One of the highest producing clones (P3G2) was selected for metabolism and cell growth analysis, and subsequent scale up.

### *P3G2 clone characterization*

In batch conditions, P3G2 clone reached cell densities of $3.6 \times 10^6$ cells $mL^{-1}$ after seven days of culture, with a viability rounding 100% along that culture period. Efficient cell culture process development for production of recombinant glycoproteins should maximize the cell productivity as well as the product yield and quality, while minimizing the process time and overall costs.[17] Cell growth and metabolism kinetic parameters were calculated: the specific growth rate was 0.005 $h^{-1}$; the cell specific glucose consumption rate was 0.140 pmol $cell^{-1}$ $h^{-1}$ and both the cell specific lactate and ammonium production rate were 0.020 pmol $cell^{-1}$ $h^{-1}$. Lactate concentration remained below 13 mM, an acceptable value considering that lactate toxic action, probably due to the effect on the pH and osmolarity, only occurs at higher concentrations (>20–30 mM). Ammonium concentration reached a maximum peak around 4 mM, but it did not seem to cause any deleterious effect, since it has been described that concentrations higher than such value are responsible of the reduction of the specific growth rate and the final cell density in batch cultures, induction of apoptotic and necrotic cell death or perturbation of protein glycosylation.[18]

In addition, P3G2 transgene copy number was determined by qPCR, indicating the existence of $2.2 \pm 0.1$ copies of the transgene, in comparison with the $42.6 \pm 2.4$ copies of the TD3 cell population from which it derives. Importantly, no copies of LP-derived components were identified in $1.0 \times 10^4$ copies of the transgene in the rhαGAL producer cell line.

### *rhαGAL production*

Cells were cultured in serum-free medium in a one-liter bioreactor in perfusion mode during 14 days, reaching cell densities up to $1.8 \times 10^7$ cell $mL^{-1}$ and cell viabilities up to 95%. Perfusion rate varied between 0.5 and 1.0 reactor volumes per day, as it was required (Figure 1), maintaining nearly constant lactate and glucose amounts. Concentrations up to 35 μg $mL^{-1}$ of rhαGAL were attained in ~10 L of harvest.

### *rhαGAL purification*

Clarified crude supernatant (~$3.8 \times 10^4$ U $mg^{-1}$) containing approximately 1.7 mg of enzyme was loaded onto a weak ionic exchange resin equilibrated with 10 mM phosphate buffer, pH 6.0. Following a wash with the same buffer, the enzyme was eluted employing isocratic NaCl gradient. The rhαGAL was efficiently bound to the resin and eluted with a recovery of 100% (Table 1). No leakage of the enzyme was observed during the loading and washing steps of this first chromatography, which allowed reaching purity around 23% (Figure 2A) and about a 13-fold increase in specific enzymatic activity. Next, a second purification step was performed, based in a hydrophobic interaction chromatography. Partially purified enzyme (~$5.0 \times 10^5$ U $mg^{-1}$) was loaded onto the resin under high ionic strength conditions and then eluted by decreasing the NaCl concentration. Most of the enzyme contaminants were removed during the washing step, while the elution condition allowed recovering the enzyme with a yield of 60%. This step was essential to remove most protein contaminants, as revealed by SDS-PAGE (Figure 2A), and it led to a fivefold increase in specific activity regarding the previous purification step. As a

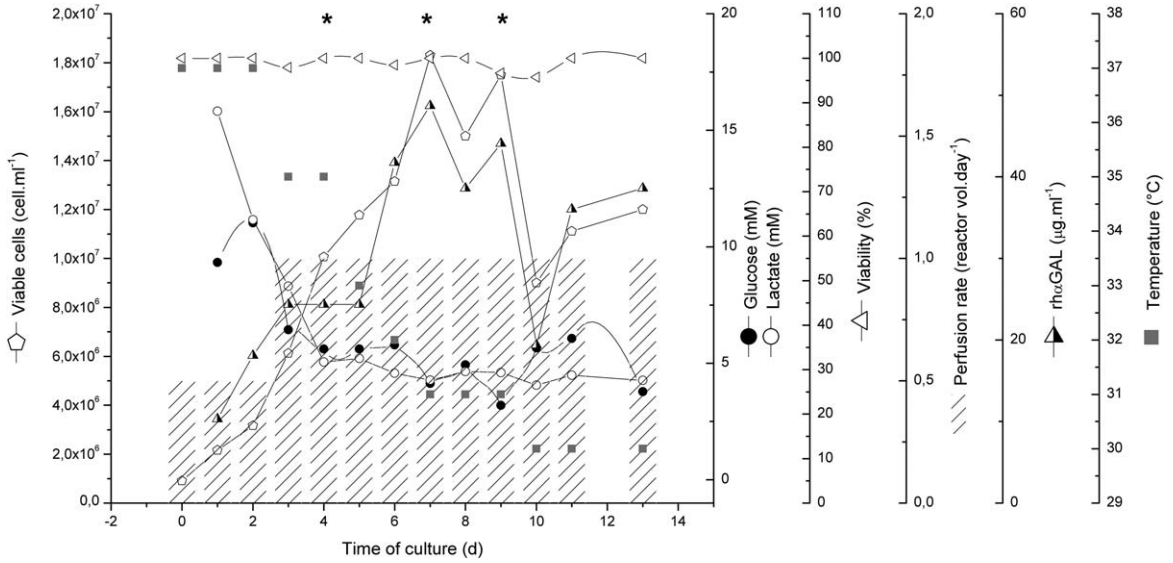


**Figure 1. Culture of P3G2 clone in 1 L bioreactor in perfusion mode. Profiles of cell density, cell viability, glucose, lactate, rhαGAL concentrations, perfusion rate and temperature are shown. Asterisk character indicates cell bleeding.**

**Table 1. Summary of Purification of rhαGAL**

| | Volume (mL) | Activity (U $mL^{-1}$) | Total Protein (mg $mL^{-1}$) | Specific activity (U $mg^{-1}$) | Recovery (%) | Enrichment (fold) | Purity (%) |
|---|---|---|---|---|---|---|---|
| **Cell harvest** | 75.0 | $4.3 \times 10^4$ | 1.1 | $3.8 \times 10^4$ | 100 | 1 | 2.0 |
| **IEX eluate** | 9.0 | $4.2 \times 10^5$ | 0.8 | $5.0 \times 10^5$ | 118 | 13 | 23.1 |
| **HIC eluate** | 1.0 | $1.9 \times 10^6$ | 0.8 | $2.4 \times 10^6$ | 59 | 63 | 102.5 |
| **Fabrazyme®** | | | | $(1.6 \times 10^6 – 4.8 \times 10^6)$* | | | |

All purification steps were performed at 4°C. Enzyme activity was determined after combining fractions using 4MU-α-Gal as a substrate. Total protein was determined using modified Bradford assay. Recovery was estimated from total enzyme activity. Enrichment was calculated as a relation between specific activities. Purity (%) was determined as a relation between enzyme concentration and total protein concentration (mg $mL^{-1}$).

*Calculated specific activity for Fabrazyme®.

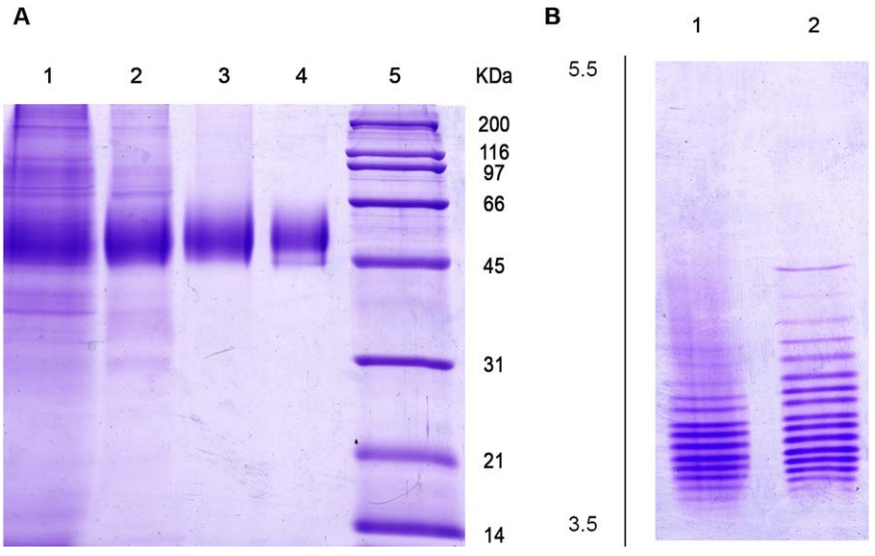


**Figure 2. (A) SDS-PAGE of fractions corresponding to different stages of the purification process of rhαGAL. Lane 1, clarified crude supernatant; lane 2, eluate from first chromatographic step; lane 3, eluate from second chromatographic step; lane 4, Fabrazyme® (reference molecule); lane 5, molecular mass standards. (B) IEF profiles of rhαGAL (1) and Fabrazyme® (2) glycoforms. The line indicates the range of pH employed.**

result, after two purification steps, the active enzyme was recovered ($2.4 \times 10^6$ U $mg^{-1}$) with high purity and 60% overall yield. A purity of 100% was obtained considering the rhαGAL amount and total protein content determined by ELISA and Bradford assay, respectively (Table 1). Here, this apparent 100% purity value can be explained as resulting from experimental errors. In addition, the purity of rhαGAL was assessed by SDS-PAGE and compared to Fabrazyme®.

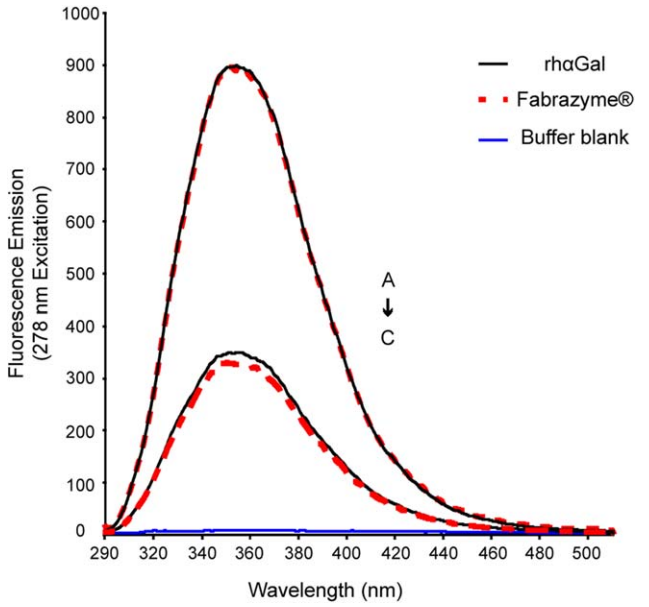


**Figure 3. Tryptophan emission spectra (278 nm excitation) of rhαGAL and Fabrazyme® in phosphate buffer. A-1 μM protein; B-0.25 μM protein; C-Buffer blank.**

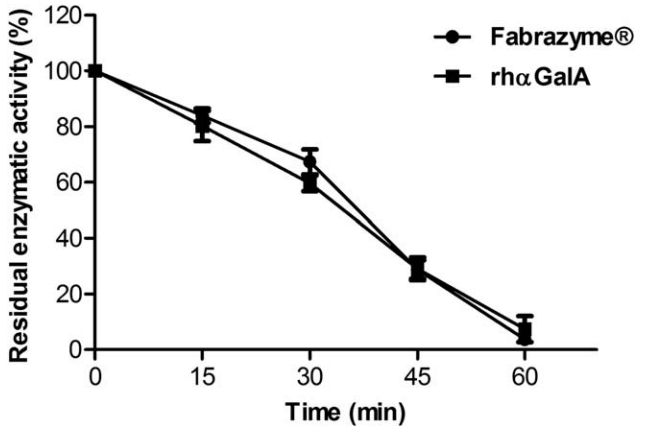


**Figure 4. *In vitro* stability of rhαGAL and Fabrazyme® following exposure to human plasma. Stability was measured as the remaining activity of the enzymes analyzed by the hydrolysis of the synthetic substrate 4MU-α-Gal after incubation in human plasma. The results are expressed as the percentage of the initial enzymatic activity. Results are an average of six measurements ± S.D.**

A purity nearly 98% was obtained for both molecules. Moreover, we did not measure process-related impurities like host cell proteins, virus, endotoxins or nucleic acids. By this means, probably an extra polishing step will be need to reach a purity higher than 99.00%. Importantly, the specific activity of rhαGAL was comprised within the range determined in this work for Fabrazyme® (Table 1), and it was also included between the values reported in literature.[19]

### *Physicochemical characterization*

*SDS-PAGE and IEF.* SDS-PAGE and IEF followed by Coomasie blue staining were performed to analyze the apparent molecular mass and isoform distribution of purified rhαGAL in comparison with Fabrazyme®. The apparent molecular mass of both molecules was nearly the same: a monomer band of about 50 kDa was observed. As Sohn et al.[12] reported, Fabrazyme® presented a second band of lower molecular mass, which could appear due to the low stability arising from lyophilized storage in the vial (Figure 2A). Besides, both enzymes showed nearly identical mobility profiles after N-deglycosylation using peptide N-glycosidase PNGase F (data not shown). Furthermore, both products exhibited complex isoelectric focusing patterns, with a wide diversity of glycoforms of low isoelectric point (Figure 2B). However, although both enzymes shared a considerable number of isoforms, rhαGAL showed a higher proportion of glycoforms concentrated in the more acidic zone of the pH range compared to Fabrazyme®.

**Table 2. Kinetic Parameters for the Hydrolysis of the Synthetic Substrate 4MU-α-Gal**

| | Vmax (nM. $min^{-1}$) | Km (mM) | Kcat ($s^{-1}$) |
|---|---|---|---|
| **Fabrazyme®** | 63.2 ± 25.3 | 1.2 ± 0.2 | 21.1 ± 8.4 |
| **rhαGAL** | 60.1 ± 12.4 | 1.6 ± 0.6 | 20.04 ± 4.14 |
| ***P*-value** | 0.7673 | 0.3363 | 0.7664 |

*Intrinsic Fluorescent Spectrometry.* Because the fluorescence profile is extremely sensitive to perturbations in the local structural environment, it provides simple and powerful evidence supporting a high degree of structural similarity between different batches of a given protein. In addition, it can provide useful insights into product comparability and biosimilarity. Molecules that exhibit fluorescence have aromatic groups, which in the case of protein drugs concern aromatic amino acids (i.e., Trp, Phe, Tyr, but primarily Trp).[20] rhαGAL has 16 Trp molecules, two of which are solvent exposed.[21] Emission spectra of rhαGAL and Fabrazyme® were recorded from 290 to 500 nm in one nm steps with excitation at 278 nm. No discernible difference was observed between fluorescence peak maximum (345 nm) of both proteins, and both spectra were practically superimposed, indicating that the purification process of rhαGAL did not alter the local polarity of the Trp and therefore did not change the protein structure (Figure 3).

### *Biochemical characterization*

*In Vitro Stability in Plasma.* The *in vitro* stability of rhαGAL and Fabrazyme® following exposure to human plasma was assessed by analysis of their residual enzymatic activity. Both molecules showed similar profiles ($P > .05$): as soon as they were diluted in plasma their activity began to fall, retaining ~29% of the initial value after 45 min of incubation (Figure 4).

*Michaelis-menten Kinetic.* To compare the kinetic parameters of rhαGAL and Fabrazyme®, enzymatic hydrolysis of the 4MU-α-Gal synthetic substrate was measured. Michaelis–Menten analysis demonstrated that rhαGAL was able to hydrolyze the synthetic substrate at a comparable rate to Fabrazyme®. Moreover, no statistical differences were found between the kinetic parameters of both molecules (Km, Vmax and Kcat) (Table 2).

### *Analysis of glycosylation*

*Sialic Acid and M6P Composition.* Sialic acid and M6P are key residues for successful enzyme replacement therapy

**Table 3. Monosaccharide Composition of rhαGal and Fabrazyme®**

| Monosaccharide (mol. mol rhαGAL$^{-1}$) | Fabrazyme® | rhαGAL |
|---|---|---|
| **M6P** | 2.9 ± 0.6 | 3.1 ± 0.3 |
| **Neu5Ac Acid** | 7.1 ± 0.7 | 9.2 ± 1.1 |
| **Neu5Gc Acid** | 0.082 ± 0.016 | 0.031 ± 0.005 |
| **Fuc** | 1.5 ± 0.1 | 2.8 ± 0.2 |
| **Gal** | 12.2 ± 0.2 | 11.8 ± 0.2 |
| **Man** | 10.6 ± 0.4 | 14.7 ± 2.5 |
| **GlcNAc*** | ND† | ND† |
| **Sialic acid: Gal** | 0.60 | 0.80 |

*N-acetylglucosamine.
†Not determined.

for Fabry disease, and thus it is crucial to determine their content. Mannose-6-phosphate is required to promote the uptake of rhαGAL into the lysosomes of cells by the M6P receptor. Sialic acid capping is essential to mask terminal galactose, reducing uptake of the protein by the asialoglycoprotein receptor in the liver. This results in proper tissue distribution of the enzyme, which exhibits a long circulatory half-life in the body.[12] In addition, the content of Neu5Gc acid should be measured. N-glycolylneuraminic is a sialic acid found in many animal cells (including CHO cells) that is not found in humans because of the lack of the enzyme required for its synthesis.[22] Hence, it constitutes an antigenic residue whose amount should be controlled to avoid therapy failure.

Both molecules presented similar levels of M6P (3.1 ± 0.3 and 2.9 ± 0.6 mol per mol protein for rhαGAL and Fabrazyme®, respectively) ($P > .05$), which is one of the major factors determining the proper lysosomal targeting of the enzyme. The acid sialic content (Neu5Ac) of rhαGAL was about 40% higher than the corresponding one for Fabrazyme® (9.2 ± 1.1 and 7.1 ± 0.7 mol per mol protein, respectively) ($P < .05$). This could represent a considerable advantage of rhαGAL over Fabrazyme®, since it has been widely demonstrated that the higher the content of sialic acid, the longer the half-life of the protein.[23–25] Regarding Neu5Gc, rhαGAL exhibited a 33% reduced content of this immunogenic type of sialic acid compared to Fabrazyme®, defining another benefit of our molecule (Table 3).

*Monosaccharide Analysis.* Monosaccharide analysis indicated that there were no differences in the amounts of Gal and Man ($P > .05$). The amount of Fuc was approximately 50% higher for rhαGAL (Table 3) ($P < .05$). It should be noted that the ratio sialic acid:galactose was higher for rhαGAL, due to the fact that both preparations displayed the same content of galactose but rhαGAL exhibited higher amounts of sialic acid. This could mean that more galactose residues may be capped in rhαGAL, favoring its slower removal from circulation and promoting a longer duration of action in comparison with Fabrazyme®.

*N-deglycosylation and Fluorescent Labeling with 2-AB.* . To evaluate the sialylation and phosphorylation pattern of rhαGAL in comparison with Fabrazyme®, the N-glycans from each enzyme were isolated and labeled with 2-AB, as described in Materials and Methods section. The untreated 2-AB labeled glycans were then applied to a WAX-HPLC column and separated according to their charge. Glycans were identified as neutral- (asialo-), mono-, di-, tri-, tetrasialylated and high-mannose mono-phosphorylated structures using proper standards.

As it can be noted in Figure 5, the elution profiles of N-glycans from each sample were quite similar. However, in comparison with Fabrazyme®, rhαGAL presented a smaller proportion of neutral structures (16.9% for Fabrazyme® and 5.2% for rhαGAL) and a higher proportion of sialylated species (67.5% for Fabrazyme® and 81.6% for rhαGAL), particularly regarding bi- and tetra-sialylated structures (Table 4). This was in accordance with the IEF assay and sialic acid quantitation, where a higher proportion of highly-sialylated glycoforms were detected for rhαGAL.

Regarding high-mannose mono-phosphorylated species, identification was performed by comparing retention times with reference glycan standards and based on the 2-AB labeled glycans treated with 2% (v/v) TFA or ALP analysis. Only two peaks were visible after treatment with 2% (v/v) TFA: the first one corresponding to neutral glycans and the second one (between thirty and forty minutes), to high-mannose mono-phosphorylated glycans. Likewise, the peak between thirty and forty minutes disappeared after treatment with ALP, but peaks corresponding to sialylated structures were observed (Supporting Information Figure S1A,B). Both molecules showed similar relative proportion of mono-phosphorylated structures (13.9% for rhαGAL and 15.5% for Fabrazyme®, respectively). This correlated with M6P quantitation, which indicated that both molecules contained nearly the same amount of that residue.

## Discussion

Drugs for rare diseases, so-called orphan drugs, are an important public health concern and a challenge for the medical community since they are often difficult to discover, develop and market. Basic research in the mechanisms of rare diseases receives relatively little attention and financial support in both academia and industry.[26]

Enzyme replacement therapies (ERTs)—in which a defective or absent enzyme is replaced by exogenous injection regularly throughout the patient's life—have been used to treat lysosomal storage disorders since the early 1990s. Gaucher's disease, caused by a deficiency in the enzyme glucocerebrosidase, was the first of such disorders for which an ERT was developed.[2] Regarding Fabry disease, two different recombinant enzymes have been developed: agalsidase beta and agalsidase alfa. In addition, another recombinant version, Pegunigalsidase alfa (PRX-102, Protalix, Biotherapeutics) is in advanced stages of development (phase III clinical trial). This therapeutic is produced in a plant cell-based protein expression system and is chemically modified with a homobifunctional polyethylene glycol molecule, resulting in a PEGylated variant.[1] However, access to orphan drugs continues to be limited by high prices. Pricing of orphan drugs is unique in that the costs of research and development must be retrieved from a small number of patients. Also, the lack of interest from companies to develop generic alternatives for such extremely small patient populations allows for a price monopoly in which the innovative manufacturer can set virtually any price. Given this, orphan drugs are relatively expensive, often exceeding €100.000 per patient per year (e.g., agalsidase alfa, costs on average US$266K per patient per year).[27]

Currently, different strategies have been applied to optimize the global production process of rhαGAL. Selection of best-performing clones is one of the major time-consuming

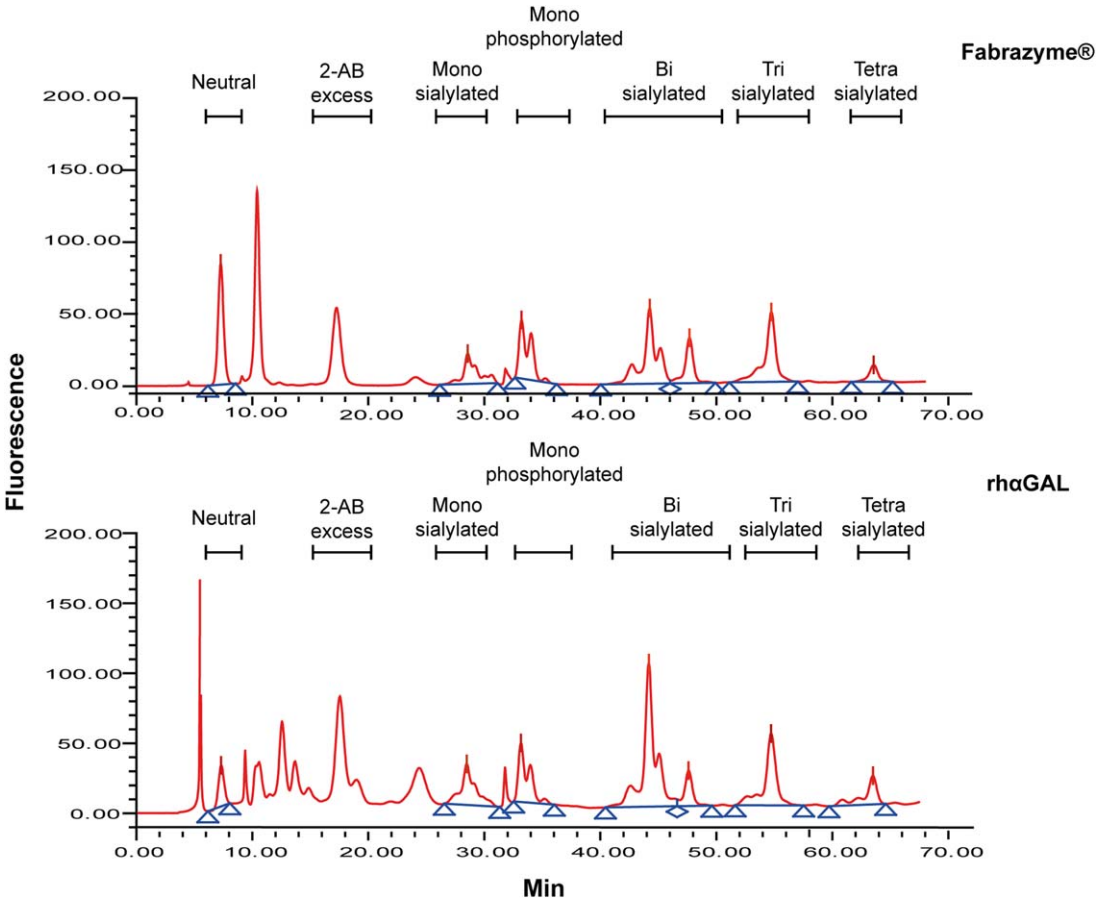


**Figure 5. Analysis of native 2-AB labeled N-glycans from Fabrazyme® and rhαGAL by WAX-HPLC. The 2AB-labelled N-glycans were separated based on their charge.**

**Table 4. Relative Amount of Charged Glycans in rhαGAL and Fabrazyme®**

| N-glycans (%) | Fabrazyme® | rhαGAL |
|---|---|---|
| **Neutral** | 16.9 | 5.2 |
| **Mono-sialylated** | 10.2 | 12.5 |
| **Mono-phosporylated** | 15.5 | 13.9 |
| **Bi-sialylated** | 34.1 | 43.4 |
| **Tri-sialylated** | 19.3 | 18.0 |
| **Tetra-sialylated** | 3.9 | 7.7 |

tasks and it further increases the cost of development and production. The highest enzyme expressing clone with the proper biochemical characteristics described until now, AGA5.3, obtained by methotrexate amplification, secreted rhαGAL levels of 7.5 pg cell$^{-1}$ d$^{-1}$ with specific enzymatic activity of 1.3 × 10$^4$ U mg$^{-1}$.[28,29] Sonh et al.[12] reported the development of CHO clones with 30-fold higher productivities of rhαGAL (more than 150 mg L$^{-1}$ d$^{-1}$), than the one achieved by AGA5.3 clone in the hollow fiber bioreactor (5 mg L$^{-1}$ d$^{-1}$).[30] However, the resulting enzyme (I303) appeared to experience incomplete sialylation due to the gap between the high level of protein production and the low capacity of the glycosylation process taking place during secretion. Despite obtaining complete sialic acid capping after carrying out an *in vitro* enzyme reaction, this inefficiency makes the production process time-consuming, laborious and more expensive, as an additional step is required to reach the final product. Transient gene expression has also been proposed as an alternative strategy to obtain rhαGAL in suspension human HEK293 cells.[6] Transfection of human HEK293 cells has been extensively used for small and large-scale transient protein production, with yields exceeding 50 mg L$^{-1}$ of recombinant protein. However, most of recombinant proteins produced for clinical trials are expressed in stable CHO cell line and their production levels are generally lower than those of HEK293 cells.[31]

Under these circumstances, we herein report the production of rhαGAL in suspension CHO-K1 cells, using third-generation LP as an optimized expression platform. LP have been demonstrated to be powerful tools for the delivery of genes or RNAi into mammalian genomes. They have advantages over other gene delivery systems in that they can efficiently transduce cells that are not undergoing cell division. They are actively transported into the nucleus and thus they do not require breakdown of the nuclear membrane for access. In addition, the copy number of the integrated viral vector can be controlled by varying the multiplicity of infection (MOI).[11] To achieve a high copy number, host cells are transduced at a high MOI so that the expression starts out at relatively high levels. In this context, serial transduction of suspension CHO-K1 cells employing rhαGAL LP was performed at a MOI of ~35. Interestingly, clone P3G2 presented two copies of the transgene, in comparison with the ~43 copies of the cell population (TD3) from which it derived, maintaining high expression levels. This could be attributed to the fact that the transgene insertion occurred in a highly stable chromosome site, and it is especially desirable because it simplifies the posterior identification of the

transgene insertion sites in the chromosome's manufacturing cell line. In addition, to ensure that the master cell bank is safe for therapeutic use, the detection of LP-derived contaminants is essential. None copy of LP component was identified in $1.0 \times 10^4$ copies of the transgene in rhαGAL producer cell line.

Since LP have been used in human gene therapy, clinical trials and safety testing assays and procedures have been developed. These could be easily applied to the development of manufacturing cell lines for protein therapeutics to ensure the absence of unwanted viral material in cell banks and biologic products.[11,32] Besides, the third-generation LP system is widely used for clinical purposes. It is a four-plasmid system, consisting of three helper plasmids and one transfer vector plasmid, which constitutes the only genetic material transferred to the target cells.[33]

The studies reported herein demonstrate, once more, the potential of lentiviral vectors to deliver the transgene in manufacturing cell lines, as we have obtained a panel of 15 clones with high productivities (3.5 to 59.4 pg cell–$^1$ d$^{-1}$) and specific enzyme activities ($3.0 \times 10^3$ to $2.4 \times 10^4$ U mg$^{-1}$). By this means, our clones achieve up to eightfold higher productivities and up to 1.8-fold higher specific enzymatic activities in culture supernatants compared to the ones corresponding to AGA5.3 clone, which constitutes the actual platform for the production of the commercially available algasidase beta, Fabrazyme®. This result is consistent with most others that report high protein production using LP as a gene delivery system. Although higher productivity values of rhαGAL have been reported by Sohn et al.,[12] the quality of the produced enzyme (I303), especially regarding glycosylation, was not appropriate to reach full therapeutic efficacy. Thus, additional *in vitro* sialylation reactions were required. Instead, our process achieves a balance between high productivity values and adequate biochemical properties, which are critical for rhαGAL *in vivo* activity without the need of further steps. Indeed, as it is discussed later, our enzyme exhibits improved glycosylation characteristics compared not only to I303 but also to Fabrazyme®, which may positively influence its therapeutic efficacy. This result is consistent with most others that report high protein production using LP as a gene delivery system.[11,16,32,34]

This platform eliminates the need of using drugs such as methotrexate or methionine sulfoximine and, as this procedure allows starting with a highly expressing pool, may reduce the number of clones that need to be screened. Indeed, it is known that the ability to adapt host cells to grow in suspension is highly desirable, as it allows volumetric scalability and permits the use of protein- and serum-free media.[35] Using third-generation lentiviral vectors as an expression platform gives the possibility to perform cell transduction directly in suspension conditions. By this, we can reduce the time and cost of the overall production process of rhαGAL, replacing a method that implies months to achieve modest protein yields by a protocol that involves less than a month, avoiding drug selection and posterior expansion of the small cell population that achieved positive integrations events.[11]

After two purification steps, the active enzyme was recovered ($2.4 \times 10^6$ U mg$^{-1}$) with 98% purity and 60% overall yield. During purification and subsequent storage, many processes can occur that may probably interfere with the biological activity of the enzyme. To assess the proper folding and enzymatic properties of rhαGAL, we compared the kinetic parameters of rhαGAL with those of Fabrazyme®. Michaelis-Menten analysis demonstrated that rhαGAL was capable of hydrolyzing the synthetic substrate (4MU-α-Gal) at a comparable rate to Fabrazyme® without significant differences between their kinetic parameters. Additionally, both proteins presented the same stability in plasma at 37°C, and no apparent differences were detected in their structural conformation, as assessed by fluorescence spectrometry.

Moreover, unlike the amino acid sequence of the therapeutic protein, glycosylation is not only determined by the sequence of the inserted gene but also by the cascade of post-translational modifications that the eukaryotic host cells can perform. Therefore, it can vary from host to host and even from clone to clone depending on the fermentation medium, specific productivity, and the physiological stage of the producer cell.[23] The glycosylation pattern assessed by IEF assay demonstrated that both enzymes presented almost the same type of charged glycans but in different proportions, since rhαGAL had a higher proportion of glycoforms situated in the more acidic zone of the gradient.

For successful enzyme replacement therapy for Fabry disease, the importance of M6P and sialic acid content in rhαGAL products has been reported.[12] Analysis of M6P glycans is important to determine the efficiency of lysosomal targeting and, therefore, therapeutic efficacy.[36] Sialic acid capping is essential to mask terminal galactose, which reduces uptake by the asialoglycoprotein receptor in the liver and thus results in proper tissue distribution with a long half-life in the body. Moreover, charge and size of proteins are important for glomerular filtration: since the glomerular filter is negatively charged, the anionic molecules are repelled and not filtered.[37] M6P content was the same for both proteins, whereas the Neu5Ac amount of rhαGAL was about 40% higher than the corresponding one for Fabrazyme®. Also, rhαGAL exhibited a 33% reduced content of the immunogenic type of sialic acid (Neu5Gc) compared to Fabrazyme®. These results were comparable to the ones obtained by WAX-HPLC, which indicated the presence of more negatively charged structures and less neutral structures for rhαGAL. Taking into account that the higher the sialic acid content, the longer the serum half-life of rhαGAL, these results may probably have a significant impact on the protein therapeutic efficacy, probably reducing the amount of drug and the frequency of administration during therapy.[24,38] However, these results must be verified in the future by *in* vivo assays.

## Conclusions

To summarize, in this study we have replaced the methotrexate amplification strategy by third-generation lentiviral transduction of CHO-K1 suspension cells for rhαGAL production. In this way, we have not only obtained clones, which demonstrated a suitable balance between high productivity and high quality of the enzyme, but we have also enhanced the efficiency of gene delivery and reduced the timeline for obtaining a producer cell line. After two purification steps, the active enzyme was recovered with 98% purity and 60% overall yield. Importantly, our product evidenced properties similar to those of the current ERT available for Fabry disease produced in CHO cells (Fabrazyme®). Moreover, our enzyme exhibited improved properties regarding glycosylation, in particular, higher sialic

acid content and a reduced percentage of antigenic Neu5Gc glycan, attributes which may derive in an enhanced *in vivo* efficacy of rhαGAL. Taken together, our results represent a significant advance that could improve the overall production process of a promising therapeutic alternative for Fabry disease.

## Conflict of Interest

The authors declare that they have no conflict of interests.

## Acknowledgments

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