

## Enhanced sialylation and *in vivo* efficacy of recombinant human $\alpha$ -galactosidase through *in vitro* glycosylation

Youngsoo Sohn<sup>1,3</sup>, Jung Mi Lee<sup>2</sup>, Heung-Rok Park<sup>3</sup>, Sung-Chul Jung<sup>4</sup>, Tai Hyun Park<sup>1,\*</sup> & Doo-Byoung Oh<sup>2,\*</sup>

<sup>1</sup>School of Chemical and Biological Engineering, Seoul National University, Seoul 151-742, <sup>2</sup>Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon 305-806, <sup>3</sup>ISU ABXIS Co. Ltd, Seoul 120-752, <sup>4</sup>Department of Biochemistry, School of Medicine, Ewha Womans University, Seoul 158-710, Korea

**Human  $\alpha$ -galactosidase A (GLA) has been used in enzyme replacement therapy for patients with Fabry disease. We expressed recombinant GLA from Chinese hamster ovary cells with very high productivity. When compared to an approved GLA (agalsidase beta), its size and charge were found to be smaller and more neutral. These differences resulted from the lack of terminal sialic acids playing essential roles in the serum half-life and proper tissue targeting. Because a simple sialylation reaction was not enough to increase the sialic acid content, a combined reaction using galactosyltransferase, sialyltransferase, and their sugar substrates at the same time was developed and optimized to reduce the incubation time. The product generated by this reaction had nearly the same size, isoelectric points, and sialic acid content as agalsidase beta. Furthermore, it had better *in vivo* efficacy to degrade the accumulated globotriaosylceramide in target organs of Fabry mice compared to an unmodified version. [BMB Reports 2013; 46(3): 157-162]**

### INTRODUCTION

Fabry disease, an X-linked genetic disease, is known to arise from a deficiency in the lysosomal enzyme  $\alpha$ -galactosidase A (GLA), which hydrolyzes the terminal  $\alpha$ -galactosyl moieties from glycolipids and glycoproteins. Defects in the encoding gene cause a failure to catabolize alpha-D-galactosyl glycolipid moieties in the lysosome. The resulting accumulation of globotriaosylceramide (Gb3) leads to progressive multi-systemic damage to the kidney, heart, and cerebrovascular system. Early symptoms of this form of glycolipid accumu-

lation include peripheral extremities, vascular skin lesions, decreased sweating and corneal opacity. With increasing age, these symptoms develop into functional failures in the heart, brain, and kidney, finally leading to death.

Several lysosomal storage diseases, including Fabry disease, can be treated with enzyme replacement therapy by employing an injection of a recombinant enzyme with proper glycans for lysosomal targeting. Currently, two different recombinant GLAs are developed and approved as therapeutic enzymes for Fabry disease. One is agalsidase beta (Fabrazyme, Genzyme) produced in CHO cells and the other agalsidase alfa (Replagal, Shire HGT) generated in human cultured fibroblasts. Comparative studies of their glycosylation patterns, enzyme activities and therapeutic effects have been reported (1, 2). Both enzymes are homodimeric glycoproteins with the same amino acid sequences having three N-glycosylation sites in each monomer. However, the differences in the glycosylation patterns between two products are observed mainly in the contents of sialic acid and mannose-6-phosphate (1, 2). These two sugar residues are essential factors for the successful targeting to the lysosomes of the tissues affected in Fabry patients. Specifically, the mannose-6-phosphate residues of lysosomal enzymes are recognized by mannose-6-phosphate receptors and the enzymes are transported to the lysosome via the endosome. However, some portions escaping this pathway are secreted into the extracellular space and can be retaken up by mannose-6-phosphate receptors located on the plasma membrane. This "secretion recapture" mechanism utilizing mannose-6-phosphate receptors has been the basis for functional complementation by an injection of recombinant enzymes. Successful targeting also requires the terminal sialic acid capping of complex type glycans, as the asialoglycoprotein receptor in the liver rapidly removes the glycoprotein containing the exposed galactose residues from blood circulation. The increased level of sialic acids of glycoproteins is well known to correlate with the prolonged half-life in the serum (3). Studies comparing both GLA products reported that agalsidase beta showed superior activity in Fabry mouse models, mainly due to the fact that it contains higher contents of mannose-6-phosphate and sialic acid (1, 2).

We expressed GLA in CHO cells using conventional re-

\*Corresponding author. Doo-Byoung Oh, Tel: +82-42-860-4457; Fax: +82-42-879-8494; E-mail: dboh@kribb.re.kr; Tai Hyun Park, Tel: +82-2-880-8020; Fax: +82-2-875-9348; E-mail: thpark@snu.ac.kr  
<http://dx.doi.org/10.5483/BMBRep.2013.46.3.192>

Received 18 September 2012, Revised 21 September 2012,  
Accepted 21 September 2012

**Keywords:** Alpha-galactosidase A, Enzyme replacement therapy, Fabry disease, *In vitro* glycosylation, Sialic acid

combinant technologies with much higher productivity compared to that of agalsidase beta. Unfortunately, the produced recombinant GLA was found to have a low level of sialic acid, which may have been caused by the biased selection of a high producer lacking the capability to add sialic acids. To enhance its sialic acid content, an *in vitro* glycosylation reaction using galactosyltransferase, sialyltransferase and their sugar substrates was established and optimized. The sialic acid content and *in vivo* efficacy of the resulting glycosylated product were analyzed in a parallel comparison with agalsidase beta and unmodified GLA.

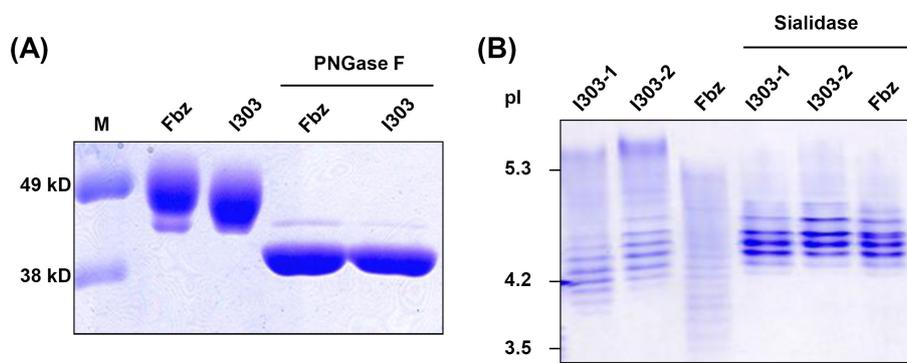
## RESULTS AND DISCUSSION

### GLA produced from high-producer CHO cells lacks sialic acid contents

The gene encoding GLA was amplified from the cDNA of human fibroblast by a polymerase chain reaction and cloned into the CHO cell expression vector containing the dihydrofolate reductase (DHFR) gene. The resulting vector was transfected to a DHFR-deficient CHO cell line (DG44) after which high-expressing clones were generated by a conventional methotrexate (MTX) amplification method. The productivity of the selected clone was measured as more than 150 mg/L/day by an enzyme-linked immunosorbent assay (data not shown), which is

>30 times higher than the productivity (5 mg/L/day) in the hollow fiber bioreactor described in the patent for agalsidase beta (EP2210947). Recombinant GLA was purified from the cell culture supernatant by three purification steps including anion exchange and hydrophobic interaction chromatography resins, and the purity after each step was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Supplementary Fig S1A). Interestingly, weak intensity of the GLA homodimer band was detected around ~95 kDa, even in the denaturing condition of SDS-PAGE, while a strong monomer band was noted between 42 and 49 kDa. In contrast, the peak of the GLA homodimer was only detected in gel permeation chromatography with more than 99% purity (Supplementary Fig. S1C). It was termed I303.

The size of I303 was found slightly smaller than that of agalsidase beta when compared in SDS-PAGE (Fig. 1A). However, after deglycosylation using peptide N-glycosidase (PNGase) F, both enzymes showed nearly identical mobility levels, indicating that the relatively smaller size of I303 resulted from the different N-linked glycosylation. Furthermore, an image of the isoelectric focusing (IEF) gel showed that the multiple bands of I303 are located in more neutral areas, in contrast to those of agalsidase beta, which are dispersed in acidic regions (Fig. 1B). Here, two different batches of I303 were compared, and both of them found in more neutral regions although slight



**Fig. 1.** Comparison of the sizes and charges of agalsidase beta and I303. (A) Sizes of agalsidase beta (Fbz; Fabrazyme) and I303 were compared in SDS-PAGE before and after deglycosylation using PNGase F digestion. Here, M represents the molecular weight marker. (B) Their isoelectric patterns were analyzed in IEF gel with/without a sialidase treatment. Here, I303-1 and I303-2 represent two different batches of the I303 product.

**Table 1.** Monosaccharide compositions of recombinant GLAs

	Monosaccharide mol/mol protein		
	Agalsidase beta	I303	I303 <sup>ES</sup>
N-acetylglucosamine	14.7 ± 0.3	16.4 ± 4.3	23.0 ± 1.2
Mannose	16.3 ± 3.6	14.2 ± 2.8	17.7 ± 3.0
Mannose-6-phosphate	2.9 ± 0.1	3.0 ± 0.1	3.2 ± 0.2
Galactose	8.3 ± 0.3	4.3 ± 0.9	5.7 ± 1.0
Sialic acid	7.3 ± 0.2	2.8 ± 0.2	6.2 ± 0.8
Sialic acid/Galactose <sup>a</sup>	0.88	0.65	1.09

<sup>a</sup>The ratios between sialic acid and galactose were calculated in order to estimate the level of sialic acid capping.

batch variations were observed. After the removal of sialic acids by a sialidase treatment, these different patterns of isoforms were converged to the same pattern of multiple bands with a neutral shift, which confirmed that the differences in the isoelectric patterns were caused by negative charges of sialic acids. Notably, although the complexities of multiple bands were greatly reduced after the sialidase treatment, multiple patterns representing charge heterogeneity still existed, suggesting that there are other factors, such as mannose-6-phosphate, contributing to the complex charge status.

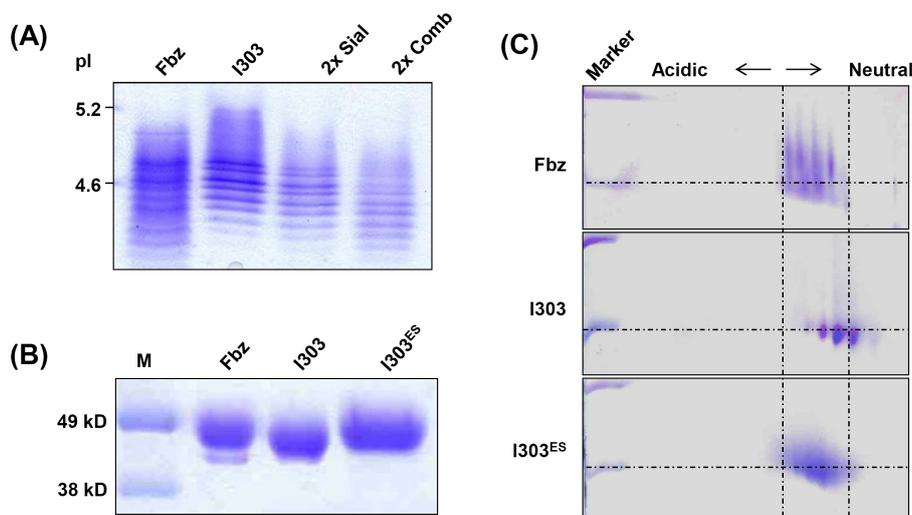
Direct analysis of the monosaccharide composition showed that one mole of I303 had only 2.8 mol of sialic acids, which was less than half of the sialic acid content of agalsidase beta (7.3 mol/mol protein, Table 1). From the ratio of the sialic acid and galactose amounts, it could be calculated that only ~65% of all galactosyl residues are capped with terminal sialic acids, in contrast to ~88% for agalsidase beta (Table 1). Taken together, I303, having an equivalent protein moiety with agalsidase beta, appears to experience incomplete sialylation possibly due to the gap between the high level of protein production and the low capacity of the glycosylation process taking place during secretion.

#### Establishment of an *in vitro* enzyme reaction to enhance sialylation

To increase the sialic acid content of I303, we initially carried out an *in vitro* enzyme reaction using  $\alpha$ (2,3)-sialyltransferase and CMP-N-acetylneuraminic acid (NANA) as the donor sugar based on a previously reported reaction condition (4). Here, NANA is the most commonly found sialic acids. However, this simple reaction scarcely affected the charge status of I303, indicating an insignificant increase in sialylation (Supplementary Fig. S2A). To avoid cases in which the activity of sialyltransferase is reduced and/or the donor sugar becomes exhausted dur-

ing a long incubation time, another aliquot of sialyltransferase and CMP-NANA was added after 12 hours of incubation, which was then followed by further incubation for 12 hours. However, the resulting sialylation increase was still insignificant. Therefore, it was postulated that an efficient increase in sialylation also requires the addition of galactose to the exposed terminal N-acetylglucosamines of the glycans as a prerequisite reaction.

Galactosylation was combined with sialylation with two different ways in an effort to find the best condition. First, in a stepwise reaction, galactosylation using  $\beta$ (1,4)-galactosyltransferase and UDP-galactose as the donor sugar was carried out and then followed by sialylation as described in the previous paragraph. On the other hand, the combined reaction represents a single-step reaction in which I303 was treated with a combination of  $\alpha$ (2,3)-sialyltransferase and  $\beta$ (1,4)-galactosyltransferase in the presence of CMP-NANA and UDP-galactose as the donor sugars. The conditions for both reactions are described in the Materials and Methods section and are summarized in Supplementary Table S1. Both reactions added more sialic acids to I303 than simple sialylation (Supplementary Fig. S2), which confirmed the prerequisite of galactosylation for efficient sialylation. However, the sialylation levels of the resulting products were still lower when compared to that of agalsidase beta. As a subsequent attempt, we increased the amount of glycosyltransferases and donor sugars by two times compared to those previously reported (4). This doubling in a combined reaction condition (2x combined) dramatically increased the sialic acid content of I303 (Supplementary Fig. S2A). Finally, we tested whether the incubation time of this reaction could be shortened because the prolonged reaction at 37°C may have harmful effects on the enzyme activity and stability of GLA. As a combined reaction has the advantage of reducing the incubation time compared



**Fig. 2.** Changes of charge and size induced by enhanced sialylation. (A) After *in vitro* glycosylation including 2x sialylation (2x Sial) and 2x combined (2x Comb) reactions, isoelectric points of I303 were moved to more acidic regions, which are comparable to those of agalsidase beta (Fbz; Fabrazyme). (B) The size of the I303<sup>ES</sup> generated by the 2x combined reaction increased slightly to have the same mobility as agalsidase beta in SDS-PAGE. (C) Changes in the size and charge induced by the 2x combined reaction were analyzed in 2D gel.

to a stepwise reaction, only 2x combined reactions with different incubation times were analyzed. Surprisingly, an enhancement of the sialylation process was almost completely achieved in only 2 hours and reached a plateau (Supplementary Fig. S2B). We selected a 6-hour incubation time for the 2x combined reaction as a sufficient time without batch variations. The I303 containing an enhanced amount of sialic acid by this reaction was renamed as I303<sup>ES</sup>.

### Comparison of I303<sup>ES</sup> with I303 and agalsidase beta

The charge and size of I303<sup>ES</sup> generated by the 2x combined reaction were compared with those of agalsidase beta and I303 by IEF, SDS-PAGE and 2D-gel analysis (Fig. 2). The charged status of I303 was shifted to a similar acidic region of agalsidase beta only by the 2x combined reaction (Fig. 2A). The incomplete increase in the sialic acid content by 2x sialylation without galactosylation clearly showed the requirement of the galactosylation as a prerequisite again. The size of I303<sup>ES</sup> was larger than that of I303, which is nearly the same size as agal-

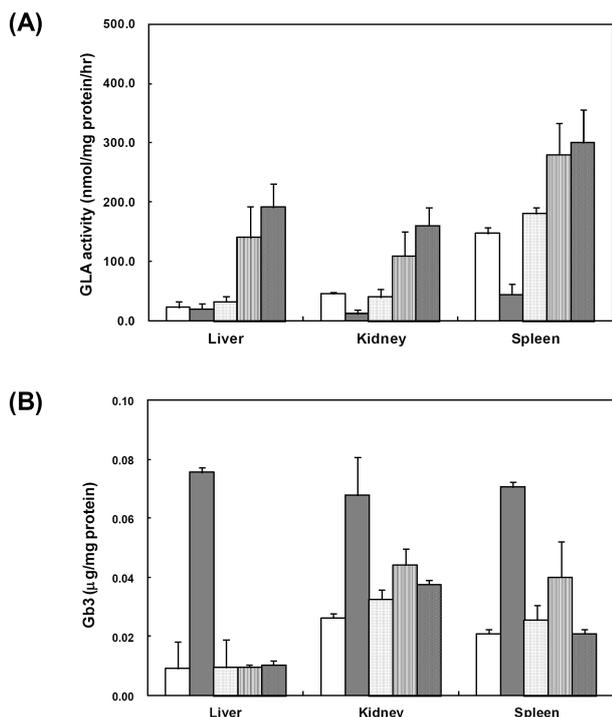
sidase beta (Fig. 2B). Interestingly, a doublet-containing weak band of a smaller size was observed in agalsidase beta, whereas the I303<sup>ES</sup> displayed a single protein band corresponding to the upper band of the doublet. Also, the charge and size of three GLAs were compared at the same time through a 2D-gel analysis (Fig. 2C). The bands of I303<sup>ES</sup> were detected in nearly the same region of the agalsidase beta bands, while I303 bands were located in a more neutral and smaller area. Notably, I303<sup>ES</sup> showed a more continuous band pattern while agalsidase beta and I303 had discrete band patterns, especially in the first IEF dimension.

Monosaccharide compositions of agalsidase beta, I303 and I303<sup>ES</sup> were determined by high-pH anion exchange chromatography (Table 1). One mol of I303<sup>ES</sup> was shown to have ~6.2 mol of sialic acid and ~5.7 mol of galactose, which were 220% and 130% increases, respectively, as compared to ~2.8 mol of sialic acid and ~4.3 mol of galactose in I303. These values were slightly lower than the ~7.3 mol of sialic acid and 8.3 mol of galactose for agalsidase beta. However, it is noteworthy that I303<sup>ES</sup> showed a higher ratio of sialic acid to galactose than agalsidase beta (Table 1). The calculated ratio of 1.09, which was more than one, represented complete sialic acid capping without exposed galactose residue. Here, the value over one can be explained as resulting from experimental errors when considering the high values of the standard deviations. All GLAs showed high levels of mannose with >14 mol per mol of protein, which represented the presence of high-mannose-type glycans considering three *N*-glycosylation sites of GLA. Also, they had similar levels of mannose-6-phosphate (2.9-3.2 mol per mol protein) which is one of the major factors determining the proper lysosomal targeting.

### *In vivo* efficacies of recombinant GLAs compared in Fabry mice

We employed Fabry mice (GLA gene knock-out) for *in vivo* efficacy analyses of agalsidase beta, I303, and I303<sup>ES</sup>. The enzyme activities of GLA in the liver, kidney and spleen were analyzed 72 hours after an injection into the tail vein of Fabry mice (Fig. 3A). As expected, the GLA activities in the kidney and spleen of the Fabry mice were strikingly lower than those of the wild type. The injections of all three recombinant GLAs into Fabry mice greatly increased these activities up to a level far over those of the wild type, but the degrees of the increases were different. In this case, I303 and I303<sup>ES</sup> showed much higher activity levels in all of the tested organs compared to agalsidase beta, which may result from the differences in the enzyme stability levels. Compared to the fresh preparations of I303 and I303<sup>ES</sup>, agalsidase beta may have low stability arising from lyophilized storage in the vial, which may be related to its doublet band as observed in SDS-PAGE.

The instances of degradation of the accumulated Gb3 by the injected GLA was also directly analyzed (Fig. 3B). Because I303 had glycans with deficient capping of the sialic acids, a significant portion of the I303 was expected to be trapped in



**Fig. 3.** Effects of agalsidase beta, I303, and I303<sup>ES</sup> in Fabry mice. (A) The GLA activities in the liver, kidney and spleen were measured. Fabry mice were sacrificed 72 hours after the single-dose injection (1 mg/kg body weight) of GLAs. (B) Levels of Gb3 in the same organs were analyzed 72 hours after the injection. Wild-type mice, open bar; Fabry mice, filled bar; Fabry mice injected with agalsidase beta, dotted bar; Fabry mice injected with I303, vertically striped bar; Fabry mice injected with I303<sup>ES</sup>, hatched bar. Error bars represent the mean  $\pm$  standard deviation (n = 3).

the liver, containing the asialoglycoprotein receptor, which results in a short half-life in serum and an unwanted body distribution pattern. As expected, the Gb3 degradation induced by the injection of I303 was lower in the target organs (kidney and spleen) as compared to agalsidase beta, while the degradation in the liver was restored up to the level of wild-type mice. However, I303<sup>ES</sup> with increased sialic acid levels via the 2x combined reaction induced more degradation of Gb3 in the kidney and spleen to the level decreased by the injection of agalsidase beta. This result clearly represents the importance of sialic acid capping for proper targeting and tissue distribution, as the only difference between I303 and I303<sup>ES</sup> is the sialic acid content generated by *in vitro* glycosylation.

For successful enzyme replacement therapy for Fabry disease, the importance of mannose-6-phosphate and the sialic acid content in GLA products has been reported (1,2). Mannose-6-phosphate is required to promote the uptake into the lysosomes of cells by the mannose-6-phosphate receptor. Sialic acid capping is essential to mask terminal galactose, which reduces uptake by the asialoglycoprotein receptor in the liver and thus results in the proper tissue distributions with a long half-life in the body. We succeeded in constructing a high-producer cell line with >150 mg/L/day productivity. Unfortunately, the produced GLA was found to have a low level of sialic acid, though it has an amount of mannose-6-phosphate similar to that of commercial agalsidase beta. Therefore, we developed and optimized an *in vitro* glycosylation process designed to increase the sialic acid content. In particular, a 2x combined reaction using high amounts of galactosyltransferase, sialyltransferase, and their sugar substrates at the same time was selected to reduce the incubation time which is also a critical factor for enzyme activity and stability. The resulting product showed almost complete sialic acid capping, which also increased the *in vivo* efficacy to degrade the accumulated Gb3 in the kidney and spleen of Fabry mice. This study provides an efficient strategy to enhance the sialylation of therapeutic enzymes *in vitro* and confirms the importance of sialic acid capping for proper tissue targeting.

## MATERIALS AND METHODS

### Construction of CHO cell lines expressing human GLA

The full-length cDNA of human  $\alpha$ -galactosidase was cloned from the human fibroblast cDNA library using PCR and the cDNA was inserted into the pMSG cloning vector. The expression vector containing the human  $\alpha$ -galactosidase gene was transfected into Chinese hamster ovary (CHO) cell lines with a Dospert<sup>TM</sup> (Roche) device using the lipo-lectin method. After 48 hours of incubation at 37°C with 5% CO<sub>2</sub>, transfected cells were collected and inoculated into 96-well plates at the initial cell density (6-7 × 10<sup>5</sup> cells/well) with fresh  $\alpha$ -MEM (Gibco, Grand Island, NY, USA) containing 10% dFBS. The adapted cell lines were subjected to amplification by increasing the concentration of methotrexate from 10 nM to 1  $\mu$ M.

Individual clones were isolated with a limiting dilution method and clones which expressed the highest levels of GLAs were selected after analysis by an enzymatic assay.

### Purification of recombinant GLA

The culture supernatant was centrifuged at 3,000 g for 20 min and then further filtered for clarification using a 0.45  $\mu$ m filter (Millipore, Billerica, MA, USA). After a buffer exchange (10 mM sodium phosphate, pH 7.0), the resulting solution was loaded onto a DEAE sepharose fast-flow column (GE Healthcare, Uppsala, Sweden) pre-equilibrated with the same buffer. Elution was carried out by applying the linear gradient (0-0.5 M sodium chloride) of sodium chloride and the fraction containing the recombinant GLA was collected. The pooled fractions were loaded on hydrophobic interaction chromatography (phenyl sepharose high-performance column, GE Healthcare) after adjusting their salt concentrations fractions to 1 M of sodium chloride. The bound recombinant GLA was eluted from the column using a linear gradient from 1 to 0 M sodium chloride. The fractions containing GLA were collected and dialyzed with formulation buffer (50 mM sodium phosphate, pH 7.0). The purity and identity were checked by SDS-PAGE and Western blotting using the anti-human  $\alpha$ -galactosidase monoclonal antibody (ISU ABXIS, Seoul, Korea), respectively.

### SDS-PAGE, IEF and 2D-gel analysis

SDS-PAGE was performed using a precast gel in the NuPAGE Gel System (Life Technologies, Carlsbad, CA, USA). For the determination of the charged status, IEF was performed using Novex pH 3-10 IEF Gel (Life technologies). For 2D-gel electrophoresis, first-dimension IEF was carried out using a pH 3-10 immobilized pH gradient (IPG) Immobiline DryStrip on the Ettan IPGphor system (GE Healthcare). For the second-dimension electrophoresis process, the resulting IPGs strips were equilibrated for 15 min by rocking in the first solution (6 M urea, 30% glycerol, 2% SDS, bromophenol blue, 1% DTT, 50 mM Tris-HCl, pH 8.8) and were equilibrated again for 15 min in the second solution (6 M urea, 30% glycerol, 2% SDS, bromophenol blue, 2.5% iodoacetamide, 50 mM Tris-HCl, pH 8.8). The equilibrated strips were laid on 12% SDS-PAGE gel with 1% agarose in a cathode buffer and electrophoresis was carried out.

### Monosaccharide composition analysis

A monosaccharide analysis for neutral and amino sugars, sialic acid and mannose-6-phosphate was carried out using high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD, DX-600, Dionex, Sunnyvale, CA, USA), as previously described (1) with slight modifications. Briefly, for the analysis of the neutral and amino sugars, 40  $\mu$ g of samples were hydrolyzed with 2 M of trifluoroacetic acid at 100°C for 4 h. The same amounts of samples were hydrolyzed under mild acid conditions with either 0.1 M

HCl at 80°C for 1 hour or 6.75 M trifluoroacetic acid at 100°C for 1.5 hour to release the sialic acid or mannose-6-phosphate, respectively. Analytes were separated with CarboPac PA1 or PA10 analytical column (Dionex).

### Experimental design of *in vitro* glycosylation

For *in vitro* glycosylation, the final formulation buffer containing the active protein ingredient was exchanged for a buffer of 50 mM of MES at pH 6.4 by repetitive ultracentrifugation using an YM-10 Microcon membrane (Millipore). For sialylation, 1 mg of GLA was incubated in 1 ml of solution in total with 10 mU recombinant rat  $\alpha(2,3)$ -sialyltransferase (Merck, Darmstadt, Germany), 5  $\mu$ mol CMP-NANA and 5  $\mu$ mol MnCl<sub>2</sub> at 37°C for 12 hours. Then, the same amounts of aliquots of  $\alpha(2,3)$ -sialyltransferase, CMP-NANA and MnCl<sub>2</sub> were added and the mixture was further incubated for another 12 hours. For stepwise galactosylation and sialylation, 1 mg of GLA was also treated with 20 mU bovine  $\beta(1,4)$ -galactosyltransferase (Merck), 10  $\mu$ mol UDP-galactose and 5  $\mu$ mol MnCl<sub>2</sub> at 37°C for 12 hours. Then, 20 mU  $\alpha(2,3)$ -sialyltransferase, 10  $\mu$ mol CMP-NANA and 5  $\mu$ mol MnCl<sub>2</sub> were added to the reaction solution and further incubated for another 12 hours. For combined galactosylation and sialylation, 10 mU  $\beta(1,4)$ -galactosyltransferase and 10 mU  $\alpha(2,3)$ -sialyltransferase together with 5  $\mu$ mol each of UDP-galactose, CMP-NANA and MnCl<sub>2</sub> were added to the GLA solution (1 mg/ml) and incubated at 37°C for 12 hours. Then, another aliquot of galactosyltransferase, sialyltransferase, nucleotide sugars and cofactor MnCl<sub>2</sub> were added and the mixture was incubated for additional 12 hours. For 2x sialylation and the 2x combined reaction, doubled amounts of glycosyltransferase, nucleotide sugars and a cofactor were used for the same strategies with the sialylation and combined reactions previously described. The amounts of reaction components used for all five reactions (sialylation, stepwise, combined, 2x sialylation and 2x combined reactions) are summarized in Supplementary Table S1. After optimization experiments to determine the best reaction condition, the 2x combined reaction with a short reaction time (6 hours) was selected for the generation of I303<sup>ES</sup>.

### *In vivo* activity assay using Fabry mice

Fabry mice, which were kindly provided by Dr. Roscoe O. Brady of the National Institute of Health (Bethesda, MD, USA), were 18 weeks old at the beginning of the study. All mice were genotyped by the PCR method, as described previously (5). All animals were treated in accordance with the Animal Care Guidelines of the Ewha Womans University School of

Medicine (Seoul, Korea). For enzyme replacement therapy, the mice received an infusion of 1.0 mg/kg body weight of agalsidase beta, I303, and I303<sup>ES</sup> via the tail vein. They were sacrificed 72 hours after the injection and their tissues were analyzed. The extraction and saponification of lipids and the extraction of the glycolytic fraction were performed as described previously (6). The glycolipid fraction was mixed with 5 ml of *N*-acetyl-galactosylsphingosine and 795  $\mu$ l of 80% dioxane and then analyzed using a liquid chromatography-mass/mass spectrometer system (LC-MS/MS, ABI 4000; Applied Biosystems, Foster City, CA, USA). Glycolipid quantitation was performed using a C8 column and an evaporative light-scattering detector. The Gb3 standard was obtained from Matreya (Pleasant Gap, PA, USA).

### Acknowledgements

This study was supported by a grant to D.-B. Oh from the Next-Generation BioGreen 21 Program (SSAC-PJ008001).

### REFERENCES

1. Lee, K., Jin, X., Zhang, K., Copertino, L., Andrews, L., Baker-Malcolm, J., Geagan, L., Qiu, H., Seiger, K., Barngrover, D., McPherson, J. M. and Edmunds, T. (2003) A biochemical and pharmacological comparison of enzyme replacement therapies for the glycolipid storage disorder Fabry disease. *Glycobiology* **13**, 305-313.
2. Sakuraba, H., Murata-Ohsawa, M., Kawashima, I., Tajima, Y., Kotani, M., Ohshima, T., Chiba, Y., Takashiba, M., Jigami, Y., Fukushige, T., Kanzaki, T. and Itoh, K. (2006) Comparison of the effects of agalsidase alfa and agalsidase beta on cultured human Fabry fibroblasts and Fabry mice. *J. Hum. Genet.* **51**, 180-188.
3. Choi, S. S. and Park, T. H. (2006) Enhancement of sialyltransferase-catalyzed transfer of sialic acid onto glycoprotein oligosaccharides using silkworm hemolymph and its 30K protein. *J. Mol. Catal. B: Enz.* **43**, 128-132.
4. Raju, T. S., Briggs, J. B., Chamow, S. M., Winkler, M. E. and Jones, A. J. (2001) Glycoengineering of therapeutic glycoproteins: *in vitro* galactosylation and sialylation of glycoproteins with terminal *N*-acetylglucosamine and galactose residues. *Biochemistry* **40**, 8868-8876.
5. Choi, J. O., Lee, M. H., Park, H. Y. and Jung, S. C. (2010) Characterization of Fabry mice treated with recombinant adeno-associated virus 2/8-mediated gene transfer. *J. Biomed. Sci.* **17**, 26.
6. Rose, H. G. and Oklander, M. (1965) Improved procedure for the extraction of lipids from human erythrocytes. *J. Lipid. Res.* **6**, 428-431.