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Production of recombinant human acid β-glucosidase with high mannose-type N-glycans in rice gnt1 mutant for potential treatment of Gaucher disease

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Author Comments:	Dear Editor, may 2, 2018 I want to submit a manuscript entitled as 'Production of recombinant human acid β-glucosidase with high mannose-type N-glycans in rice gnt1 mutant for potential treatment of Gaucher diseasee' to be considered as a publication at Plant Cell reports. This manuscript was prepared from the work enhancing the production of recombinant human acid β-glucosidase in rice gntI mutant rice cell suspension culture. This manuscript was sent to professional textcheck company and English in this manuscript was proofread by the specialist majoring related field of this study. The authors signify that the contents have not been published elsewhere and the paper is not being submitted elsewhere, and consent to the paper being submitted for consideration for publication. Please inform the corresponding author of any additional requirements or information that may be needed. Thank you very much for your courtesy. Sincerely yours, Nan-Sun Kim
Suggested Reviewers:	Strasser Richard Universitat fur Bodenkultur Wien richard.strasser@boku.ac.at He is the professional in field of N-glycosylation pathway and engineering.
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Production of recombinant human acid β-glucosidase with high mannose-type *N*-glycans in rice *gnt1* mutant for potential treatment of Gaucher disease

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Key message

This work is the first attempt to express recombinant human acid β -glucosidase with terminal mannose residues in rice *gnt1* mutant. It will be useful for the treatment of Gaucher disease.

Abstract

Gaucher disease is an inherited metabolic disease caused by genetic acid β -glucosidase (GBA) deficiency and is currently treated by enzyme replacement therapy. For uptake into macrophages, GBA needs to carry terminal mannose residues on their *N*-glycans. Knockout mutant rice of *N*-acetylglucosaminyltransferase-I (*gnt1*) have a disrupted N-glycosylated pathway and produce only glycoproteins with high mannose residues. In this study, we introduced a gene encoding recombinant human GBA into both wild-type rice (WT) and rice *gnt1* calli. Target gene integration and mRNA expression were confirmed by genomic DNA PCR and Northern blotting, respectively. Secreted rhGBAs in culture media from cell lines originating from both WT (WT-GBA) and rice *gnt1* (*gnt1*-GBA) were detected by Western blotting. Each rhGBA was purified by affinity and ion exchange chromatography. *In vitro* catalytic activity of purified rhGBA was comparable to commercial Chinese hamster ovary cell-derived rhGBA. *N*-glycans were isolated from WT-GBA and *gnt1*-GBA and analyzed by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The amounts of high mannose-type *N*-glycans were highly elevated in *gnt1*-GBA (almost 86%) compared to WT-GBA (almost 1%).

Keywords: Gaucher disease, acid β-glucosidase (GBA), rice cell suspension culture, *N*-glycosylation, *N*-acetylglucosaminyltransferase-I (GnTI), rice *gnt1* mutant.

Introduction

Gaucher disease is a lysosomal storage disorder that causes skeletal deterioration, hepatosplenomegaly, anemia, and thrombocytopenia. It is a chronic multisystem disease resulting from deficient activity of acid beta-glucosidase (GBA), which catalyzes the hydrolysis of glucosylceramide (glucocerebroside, GlcCer). This defect causes the accumulation of GlcCer in cells, especially lysosomes in macrophages, resulting in the manifestation of clinical symptoms (Grabowski and Hopkin 2003; Jmoudiak and Futerman 2005).

Enzyme replacement therapy (ERT) can be applied to patients to improve symptoms (Barton et al. 1991; Beutler 2004; Grabowski et al. 1995; Grabowski and Hopkin 2003; Pastores et al. 1993; Weinreb et al. 2002). Currently, recombinant human GBAs (rhGBAs) expressed in Chinese hamset ovary (CHO) cells (Cerezyme®) and carrot cells (Elelyso®) have been approved for ERT for Gaucher disease (Grabowski et al. 1995; Jmoudiak and Futerman 2005; Shaaltiel et al. 2007; Weinreb et al. 2002).

GBA binding and subsequent internalization into target cell macrophages is mediated by interactions between terminal mannose residues on GBA and mannose receptors on the cell surface (Bijsterbosch et al. 1996; Sato and Beutler 1993). GBA is known to contain five potential *N*-glycosylation sites (Berg-Fussman et al. 1993; Brumshtein et al. 2006). Exposure of terminal mannose residues by a sequential *in vitro* deglycosylation strategy in Cerezyme® production dramatically improved its uptake into target cells (Bijsterbosch et al. 1996; Doebber et al. 1982; Friedman et al. 1999; Furbish et al. 1981). Elelyso®, the first FDA-approved plant-derived pharmaceutical protein, was used for targeted sorting into protein storage vacuoles, which are expected to possess activities that expose terminal mannose residues on pauci-mannosidic *N*-glycan (Shaaltiel et al. 2007).

Plant cell suspension culture has many advantages such as safety and cost-effectiveness. No animal-derived components are used in the entire production procedure, complementing the safety advantages associated with plant culture processing (Corbin et al. 2016). The rice alpha-amylase 3D (*RAmy3D*) promoter and terminator constitute a powerful system for recombinant protein production, and secretion of target proteins into media plays a role in increasing the simplicity and ease of purification (Chung et al. 2014; Kim et al. 2014; Kim et al. 2011).

rhGBA was first expressed and purified in rice cell suspension culture by Nam (2017). rhGBA was also previously expressed and secreted into culture media. However, in some cases, glycoproteins produced in wild-type (WT) rice suspension culture possessed relatively small amounts of terminal mannose residues and

other undesirable *N*-glycans (Jung et al. 2016; Shin et al. 2010). Hence, additional glycoengineering steps or modification of the glycosylation pathway is needed to achieve any therapeutic potential.

Recently, recombinant acid alpha-glucosidase with only high mannose residues was produced in rice *gnt1* calli (Jung et al. 2017). *N*-acetylglucosaminyltransferase-I (GnTI) can be used to initiate further processing of *N*-glycans such as fucosylation, xylosylation, galactosylation, and/or sialylation in the Golgi apparatus by adding *N*-acetylglucosamine onto the *N*-glycan core structure. Loss of GnTI function blocks the glycosylation pathway mentioned above and results in the presence of only high mannose-type *N*-glycans (Fanata et al. 2013; He et al. 2012; Strasser et al. 2008).

In this study, we expressed rhGBA in both WT and rice *gnt1* calli using a rice alpha-amylase expression system to compare their characteristics. In addition, we analyzed the enzymatic activities and *N*-glycan patterns of target proteins to prove their therapeutic potential.

Materials and Methods

Vector construction and cloning

cDNA clone of the human acid β-glucosidase (*hGBA*) gene (Genbank accession no: NM 000152) was purchased from Sino Biological Inc. (Beijing, China). The *hGBA* gene without a signal peptide-coding region was amplified by a standard PCR procedure using a pair of primers (sig-GBA-F : 5‘- AAC TTG ACA GCC GGG GCC CGC CCC TGC ATC-3’; GBA-His-R(*Sac*I) : 5’-TAT CTC TCA GTG GTG ATG GTG ATG ATG CTG GCG ATG CCA CAG). The signal peptide region of the rice α-amylase 1A gene was amplified by PCR using a pair of primers (Sig-F (*Xba*I) : 5’- TCT AGA ATG CAG GTG CTG AAC ACC ATG-3’; Sig-GBA-R : 5’-GAT GCA GGG GCG GGC CCC GGC TGT CAA GTT-3’) and fused to the 5’end of the *hGBA* gene by overlapping PCR. The prepared DNA fragment 1Asp-GBA-His was excised by *Xba*I and *Sac*I and then introduced into the same site of pCAMBIA1300 (Hajdukiewicz et al. 1994) under control of the rice α-amylase 3D promoter using the 3’UTR of the *Ramy3D* gene as a terminator to form pMYD94.

The gene expression cassette was digested with *Hin*dIII and *Eco*RI from pMYD94. The digested fragments were ligated into rice expression vector pMYP130 (Kim et al. 2013) by using T4 DNA ligase (Takara Bio, Shiga, Japan), yielding plasmid pMYD96.

Rice Transformation and genomic DNA PCR analysis

WT (*Oryza sativa* L. cv. Dongjin) and *gnt1* rice calli (SAI2G12, http://signal.salk.edu/cgi-bin/RiceGE) were propagated on calli induction medium and transformed using the individual plant expression vectors pMYD94 and pMYD96 by particle bombardment-mediated transformation, as described previously (Chen et al. 1994). After bombardment, calli were cultured on N6 co-culture medium supplemented with 2,4-D (2 mg/L), sucrose (30 g/L), kinetin (0.2 mg/L), and glucose (10 g/L) without antibiotics for 3 days under dark conditions and then transferred to N6 selection (N6SE) medium supplemented with 2 mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D), 0.02 mg/L of kinetin, 3% sucrose, and either hygromycin B (50 mg/L) or DL-phosphinothricin (PPT; 4.4 ppm). After 3~4 weeks, putative transgenic calli surviving on selection medium were transferred onto new N6 selection medium based on their name (D94 and D96) and number and then further propagated. Genomic DNA PCR was conducted after genomic DNA extraction using a Zymobead™ genomic DNA kit (Zymoresearch, Irvine, USA). A pair of primers (HGBA-PCR F: 5’-GCA

GCC AGA ACA GAA GTT CC -3' and GBA-His R1 (*Sac*I): 5'-GAG CTC TCA GTG GTG ATG GTG ATG ATG CTG GCG ATG CC -3') was used to confirm integration of the *hGBA* gene into the putative transgenic cell lines. Thermal cycling was performed for 30 cycles, consisting of 1 min at 94 °C 1 min at 56 °C and 1 min 30 sec at 72 °C. The PCR products were electrophoresed on a 1.0% (w/v) agarose gel, visualized by staining with ethidium bromide, and observed under UV light.

Northern blot analysis

Expression of the *hGBA* gene under control of the *RAmy3D* promoter in transgenic rice calli was induced for 3 days under sugar starvation conditions. Total RNA was extracted from WT rice, rice *gnt1*, and transgenic rice calli using TRI Reagent (Molecular Research Center, Cincinnati, OH), according to the supplier's protocol. To prepare the specific probe, an approximately 800-bp fragment of the *hGBA* gene was amplified from plasmid pMYD94 using forward primer (5'-CTC ACC ACA ATG TCC GCC TAC TC -3') and reverse primer (5'-GAG CTC TCA CTG GCG ATG CC -3'). The fragment was amplified using primeSTAR HS DNA polymerase (Takara Bio, Shiga, Japan). Thermal cycling was performed for 30 cycles, consisting of 30 sec at 94 °C, 30 sec at 56 °C, and 45 sec at 72 °C. The RNA samples (20 μg) were separated by electrophoresis onto a 1.2% formaldehyde–agarose gel (Lehrach et al. 1977) and capillary-blotted onto an Hybond N+ membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The blot was hybridized overnight in a Hybridization Incubator (Finemould Precision Ind. Co., Seoul, Korea) with a ^{32}P-labeled, randomly primed fragment of *hGBA* probe (Promega, WI, USA) at 65 °C in buffer (pH 7.4) containing 1 mM EDTA, 250 mM $Na_2HPO_4.7H_2O$, 1% hydrolyzed casein, and 7% SDS. The blot was washed twice with washing buffer A (2 X SSC, 0.1% SDS), twice with washing buffer B (2 x SSC, 1 % SDS), and twice with washing buffer C (0.1 X SSC, 0.1 % SDS) for 15 min, each at 65 °C. Hybridized bands were detected by autoradiography using X-ray film (Fuji Photo Film Co. HR-G30, Tokyo, Japan).

Propagation and induction of rice cell suspension culture

Transgenic rice calli showing strong signals for *hGBA* mRNA on the Northern blot were selected, and cell suspension culture was setup at 28 °C in the dark using a rotary shaker with a rotation speed of 110 rpm. To maintain the cell line, the cell suspension was cultured in 500 mL flasks using N6SE medium. Transgenic rice cell lines were sub-cultured every 9 days at 20% (v/v) concentration. To induce expression of the target

gene under control of *Ramy3D* promoter, N6 medium was removed from the cell suspension via aspiration, and 3 g of rice cells was inoculated into 30 mL of fresh N6 (CHU) medium without sucrose (N6S-).

Expression of recombinant hGBA

Secreted WT-GBA and *gnt1*-GBA in the transgenic rice cell culture media were identified by SDS-PAGE and Western blotting. Each sample (30 µL) cultured for 9 days in N6S media was loaded onto an 8% polyacrylamide gel and separated using electrophoresis, followed by staining with Coomassie Brilliant Blue R-250. For Western blotting, polyacrylamide gel was electroblotted onto a 0.45-µm polyvinylidene difluoride (PVDF) transfer membrane (Merck Millipore, Darmstadt, Germany). Detection of rhGBA was conducted using mouse anti-hGBA monoclonal antibody (Abnova, CA, USA) at 1:1000 dilution for 3 h at room temperature. Primary antibody was detected using peroxidase-labeled goat anti-mouse IgG (Thermo Fisher Scientific, MA, USA) at 1:2000 dilution for 1 h at room temperature. The PVDF membrane was developed using TMB peroxidase substrate (KPL, MD, USA).

Purification of rhGAA

Suspension cultured media were recovered at 9 days after induction (dai) and filtered through a 0.22-µm Millipore Express® PLUS membrane filter (Merck Millipore). Purification of secreted rhGBA in rice cell culture medium was performed on an ÄKTA-FPLC system (GE Healthcare, Buckinghamshire, UK). Initial purification step was conducted by His-tagged affinity chromatography using Ni-NTA Superflow Cartridges (Qiagen, CA, USA) according to the manufacturer's protocols. Following column washing using 5 CV (Column volume) of distilled water, column equilibration was performed using 5 CV of equilibration buffer (20 mM sodium phosphate, 1.5 M ammonium sulfate, pH 6.3). Before the first sample loading, the sample was adjusted to 5 mM imidazole at pH 7.8. Column was washed with 5 CV of equilibration buffer, and bound proteins were eluted by elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 250 mM imidazole, pH 7.8). Protein peak was detected using a UV detector (GE Healthcare). For the next purification step, buffer containing purified protein was exchanged for MES buffer (25 mM 2-(N-morpholino) ethanesulfonic acid, pH 6.3) using HiPrepTM 26/10 Desalting (GE Healthcare). The second purification step was conducted by ion exchange chromatography using a HiTrapTM SP Sepharose Fast Flow IEX Column (GE Healthcare) to increase purity according to the manufacturer's protocols. Column washing and equilibration were

performed using 5 CV of MES buffer. Final rhGBA was eluted with a salt gradient using 10%, 15%, 20%, 25%, 50%, and 100% elution buffer (25 mM MES, 1M NaCl, pH 6.3). The purified recombinant proteins were quantified by Bradford protein assay (Bio-Rad, CA, USA) and identified by separate SDS-PAGE and Western blot analyses using a mouse anti-hGBA antibody (Abnova).

Acid β-glucosidase enzyme activity

Catalytic activity of GBA was assayed using a synthetic substrate, *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG) (Santa Cruz Biotechnologies, Dallas, TX, USA). The assay buffer contained 4 mM β-mercaptoethanol, 1.3 mM EDTA, 0.15% Triton X-100, 0.125% sodium taurocholate, and 60 mM phosphate–citrate buffer and was adjusted to pH 6.0. The assay was performed by 1/2 serial dilution of 50 ng of either purified rhGBA from D94, D96, or CHO-GBA (Genzyme, MA, USA) in a final volume of 200 μL of assay buffer. After 60 min of incubation at 37 °C, the reaction was terminated by adding 6 μL of 5 N NaOH. The amount of reaction product, *p*-nitrophenol, was measured based on its absorbance at 405 nm (Friedman et al. 1999).

N-glycan analysis

The *N*-glycan structures of rhGBA were determined using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), followed by 2-aminobenzamide (2-AB) (Sigma, MO, USA) labeling according to the method described by Hwang (2014) with slight modification. The rhGBA samples were digested with trypsin (Sigma-Aldrich, St. Louis, MO, USA) and chymotrypsin (Sigma-Aldrich) in 10 mM Tris–HCl buffer (pH 8.0) at 37 °C for 18 h and deactivated by heating at 100 °C for 10 min. *N*-glycans were released by incubation with glycoamidase A (Roche Diagnostics, Mannheim, Germany) in citrate-phosphate buffer (pH 5.0) at 37 °C for 18 h and purified using a graphitized carbon cartridge (Alltech, IL, USA) according to Packer (1998). The labeling solution was freshly prepared by subsequently dissolving 2-AB with sodium cyanoborohydride (Sigma-Aldrich) in 30% v/v acetic acid in dimethyl sulfoxide and directly added to the lyophilized *N*-glycan samples. The mixture was shaken and incubated for 3 h at 65 °C. The reaction mixture was purified by hydrophilic interaction chromatography involving an SPE cartridge packed with microcrystalline cellulose (Sigma-Aldrich). The 2AB-labeled *N*-glycans were lyophilized and stored at −20 °C until use. MALDI-TOF/MS analysis was carried out using the Ultraflex™ III system (Bruker Daltonik, Bremen, Germany) in reflector positive ionization mode. The dried 2-AB-labeled *N*-

glycans were dissolved in water and mixed with 2,5-dihydroxybenzoic acid (Sigma) matrix solution (10 mg/mL in acetonitrile/water (50:50, v/v)). These samples were then loaded onto the stainless steel target and dried at room temperature.

Results

Transgenic cell line

The expression cassette for the *hGBA* gene was cloned into two individual plant expression vectors the harboring *hpt* and *bar* genes as selection markers, respectively. Expression of *GBA* gene was controlled by the *RAmy3D* promoter and *RAmy3D* terminator (Fig. 1A). Each expression vector was transformed into WT and *gnt1* rice calli using the particle bombardment-mediated transformation method. The putative transgenic calli appeared on selection media containing 50 mg/L of hygromycin B or 4.4 ppm of PPT as a selection marker after 3–4 weeks. PCR of the genomic DNA confirmed 10 and three putative transgenic lines from D94 and D96, respectively, with amplicons corresponding to the size of the *hGBA* gene (Fig. 1B). These amplicons were not amplified from genomic DNA from WT and *gnt1* rice calli.

hGBA mRNA expression

Target mRNA expression of the D94 and D96 cell lines under control of the inducible *RAmy3D* promoter was induced for 3 days on solid N6 media without sucrose (N6S-). Northern blot analysis was conducted to confirm mRNA transcription levels using a ^{32}P-labeled specific 800-bp *hGBA* probe. Positive signals were detected from transgenic lines while no signal was detected in WT rice and rice *gnt1* calli (Fig. 1C). The differences in target mRNA expression levels between each cell line may be attributed to the combination of position effects and copy number of gene insertion (Peach and Velten 1991; Streatfield 2007). Four D94 cell lines (#4, 6, 8, and 10) and two D96 cell lines (#2 and 3) were selected for the establishment of cell suspension culture.

Production of rhGBA

To measure rhGBA expression and secretion by sucrose depletion, suspension culture media were used for analysis. The samples were collected at 7 dai and subjected to protein electrophoresis. SDS-PAGE showed rice α-amylase bands at 44 and 46 kDa (Chen et al. 1994) as secreted reference proteins. Expression of rhGBA was detected using anti-GBA antibody in Western blot analysis (Fig. 2). The detected band sizes were approximately 60 kDa, which is the expected size of hGBA (Shaaltiel et al. 2007). Based on the Western blot results, the highest production of rhGBA in culture media was observed in the transgenic cell lines D94#10 and D96#3 (Fig. 2). These cell lines were propagated and used for further experiments.

Maximum expression of rhGBA was measured by analyzing culture media harvested at 2-day intervals (Fig. 3). Maximum rhGBA expression date of D94#10 was maximized at 6 dai, whereas that of D96#3 was 10 dai. This difference in expression pattern may depend on changes in protein structure affecting susceptibility to endogenous proteases (Ko et al. 2003; Lee et al. 2015).

Purification of rhGBA

For purification of rhGBA from suspension culture of the D94#10 and D96#3 cell lines, cultured media were harvested at 7 dai and 10 dai, respectively. Despite efficient binding of rhGBA to the Ni-NTA column, endogenous host cell proteins (HCPs), especially protein with a molecular mass of approximately 30 kDa, remained in the eluted fraction. Further purification was used to increase the purity of rhGBA in the eluted fraction (Fig. 4). rhGBA was successfully separated from the 30 kDa rice-derived HCPs by strong cation exchange chromatography. The purified proteins were detected from each elution fraction by SDS-PAGE (Fig. 4A) and Western blot analysis (Fig. 4B). In rhGBA derived from WT rice cell line (D94#10), most 30 kDa rice-derived HCPs were eliminated by 10% elution buffer (E1), whereas a small amount of HCPs was eliminated by 15% elution buffer (E2). On the other hand, 30 kDa rice-derived HCPs of the glycoengineered rice cell line (pMYD96#3) were mostly eliminated by 15% elution buffer, whereas a small amount of HCPs was eliminated by the elution fraction of E1, indicating that mutation of *gnt1* in rice cells may affect the characteristics of proteins with different *N*-glycan structures. Finally, we recovered rhGBA from the eluted fractions of (E3 and E5) for D94#10, (E3, E4, and E5) for D96#3, respectively.

Biological activity of rhGBA

The activities of WT-GBA and *gnt1*-GBA were analyzed by measuring the absorbance of p-NP cleaved from p-NPG and compared with that of CHO-GBA. WT-GBA and CHO-GBA showed similar activities, whereas *gnt1*-GBA showed slightly higher activity. GBA activity gradually increased with protein amount (Fig. 5).

N-glycan analysis

N-glycans of rhGBA derived from WT and *gnt1* rice cells were released by glycoamidase A, fluorescently labeled, and analyzed by MALDI-TOF MS (Fig. 6 and Table. 1). As shown in Fig. 6A, rhGBA produced by

WT rice cells were exclusively glycosylated with complex-type *N*-glycans (99.0%), which consisted of mainly MMXF^3 (54.2%), followed by GnMXF^3 (16.9%). In addition to these two major glycan structures, various complex-type *N*-glycans such as MMX (9.3%), GnMXF^3 (8.0%), and GnGnXF^3 (5.2%) were identified. There were very little amounts of high mannose-type *N*-glycans, M6 (1.0%) in rhGBA from WT rice cells. On the other hand, Fig 6B indicates that rhGBA from *gnt1* rice cells displayed mainly high mannose-type *N*-glycans (86.0%) with a small amount of complex-type *N*-glycans (14.0%). The most abundant high mannose-type *N*-glycan was M5 (68.2%), followed by M6 (14.0%). Complex-type *N*-glycans of rhGBA from *gnt1* rice cells were mainly composed of AGn (6.7%) and MMF^3 (4.3%), which were scarce or absent in rhGBA from WT rice cells.

Discussion

In this study, we demonstrated the expression and characterization of GBA with only high mannose residues on its *N*-glycosylation site using *gnt1* mutant rice suspension culture for treatment of Gaucher disease. In a previous study, GBA was expressed in rice cell suspension culture and showed similar enzyme activity to that of CHO-GBA (Nam et al. 2017). However, until now, there have been no statements about *N*-glycosylation patterns. A similar type of GBA with six histidine residues (WT-GBA) was assayed in this study and was shown to contain a high mannose-type *N*-glycan on its *N*-glycosylation site. Plant-specific α-1,3-fucose, β-1,2-xylose, β-1,3-galactose, and α-1,4-fucose were detected in the *N*-glycan analysis (Fig. 5). So, additional *N*-glycan engineering strategies may be required for its therapeutic application.

rhGBA was successfully expressed and secreted from rice *gnt1* cells (Fig. 2). Even though the expression level of rhGBA was quite low, it can be improved by using additional strategies such as media optimization or protease inhibition (Kim et al. 2008; Kim et al. 2014). The size of secreted *gnt1*-GBA in medium was almost similar to that of CHO-GBA, whereas WT-GBA was slightly larger on the nitrocellulose membrane (Figs. 2-3). This difference may be due to the characteristics of *N*-glycans. Commercial rhGBAs such as Cerezyme® (Grabowski et al. 1995; Jmoudiak and Futerman 2005; Weinreb et al. 2002) and Elelyso® (Aviezer et al. 2009) mainly possess *N*-glycans with terminal mannose residues with molar weights (MM : ~1127 Da, MMXF : ~1289 Da) that are similar to those of Man_4 and Man_5 (~1173 Da and ~1335 Da) abundant in *gnt1*-GBA. However, the slightly larger size of WT-GAA was due to its hybrid or complex-type *N*-glycans (about 1638 ~ 2311 Da). The *N*-glycan analysis results also corroborated the results (Fig. 5).

gnt1-GBA has many advantages such as scalability, non-contamination by animal pathogens, downstream purification, and *N*-glycan engineering. *gnt1*-GBA can be expressed and secreted into culture media with high mannose residues without any downstream processing such as sequential *in vitro* deglycosylation by using glycosidase in Cerezyme® production or protein extraction from cultured cells in Elelyso® production. Additionally, production of plant-specific *N*-glycans present in heterologous protein expression in plant systems is dramatically decreased. *N*-glycan structures containing plant-specific *N*-glycans, especially α-1,3-fucose and β-1,2-xylose, cause allergic IgE immune responses in mammals (Bardor et al. 2003; Piron et al. 2015; van Ree et al. 2000). In contrast, a recent study proved that plant-specific *N*-glycans have no adverse effects in humans (Shaaltiel and Tekoah 2016). Even though conflicts remain, use of *gnt1*-GBA could avoid these concerns. Loss of GnTI activity affects the function of downstream pathways such as attachment of α-1,3-fucose and β-1,2-xylose to *N*-glycans (Fanata et al. 2013; Jung et al. 2017). The effect of mannose chain

length on targeting of GBA to macrophages was examined in a previous study (Van Patten et al. 2007). Various GBAs harboring Man_2 to Man_9 have similar activities and targeting behaviors. Thus, all *N*-glycan types we detected in the *N*-glycan analysis (Man_{3-5}) could act as ligands for mannose receptor.

We described the expression of rhGBA in *gnt1* mutant rice cell suspension culture. *gnt1*-GBA was found to be highly similar to Cerezyme® in terms of enzymatic activity and possessed only high mannose-type *N*-glycans for targeting macrophages. Although many products were developed for expression of GBA, *gnt1*-GBA could be an alternative therapeutic for ERT of Gaucher disease.

Author contribution statement

JW Jung, HY Choi, DI Kim, and NS Kim conceived, designed, and performed the overall study. NX Huy, HJ Park and SH Kang provided technical assistance and performed the experiments. HH Kim and MS Yang were involved in devising the work and interpretation of results. JW Jung and HY Choi wrote the manuscript. All authors read and approved the manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

Figure 1. Vector construction (A). The *hGBA* gene with 6-histidine residues following the signal peptide of rice amylase 1A gene was located between the rice amylase 3D promoter (*Ramy3D*-p) and the 3'untranslated region (3'UTR). Transferred DNA (T-DNA) of each plasmid is shown. RB, T-DNA right border; 35S-p., CAMV35S promoter; *hpt*, hygromycin phosphotrasferase (Hyg^R); *bar*, bialaphos resistance gene; 35S polyA, terminator of 35S gene; LB, T-DNA left border. Detection of the *hGBA* gene in transgenic callus via PCR (B). Lane PC, pMYD94 plasmid as positive control; Numbered lanes indicate PCR product obtained from genomic DNA of transgenic callus. Northern blot analysis used to determine expression of hGBA mRNA in transgenic rice cell suspension culture (C). Lane NC, total RNA extraction of non-transformed WT and *gnt1* rice cells as negative control individually; Numbered lanes are the total RNA extracts of transgenic rice suspension cells. Loading standard are indicated by ethidium bromide-stained rRNA.

Figure 2. Detection of rhGBA expression and secretion by SDS-PAGE (A) and Western blot analysis (B). Lane PC, 250 ng (A) or 25 ng (B) of CHO-GBA; Lane NC1, Culture medium from WT rice cell line ; Lane NC2, Culture medium from non-transformed *gnt1* rice cell line; Numbered lane, Culture medium from transgenic cell line (D94 and D96). Arrowheads indicate secreted rhGBA; Asterisk indicates rice alpha-amylase.

Figure 3. Time course study of rhGBA production in transgenic rice culture medium by SDS-PAGE (A) and Western Blot (B). Culture medium was used to measure hGBA production from 2~12 dai. PC, 250 ng (A) or 25 ng (B) of CHO-GBA; Arrowheads indicate rhGBA. Asterisk indicates rice alpha-amylase, respectively.

Figure 4. Analysis of purified rhGBA in transgenic rice suspension cell culture medium. SDS-PAGE (A) and Western blot analysis (B) were conducted to evaluate the ion exchange chromatography of rhGBA using a HiTrapTM SP Sepharose Fast Flow IEX Column. PC, 250 ng (A) or 25 ng (B) of CHO-GBA; His, eluted fraction after His-tagged affinity chromatography using Ni-NTA Superflow Cartridges; FT, flow through from ion exchange chromatography column; W, wash from ion exchange chromatography column; from E1 to E6, eluted fraction from ion exchange chromatography column with salt gradient of NaCl, E1; 10%, E2;

15%, E3; 20%, E4; 25%, E5;50%, E6;100%. Arrowheads indicate rhGBA. Single asterisk indicates rice α-amylase, and double asterisk indicates 30 kDa host cell proteins of rice cells, respectively.

Figure 5. Representative curve of acid β -glucosidase activity. GBA activity of purified WT-GBA, *gnt1*-GBA, and CHO-GBA were measured with *p*-NPG synthetic substrate. Release of *p*-NPG was observed at 400 nm in a spectrophotometer. Error bars indicate the standard error of triplicate cultures.

Figure 6. MALDI-TOF MS spectra of *N*-glycans of WT-GBA (A) and *gnt1*-GBA (B). ■, *N*-acetylglucosamine; ●, mannose; ●, galactose; ▲, fucose; ☆, xylose.

Table 1. Comparison of *N*-glycans from WT-GBA and *gnt1*-GBA.

Peak no.[a]	*N*-glycan	Theoretical m/z[b]	WT-GBA		*gnt1*-GBA	
			Observed m/z	Relative quantity[c] (%)	Observed m/z	Relative quantity (%)
Complex type *N*-glycan						
a	MM	1053.386	1053.461	0.5	ND	-
b	MMX	1185.428	1185.492	9.3	ND	-
c	MMF^3	1199.444	1199.581	0.6	1199.515	4.3
e	$MMXF^3$	1331.486	1331.569	54.2	1331.711	1.6
g	GnMX	1388.507	1388.604	1.7	ND	-
h	$GnMF^3$	1402.523	1402.590	8.0	ND	-
i	$GnMXF^3$	1534.565	1534.731	16.9	ND	-
k	GnGnX	1591.587	1591.725	0.8	ND	-
l	$GnGnF^3$	1605.602	1605.739	0.4	1605.852	1.4
m	AGn	1621.597	ND	-	1621.723	6.7
n	$AMXF^3$	1696.618	1696.757	0.5	ND	-
o	$GnGnXF^3$	1737.645	1737.787	5.2	ND	-
p	$(FA)MXF^3$	1842.676	1842.909	0.2	ND	-
q	$AGnXF^3$	1899.697	1899.863	0.3	ND	-
s	$(FA)GnXF^3$	2045.755	2045.981	0.4	ND	-
			Total	99.0	Total	14.0
High mannose type *N*-glycan						
d	M4	1215.439	ND	-	1215.453	2.5
f	M5	1377.491	ND	-	1377.512	68.2
j	M6	1539.544	1539.718	1.0	1539.578	14.0
r	M9	2025.703	ND	-	2026.767	1.3
			Total	1.0	Total	86.0

[a] Peak no. from MALDI-TOF spectrum in Fig. 6.

[b] Mass values of $[M+Na]^+$ ions of each peak

[c] Relative abundance of each glycan calculated according to ratio of each peak intensity per total peak intensities in Fig. 6.

ND : Not detected.

Fig. 1

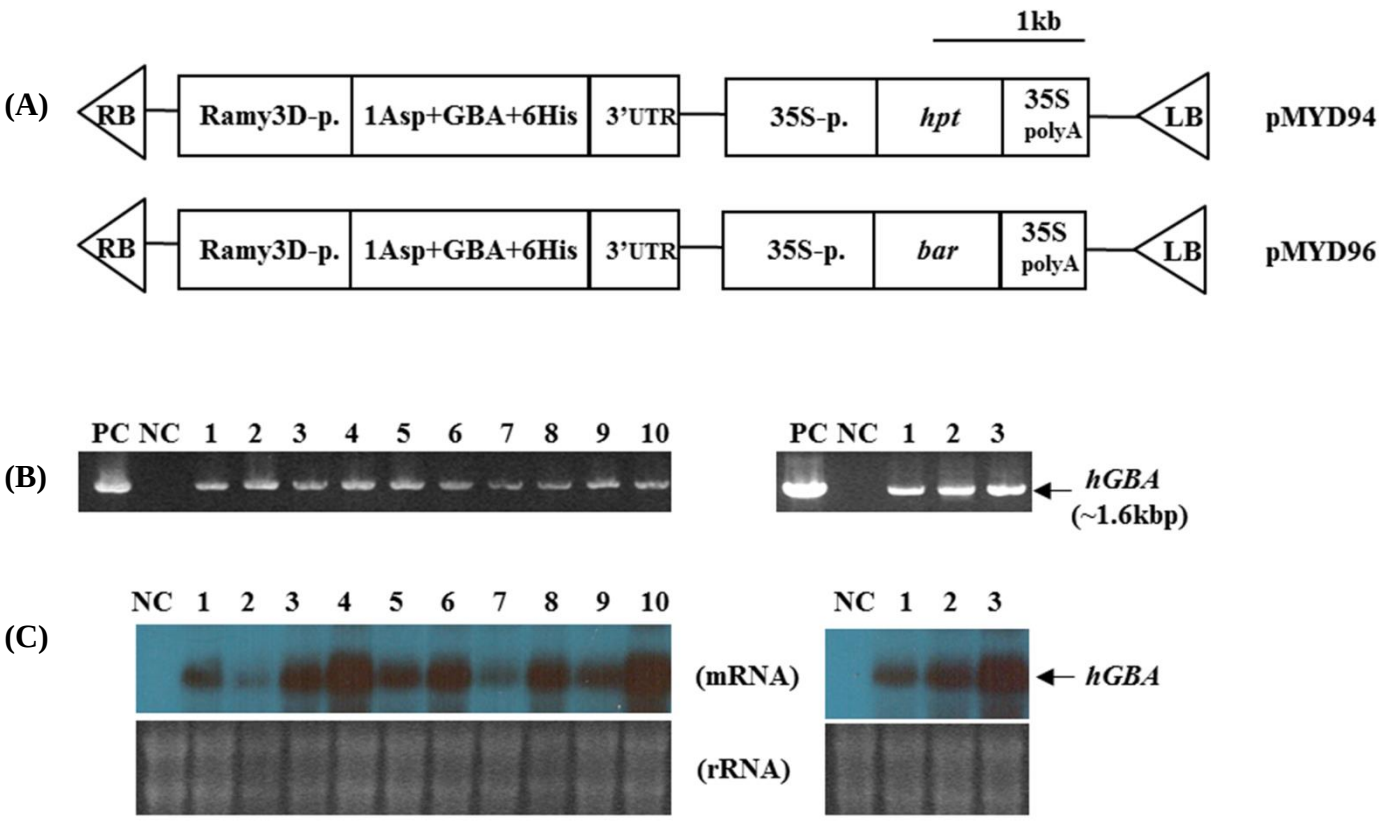


Fig. 2

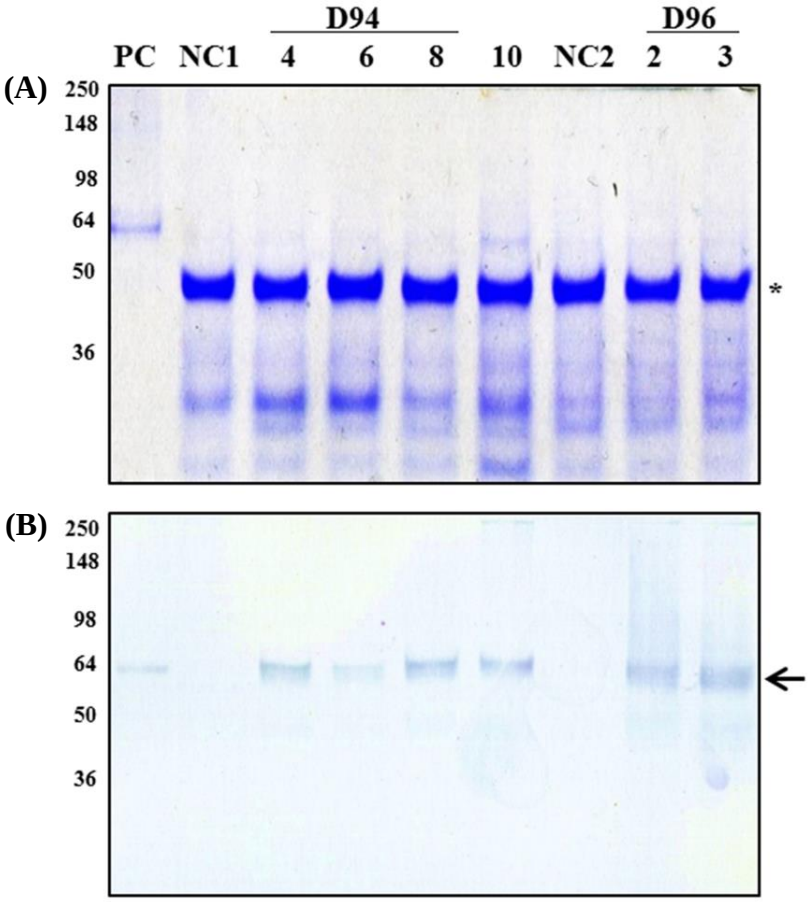

D94
D96
PC
NC1
4
6
8
10
NC2
2
3
(A)
250
148
98
64
50
36
*
(B)
250
148
98
64
50
36

Fig. 3

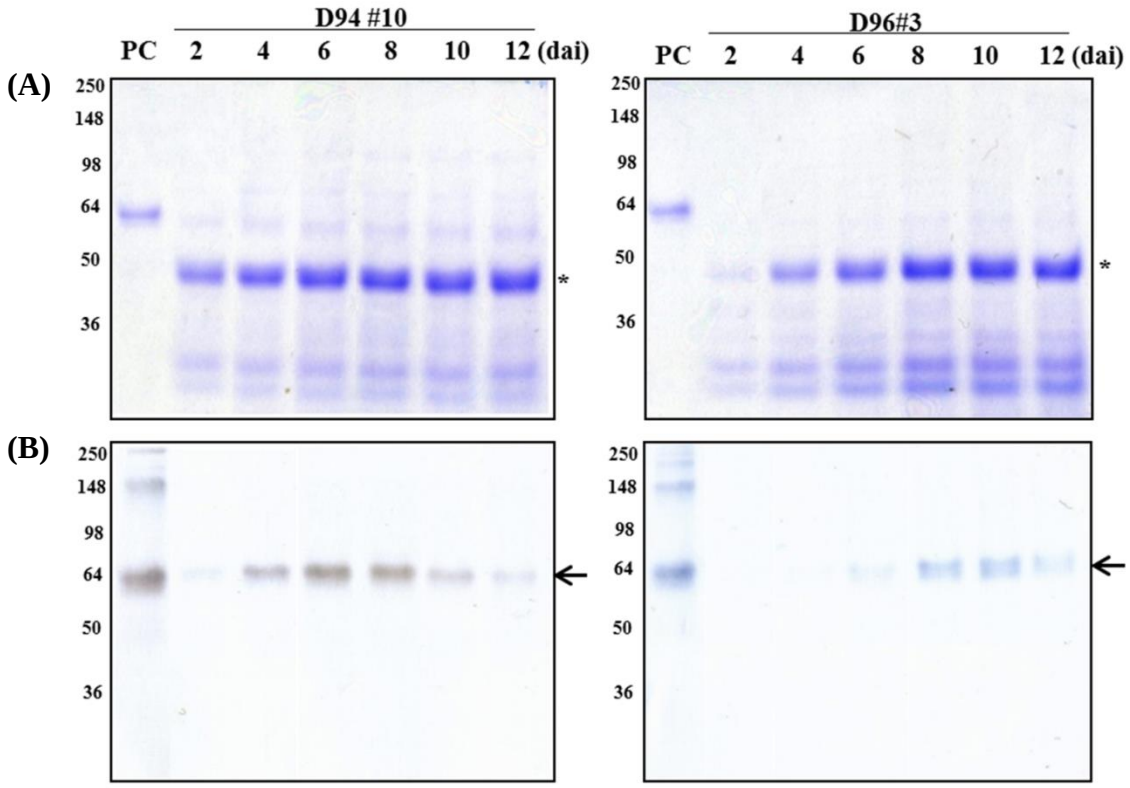

D94 #10
PC
2
4
6
8
10
12 (dai)
D96#3
PC
2
4
6
8
10
12 (dai)
(A)
(B)
250
148
98
64
50
36
*
*

Fig. 4

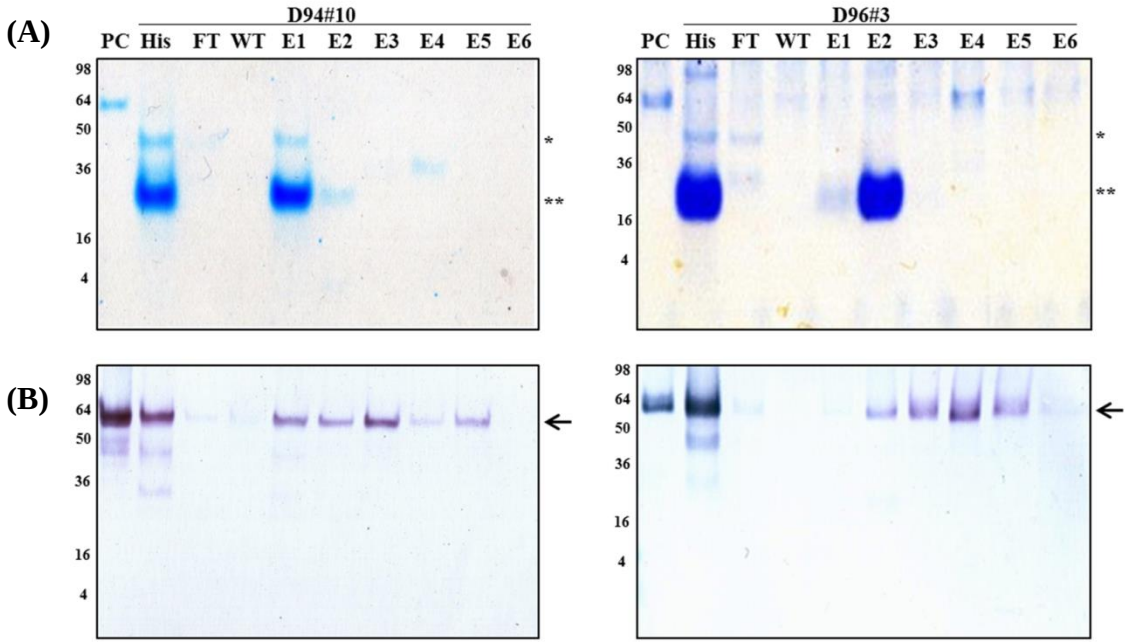

(A)
D94#10
PC His FT WT E1 E2 E3 E4 E5 E6
98
64
50
36
16
4
*
**
D96#3
PC His FT WT E1 E2 E3 E4 E5 E6
98
64
50
36
16
4
*
**
(B)
98
64
50
36
16
4
98
64
50
36
16
4

Fig. 5

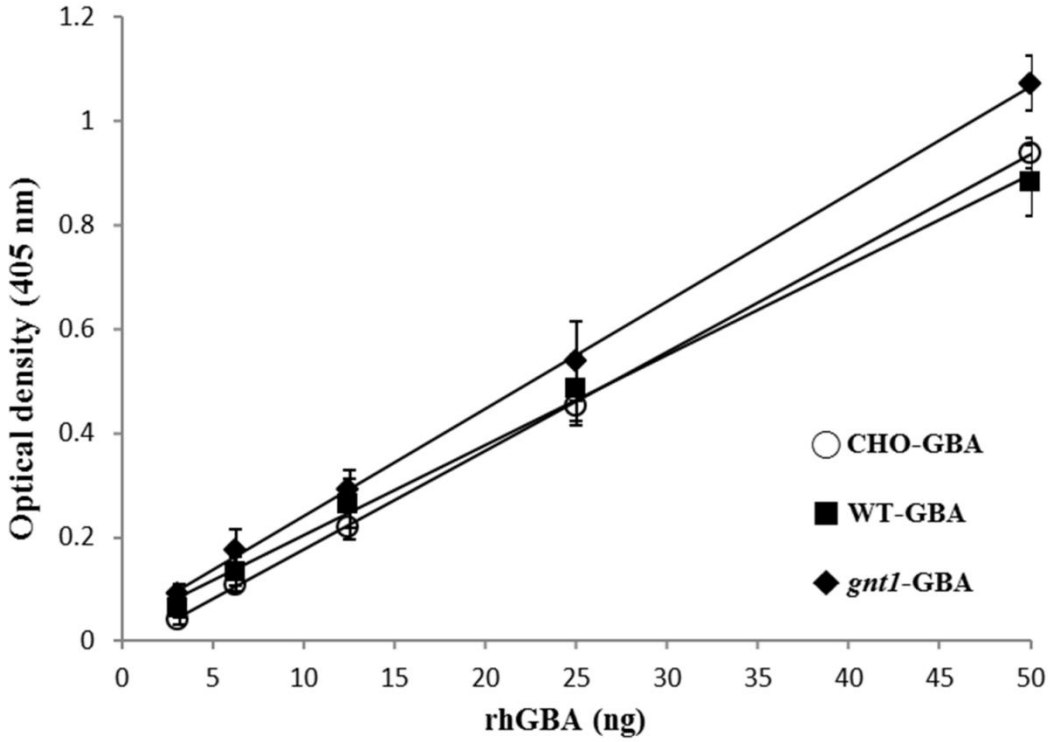
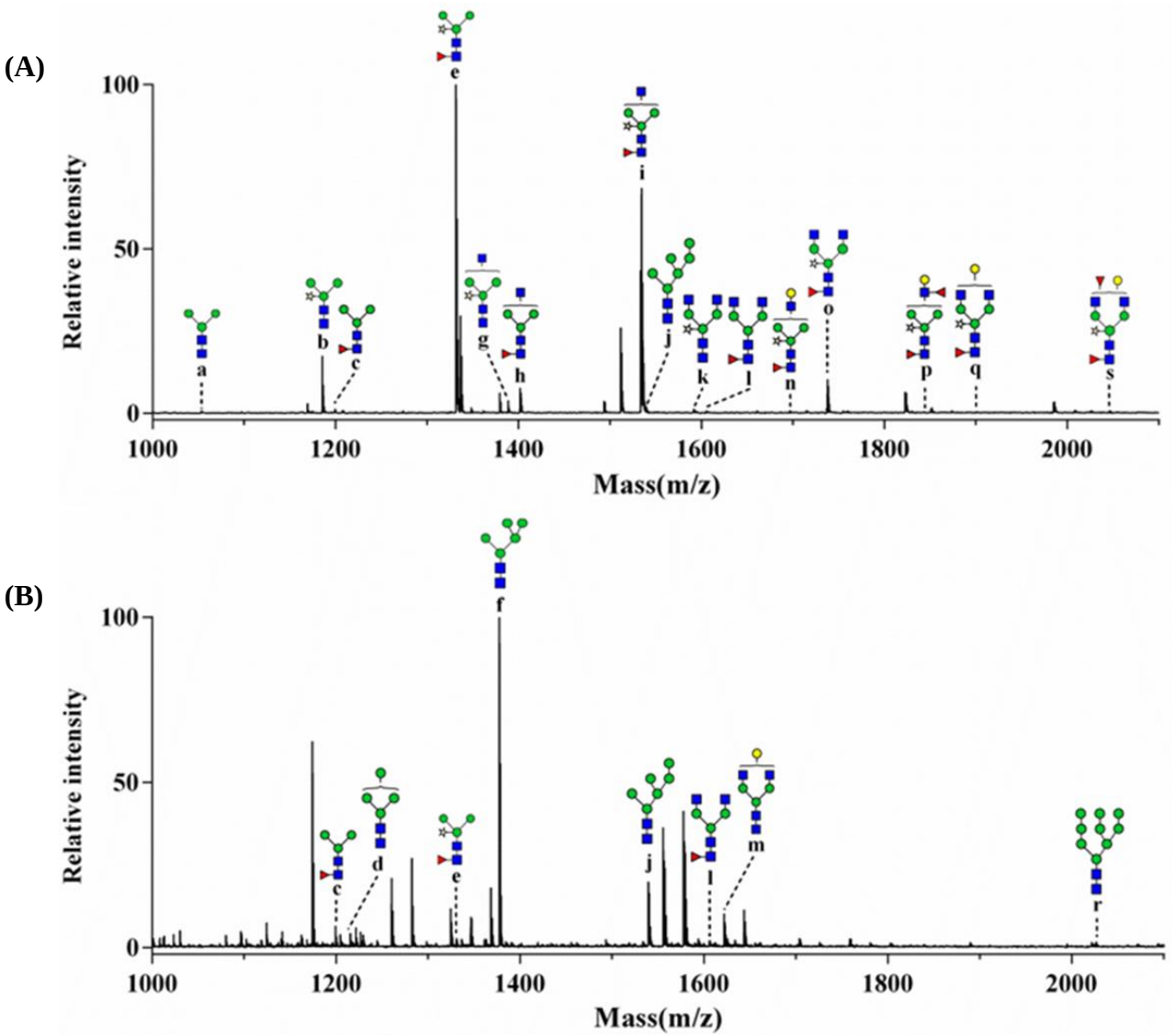

1.2
1
0.8
0.6
0.4
0.2
0
Optical density (405 nm)
0
5
10
15
20
25
30
35
40
45
50
rhGBA (ng)
CHO-GBA
WT-GBA
gnt1-GBA

Fig. 6


(A)
Relative intensity
100
50
0
1000
1200
1400
1600
1800
2000
Mass(m/z)
a
b
c
e
g
h
i
j
k
l
n
o
p
q
s
(B)
Relative intensity
100
50
0
1000
1200
1400
1600
1800
2000
Mass(m/z)
c
d
e
f
j
l
m
r