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Identification of a GH110 Subfamily of α 1,3-Galactosidases NOVEL ENZYMES FOR REMOVAL OF THE α 3GAL XENOTRANSPLANTATION ANTIGEN^{*§}

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In search of α -galactosidases with improved kinetic properties for removal of the immunodominant α 1,3-linked galactose residues of blood group B antigens, we recently identified a novel prokaryotic family of α -galactosidases (CAZy GH110) with highly restricted substrate specificity and neutral pH optimum (Liu, Q. P., Sulzenbacher, G., Yuan, H., Bennett, E. P., Pietz, G., Saunders, K., Spence, J., Nudelman, E., Levery, S. B., White, T., Neveu, J. M., Lane, W. S., Bourne, Y., Olsson, M. L., Henrissat, B., and Clausen, H. (2007) *Nat. Biotechnol.* 25, 454–464). One member of this family from *Bacteroides fragilis* had exquisite substrate specificity for the branched blood group B structure Gal α 1–3(Fuc α 1–2)Gal, whereas linear oligosaccharides terminated by α 1,3-linked galactose such as the immunodominant xenotransplantation epitope Gal α 1–3Gal β 1–4GlcNAc did not serve as substrates. Here we demonstrate the existence of two distinct subfamilies of GH110 in *B. fragilis* and *thetaitoamicon* strains. Members of one subfamily have exclusive specificity for the branched blood group B structures, whereas members of a newly identified subfamily represent linkage specific α 1,3-galactosidases that act equally well on both branched blood group B and linear α 1,3Gal structures. We determined by one-dimensional ¹H NMR spectroscopy that GH110 enzymes function with an inverting mechanism, which is in striking contrast to all other known α -galactosidases that use a retaining mechanism. The novel GH110 subfamily offers enzymes with highly improved performance in enzymatic removal of the immunodominant α 3Gal xenotransplantation epitope.

We recently identified a new prokaryotic α -galactosidase gene family (designated GH110 in the carbohydrate-active enzymes (CAZy)² data base, available on the web; Ref. 45), which contains enzymes with surprisingly narrow substrate specificity for the branched blood group B oligosaccharide (Gal α 1–3(Fuc α 1–2)Gal) (1). Family GH110 enzymes exhibit no significant sequence similarity to other glycosidase families, including CAZy families GH4, GH27, GH36, and GH57, which contain all other reported α -galactosidases, including enzymes previously used for the enzymatic removal of the immunodominant terminal α 1,3-linked Gal residues of the blood group B antigen and the related non-fucosylated xenotransplantation epitope (Gal α 1–3Gal β 1–4GlcNAc) (2–6). Family GH27 (7), together with family GH36, hosts all α -galactosidases from eukaryotic organisms, including the clinically important human lysosomal α -galactosidase and α -N-acetylgalactosaminidase enzymes. In addition to families GH27 and GH36, α -galactosidases from microbial sources are also found in families GH4 and GH57 as well as GH110. The α -galactosidases from families GH4, GH27, GH36, and GH57 studied so far do not exhibit substrate linkage specificities, and have all been shown to function with a retaining mechanism of catalysis and use an aspartic acid or a glutamic acid as the catalytic nucleophile (8–10), with the exception of family GH4 enzymes that utilize NAD⁺ and Mn²⁺ as cofactors (11). Furthermore, families GH27 and GH36 are thought to share a common ancestor with a (β/α)₈-barrel fold of the catalytic domain, forming the glycoside hydrolase clan GH-D (12, 13).

Linkage specificity of glycosidases is highly desirable for use of these enzymes in modifying glycans on therapeutic glycoproteins or cells and as tools in structural analysis of glycoconjugates (14, 15). Many glycosidases are linkage-specific and, *e.g.*

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AM259273.

[§] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental text, Figs. S1 and S2, Table S1, and additional references.

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² The abbreviations used are: CAZy, carbohydrate-active enzymes; Gal, galactose; Fuc, fucose; Fru, fructose; AMC, 7-amino-4-methylcoumarin; B-tetra-AMC, AMC-labeled blood group B tetrasaccharide; Gal α -pNP, *para*-nitrophenyl α -D-galactopyranoside; RBC, red blood cell; pRBC, packed RBC; ECO, enzyme-converted to group O; FACS, fluorescence-activated cell sorting; BfGal110A and BfGal110B, two α -galactosidase variants from *B. fragilis*; BtGal110A and BtGal110B, two α -galactosidase variants from *B. thetaiotaomicron*; SaGal110A, α -galactosidase from *S. avermitilis*; SgGal110A, α -galactosidase from *S. griseoplanus* 2357; PBS, phosphate-buffered saline.

Novel GH110 Subfamily of α 1,3-Galactosidases

β -galactosidases with β 1,3 (GH43)-, β 1,4-, or β 1,4/6 (GH35)-linkage specificity have been identified (16–18). In contrast, α -galactosidases with linkage specificity have not been reported so far, although we recently found that family GH110 contains enzymes with highly restricted specificity for the blood group B structure (1). The molecular basis for this restricted specificity is currently unknown, but the BfGal110A α -galactosidase (formerly designated FragA) characterized is not simply an α 1,3-linkage-specific enzyme, because linear oligosaccharides, terminated with an α 1,3Gal residue and devoid of the fucose substitution on the penultimate galactose as in blood group B, did not serve as substrates.

In this study, we report the identification of a subfamily of GH110 α -galactosidases, comprising paralogs from *Bacillus fragilis* and *B. thetaiotaomicron* that display distinct α 1,3-linkage substrate specificity as evaluated with a panel of oligosaccharides. We further demonstrate by NMR spectroscopy that family GH110 glycosidases function with an inverting mechanism, which is in contrast to all other known α -galactosidase families that exclusively use a retaining mechanism. Finally, we applied one of the novel isoforms derived from *B. fragilis* for enzymatic removal of the major α 3Gal xenotransplantation antigen using porcine and rabbit red blood cells as a model and demonstrate that this enzyme can be used with much lower enzyme consumption than other α -galactosidases.

EXPERIMENTAL PROCEDURES

Phylogenetic Analysis—Amino acid sequences were aligned using MUSCLE (19), and the neighbor-joining tree was made from the resulting distance matrix using Blosum62 substitution parameters. Visualization of the tree was made with an in-house program.

Cloning and Expression of Selected Members of Family GH110 Glycosidases—One novel variant from *B. fragilis* (designated BfGal110B), two from *B. thetaiotaomicron* (designated BtGal110A and BtGal110B), and one from *Streptomyces avermitilis* (designated SaGal110A) were identified and amplified from their corresponding genomic DNA by PCR as described previously for the *B. fragilis* isoform designated BfGal110A (1). The gene for the α -galactosidase from *Streptomyces griseoplanus* (designated SgGal110A), which was identified, purified, and partially sequenced (1), was sequenced as described in supplemental results and then amplified similarly as other α -galactosidase genes. The amplified genes were introduced into the pET28 vector for expression of the His₆-tagged or non-tagged proteins using appropriate restriction sites for protein expression in *Escherichia coli* Rosetta2(DE3)pLysS (Novagen, Madison, WI). Primers and vectors used for generation of expression constructs are shown in the supplemental Table S2. The transformants were screened for the correct size of the insert, and all constructs were fully sequenced on a 377 ABI Prism instrument (Applied Biosystems, Foster City, CA).

Expression and Purification—*E. coli* cultures were grown at 37 °C, 220 rpm, in 1× Terrific Broth (Sigma-Aldrich), supplemented with 34 μ g/ml chloramphenicol, 30 μ g/ml kanamycin, induced at an $A_{600\text{ nm}} \sim 0.6$ for 3–4 h with 0.25 mM isopropyl-1-thio- β -D-galactopyranoside, harvested by centrifugation for 15 min at 3450 \times g, 4 °C, and the resultant cell pellets stored at

–20 °C until use. The cell pellet from 1 volume of culture was lysed in 0.05–0.1 volume of Buffer A (40 mM NaPO₄, pH 6.8, 10 mM NaCl), supplemented with 1× BugBuster, and 5,000 units of benzonase nuclease (Novagen products) per liter culture, for 2 h at room temperature while stirring. To the crude lysates of SaGal110A and SgGal110A α -galactosidases sodium chloride was added to 400 mM, and stirring was continued for another hour. The crude lysates were centrifuged for 30 min at 35,000 \times g, 4 °C (high speed centrifugation), and the expressed proteins were found either in the supernatant or cell debris pellet. Extraction of the protein from the cell debris pellet was performed by re-suspending the cellular debris in Buffer B (40 mM Tris-HCl, pH 7.5, 400 mM NaCl), and stirring the suspension for at least 1 h to form a homogeneous suspension, followed by high speed centrifugation to recover the soluble proteins. The recombinant proteins were purified with two or more of the following chromatographic steps: anion-exchange chromatography (Macro-Prep High Q, Bio-Rad), hydrophobic interaction chromatography (phenyl-Sepharose high performance, Amersham Biosciences), hydroxyapatite (Bio-Gel HTP hydroxyapatite, Bio-Rad), and Co²⁺-immobilized metal affinity chromatography (Talon Metal Affinity Resin, BD Biosciences Clontech, Palo Alto, CA). All concentrations and buffer exchanges of protein solutions were performed using Centricon Plus-20 or -70 centrifugal filter devices (10,000-Da molecular mass cut-off, Millipore Corp., Bedford, MA) as described by the manufacturer. All steps were performed at room temperature unless otherwise indicated. All final products were dialyzed into Buffer C (20 mM NaPO₄, pH 7.0, 300 mM NaCl), and protein concentrations were determined from absorbance at 280 nm and the calculated extinction coefficient of each protein. Details of conditions for purification of each of the enzymes were as follows: BfGal110B and BtGal110A α -galactosidases were purified from the soluble fraction of the cellular debris extracts. After adjustment to pH 8.5 with 1 M free Tris and double dilution with water, the protein solution was allowed to pass an anion-exchange chromatography column, pre-equilibrated with Buffer D (40 mM Tris-HCl, pH 8.5, 10 mM NaCl), and the column was washed with 4 bed volumes of the same buffer. The anion-exchange chromatography flow-through and wash were pooled, adjusted to 500 mM with 5 M NaCl, lowered to pH 7.5 with acetic acid, and loaded onto a hydrophobic interaction chromatography column, pre-equilibrated with Buffer B, and the column was washed with 4 bed volumes of the same buffer. The hydrophobic interaction chromatography flow-through and wash were pooled, concentrated, and buffer-exchanged into Buffer C. The resultant protein solution was diluted with an equal volume of water and loaded onto a hydroxyapatite column, pre-equilibrated with 10 mM NaPO₄, pH 7.0, 100 mM NaCl, and the column was washed extensively with the equilibration buffer. The bound protein was gradient-eluted with 20–250 mM NaPO₄, pH 7.0, 100 mM NaCl. The enzyme fractions, typically eluted at 100–150 mM NaPO₄, were pooled, and buffer-exchanged into Buffer D plus 100 mM NaCl. The resultant protein solution was diluted with 3 volumes of Buffer D, and loaded onto an anion-exchange chromatography column, pre-equilibrated with Buffer D. The column was washed with 4 bed volumes of Buffer D to recover the

residual bound protein. The flow-through and wash were pooled, adjusted to 300 mM NaCl, concentrated, and buffer-exchanged. The His₆-tagged proteins, BtGal110B in the soluble fraction of the cellular debris extract, SaGal110A and SgGal110A in the supernatants of crude lysates, were purified by sequential Co²⁺-immobilized metal affinity chromatography and hydroxyapatite steps. Briefly, the protein solution was loaded onto an Co²⁺-immobilized metal affinity chromatography column, pre-equilibrated with Buffer A plus 300 mM NaCl, and the column was washed extensively with the equilibration buffer plus 20 mM imidazole. The bound protein was eluted with 20 mM NaPO₄, pH 7.0, 100 mM NaCl, and 150 mM imidazole. The eluted protein was loaded directly onto a hydroxyapatite column, and the protein was eluted similarly as described above for the purification of BtGal110A and BfGal110B α -galactosidases.

Enzyme Assays—Carbohydrate substrates (Tables 1 and 2) were obtained from Sigma-Aldrich, V-LABS (Covington, LA), Oxford Glyco Sciences (Abingdon, UK) and IsoSep (Tullinge, Sweden). The blood group B tetrasaccharide 7-amino-4-methylcoumarin-labeled derivative (B-tetra-AMC) was custom synthesized by Alberta Research Council (Edmonton, Alberta, Canada). Unless otherwise indicated, all assays were performed at 26 °C in 100 mM NaPO₄, pH 6.8, 50 mM NaCl, supplemented with 0.1–0.2 mg/ml bovine serum albumin.

Assays using the blood group B antigen, B-tetra-AMC, were performed at 0.1 mM concentration in volumes of 10 μ l with pre-determined amount of enzyme. Aliquots of 2 μ l were sampled at time points 5–20 min, which included complete digestion of the substrate. Reactions were spotted onto a silica-gel coated TLC plate (EMD Chemicals Inc., Gibbstown, NJ), which was developed in chloroform/methanol/water (60:35:8, v/v) for 10 min and photographed by a Bio-Rad Fluor-S MultiImager with Quantity One-4.1.1 software. One unit of enzyme activity is defined as the amount of enzyme required to cleave 1 μ mol of B-tetra-AMC per minute under the defined condition. Evaluation of cleavage of B-tetra-AMC and oligosaccharides were based on time point for complete digestion of substrate. Assays with other oligosaccharides for substrate specificity studies were modified to be carried out at 5 mM concentration in 10 mM NaPO₄, pH 6.8, 2.5 mM NaCl. The TLC plate was developed in chloroform/methanol/water (30:60:10, v/v) and stained with 0.05% orcinol in 0.5 M H₂SO₄ (w/v).

A continuous substrate depletion assay, coupled with a NADP⁺-mediated oxidation of released galactose by glucose dehydrogenase, was used to determine the k_{cat}/K_m values of the oligosaccharide substrate, which was carried out in 400- μ l reaction volumes containing 0.05 mM substrate, 0.5 mM NADP⁺, and 2 units of *Thermoplasma acidophilum* glucose dehydrogenase (Sigma-Aldrich), and absorption at 340 nm was monitored over a period of 5–60 min (20).

Initial rate assays were performed for analysis using with *para*-nitrophenyl α -D-galactopyranoside (Gal α -pNP) for 5 min to 2 h in 400- μ l reactions containing 2.5 mM substrate and proper amount of enzyme ensuring less than 10% consumption of the substrates, and terminated with 600 μ l of 1 M Na₂CO₃ and read at 405 nm for released nitrophenol ($\epsilon = 18.3 \text{ mM}^{-1} \text{ cm}^{-1}$). One unit of enzyme activity for Gal α -pNP is defined

similarly as for B-tetra-AMC substrate but obtained from the initial rate analysis. Kinetic parameters were obtained by fitting the initial rate data, acquired in the range of 0.1–10 mM Gal α -pNP, to the Michaelis-Menten equation using non-linear regression analysis with Prism 4.0 software (GraphPad, San Diego, CA).

Stereochemistry of the Hydrolysis of Gal α -pNP by BfGal110B α -Galactosidase—¹H NMR experiments were acquired at 25 °C on a Varian Inova 500-MHz spectrometer, using standard acquisition software, including residual solvent suppression via long presaturation pulse, available in the Varian VNMR software package. Proton chemical shifts were measured relative to an external standard of acetone (trace) in D₂O (the acetone H₃C signal was set to $\delta = 2.225$ ppm). After recording the reference spectrum ($t = 0$ min) of deuterium-exchanged Gal α -pNP (1.2 mg) in 0.50 ml of D₂O, deuterium-exchanged BfGal110B α -galactosidase (~0.1 mg in 200 μ l of D₂O) was added to initiate the reaction. NMR spectra were acquired at $t = 3, 6, 9, 20, 25, 40, 60, 120, 140, 160, 180,$ and 300 min after initiation of the reaction. Peak integrations were calculated by standard software and are judged accurate to within 2%.

Enzymatic Treatment of Red Cells with the BfGal110B α -Galactosidase—Fresh human whole blood was obtained from the Oklahoma Blood Institute (Oklahoma City, OK), rabbit whole blood and porcine whole blood were from Pel-Freez Biologicals (Rogers, AR), and buffy coats were removed. Enzymatic conversions were performed in 1-ml reaction mixtures containing 200 mM glycine, pH 6.8, 3 mM NaCl (conversion buffer), with 30% packed RBCs as described previously (1). Briefly, RBCs were prewashed 1:1 and 1:4 (v/v) in conversion buffer before addition of enzyme, and reactions were incubated for 60 min with gentle mixing at 26 °C, followed by four repeat washing cycles with 1:4 (v/v) of PBS by centrifugation at 900 $\times g$ for 5 min. The washed enzyme-treated RBCs were tested by agglutination analysis using licensed anti-B-typing reagents according to standard blood banking techniques (21) and the *Griffonia (Bandeiraea) simplicifolia* lectin (B4, IB4, or BS4, Vector Laboratories, Burlingame, CA, Sigma-Aldrich).

Flow Cytometry—Flow cytometric analysis of native and enzyme-converted RBCs of porcine and rabbit source was performed using a FACScan flow cytometer (BD Biosciences) and fluorescein IB4 lectin. A 96-well plate (NUNCTM Apogent, Denmark) was used for sample preparation: briefly, ~500,000 RBCs were added to each well containing 50 μ l of PBS. Cells were fixed for 10 min at room temperature under gentle agitation by the addition of 100 μ l of 0.1% glutaraldehyde (w/v, Sigma-Aldrich) in PBS to prevent agglutination of antigen-positive cells. The plate was then centrifuged at 200 $\times g$ for 1 min in a plate centrifuge and the supernatant discarded. To each well lectin in 50 μ l of PBS was added to the final concentrations of 5 or 25 μ g/ml, and the reactions were incubated for 5 min at room temperature under gentle agitation and then 2 h at 4 °C. The RBCs were then washed three times with 150 μ l of cold PBS and re-suspended in 300 μ l of the same buffer. All incubations were carried out in darkness. A total of 10,000 events were evaluated, and an RBC gate corresponding to ~90% of all cells was set during analysis of the data.

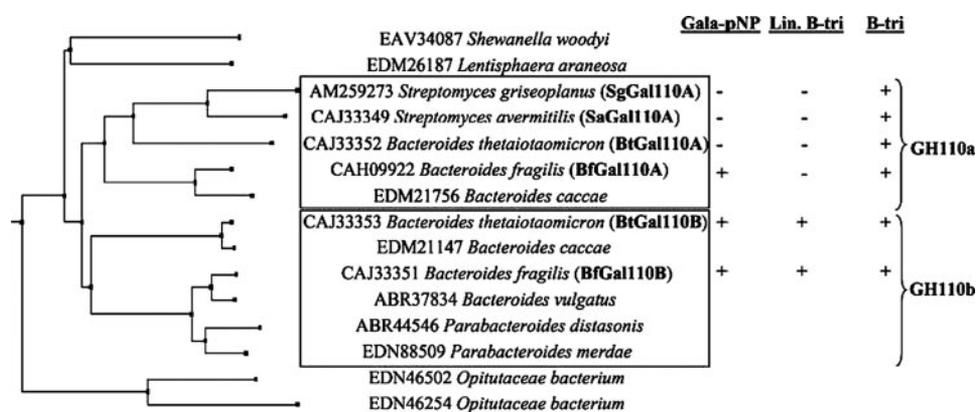


FIGURE 1. **Phylogenetic analysis of family GH110 proteins.** The GenBank™ accession numbers for proteins are indicated except for the *S. griseoplanus* gene, where accession number to the nucleotide sequence is indicated. Designated abbreviated names for the characterized proteins are shown in parentheses. The members assigned to subfamilies GH110a and GH110b are boxed, respectively. Substrate specificities of the characterized enzymes with respect to Gal α -pNP, branched blood group B (B-tri) and linear B trisaccharide (Lin. B-tri), as found in this study or reported previously (1) are indicated by “+” (active) and “-” (inactive). The neighbor-joining tree was made from the resulting distance matrix using Blossum62 substitution parameters by aligning the amino acid sequences using MUSCLE (19) and displayed with an in-house program. For an updated list of enzymes, the reader should consult the CAZy data base, which features continuously updated listings of the members of glycoside hydrolase families including GH110.

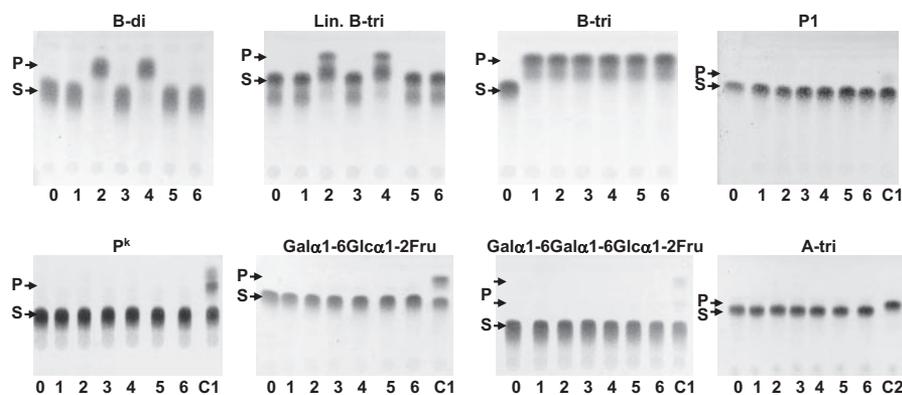


FIGURE 2. **Substrate specificity of α -galactosidases analyzed by TLC assay.** Analysis of reactions after 2-h incubation of the substrates with the enzymes is shown. Cleavage of the active substrates was complete in 5–10 min (not shown). Structures of oligosaccharide substrates are shown in Table 1. The following enzymes were used: none (0), \sim 0.2 μ g of BfGal110A (1), BfGal110B (2), BtGal110A (3), BtGal110B (4), SaGal110A (5), and SgGal110A (6), or 1.2 μ g of recombinant coffee bean α -galactosidase (C1) (44) or *Elizabethkingia meningoseptica* α -N-acetylgalactosaminidase (C2) (1). Arrows indicate the mobilities of substrates (S) and products (P).

Glycolipid Analysis—Glycolipids were prepared essentially as previously described (22). Briefly, RBC membranes were extracted by homogenization of the RBCs, either native or treated with BfGal110B α -galactosidase, in 10 volumes of isopropanol/hexane/water (55:25:20, v/v, lower phase), filtered, evaporated, and partitioned by the Folch extraction method. The upper phase was collected, dialyzed, evaporated, and applied to a DEAE-Sephadex column. The flow-through containing the total upper neutral lipids was evaporated to dryness and brought up in chloroform/methanol (2:1, v/v), 1/60th volume of the original packed RBC. Total upper neutral glycolipid fractions were analyzed by TLC, which was developed in chloroform/methanol/water (30:60:8, v/v) for 40 min and stained with orcinol.

RESULTS

Phylogenetic Analysis—It has long been known that sequence-based glycoside hydrolase families often group

together enzymes with different substrate specificities (12). This may pose problems for the functional annotation of homologous genes and stresses the need for experimental characterization of functions (23, 24). Our phylogenetic analysis of available family members of GH110 suggested the existence of at least two distinct subfamilies designated GH110a and GH110b (Fig. 1).

Cloning and Expression of Family GH110 α -Galactosidases—To further explore this finding we proceeded with a detailed functional analysis of enzymes derived from *B. fragilis* and *B. thetaiotaomicron*, each containing two isoforms in their genomes, and *S. avermitilis* and *S. griseoplanus*, each containing only one isoform. Recombinant enzymes were expressed in *E. coli* Rosetta2(DE3)pLysS cells. The *Bacteroides* enzymes were found as active proteins associated with the cell debris when lysis was performed under low salt conditions, and following extraction in high salt buffer the enzymes were soluble. The *Streptomyces* α -galactosidases were expressed mainly as inclusion bodies, and only a small amount of active enzyme could be purified from the supernatant of the crude lysates. All the recombinant enzymes used in the study were purified to apparent homogeneity as evaluated by SDS-PAGE analysis.

Substrate Specificity of Family GH110 α -Galactosidases—The BfGal110A enzyme was previously shown to exhibit similar specificity to the purified endogenous enzyme originally isolated from *S. griseoplanus* 2357 (1). Both enzymes group into subfamily GH110a in the phylogenetic analysis (Fig. 1). Analysis of the substrate specificities of one additional member of the subfamily GH110a (BtGal110A) confirmed that all enzymes exhibited exclusive specificity for the branched blood group B structures (Fig. 2 and summarized in Table 1). In striking contrast, the two tested members of subfamily GH110b (BfGal110B and BtGal110B) were equally active with the branched blood group B substrates and the linear substrates (Fig. 2 and Table 1). All enzymes exhibited exclusive α 1,3 linkage specificity and none cleaved α 1,4- or α 1,6-linked Gal residues. Thus, the predicted subfamilies in family GH110 based on sequence analysis resolved two functionally different α 1,3-galactosidase activities with dramatic difference in specificity for linear Gal α 1–3Gal β 1–4GlcNAc substrates.

TABLE 1
Substrate specificity of family GH110 α -galactosidases

Substrates	Blood group specificity	α -Galactosidases					
		BfGal110A	BfGal110B	BtGal110A	BtGal110B	SaGal110A	SgGal110A
Galα1-3Gal structures							
Gal α 1-3Gal	B-di	— ^a	+ ^b	—	+	—	—
Gal α 1-3Gal β 1-4GlcNAc	Linear B-tri	—	+	—	+	—	—
Gal α 1-3(Fuca1-2)Gal	B-tri	+	+	+	+	+	+
Gal α 1-3(Fuca1-2)Gal β 1-4GlcNAc β 1-AMC	B-tetra	+(4-8) ^c	+(4-8)	+	+	+	+
Galα1-4Gal structures							
Gal α 1-4Gal	P1	—	—	—	—	—	—
Gal α 1-4Gal β 1-4Glc	P ^k	—	—	—	—	—	—
Galα1-6Gal structures							
Gal α 1-6Glc α 1-2 β Fru (Raffinose)		—	—	—	—	—	—
Gal α 1-6Gal α 1-6Glc α 1-2 β Fru (Stachyose)		—	—	—	—	—	—
GalNAcα1-3Gal structures							
GalNAc α 1-3(Fuca1-2)Gal	A-tri	—	—	—	—	—	—
GalNAc α 1-3(Fuca1-2)Gal β 1-4Glc β 1-AMC	A-tetra	—	—	—	—	—	—

^a No cleavage of substrate after incubating with the enzyme for 2 h.^b Cleavage of substrate completed after incubating with the enzyme for 5–10 min.^c Specific activity (units/mg) determined using quantification by fluorography of TLC analysis.**TABLE 2**
Kinetic parameters of BfGal110A and BfGal110B α -galactosidase

Substrates	BfGal110A				BfGal110B	
	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m
	s^{-1}	mM	$s^{-1} mM^{-1}$	s^{-1}	mM	$s^{-1} mM^{-1}$
Gal α 1-3Gal β 1-4GlcNAc	— ^a	—	—	ND ^b	ND	79 \pm 11
Gal α 1-3(Fuca1-2)Gal	ND	ND	65 \pm 10	ND	ND	84 \pm 8
Gal α -pNP	0.38 \pm 0.01	0.70 \pm 0.04	0.54	3.9 \pm 0.1	5.2 \pm 0.1	0.8

^a No cleavage of substrate after incubating with the enzyme for 2 h.^b ND, not determined.

Kinetic Analysis of BfGal110A and BfGal110B α -Galactosidases—To further study the subtle differences of substrate specificity between the two enzyme subfamilies, the k_{cat}/K_m values of BfGal110A and BfGal110B were obtained for linear and branched blood group substrates (Table 2). BfGal110A and BfGal110B demonstrated similar catalytic efficiency for the branched blood group B substrate, and while BfGal110B was essentially equally active on the linear substrate compared with the branched substrate, BfGal110A was completely inactive with the linear substrate.

Analysis of BfGal110B and BtGal110B with the Gal α -pNP substrate showed that these have readily detectable activities when analyzed under standard conditions (2.5 mM substrate), although this activity is considerable lower than that for the blood group B substrates (Table 2). We previously found that BfGal110A also exhibited low activity with the Gal α -pNP substrate (1), and analysis of the activity of all four members of the GH100a subfamily showed that only BfGal110A had readily detectable activity with this substrate, whereas the other enzymes all had extremely low activities with Gal α -pNP and only very weak color development with the Gal α -pNP substrate was observed after prolonged incubation with excess amount of enzyme (not shown). This low level of activity after prolonged incubation is at least four orders of magnitude lower than that found with the blood group B substrates. The kinetic parameters of BfGal110A and BfGal110B with the Gal α -pNP substrate are shown in Table 2. The k_{cat} and K_m values of BfGal110B were \sim 10- and 7-fold higher, respectively, than those of BfGal110A, translating into a slightly higher catalytic efficiency of BfGal110B than BfGal110A for the aryl substrate (0.8 versus 0.54 mM⁻¹ s⁻¹).

Not surprisingly, the catalytic efficiency of both enzymes for the aryl substrate was much lower than that for oligosaccharides, \sim 100-fold lower efficiency on Gal α -pNP compared with branched or linear B structures.

Stereochemistry of the Hydrolysis of Gal α -pNP by BfGal110B α -Galactosidase—Prior to addition of the enzyme, a one-dimensional ¹H NMR spectrum of the substrate, Gal α -pNP, was acquired in D₂O at 25 °C (Fig. 3A). Within 3 min of addition of the enzyme, evidence for the initial product appeared in the NMR spectrum in the form of a doublet at 4.576 ppm (³J_{1,2} = 8.0 Hz), essentially identical in chemical shift to the H-1 resonance of free β -galactose (Fig. 3B). This signal increased in intensity steadily over the next 5 h, while the H-1 and other signals from the substrate decreased concomitantly (Fig. 3, C–E). The onset of mutarotatory equilibration was not apparent in the NMR spectra until later time points, e.g. between 40 and 60 min, mutarotation of the initial product to α -galactose was observed, indicated by the appearance and steady increase in intensity of another H-1 signal at 5.258 ppm (³J_{1,2} = 3.3 Hz) (Fig. 3C). The published values for H-1 of the β - and α -anomers of free galactose are 4.58 and 5.26 ppm, respectively, when corrected for the difference in reference chemical shift of the acetone standard (25). Integration showed that at 60 min (Fig. 3C) the reaction was \sim 60% complete, and relative amounts of α - and β -galactose were 8 and 92%, respectively; while at 120 min (Fig. 3, D and F), when the reaction was \sim 90% complete, the relative amounts of α - and β -galactose were 13 and 86%, respectively (\sim 1% of another species, probably the β -furanose form of galactose, appeared to be formed by this time). By 300 min (Fig. 3, E and F) the reaction was \sim 99% complete, and relative amounts of α - and β -galactose were 25 and 73%,

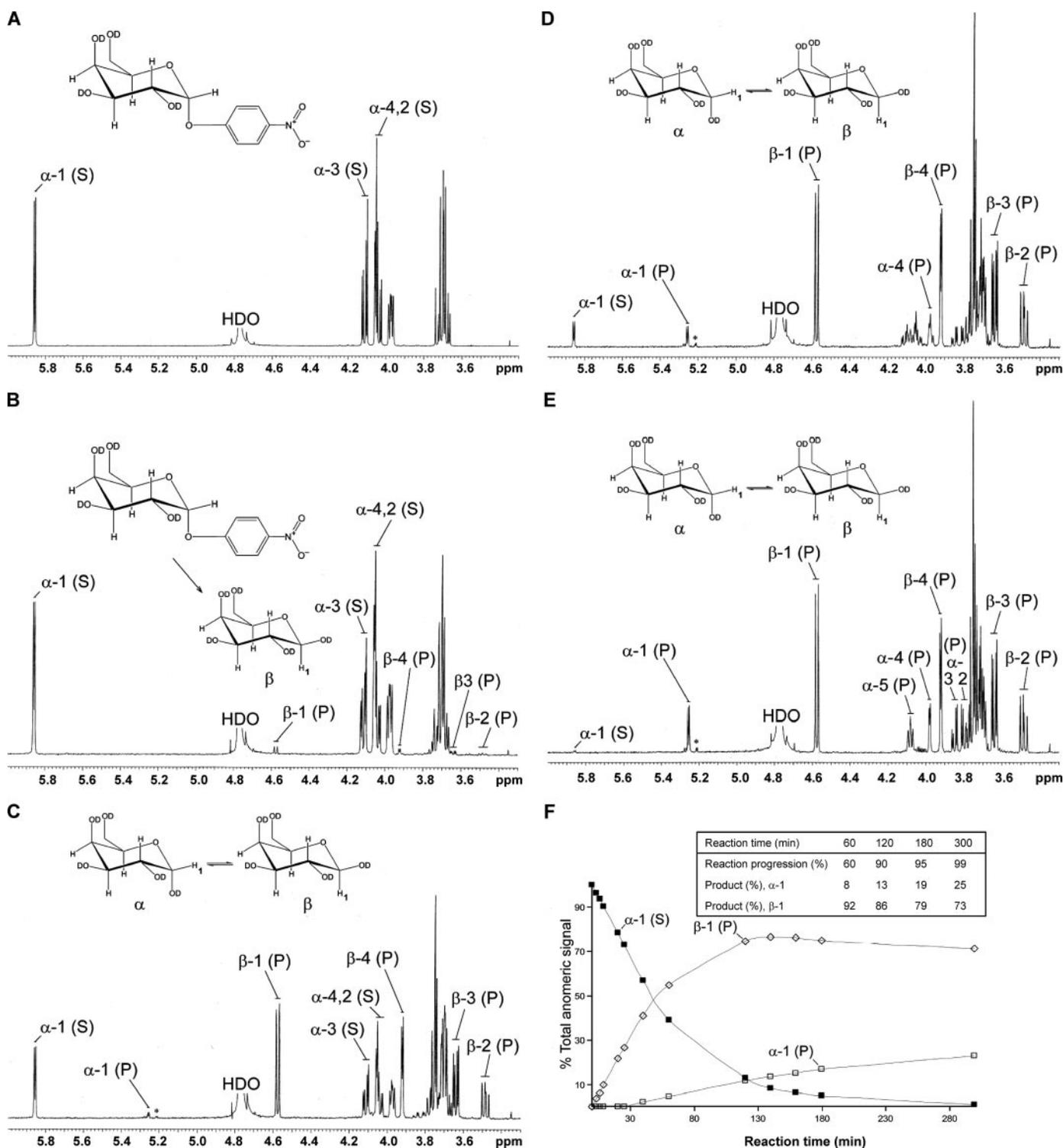


FIGURE 3. NMR spectroscopic monitoring of the hydrolysis of Gal α -pNP by BfGal110B α -galactosidase. In these one-dimensional ^1H NMR spectra of deuterium-exchanged Gal α -pNP, only the monosaccharide resonances are shown; downfield aromatic resonances from the nitrophenol are omitted. Spectra were acquired at $t = 0$ (A, prior to enzyme addition), 3 (B), 60 (C), 120 min (D), and 300 min (E) after addition of the enzyme. Structures of predominant species are superimposed on each panel; proton resonance assignments are marked as follows: (S) = signals from substrate, (P) = signals from products, * = probable H-1 from β -furanose form of galactose, HDO = residual monodeuterated water. The reaction time-dependent changes in amplitude of H-1 signals from substrate, α -1 (S), and products, α -1 (P), and β -1 (P), are plotted in F as % of total anomeric (H-1) signal. Inset, table shows progress of reaction for selected time points (min). Tabulated are: approximate α -1 (S) amplitude as a percentage of that at $t = 0$ (reaction progression), and amplitudes of α -1 (P) and β -1 (P) as percentages of total product H-1 signals (product α -1 and β -1, respectively). Note in F the initial lag in appearance of observable signal for α -1 (P) until after the 25-min time point, whereas that for β -1 (P) rises rapidly, levels off, and then begins to decline after 140 min, as the amplitudes of the α -1 and β -1 (P) signals approach equilibrium proportions (see Table, inset).

respectively ($\sim 2\%$ β -furanose form of galactose), approaching the equilibrium concentrations of α - and β -galactose (~ 28 and 72% , respectively). These observations indicate that the initial

product must be β -galactose, and the enzyme proceeds with a mechanism leading to inversion of the anomeric configuration of the substrate.

TABLE 3
Blood group typing of pig and rabbit RBCs with BfGal110B enzyme dose titrations

RBC source	Enzyme dose	ABO typing results ^a				Lectin typing results	
		Diagast anti-B		Immucor anti-B		IB4-lectin	
		IS ^b	4 °C	IS	4 °C	10	50
	mg/ml pRBC						
Pig	0	1+	2+	W+	3+	4+	4+
	0.21	0	0	0	0	0	0
	0.11	0	0	0	0	0	0
	0.053	0	0	0	0	0	0
	0.026	0	0	0	0	0	0
	0.013	0	0	0	0	0	0
	0.007	0	0	0	0	0	ND ^c
	0.003	0	0	0	0	0	ND
	0.002	0	0	0	0	0	ND
	0.001	0	0	0	0	0	ND
Rabbit	0	4+	4+	4+	4+	1+	2+
	0.21	0	vw+	0	vw+	0	vw+
	0.11	0	w+	0	w+	0	1+
	0.053	0	w+	0	1+	0	2+
	0.026	0	1+	0	1+	0	2+
	0.013	0	1+	0	2+	0	2+

^a Typing with licensed ABO typing reagents and methods, or lectin as indicated using agglutination score 0, vw (very weak), w (weak), and 1+ to 4+.

^b Immediate spin reactions.

^c ND, Not determined.

Enzymatic Removal of the α 3Gal Antigens on Porcine and Rabbit RBCs—We previously developed a process for enzymatic removal of blood group B antigens from human RBCs using the BfGal110A α -galactosidase (1). Using the same protocol BfGal110B was shown to be equally effective in converting human blood group B RBCs to RBCs that type as group O (not shown). We then tested if BfGal110B could remove the α 3Gal xenotransplantation epitope from rabbit and porcine RBCs (Table 3) using the IB4 lectin as well as blood group anti-B typing reagents to monitor the conversion. Both reagents were shown to react strongly especially with rabbit RBCs, and to our knowledge reactivity of anti-B typing reagents with the linear α 3Gal epitope has not been reported previously. The enzymatic treatment removed essentially all reactivity with the lectin, although some residual reactivity was found with rabbit RBCs at the highest lectin concentration (50 μ g/ml). Similarly, reactivity with the blood group B typing reagents was essentially eliminated, although very weak reactivity at 4 °C was observed with rabbit cells at the highest concentration of the IB4 lectin tested. Interestingly, removal of the antigens from rabbit RBCs required considerably higher concentration of enzyme than previously used for efficient removal of blood group B from human RBCs (0.01 mg/ml pRBCs) or pig RBCs (<0.001 mg/ml pRBCs) (1) (Table 3). These differences could reflect variations in structures of carbohydrates and glycoconjugates. Glycosphingolipids from rabbit RBCs have been particularly well characterized (26). They consist mainly of type 2 chain elongated and branched polyactosamine structures terminated with Gal α 1,3Gal epitopes, which should serve as efficient substrates for the enzyme. Another possibility for these differences could be enzyme accessibility to substrates on the cell surface. The buffer system, developed for efficient conversion of human group B RBCs optimized for ionic interactions between enzyme

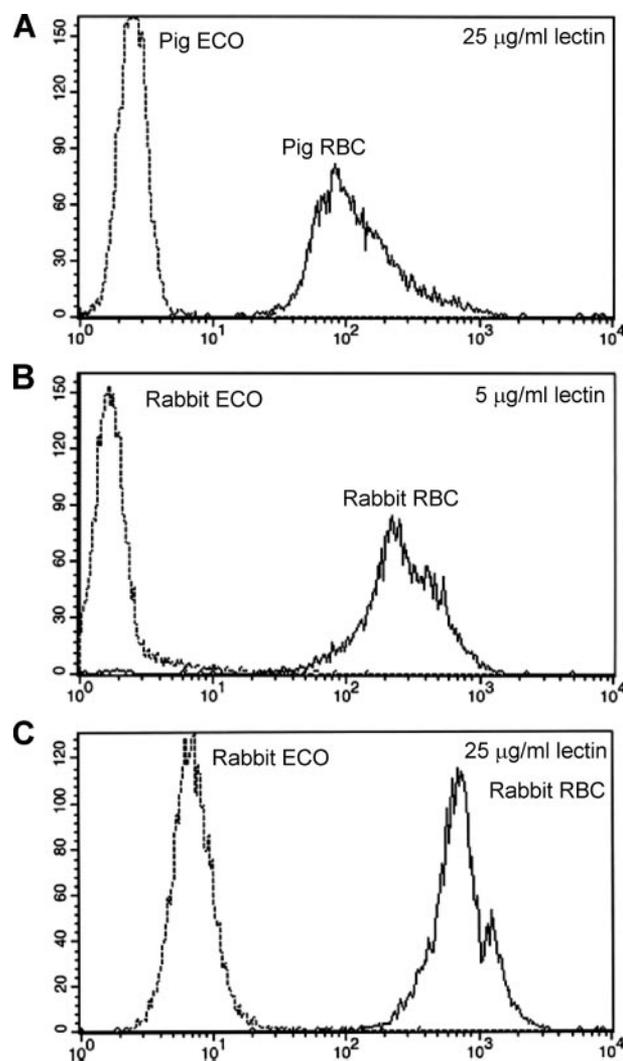


FIGURE 4. FACS analysis of BfGal110B enzyme-treated porcine and rabbit RBCs. FACS histograms show the relative antigen site density as measured by IB4 (BS-4) lectin on native (solid lines) and enzyme-converted (dashed lines) RBCs from pig (A) or rabbit (B and C). The x-axis represents the fluorescence intensity on a logarithmic scale, whereas the y-axis shows number of RBCs evaluated. The lectin concentration used was 25 μ g/ml in A and C, but 5 μ g/ml in B. Unstained porcine and rabbit RBCs and human group O RBCs served as additional negative controls and gave similar mean fluorescence intensity values compared with the converted animal RBCs in A and B. In C, higher mean fluorescence intensity was noted for the native rabbit RBCs compared with native porcine RBCs at the same lectin concentration. Post-conversion rabbit RBCs indicate low amounts of remaining Gal α 1,3Gal epitopes detectable at the higher but not lower lectin concentration shown in C and B, respectively.

and human cells (1), may not be the optimal condition for pig or rabbit cells.

FACS Analysis—Native and enzyme-treated porcine and rabbit RBCs were subjected to flow cytometric analysis with fluorescein-labeled IB4 lectin to evaluate relative antigen site density, conversion efficacy, and homogeneity similarly to what we previously reported with ABO blood group typing reagents and human RBCs (1). In general, higher mean fluorescence intensity values were obtained with native rabbit RBCs (Fig. 4C) compared with porcine RBCs (Fig. 4A) at the same lectin concentration. Although an apparently complete conversion was observed for porcine RBCs (Fig. 4A), post-conversion rabbit RBCs indicate low amounts of remaining Gal α 1,3Gal epitopes

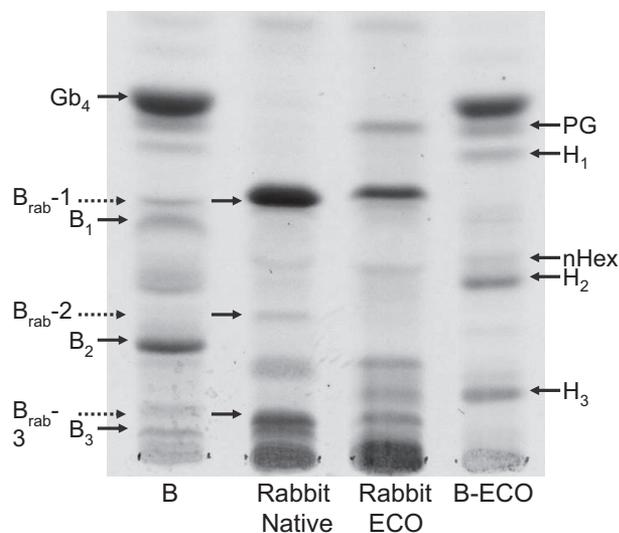


FIGURE 5. TLC analysis of the glycolipids from BfGal110B enzyme-treated rabbit RBCs. Total upper neutral glycolipid fractions from human blood group B RBCs (native B and B-ECO) and rabbit RBCs (native and rabbit ECO) were separated on TLC plates and glycolipids visualized by orcinol staining. Arrows indicate mobilities of major human blood group ABH active glycolipids (B1–B3: Gal α 1-3(Fuca1-2)(Gal β 1-4GlcNAc β 1-3) $_n$ = $_{1-3}$ Gal β 1-4Glc β 1-Cer, and H1–H3: Fuc α 1-2(Gal β 1-4GlcNAc β 1-3) $_n$ = $_{1-3}$ Gal β 1-4Glc β 1-Cer], rabbit linear B glycolipids (B $_{rab-1}$ –B $_{rab-3}$: Gal α 1-3(Gal β 1-4GlcNAc β 1-3) $_n$ = $_{1-3}$ Gal β 1-4Glc β 1-Cer), Gb $_4$ (globoside: GalNAc β 1-3Gal α 1-4Gal β 1-4Glc β 1-Cer), PG (paragloboside: Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc β 1-Cer), and nHex (nor-hexosylceramide: Gal β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc β 1-Cer).

detectable only at the highest lectin concentration tested (Fig. 4C). An apparently homogenous conversion of cells was observed in all experiments.

Glycolipid Analysis—TLC analyses of total upper neutral glycolipid of native and enzyme-treated rabbit RBCs showed significant cleavage of B $_{rab}$ glycolipids (Fig. 5). Interestingly, the shortest glycolipid species, B $_{rab-1}$ (Gal α 1-3Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc β 1-Cer), was cleaved only partially, whereas more extended species, B $_{rab-2}$ and B $_{rab-3}$ with additional *N*-acetylglucosamine units, were cleaved more completely. The apparent incomplete cleavage was in agreement with the results from agglutination typing and FACS analysis (Table 3 and Fig. 4). Similar analyses of enzyme-treated human red cells show complete cleavage of all branched blood group B glycolipid species (Fig. 5) (1). Further direct enzyme digest of the residual B $_{rab-1}$ glycolipid band isolated by preparative TLC from enzyme-treated rabbit RBCs showed complete digestion to the expected tetrasaccharide PG (paragloboside) (not shown), suggesting that the inefficiency of the enzyme reaction is related to the accessibility of the enzyme to cell surface substrates on the rabbit red cell membrane. It is likely that further improvement in conversion could be achieved by optimization of the conversion buffer system, similar to what was found for the conversion of human red cells (1). Analysis of porcine RBCs was not performed since the structures of their glycolipids have not been characterized in detail.

DISCUSSION

Our further analysis of the recently discovered GH110 glycosidase family resolved two distinct subfamilies of enzymes with different substrate specificities that are of substantial biomedical

interest. While enzymes from one subfamily exhibited unprecedented restricted substrate specificity for the branched blood group B structure, we found that members of the second subfamily of enzymes were general α 1,3-linkage-specific galactosidases. Remarkably, the enzymes from the newly identified subfamily, GH110b, had essentially identical kinetic properties with both branched and linear substrates with terminal α 1,3-galactose residues.

In the CAZy sequence-based classification of glycosidases, α -galactosidases found in families GH4, -27, -36, and -57 do not have particular substrate-linkage specificities. The recently reported BfGal110A α -galactosidase found in family GH110 was the first with a restricted substrate specificity limited to the α 1,3-linked Gal residue in the branched B structures (1). However, the extent of this linkage specificity could not be predicted, because other structures tested, including linear oligosaccharides terminated with α 1,3-linked galactose but devoid of the fucose substitution on the penultimate galactose, did not serve as substrates. The analysis of the novel GH110b subfamily isoforms, BfGal110B and BtGal110B, indicated that GH110 members in fact represent α 1,3-linkage-specific α -galactosidases. The strict substrate specificity of the GH110a α -galactosidases may suggest that these enzymes have entirely different structure and catalytic mechanism compared with previously reported α -galactosidases in families GH4, -27, -36, and -57. In agreement with this, analysis of the catalytic mechanism utilized by the BfGal110B enzyme showed that this leads to overall inversion of the stereochemistry at the site of cleavage, a feature that differs from all other known α -galactosidases. A search for invariant Asp and Glu residues in a multiple sequence alignment of all presently known family GH110 members identified Asp-141, Asp-304, Asp-327, Asp-328, and Glu-473 (BfGal110A numbering) as candidate catalytic residues.

Considering their stringent linkage specificity for the naturally occurring substrates, the readily detectable activity for Gal α -pNP observed with some members of the family GH110 enzymes is intriguing, but not unprecedented. For example, the β -galactosidases with β 1,4- and β 1,4/6-linkage specificity were shown to be active on Gal β -pNP substrates, whereas other linkage-specific glycosidases such as β 1,3-galactosidase and α 1,2-L-fucosidase demonstrated no activity for their corresponding aryl monosaccharide substrates (16–18, 27). However, the moderate apparent K_m values of BfGal110A and BfGal110B for the monosaccharide (0.70 and 5.2 mM, respectively) do not appear to suggest that the penultimate saccharide plays a critical role in the enzyme-substrate binding. Structural studies on representative members of family GH110 enzymes are in progress, and we hope they will shed light on the molecular basis for the differences in substrate specificity, the mechanism and evolution of this family of enzymes.

Almost all family GH110 α -galactosidases are predicted to be secreted proteins using SignalP 3.0 (28). In agreement with this prediction the GH110 *S. griseoplanus* α -galactosidase was originally isolated from spent culture medium (1). Most of the GH110 glycosidases are found in symbiotic bacteria such as *Bacteroides* or *Parabacteroides*, or soil-borne bacteria such as

Streptomyces, which are known to be over-represented with secreted enzymes, especially glycosidases (29, 30).

Glycosidases are useful for remodeling glycan structures. They are widely used for structural determination of complex glycans, as therapeutics in enzyme replacement therapies of lysosomal storage diseases such as Fabry's disease (31), and they have potential use for the production of biomedical products such as universal RBCs (32) and xenotransplants such as pig cruciate ligaments (33). For the latter applications, it is important that the enzymes have restricted substrate specificity, have high specific activity, and work optimally under normal physiological conditions including at neutral pH. A number of investigators have in the past considered use of α -galactosidases for procurement of tissues for use in animal to human xenotransplantation to remove the immunodominant α 3Gal residues found on many glycoconjugates in animals (34–36). This approach is particularly attractive for tissues and tissue-derived acellular products with little or no ability to replace the glycoconjugates (36). Family GH110 offers α -galactosidases with improved characteristics, and the subfamily GH110b enzymes represented by BfGal110B may substantially enhance our ability to produce animal tissues for use in xenotransplantation. Currently, the only enzyme tested for the production of xenotransplants in clinical studies has been the GH27 α -galactosidase derived from green coffee beans (33). This enzyme has an acidic pH optimum and relatively poor kinetic properties with α 3Gal-terminated oligosaccharides compared with GH110 enzymes (1). The recombinant coffee enzyme was used at \sim 3 mg/ml (100 units/ml with Gal α -pNP substrate) for removal of the α 3Gal residues of porcine ligaments for use in humans (33). The need for this high enzyme dose is due to the fact that the coffee bean enzyme has a specific activity of only 17 milliunits/mg with oligosaccharide structures such as blood group B antigens (1). In contrast, BfGal110B has a specific activity of 4–8 units/mg with the same substrates, and therefore considerably less enzyme will be required for the enzymatic treatment. Another potential problem with the coffee enzyme is that it is a broad α -galactosidase with α 1,4-galactosidase activity and hence will digest the Gal α 1,4-linkages found in glycolipids (Gal α 1,4Gal β 1,4Glc-ceramide). It is also noteworthy that all α -galactosidases that function with a retaining mechanism except for those from family GH4 are prone to transglycosylation reaction, a known side-reaction capable of giving rise to non-natural structures at high substrate concentration. The inverting nature of family GH110 enzymes excludes such undesired reactions. Considerable efforts have been applied to testing the use of an endo- β -galactosidase with strict specificity for the Gal α 1–3Gal β 1–4GlcNAc structure (35, 37–39). The action of this enzyme results in exposure of β GlcNAc residues rather than the Gal β 1–4GlcNAc structures exposed with exo- α -galactosidase strategies, and this may result in other immune problems. Several other strategies for dealing with living tissues with renewal potential are being explored, including transgenic animals with competing glycosylation pathways and knock-outs of the α 1,3-galactosyltransferase responsible for the synthesis (for a review see Ref. 40). Importantly, while the use of animals deficient in the α 1,3-galactosyltransferase may appear to be the most attractive option for animal to human xenotrans-

plantation, studies have suggested that some tissues, including the vascular endothelium of deficient animals, may in fact still express minor quantities of Gal α 1–3Gal epitopes (41, 42). The most likely explanation for this has been identified as expression of the isogloboside glycolipid (Gal α 1–3Gal β 1–4Glc β 1-Cer) (42), which is controlled by another homologous glycosyltransferase of the CAZy GT6 family, the iGb3 synthase (UDP-Gal:lactosylceramide α 1,3-galactosyltransferase) (43).

Interestingly, the phylogenetic tree of the entire family GH110 enzymes suggests that other subfamilies (Fig. 1) will most likely emerge in the future and that characterization of the fine substrate specificity of these enzymes may reveal new enzymes with important functions. In summary, we describe novel bacterial α 1,3-galactosidases from family GH110 with potential important biomedical applications in enzymatic conversion of red cells and xenotransplantation.

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