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Human chitotriosidase CHIT1 cross reacts with mammalian-like substrates

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ABSTRACT

Humans do not synthesize chitin, yet they produce a number of active and inactive chitinases. One of the active enzymes is chitotriosidase whose serum levels are elevated in a number of diseases such as Gaucher's disease and upon fungal infection. Since the biological role of chitotriosidase in disease pathogenesis is not understood we screened a panel of mammalian GlcNAc-containing glycoconjugates as alternate substrates. LacNAc and LacdiNAc-terminating substrates are hydrolyzed, the latter with a turnover comparable to that of *p*NP-chitotriose. Glycolipids or glycoproteins with LacNAc and LacdiNAc represent potential chitinase substrates and the subsequent alteration of glycosylation pattern could be a factor in disease pathogenesis.

1. Introduction

Chitin, a linear polymer of β1,4 linked N-acetylglucosamine (GlcNAc) is found in insects, crustaceans and most fungi, but not in plants, vertebrates or prokaryotes. Chitinases that hydrolyze chitin are ubiquitous enzymes produced by a wide variety of organisms, including some that do not contact or produce chitin [1]. These include humans which produce two catalytically active chitinases, chitotriosidase (CHIT1) and acidic mammalian chitinase (AMCase) and four catalytically inactive chitinase-like proteins or chitolectins with mutations in the putative active site [2–5]. Human chitinases and chitolectins are all members of CAZy glycoside hydrolase family 18 [6]. The catalytically active enzymes CHIT1 and AMCase are both 50 kDa proteins, comprised of a 39 kDa catalytic region and C-terminal CBM 14 chitin binding module [3,7].

CHIT1 is produced in mature macrophages, neutrophils, Gaucher's cells and lung macrophages [4,5,7] and CHIT1 plasma levels are elevated in pathological conditions including Gauchers disease where it is secreted as the active full-length 50 kDa form [8]. An active proteolyzed 39 kDa form is found in lysosomes. The biological basis for the roles of chitotriosidase in human disease remains unclear since no cellular targets have been identified. In this study we survey a panel of GlcNAc containing glycoconjugates as alternate substrates for CHIT1. The structures are fluorescently labelled conjugates to facilitate screening and they contain terminal LacNAc (Galβ1-4GlcNAc), LacdiNAc (GalNAcβ1-4GlcNAc) or Type 1 (Galβ1-3GlcNAc) motifs (Fig. 1 and Supplementary Fig. 1). Several of these conjugates were hydrolyzed including LacdiNAc at a comparable rate to that of *p*NP-chitotriose. Hydrolysis of cellular glycoconjugates with these terminal structures might be associated with chitotriosidase pathologies.

Abbreviations: CHIT1, chitotriosidase; AMCase, acidic mammalian chitinase

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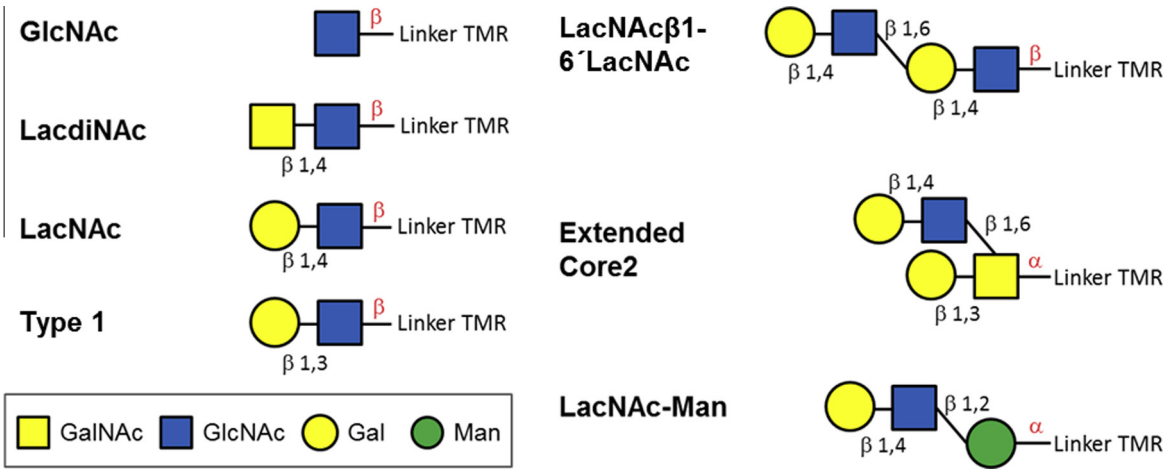

Fig. 1. Fluorescent substrates used in this study. Their exact structures are shown in Supplementary Fig. 1.

2. Materials and methods

2.1. Production of CHIT1 in HEK293 EBNA cells

Full-length CHIT1 cDNA (BC105682) for the 50 kDa form of enzyme was cloned into the StrepII C-term vector pCPR0051 (pCEP4-CTStrepII) and the construct verified by sequencing. After transformation into Mach1-T1 cells (Invitrogen) DNA was purified using the Nucleobond PC 10000 EF kit (Macherey–Nagel). Cells were grown in unsupplemented Freestyle 293 expression medium, and incubated in an orbital shaker incubator at 37 °C, 70% humidity, 5% CO_2 and 120 rpm (Ø50 mm). 1 day prior to transfection, HEK293 EBNA cells were re-suspended in fresh media to a cell density of 1.2×10^6 cells/mL and incubated overnight. Approximately 15 min before transfection, cells were harvested, re-suspended to 50 mL in fresh media at a cell density of 20×10^6 cells/mL and incubated until transfection. 2500 μg DNA (50 μg/mL final) and 5 mL of polyethylenimine "MAX" (PEI) (Polysciences) (100 μg/mL solution final) was added directly to the cell suspension. 4 h post transfection, 950 mL of media was added to a final volume of 1 L of cell suspension.

Five days post transfection the supernatants were collected by centrifugation at 6500 rpm for 10 min at 4 °C. After addition of 2 tablets of cOMPLETE protease inhibitor (Roche), the supernatants were stored at −80 °C.

2.2. Purification

CHIT1 was purified using Strep-tag/Strep-Tactin purification system (IBA Technologies). Prior to the purification the supernatant was concentrated by 65% ammonium sulfate precipitation or was supplemented with 2 units of biotin decoy solution, Avadin (IBA) and 10% buffer W (IBA) to adjust to pH 7 (untreated supernatant). Precipitated protein dissolved in 50 mL buffer W (pH 7) or untreated supernatant (240–300 mL) was loaded onto a 5 mL Strep-Tactin superflow H-PR cartridge (IBA, 2-1232-001) at a flow rate of 1–1.5 mL/min using a peristaltic pump. Washing and elution of protein was performed according to the manufacturer's instructions. Fractions containing protein were pooled followed by concentration and exchanged into 50 mM sodium phosphate buffer with Vivaspin (30 000 Da cutoff, GE Healthcare). The protein concentration was determined by the Bradford method using a commercial kit (Bio-Rad) with bovine γ-globulin as a protein reference.

2.3. Examination of enzyme activity towards chitin and cellulose pseudo-substrates

The activity of CHIT1 was determined using the chitin pseudo-substrates; 4-nitrophenyl N-acetyl-β-D-glucosaminide (*p*NP-GlcNAc), 4-nitrophenyl-N,N′-diacetyl-β-D-chitobioside (*p*NP-$(GlcNAc)_2$), 4-nitrophenyl-N,N′,N″-triacetyl-β-D-chitotriose (*p*NP-$(GlcNAc)_3$) and the cellulose pseudo-substrate 4-nitrophenyl-β-D-cellobioside (*p*NP-cellobioside) with a commercial chitinase kit (Sigma). In a standard assay 5 μL enzyme diluted in 50 mM sodium phosphate buffer, pH 6.0, and 45 μL 1 mg/mL substrate dissolved in 50 mM sodium phosphate buffer, pH 6.0 were incubated at 30 °C. Samples (50 μL) were removed after 30 min to 24 h and the reaction was quenched by adding 250 μL of sodium carbonate (0.4 M, pH 11). To limit the conversion of substrates to a maximum of 15%, the enzyme was diluted to 111 μg/mL in the *p*NP-$(GlcNAc)_2$ assay and *p*NP-$(GlcNAc)_3$ assay. Absorbance was measured in a plate reader at 405 nm and corrected for absorption in a control sample with added sodium phosphate buffer, pH 6.0, instead of enzyme. Absorption values were converted into concentrations by the use of a *p*-nitrophenol (Sigma) standard curve.

2.4. Examination of CHIT1 with LacNAc-TMR, LacdiNAc-TMR, Type1-TMR and GlcNAc-TMR substrates

The fluorescently labeled substrates had a tetramethylrhodamine (TMR) tag covalently linked to carbohydrates via a hydrophobic linker (Fig. 1 and Supplementary Fig. 1). Their preparation has been described previously [10,11]. 2 μL of the respective substrates, GlcNAcβ-O-$(CH_2)_8CONH(CH_2)_2NHCO$-TMR (GlcNAc-TMR) (1.5 mM), Gal-β1,4-GlcNAc-O-$(CH_2)_8CONH(CH_2)_2NHCO$-TMR (LacNAc-TMR) (1.9 mM), GalNAc-β1,4-GlcNAc- O-$(CH_2)_8CONH(CH_2)_2$-NHCO-TMR (LacdiNAc-TMR) (1.9 mM), or Gal-β1,3-GlcNAc-O-$(CH_2)_8CONH(CH_2)_2NHCO$-TMR (Type 1-TMR) (1 mM) were incubated with 4 μL of enzyme (CHIT1 68 μg/mL) at room temperature for 30 min to 20 h. Reaction progress was monitored by removing 0.5 μL aliquots for thin-layer chromatography on silica gel plates developed with $CHCl_3$/MeOH/H_2O (65/35/5). TMR-labeled compounds have a bright red color and thus are visible by eye. When appropriate, supernatants from cells lacking expression of CHIT1 were used as a negative control.

Kinetic parameters for LacNAc-TMR and LacdiNAc-TMR were determined by monitoring product formation with 8 different concentrations of substrate ranging from 0.015 to 0.80 mM (LacNAc-

TMR) and 0.0375 to 0.80 mM (LacdiNAc-TMR) at 30 °C by capillary electrophoresis [12]. The reaction volume was 10 μL in 50 mM sodium phosphate buffer, pH 6.0 and enzyme concentrations were 3–45 μg/mL. Aliquots (1 μL) were removed at 15–60 min and quenched with 50 μL of CE running buffer (50 mM borate, pH 9.3, and 150 mM sodium dodecyl sulfate). Analyses were performed on an automated PrinCE 560 CE system from PrinCE Technologies B.V. (Emmen, The Netherlands). Separations were carried out in an uncoated fused-silica capillary of 75 μm i.d. with CE running buffer. TMR-labeled compounds were detected and quantitated using an Argos 250B fluorescence detector (Flux Instruments) equipped with an excitation filter of 546.1/10 nm and an emission filter of 570 nm. All experiments were carried out at a normal polarity, i.e. inlet anodic. Data were processed with PrinCE 7.0 software.

2.5. Examination of CHIT1 activity with LacNAcβ1-6′LacNAc, LacNAcβ1-6Galβ1-3-GalNAc and LacNAcβ1-2-Man-(sp)(Gly)Thr(Gly) monitored by UPLC–MS

For derivatization, oligosaccharides were dissolved in PBS (pH8.5) at 50 μM (LacNacβ1-6′LacNAc and extended core 2 [13,14] or 100 μM (LacNAc-Man, [15]). TMR-N-hydroxysuccinimide ester (3 equiv., 3 μL in DMSO) was added to each oligosaccharide solution [LacNAcβ1-6′LacNAc: 15 μL (750 nmol), Extended Core 2: 10 μL (500 nmol) or LacNAc-Man (20 μL (2000 nmol)] (Supplementary Fig. 2). The reaction mixture was covered with foil to prevent exposure to light and was incubated overnight at room temperature. The derivatized product was purified by binding and elution from a C18 Sep-Pak cartridge (Waters, Milford, MA) and by silica TLC [solvent system: CHCl3/MeOH/water (65:35:5)].

Enzyme reactions consisted of TMR-labeled compound (1.25 μM), CHIT1 (1 μg), 25 mM sodium phosphate buffer, pH 6.0, in a total volume of 4 μL. After incubation for 18 h at room temperature, the reaction mixture was diluted with 300 μL of 10 mM ammonium formate, pH 4.5 (22%)/acetonitrile (88%) (v/v) for UPLC–MS analysis.

UPLC–MS analysis was performed on a Waters Acquity UPLC–MS system with fluorescence detection (λ_{ex} = 540 nm; λ_{em} = 580 - nm) using a Waters Acquity UPLC GST Amide Column (1.7 mm, 2.1 × 150 mm). Buffer A (10 mM ammonium formate, pH 4.5) and B (acetonitrile) were used. The mobile phase flow rate was set at 0.2 mL/min, with elution starting at 20% buffer A and 80% buffer B, followed by a linear gradient to 40% buffer B in 15 min. The eluted peaks were identified with a coupled mass spectrometer, Waters Micromass Quattro Premier Tandem quadrupole mass spectrometer. The instrument was operated using an electrospray source in positive mode. The ionization source conditions were as follows: capillary voltage of 4 kV, source block temperature of 120 °C and solvent evaporation temperature of 250 °C. The cone voltage was 10–50 V. The data acquisition and processing were performed using MassLynx V4.1 software (Micromass, Manchester, UK).

3. Results

3.1. CHITI activity with chitin and cellulose pseudo-substrates

Human CHIT1 were obtained from HEK293 EBNA cells with 23.4 mg of purified protein obtained per liter of supernatant (Supplementary Fig. 3). CHIT1 activity was evaluated with a panel of chitin and cellulose pseudo-substrates and hydrolyzed *p*NP-$(GlcNAc)_2$ and *p*NP-$(GlcNAc)_3$ but not *p*NP-GlcNAc or *p*NP-cellobioside. In agreement with the literature [9], a decrease in reaction rate was observed at high substrate concentrations of *p*NP-$(GlcNAc)_2$ attributed to transglycosylation (Fig. 2). The turnover number for *p*NP-$(GlcNAc)_2$ before the reduction of activity by transglycosylation is 1.5 s^{-1}. For *p*NP-$(GlcNAc)_3$ a turnover of 0.5 s^{-1} was obtained for saturating substrate with a K_m of 95 ± 25 μM (Fig. 2).

3.2. CHITI activity with LacNAc-TMR and LacdiNAc-TMR

Activity was tested towards the mammalian model substrates LacNAc-TMR, Type I-TMR and LacdiNAc-TMR (Table 1). CHIT1 was active with both LacdiNAc-TMR and LacNAc-TMR as monitored by TLC (Fig. 3 and Table 1). Complete hydrolysis of LacdiNAc-TMR to the linking arm occurred within 30 min, while only a

Table 1
Summary of CHIT1 catalytic activities.

	Substrate[a]	Chitotriosidase
*p*NP-assay	*p*NP-GlcNAc	–
	*p*NP-$(GlcNAc)_2$	+*
	*p*NP-$(GlcNAc)_3$	+
	*p*NP-cellobioside	–
TLC-assay	GlcNAc-TMR	–
	Type I-TMR	–
	LacdiNAc-TMR	+
	LacNAc-TMR	+

For *p*NP-assay: +: positive reaction (ABS_{405} > 0.08), –: negative reaction (ABS_{405} < 0.08). For TLC-assay: +: bright red spot visible to the eye, –: no spot visible to the eye.

* Apparent decrease in enzyme activity with increasing substrate concentration due to transglycosylation.

[a] *p*NP-GlcNAc: 4-nitrophenyl-N-acetyl-β-D-glucosaminide, *p*NP-$(GlcNAc)_2$: 4-nitrophenyl-N,N′-diacetyl- β-D-chitobioside, *p*NP-$(GlcNAc)_3$: 4-nitrophenyl-β-D-N,N′,N″-triacetylchitotriose, *p*NP-cellobioside: 4-nitrophenyl-β-D-cellobioside.

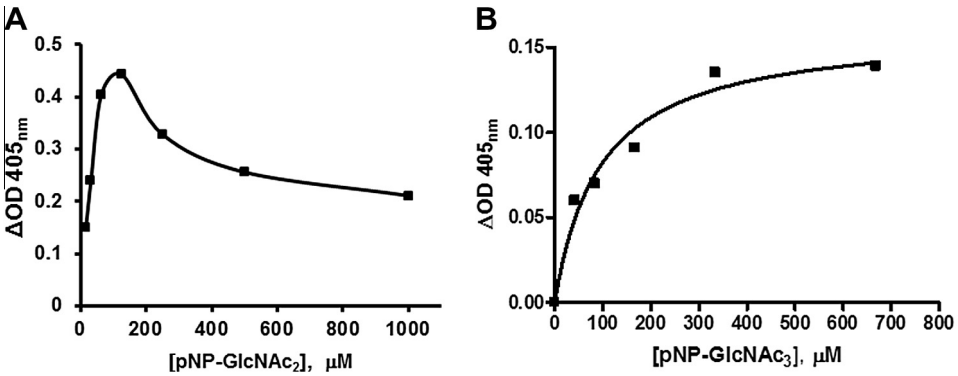

Fig. 2. Hydrolysis of *p*NP-$(GlcNAc)_2$ and *p*NP-$(GlcNAc)_3$. 111 μg/mL CHIT1 was incubated with the respective substrates for 30 min at 30 °C.

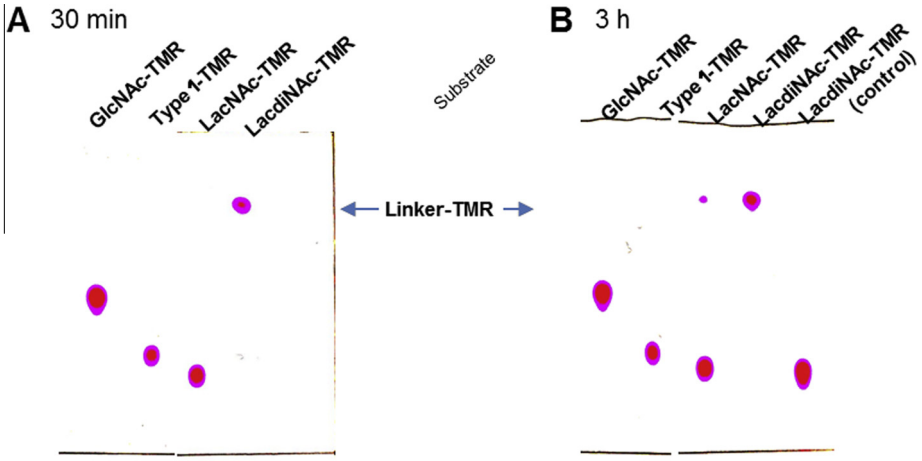

Fig. 3. TLC of reaction mixtures incubated for (A) 30 min and (B) 3 h reactions with CHIT1. The TMR-labeled compounds have a bright red color and thus are visible by eye. From the left, the TMR-labeled substrates were GlcNAc-TMR, Type 1-TMR, LacNAc-TMR, LacdiNAc-TMR. The fast moving spot at the top corresponds to Linker-TMR demonstrating that LacdiNAc and LacNAc are removed from the linker as disaccharides. The rate of hydrolysis of LacdiNAc-TMR is considerably more rapid than LacNAc-TMR.

small amount of conversion was seen after 3 h for LacNAc-TMR. There was no GlcNAc-TMR intermediate detected by TLC, thus disaccharides are hydrolyzed from the linker. CHIT1 did not hydrolyze the isomeric Type I-TMR or GlcNAc-TMR. The kinetics with both substrates was also examined by capillary electrophoresis. The turnover for LacNAc-TMR was $0.003 \pm 0.0002\ s^{-1}$ with a K_m of $34 \pm 6\ \mu M$ and with rates decreasing at concentrations above 500 μM (Supplementary Fig. 4). This highly unusual profile was reproducible and is attributed in part to competition between substrate hydrolysis and transglycosylation. The k_{cat} for LacdiNAc-TMR was $0.40 \pm 0.01\ s^{-1}$, with a K_m of $57 \pm 7\ \mu M$ with rates decreasing at concentrations above 400 μM (Fig. 4). The k_{cat} for LacdiNAc-TMR is comparable to that for *p*NP-$(GlcNAc)_3$.

3.3. CHITI activity with larger oligosaccharides LacNAcβ1,6LacNAc-TMR and extended core 2-TMR

Larger oligosaccharides terminating in LacNAc were only available in small amounts thus UPLC–MS was used to monitor their enzymatic conversions. LacNAcβ1,6LacNAc-TMR was converted to LacNAc-TMR disaccharide (40%) and the TMR linker (59%) in overnight incubations demonstrating that disaccharide units were sequentially removed by CHIT1 (Fig. 5). Only a small amount (less than 0.2%) of the extended Core 2-TMR was converted to Core 1-TMR (Galβ1, 3GalNAc-TMR). Larger concentrations of enzyme and longer incubation times did not increase the amount of conversion of extended Core2-TMR. LacNAcβ1, 2Man-TMR was not a substrate for the enzyme (Fig. 5).

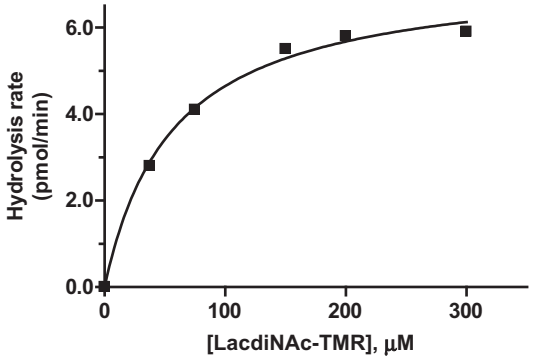

Fig. 4. Kinetics of LacdiNAc-TMR hydrolysis by CHIT1 (0.3 pmol). The hydrolysis product (Linker-TMR) was monitored by capillary electrophoresis. Substrate inhibition was observed above 400 μM substrate.

4. Discussion

We examined a panel of GlcNAc-containing disaccharides and oligosaccharides as potential substrates for human CHIT1. The full-length 50 kDa recombinant enzyme produced in HEK293 EBNA cells exhibited substrate inhibition at higher concentrations of *p*NP-$(GlcNAc)_2$ attributed to transglycosylation as reported in the literature [9]. Turnover before substrate inhibition was $1.5\ s^{-1}$. For *p*NP-$(GlcNAc)_3$ a turnover of $0.5\ s^{-1}$ was obtained giving a ratio for chitobioside/chitotrioside activity of 3. This is in agreement with the ratios of 3.9 and 3 obtained for the truncated 39 kDa and 29 kDa chitotriosidases [2]. It has also been shown that 4-methylumbelliferyl-(4″-deoxy) chitobiose which lacks the hydroxyl group at C-4 of the non-reducing GlcNAc is a better substrate for CHIT1 than 4-methylumbelliferyl-chitobiose (4MU-$(GlcNAc)_2$) (9). The deoxy-analog has a K_m of 50 μM and 4–5-fold greater activity than 4MU-$(GlcNAc)_2$, in part due to the inability to undergo transglycosylation. This also suggests that CHIT1 has some latitude with respect to recognition of the terminal 4-OH in substrates. Thus we examined LacdiNAc-TMR, which is the 4″-epimer of chitobiose as a potential substrate. While hydrolases can have strict specificity for a given epimer, LacdiNAc was cleaved by CHIT1. The turnover of LacdiNAc-TMR substrate was $0.4\ s^{-1}$ which is comparable to that of $0.5\ s^{-1}$ for *p*NP-$(GlcNAc)_3$. While this is lower than 4MU-$(GlcNAc)_3$ with a k_{cat} of $15.7\ s^{-1}$ and a K_m of 53 μM [16], turnover is in line with chitin pseudo substrates and demonstrates the differential effects of aglycones on CHIT1 reaction rates.

LacNAc-TMR which contains an N-acetyl group (acetamido) at the reducing end but lacks an N-acetyl group at the non-reducing end (Fig. 1 and Supplementary Fig. 1) was also a substrate though its turnover was considerably slower ($0.003\ s^{-1}$). The catalytic reaction for family 18 chitinase such as CHIT1 is believed to occur with substrate assisted catalysis with attack of the N-acetyl group on C-1 of substrate to cleave the glycosidic bond and generate an oxazoline intermediate. LacNAc-TMR contains the requisite N-acetyl for substrate assisted catalysis. The greater turnover of LacdiNAc is attributed to additional binding interactions since it contains two N-acetyl groups. Interactions with the N-acetyl group

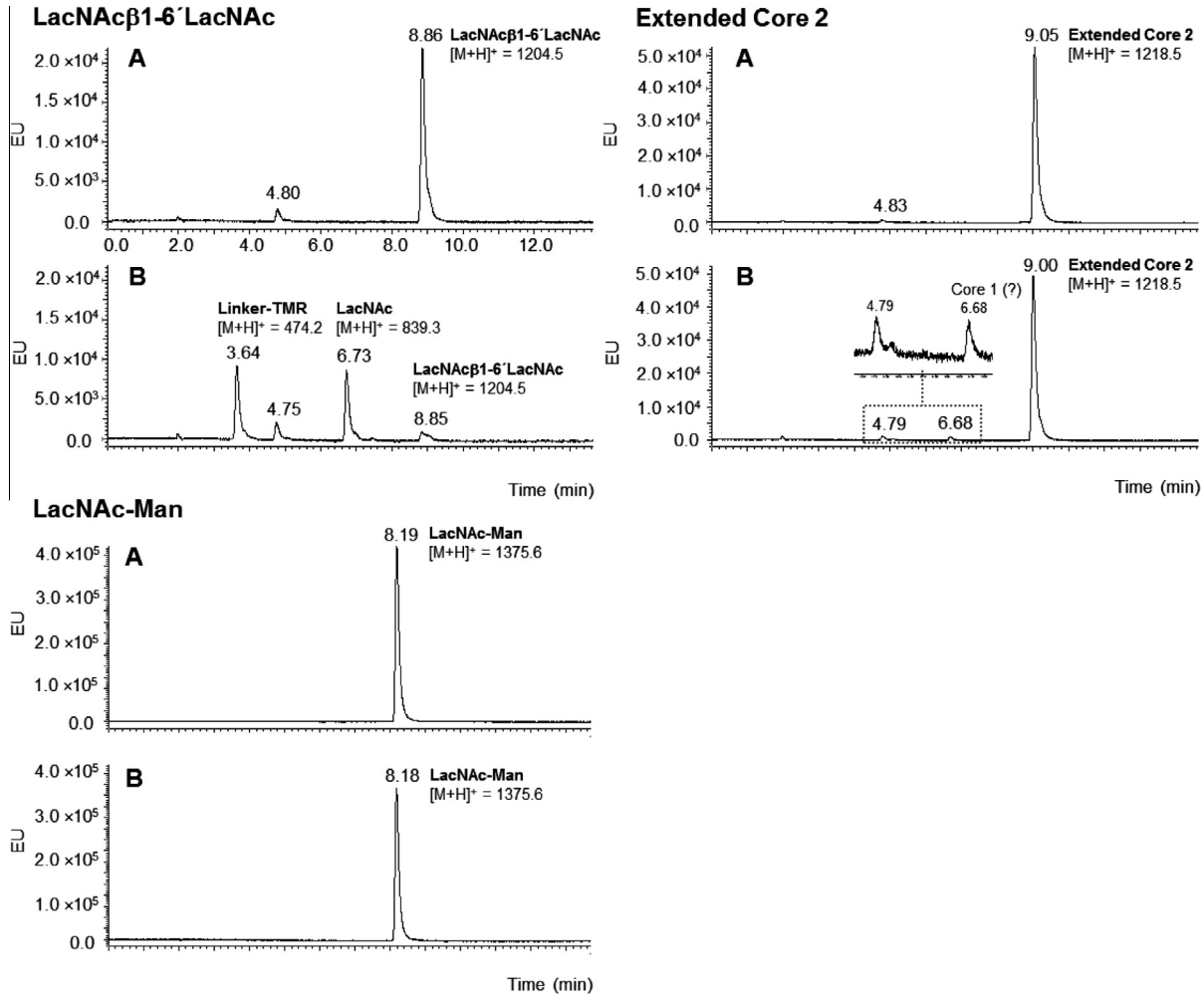

Fig. 5. UPLC–Mass Spectrometry analysis of potential oligosaccharide substrates, before (A) and after the reaction with CHIT1 (B).

in the -2 subsite were seen in the crystal structure of CHIT1 in allosamidine inhibitor complexes [17,18]. The -2 subsite corresponds to the non-reducing end of LacNAc-TMR thus accounting for its lower reactivity compared to LacdiNAc-TMR which has an N-acetyl substituent in the -2 subsite. The isomeric Type 1-TMR (Galβ1,3GlcNAc) was not a substrate, as expected for chitinases that hydrolyze β1,4 linked chitin.

There are limited sources of oligosaccharides terminating in LacNAc or LacdiNAc. We found that disaccharide units were sequentially hydrolyzed from LacNAcβ1-6′LacNAc-TMR. While oligosaccharides with β1,4 linkages are expected to be substrates for CHIT1, the hydrolysis of LacNAcβ1-6′LacNAc-TMR is attributed to the flexibility of the β1,6 linkage. There was minimal activity on the extended Core 2 substrate and no activity with LacNAcMan. Thus terminal LacNAc or LacdiNAc structures linked β1,4 or β1,6 in oligosaccharides, glycoproteins or glycolipids are potential substrates for CHIT1. The altered glycosylation upon hydrolysis by CHIT1 might be associated with pathologies.

It has been suggested that CHIT1 has a role in the defense towards chitin containing pathogens [19] however elevated expression of CHIT1 has also been associated with a range of human diseases that have no obvious connection to chitin containing pathogens. These include Gaucher type I disease [20], Alzheimer's disease [21–23], type B Niemann-Pick disease [24], sarcoidosis [25,26], atherosclerosis [27], multiple sclerosis [28,29] and scleroderma-associated lung disease where CHIT1 has even been proposed as a potential therapeutic target [30]. The specific effects of the CHIT1 in these diseases are not known.

Our results suggest that CHIT1 might adopt an activity towards GlcNAc containing substrates present in the human host, as perhaps glycan substrates found in association with the surface or cytosol of epithelial cells and/or macrophages. This study provides the first demonstration that mammalian like glycans may provide potential substrates for CHIT1. It should be considered that there may not be a common target for the actions of CHIT1 in relation to the diseases described above but rather that distinct targets might be relevant under different circumstances. It can be perceived, however, that glycans that contain either a LacNAc or LacdiNAc motif might be a part of the target with the concomitant alterations in glycosylation contributing to the pathogenic response. It is of considerable interest that chitinases produced by bacterial pathogens such as *Legionella pneumophila*, *Listeria monocytogenes* and *Salmonella* may act as virulence factors by targeting similar substrates [31].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.12.035.

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