α-L-Fucosidase from Bombyx mori has broad substrate specificity and hydrolyzes core fucosylated N-glycans

α-L-Fucosidase from *Bombyx mori* has broad substrate specificity and hydrolyzes core fucosylated *N*-glycans

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Abstract

N-glycans play a role in physiological functions, including glycoprotein conformation, signal transduction, and antigenicity. Insects display both α-1,6- and α-1,3-linked fucose residues bound to the innermost *N*-acetylglucosamine of *N*-glycans whereas core α-1,3-fucosylated *N*-glycans are not found in mammals. Functions of insect core-fucosylated glycans are not clear, and no α-L-fucosidase related to the *N*-glycan degradation has been identified. In the genome of the domestic silkworm, *Bombyx mori*, a gene for a protein, BmFucA, belonging to the glycoside hydrolase family 29 is a candidate for an α-L-fucosidase gene. In this study, BmFucA was cloned and recombinantly expressed as a glutathione-*S*-transferase tagged protein (GST-BmFucA). Recombinant GST-BmFucA exhibited broad substrate specificity and hydrolyzed *p*-nitrophenyl α-L-fucopyranoside, 2′-fucosyllactose, 3-fucosyllactose, 3-fucosyl-*N*,*N*′-diacetylchitobiose, and 6-fucosyl-*N*,*N*′-diacetylchitobiose. Further, GST-BmFucA released fucose from both pyridylaminated complex-type and paucimannose-type glycans that were core-α-1,6-fucosylated. GST-BmFucA also shows hydrolysis activity for core-fucosylated glycans attached to phospholipase A_2 from bee venom. BmFucA may be involved in the catabolism of core-fucosylated *N*-glycans in *B. mori*.

Keywords: *Bombyx mori* / fucose / glycoprotein / glycoside hydrolase family 29 / *N*-glycan

Abbreviations: ER, endoplasmic reticulum; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Fuc, fucose; Man, mannose; GH29, Glycoside hydrolase family 29; FucA. α-L-fucosidase; FucT, fucosyltransferase; 2′-FL, 2′-fucosyllactose; 3-FL, 3-fucosyllactose; 6-FGn_2, GlcNAcβ(1-4)[Fucα(1-6)]GlcNAc; 3-FGn_2, GlcNAcβ(1-4)[Fucα(1-3)]GlcNAc; PA, pyridylamino; PNGase F, peptide:*N*-glycanase F; pNP, *p*-nitrophenyl; PLA_2, Phospholipase A_2; AAL, *Aleuria aurantia* lectin

1. Introduction

Protein glycosylation is a common post-translational modification (Varki, 2017). Glycoproteins have various glycan structures that affect various physiological functions, such as protein folding, stability, transport, and activity (Moremen et al., 2012; Ohtsubo and Marth, 2006; Varki et al., 2009b). *N*-glycans are attached to an asparagine residue at the Asn-X-Ser/Thr sequon of glycoproteins, where X is any amino acid residue except Pro, and preferably at an Asn-X-Ser sequon rather than an Asn-X-Thr sequon (Reddy et al., 1999; Zielinska et al., 2010). *N*-glycans are composed mainly of fucose (Fuc), galactose, glucose (Glc), *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine, mannose (Man), and sialic acid (Moremen et al., 2012).

N-glycan structure differs between insects and mammalians. Mammalian cells produce complex-type *N*-glycans, and insect cells mainly produce paucimannose-type *N*-glycans. Biosynthesis of mammalian complex-type *N*-glycans involves an *N*-glycan precursor ($Glc_3Man_9GlcNAc_2$) first synthesized as a lipid-linked oligosaccharide in the endoplasmic reticulum (ER). This precursor is transferred to an asparagine residue in the sequon and is trimmed and elongated by enzymes located in the ER and Golgi apparatus. $GlcNAcMan_3GlcNAc_2$, an intermediate in *N*-glycan synthesis, is extended by Golgi-resident glycosyltransferases (Moremen et al., 1994). Insects display similar *N*-glycan biosynthesis; a $GlcNAcMan_3GlcNAc_2$ glycan intermediate is also formed cultured cells. Insect cells generally show low activity for β-1,2-*N*-acetylglucosaminyltransferase II (GnT-II) and galactosyltransferase and no detectable activity for sialyltransferase (Altman et al., 1993; Geisler and Jarvis, 2012; van Die et al., 1996; Walski et al., 2017b). Also, insect cells possess *N*-acetylglucosaminidase (fused lobes) that removes terminal GlcNAc residues from $GlcNAcMan_3GlcNAc_2$, a process required to synthesize paucimannose-type *N*-glycans (Geisler and Jarvis, 2008). Homologs of mammalian glycosyltransferases involved in complex glycan biosynthesis have been identified in insects (Geisler and Jarvis, 2012; Haines and Irvine, 2005; Kajiura et al., 2015; Koles et al., 2004; Miyazaki et al., 2019a; Miyazaki et al., 2019b; Vadaie and Jarvis, 2004), and complex glycans other than paucimannose-type are found in *N*-glycomes of some insects and their culture cells (Hykollari et al., 2019; Koles et al., 2007; Kurz et al., 2015; Stanton et al., 2017). Sialylated *N*-glycans were detected in some insect cells and tissues (Aoki et al., 2007; Aoki and Tiemeyer, 2010; Cime-Castillo et al., 2015; Walski et al., 2017a) and in the envelop protein of mature dengue type 2 virus derived from insect cells (Lei et al., 2015). Thus, the *N*-glycan pathway in insects is complicated and is not completely understood.

Some insect *N*-glycans can be further processed with α-1,3- and/or α-1,6-linked fucose residues on the innermost GlcNAc residue of the core structure. α-1,6-Fucosylation is catalyzed by α-1,6-fucosyltransferase (FucT6), and core α-1,6-fucosylated *N*-glycans are further processed by core α-1,3-fucosyltransferase (FucTA) to produce core α-1,6- and α-1,3-fucosylated glycans unique to insects and nematodes but absent in mammals (Aoki et al., 2007; Fabini et al., 2001; Minagawa et al., 2015; Yan et al., 2013). FucTA fucosylates non-α-1,6-fucosylated glycans, but FucT6 cannot transfer fucose to core α-1,3-fucosylated glycans (Paschinger et al., 2005). FucT6 and FucTA require a GlcNAc residue on the non-reducing end of *N*-glycans (Fabini et al., 2011; Paschinger et al., 2005). Analyses of *N*-glycan structures revealed that the core-fucosylation is universal among insects, including beetles (Walski et al., 2016; Liu et al., 2019), moths (Kajiura et al., 2015; Mabashi-Asazuma et al., 2015; Soya et al., 2016; Stanton et al., 2017), and bees (Kubelka et al., 1993; Kubelka et al., 1995). The levels of core fucosylation were reported to be different between males and females of invertebrates including insects (Scheys et al., 2019; Scheys et al., 2020). Core α-1,6-fucosylated glycans in mammals affect tissue development and signal transduction, but the role of insect core-fucosylated glycans is less clear (Becker and Lowe, 2003; Miyoshi et al., 2008; Walski et al., 2017b). Further, enzymes related to the degradation of insect *N*-glycans are poorly characterized.

α-L-Fucosidase is classified as a member of the glycoside hydrolase family (GH) 29, 95, 141, and 151 according to the CAZy database (http://www.cazy.org/, Katayama et al., 2004; Lombard et al., 2014; Ndeh et al., 2017; Sela et al., 2012) GH29 and GH95 account for most α-L-fucosidases currently registered. Hydrolysis mechanisms of GH29 and GH95 enzymes are retaining and inverting, respectively (Nagae et al., 2007; Sulzenbacher et al., 2004). GH29 is comprised of α-L-fucosidases from archaea, bacteria, fungi, plants, and mammals, whereas GH95 α-L-fucosidases are found in bacteria, fungi, and plants but not in animals (Intra et al., 2006; Pogorelko et al., 2016). Structural differences in catalytic domains are observed; GH29 enzymes adopt a $(\beta/\alpha)_8$-barrel architecture, and domains of GH95 enzymes show an $(\alpha/\alpha)_6$-barrel fold (Nagae et al., 2007; Sakurama et al., 2012). GH29 α-L-fucosidases are divided into two subfamilies, GH29-A and GH29-B, based on differences in substrate specificity (Grootaert et al., 2020; Sakurama et al., 2012). GH29-B enzymes are more specific for α-1,3/4-fucosylated substrates than GH29-A enzymes. GH29-A α-L-fucosidases have been isolated and identified from a wide range of organisms, including bacteria (Intra et al., 2007).

The physiological function of two arthropodal GH29 α-L-fucosidases has been reported. *Drosophila melanogaster* α-L-fucosidase (DmFucA) is expressed in testis, accessory gland, midgut, and brain (Pasini et al., 2008). DmFucA on the sperm plasma membrane participates, during fertilization, in gametophyte recognition possibly through interaction with glycoside ligands on egg surfaces (Intra et al., 2006). In contrast, an α-L-fucosidase from the spider, *Nephilingis cruentata* (NcFuc), is expressed in midgut diverticula (Perrella et al., 2018a). NcFuc is associated with digestive processes and likely removes fucose residues from food sources for metabolic purposes (Fuzita et al., 2016; Perrella et al., 2018a) Physiological functions and localization of arthropodal α-L-fucosidases are reported, but substrate specificity of insect α-L-fucosidases and their relationship with *N*-glycan metabolism remain unknown. In this study, GH29 α-L-fucosidase (named BmFucA) from *Bombyx mori* is first cloned, deeply investigated for substrate specificity, and identified as an enzyme that hydrolyzes both the core α-1,3- and α-1,6-fucoside linkages of *N*-glycans.

2. Materials and methods

2.1. Chemical

p-Nitrophenyl α-L-fucopyranoside (pNP-α-L-Fuc) was purchased from Merck (Darmstadt, Germany), 2′-fucosyllactose (2′-FL) and 3-fucosyllactose (3-FL) were from Carbosynth Limited (Berkshire, UK), and GlcNAcβ(1-4)[Fucα(1-6)]GlcNAc (6-FGn$_2$) and GlcNAcβ(1-4)[Fucα(1-3)]GlcNAc (3-FGn$_2$) were from Tokyo Chemical Industry (Tokyo, Japan). Fucosylated substrate structures were described in Supplementary data, Fig. S1. The pyridylaminated (PA) glycans were purchased from Masuda Chemical Industries (Takamatsu, Japan). All other reagents were of analytical grade and purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan) or Merck unless otherwise stated.

2.2. Cloning, expression, and purification of BmFucA

Total RNA of a *B. mori* fifth-instar larva was extracted using Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA) and cDNA synthesized using a PrimeScript RT reagent kit (Takara Bio, Kusatsu, Japan). DNA encoding BmFucA without the signal sequence (Met1–Gly15), predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP/, Petersen et al., 2011) as amplified by PCR using cDNA as a template and a set of primers, 5′-TTTCCATGGCCATACACAATCGTCGTGATC-3′ and 5′-TTTTCTCGAGTTATGTGGTCGCGAGTTTGAGG-3′. Amplified DNA was subcloned into

a pET41a (+) vector (Merck) at NcoI and XhoI restriction enzyme sites, and *E. coli* BL21 (DE3) was transformed with the plasmid to generate a recombinant protein containing N-terminal glutathione *S*-transferase- and His-tags (GST-BmFucA). *E. coli* BL21(DE3) harboring the expression plasmid was incubated at 37℃ in Luria–Bertani medium containing 50 μg/mL kanamycin, until optical density reached 0.6–0.8. At this point, enzyme synthesis was induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside for 24 h at 20℃. The cells were harvested by centrifugation (5,000 × g, 4℃, 5 min) and resuspended in 50 mM sodium phosphate buffer (pH 7.5) containing 300 mM NaCl and 20 mM imidazole. Cells were disrupted by sonication on ice for 20 min. After centrifugation (20,640 × g, 4℃, 15 min) to remove the insoluble fraction, the supernatant was loaded onto a Ni-Sepharose excel column (GE Healthcare, Chicago, USA) pre-equilibrated with the same buffer. The column was washed with buffer, and recombinant protein was then eluted with 50 mM sodium phosphate buffer (pH 7.5) containing 300 mM NaCl and 250 mM imidazole. Protein purity was confirmed by SDS-PAGE and Coomassie brilliant blue staining. Fractions containing protein were dialyzed against 20 mM sodium citrate buffer (pH 5.5) containing 300 mM NaCl. GST-BmFucA concentration was determined by absorbance at 280 nm based on a molar absorptivity calculated using ExPASy ProtParam (http://web.expasy.org/protparam/).

2.3. Enzyme assay

Enzyme activity for pNP-α-L-Fuc was measured in 50 μL reaction mixtures containing 1 μg/mL of GST-BmFucA and 1 mM pNP-α-L-Fuc. Reactions were stopped by two volumes of 1 M Na_2CO_3, and liberated *p*-nitrophenol was quantified by absorbance at 405 nm. pH dependence was investigated at 30℃ in 50 mM sodium citrate buffer (pH 3.0–8.0), 50 mM MES–NaOH buffer (pH 6.0–6.5), and 50 mM sodium phosphate buffer (pH 6.5–8.0). Temperature dependence was assayed at various temperatures (25℃–80℃) in 50 mM sodium citrate buffer (pH 5.0). Kinetic parameters were determined using GST-BmFucA (60 nM) and various concentrations (0.1–2 mM) of pNP-α-L-Fuc in sodium citrate buffer (pH 5.5) at 30℃. Kinetic parameters were calculated using non-linear regression analysis with Kaleida Graph (Synergy Software, Kentucky, USA).

When 2′-FL, 3-FL, and 6-FGn_2 were used as substrates, hydrolysis activity of GST-BmFucA was analyzed by TLC with reaction condition as 10 mM substrates, 95 μg/mL GST-BmFucA, 30°C, and 17 h. Reaction solution and authentic standards (fucose, lactose, and chitobiose) as controls were spotted on TLC aluminum sheet silica gel 60 F254 and developed

with 1-butanol:acetic acid:water = 2:1:1. To calculate specific activity of GST-BmFucA for 2′-FL, 3-FL, 6-FGn_2, and 3-FGn_2, fucose release was quantified using a K-FUCOSE kit (Megazyme, Dublin, Ireland). Enzyme reaction volume was 50 µL containing 95 µg/mL GST-BmFucA and 1 mM each substrate. Reaction mixtures were incubated at 30℃.

To measure hydrolytic activity toward free core α-1,6-fucosylated *N*-glycans, reaction mixtures containing 50 µg/mL GST-BmFucA, 1 µM PA-glycan (MMF^6-PA, $GnGnF^6$-PA, or $NaNaF^6$-PA), and 50 mM sodium citrate buffer (pH 5.0) were prepared and incubated at 30℃. Reaction products were separated and detected by reverse phase high-performance liquid chromatography (HPLC) using a TKSgel ODS-80_{TM} column (4.6 mm × 250 mm, Tosho, Tokyo, Japan) as described previously (Miyazaki et al., 2019a; Miyazaki et al., 2019b).

2.4. Lectin blotting

Phospholipase A_2 (PLA_2) from *Apis mellifera* bee venom (0.5 mg/mL, Cayman Chemical Co., Michigan, USA) was dissolved in sodium citrate buffer (pH 5.0) containing 10 mM dithiothreitol and denatured at 95℃ for 15 min, followed by addition of 1 µg of BmFucA and further incubation at 30℃ for 40 h. PLA_2 samples were treated with peptide:*N*-glycanse F (PNGaseF ,Takara Bio, Kusatsu, Japan) at 37℃ for 17 h and proteins separated by SDS-PAGE and electroblotted onto polyvinylidene fluoride membranes with a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). Lectin blotting used a fluorescein isothiocyanate-conjugated *Aleuria aurantia* lectin (AAL, Vector Laboratories Inc., Burlingame, USA) to evaluate defucosylation ratio after α-L-fucosidase treatment. AAL band intensity was quantified by ImageJ software, and significant differences were calculated by the Student's t-test (Schneider et al., 2012). Lectin blotting was performed in triplicate.

3. Results

3.1. Expression and general properties of recombinant BmFucA

A BLAST search was performed using amino acid sequences of characterized α-L-fucosidases to identify an α-L-fucosidase (BmFucA) gene in the *B. mori* genome. BmFucA was found and has 51% and 53% sequence identities with *Homo sapiens* α-L-fucosidase (HsFucA1) and DmFucA, respectively. The open reading frame of BmFucA encodes 477 amino acid residues with a predicted signal peptide of 15 amino acids. A phylogenetic analysis exhibited that BmFucA is classified in subfamily GH29-A (Fig. 1). Recombinant BmFucA without the signal peptide was successfully expressed in *Escherichia coli* as a GST-BmFucAand purified

to homogeneity (Fig. 2A). The molecular weight of GST-BmFucA estimated from SDS-PAGE analysis is 89 kDa, which was almost identical kDa calculated from its amino acid sequence (82 kDa).

GST-BmFucA exhibited hydrolytic activity for pNP-α-L-Fuc. Optimum pH of GST-BmFucA was pH 4.5–5.0, and optimum temperature was 45°C–60°C (Fig. 2B, C). Kinetic parameters were K_m = 0.2 ± 0.02 mM, k_{cat} = 12.5 ± 0.5 s^{-1}, and k_{cat}/K_m = 62.7 s^{-1} mM^{-1} (Table 1 and Fig. S2).

3.2. Specificity of GST-BmFucA toward fucose-containing oligosaccharides

Commercially available fucose-containing oligosaccharides, 2′-FL, 3-FL, 6-FGn_2, and 3-FGn_2, were tested for hydrolysis by GST-BmFucA. Thin-layer chromatography (TLC) analysis showed release of fucose from 2′-FL, 3-FL, and 6-FGn_2 after incubation with the enzyme (Fig. 3). All oligosaccharides including 3-FGn_2 were hydrolyzed using a K-FUCOSE kit (see Materials and Methods). Specific activities of GST-BmFucA for 2′-FL, 3-FL, 6-FGn_2, and 3-FGn_2 were 168.4 ± 9.9 nmol min^{-1} mg^{-1}, 35.3 ± 8.7 nmol min^{-1} mg^{-1}, 127.3 ± 12.2 nmol min^{-1} mg^{-1}, and 19.9 ± 3.3 nmol min^{-1} mg^{-1}, respectively. GST-BmFucA hydrolysis the showed substrate preferences as 2′-FL > 6-FGn_2 > 3-FL > 3-FGn_2. Activity toward these substrates was less than activity of pNPα-L-Fuc (Table 2).

3.3. Activity of GST-BmFucA toward core-fucosylated glycans

GST-BmFucA was assayed with core α-1,6-fucosylated fluorescent PA glycans, and the reaction products were analyzed using reversed-phase HPLC. The retention times of glycan substrates, MMF^6-PA, $GnGnF^6$-PA, and $NaNaF^6$-PA, were 15, 19, and 22 min, respectively (Fig. 4). Incubation with substrates induced new peaks with shorter retention times, increasing peak areas with time, indicating that GST-BmFucA hydrolyzed α-1,6-fucosyl linkages (Fig. 4). Specific activities with MMF^6-PA, $GnGnF^6$-PA, and $NaNaF^6$-PA were 26.9, 24.4, and 26.4 pmol min^{-1} mg^{-1}, respectively.

GST-BmFucA activity on core α-fucosylated glycoproteins was assessed using lectin blotting. PLA_2 from *Apis mellifera* bee venom (15.7 kDa), which contains core α-1,3-fucosylated and core α-1,6-fucosylated *N*-glycans, and PNGaseF were used as a model glycoprotein in the assay (Li et al., 2018). PNGaseF is active on non-fucosylated or core α-1,6-fucosylated glycans but not on α-1,3-fucosylated glycans (Tretter et al., 1991). Using AAL, which has a high affinity for fucose linked to α-1,3- and α-1,6-GlcNAc (Yamashita et al., 1985),

PLA_2 was found to be fucosylated (Fig. 4D). Band intensity of AAL for PLA_2 was reduced by 55% after GST-BmFucA treatment. Thus, GST-BmFucA hydrolyzed core α-1,3- and/or α-1,6-fucosylated *N*-glycans of PLA_2. When PLA_2 was treated with GST-BmFucA and PNGaseF, band intensity of PLA_2 was reduced by approximately 22% compared with the PNGaseF treatment alone, suggesting that GST-BmFucA might remove core α-1,3-fucose from *N*-glycans of PLA_2.

4. Discussion

In this study, BmFucA was cloned and enzymatically characterized to reveal its substrate specificity and function. The optimal pH of GST-BmFucA is like that of DmFucA and NcFuc (Intra et al., 2006; Perrella et al., 2018b) as well as rat and human lysosomal fucosidases (Dawson and Tsay, 1977; Opheim and Touster, 1977). Kinetic parameters for pNP-α-L-Fuc are comparable with those of HsFucA1, NcFuc, and TmFuc belonging to GH29-A (Table 1) (Dawson and Tsay, 1977; Perrella et al., 2018b; Tarling et al., 2003). Moreover, GST-BmFucA activity differs from GH29-B enzymes because GST-BmFucA efficiently hydrolyzes pNP-α-L-Fuc compared with GH29-B α-L-fucosidases from *Bifidobacterium bifidum*, *Bacteroides thetaiotaomicron*, and *Arabidopsis thaliana* (Ashida et al., 2009; Kato et al., 2018; Sakurama et al., 2012). These latter enzymes reportedly do not act on pNP-α-L-Fuc (Table 1). The broad specificity of GST-BmFucA for various fucosyl linkages and the greatest activity for pNP-α-L-Fuc among tested substrates are similar to GH29-A α-L-fucosidases.

2′-FL and 3-FL, found in human milk, are good substrates for GH95 α-L-fucosidase and GH29-B α-1,3/4-fucosidase from bifidobacteria, respectively (Ashida et al., 2009; Sakurama et al., 2012; Sela et al., 2012). These bifidobacterial α-L-fucosidases seem to release fucose from fucose-containing oligosaccharides for subsequent use in cellular metabolism. *Elizabethkingia meningoseptica* α-L-fucosidase cFase I, whose amino acid sequence identity to BmFucA is 25%, has high activity for the core α-1,3-fucosyl linkage in *N*-glycans as well as for 3-FL (Li et al., 2017). To date, oligosaccharides having 2′-FL and 3-FL components have not been identified in *B. mori* and other insects yet. Further, few reports are available of α-L-fucosidases exhibiting hydrolytic activity toward trisaccharides 6-FGn_2 and 3-FGn_2, though some studies report that disaccharides Fucα-1,6-GlcNAc and Fucα-1,3-GlcNAc are substrates. *Lactobacillus casei* α-L-fucosidases, AlfC and AlfB, show high activity for Fucα-1,6-GlcNAc and Fucα-1,3-GlcNAc, respectively. They also release core fucose from *N*-glycans contained in human mucosal surfaces and breast milk. *L. casei* may utilize this fucose (Becerra et al., 2020;

Rodríguez-Díaz et al., 2011). GST-BmFucA activity on trisaccharides, 6-FGn_2 and 3-FGn_2, was lower than AlfB and AlfC activity toward disaccharides. HsFucA1, located in lysosomes, also hydrolyzes Fucα-1,6-GlcNAc. HsFucA1 also has broad substrate specificity for 2′-FL and oligosaccharides containing fucose with α-1,3- and α-1,4-linkages (Dawson and Tsay, 1977). GST-BmFucA shows similar properties as HsFucA1 on various linked fucosyloligosaccharides (Table 2).

To date, few studies of α-L-fucosidase using core-fucosylated *N*-glycans as substrates are available. *Bacteroides fragilis* α-L-fucosidase (BfFucH) was used with fluorescent 2-aminobenzamide-labeled core α-1,6-fucosylated *N*-glycans. BfFucH releases fucose from paucimannose and complex *N*-glycans, but it removed only 26%–35% of fucose in 1 h. *Omnitrophica bacterium* α-L-fucosidase releases fucose from core α-1,3- and α-1,6-fucosylated glycans, respectively (Tsai et al., 2017; Vainauskas et al., 2018). In contrast, α-L-fucosidase from *Streptomyces* sp. 142 did not release fucose from core α-1,6-fucosylated complex *N*-glycans (Sano et al., 1992). Compared with BfFucH activity, GST-BmFucA displays higher activity on core α-1,6-fucosylated *N*-glycans. The present study indicates that the nonreducing end structure of *N*-glycans does not affect GST-BmFucA activity. GST-BmFucA represents the first case of activity of an arthropodal α-L-fucosidase for core-fucosylated *N*-glycans. PLA_2 has a single sequon (Asn13-Lys14-Ser15) with α-1,6 or α-1,3 core fucose (Staudacher et al., 1992). As described above, insects also exhibit core-α-1,3-fucosylated glycans (Fabini et al., 2001; Kajihura et al., 2015; Kubelka et al., 1993, Kubelka et al., 1995; Liu et al., 2019; Mabashi-Asazuma et al., 2015; Minagawa et al., 2015; Soya et al., 2016; Stanton et al., 2017; Walski et al., 2016). Tomato and *Arabidopsis* α-fucosidases classified as GH29-B are involved in *N*-glycan degradation and hydrolyze α-1,3-fucosyl linkages of 3-FGn_2 but not 3-fucosyl-*N*-acetylglucosamine and longer core-α-1,3-fucosyl *N*-glycan substrates (Kato et al., 2018; Rahman et al., 2017; Rahman et al., 2018). In addition, almond α-L-fucosidase can hydrolyze 3-FL and lacto-*N*-fucopentanose II but not showed activity core-α-1,3-fucosylated *N*-glycans (Zeleny et al., 2006). Since GST-BmFucA exhibited hydrolytic activity on core-α-1,6-fucosylated *N*-glycans and might act on core-α-1,3-fucosylated *N*-glycans (Fig. 4), BmFucA is likely to be involved in the catabolism of core-fucosylated *N*-glycans in *B. mori* cells. α-L-Fucosidase activity on both core α-1,3- and α-1,6-fucosylated *N*-glycans is reported in bacteria (Vainauskas et al., 2018) but not for eukaryotic α-L-fucosidases.

Catabolism of *N*-glycan of glycoproteins occurs mainly in lysosomes. pH in lysosomes is low, which may partially denature glycoproteins (Winchester, 2005). Glycoproteins are

degraded by various proteases and digested into polypeptides. *N*-glycans bound to polypeptides are cleaved with glycosylasparaginase to release the sugar chain (Aronson, 1999). α-L-Fucosidase, α-mannosidase, and β-*N*-acetylglucosaminidase act on released sugar chains to produce monosaccharides. These sugars are then used for re-synthesis of new *N*-glycans (Abraham et al., 1983; Barker et al., 1988; Baussant et al., 1986; Johnson and Alhadeff, 1991; Kuranda and Aronson, 1986). Considering the pH dependence of GST-BmFucA together with the above discussion, BmFucA is proposed to act on free *N*-glycans in lysosomes and release fucose for *N*-glycan turnover. This study provides new insights into carbohydrate metabolism in insects. Also, BmFucA may be used in glycoengineering to remove fucose from core-fucosylated *N*-glycans of glycoproteins, such as immunoglobulins, that are expressed in both insect and mammalian cells.

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Legends to figures

Figure 1. Phylogenetic tree of BmFucA and characterized GH29 α-L-fucosidases.

Amino acid sequences of all enzymes were obtained from GenBank. Sequence alignment was performed using Clustal Omega (Sievers et al., 2011) and a phylogenetic tree generated using MEGA 7 (Kumar et al., 2016). Sequences used were from *Bombyx mori* (XP_004923234.1), *Drosophila melanogaster* (AAM50292.1), *Homo sapiens* (AAA52481.1), *Nephilingis cruentata* (Perrella et al., 2018a), *Omnitrophica bacterium* (KXK31601.1), *Xanthomonas campestris pv. campestris* (AAM42160.1), *F. graminearum* (AFR68935.1) *Bi. longum* subsp. *infantis* (ACJ51546.1), *L. casei* (CAQ67877.1), *L. casei* (CAQ67984.1), *Thermotoga maritima* (AAD35394.1), *Ba. thetaiotaomicron* (AAO78076.1), *E. anopheles* (WP_047034007.1), *A. thaliana* (NP_180377.2), *Ba. thetaiotaomicron* (AAO77299.1), and *S. pneumoniae* (AAK76203.1). Bootstrap values are indicated, and the scale bar shows the number of assumed amino acid replacements per site.

Figure 2. General properties of GST-BmFucA.

(A) SDS-PAGE analysis of purified GST-BmFucA with CBB staining. Lane 1, molecular weight marker; lane 2, supernatant of sonicated cell extract; and lane 3, purified GST-BmFucA. Black arrow indicates GST-BmFucA (89 kDa). pH dependence (B) and temperature dependence (C) of GST-BmFucA. pH dependence was measured at 30℃ using 50 mM sodium citrate buffer (pH 3.0–6.0, circles), MES–NaOH buffer (pH 6.0–6.5, diamonds), or sodium phosphate buffer (pH 6.5–8.0, squares). Temperature dependence was measured at 25℃–80℃ using 50 mM sodium citrate buffer (pH 5.0).

Figure 3. Specificity of GST-BmFucA toward fucose-containing oligosaccharides.

The substrates were assayed with GST-BmFucA and analyzed by TLC. Abbreviations used: Fuc, L-fucose; 2′-FL, 2′-fucosyllactose; 3-FL, 3-fucosyllactose; Lac, lactose; 6-FGn$_2$, GlcNAcβ(1-4)[Fucα(1-6)]GlcNAc. Black arrows indicate standards of L-fucose and lactose.

Figure 4. Activity of GST-BmFucA toward core fucosylated *N*-glycans.

(A–C) HPLC analysis of the reaction products generated by GST-BmFucA. Reaction mixtures containing MMF^6-PA (A), GnGnF^6-PA (B), and NaNaF^6-PA (C) as substrates and GST-BmFucA were incubated in 50 mM sodium citrate buffer (pH 5.0) for 0, 20, and 60 min and analyzed using reversed-phase HPLC (see Materials and Methods). Substrates are shown

using symbols from the literature (Varki et al., 2009a). (D) Defucosylation of *N*-glycans attached to PLA_2 by GST-BmFucA. PLA_2 was incubated with GST-BmFucA and PNGaseF and analyzed using SDS-PAGE and lectin blotting (see Materials and Methods). Intact and deglycosylated PLA_2 are indicated with black and white arrows, respectively. Error bars indicate standard deviation. Asterisks indicate significant differences based on t-tests (*$p < 0.05$ and **$p < 0.01$).

Table 1. Kinetic parameters of GST-BmFucA and GH29 α-L-fucosidases from different species for pNP-α-L-Fuc.

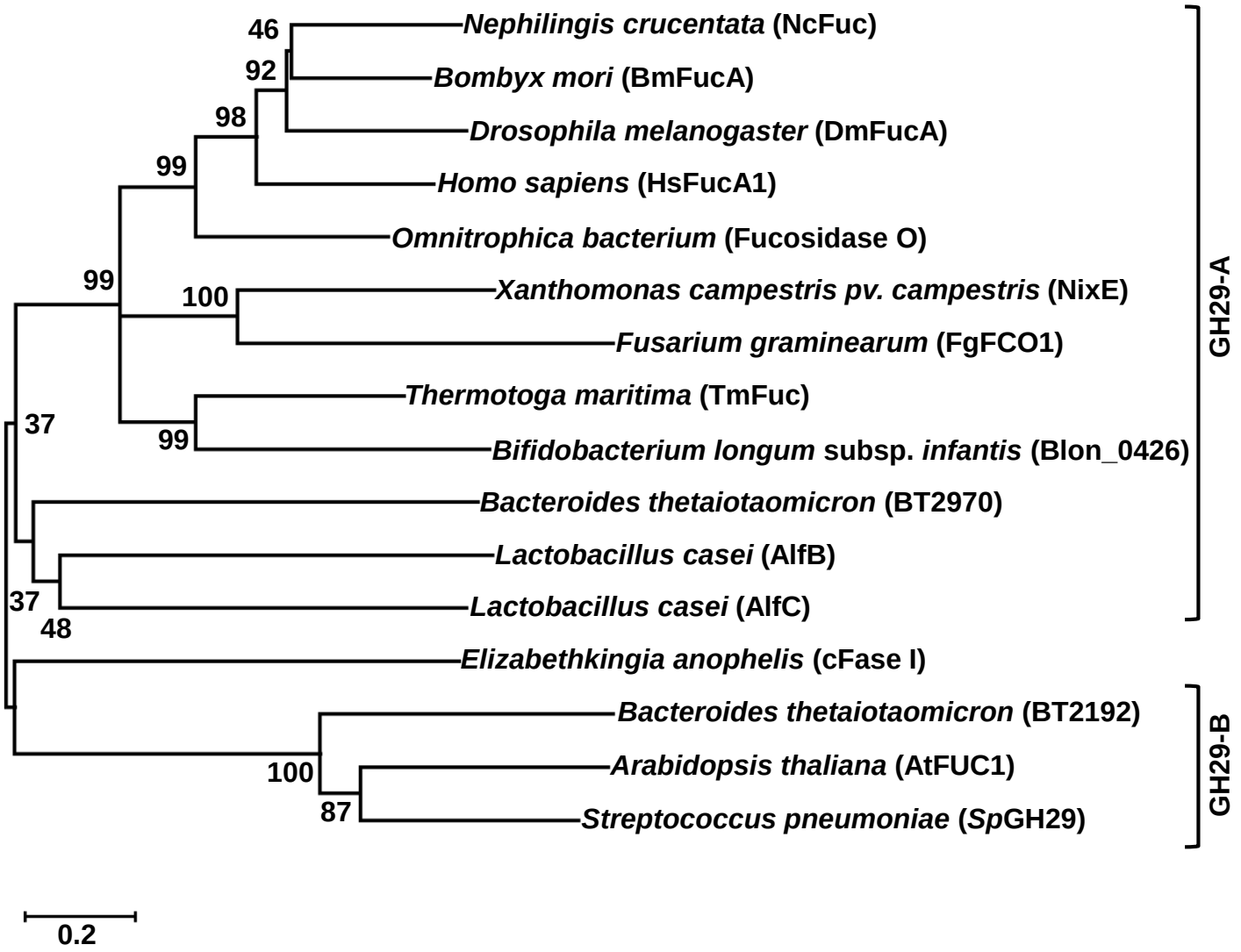
Species (Enzyme)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
Bombyx mori (BmFucA)	12.5±0.5	0.2±0.02	62.7
Nephilingis cruentata (NcFuc) [a]	11±1	0.4±0.01	27.5
Homo sapiens (HsFucA1) [b]	17.1±0.3	0.28±0.02	61.1
Thermotoga maritima (TmFuc) [c]	14.3±0.3	0.05±0.003	280
Elizabethkingia meningoseptica (cFase I) [d]	8.4±0.2	0.6±0.05	13.9

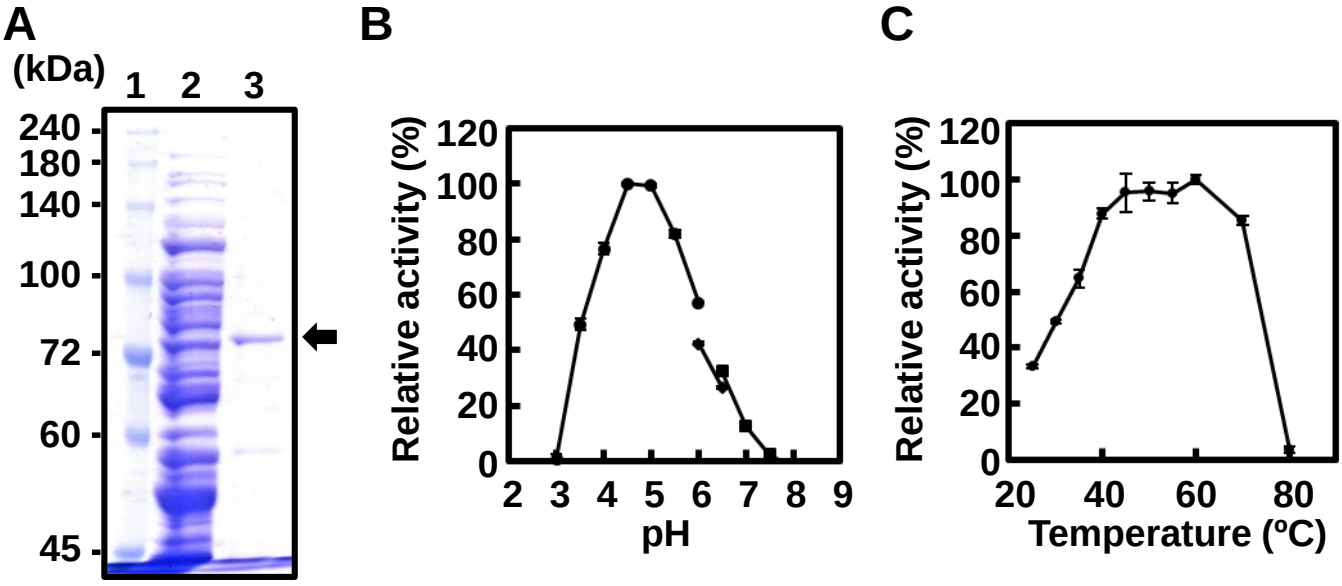
[a]Perrella et al. (2018). [b]Liu et al. (2009). [c]Sulzenbacher et al. (2004). [d] Li et al., (2018).

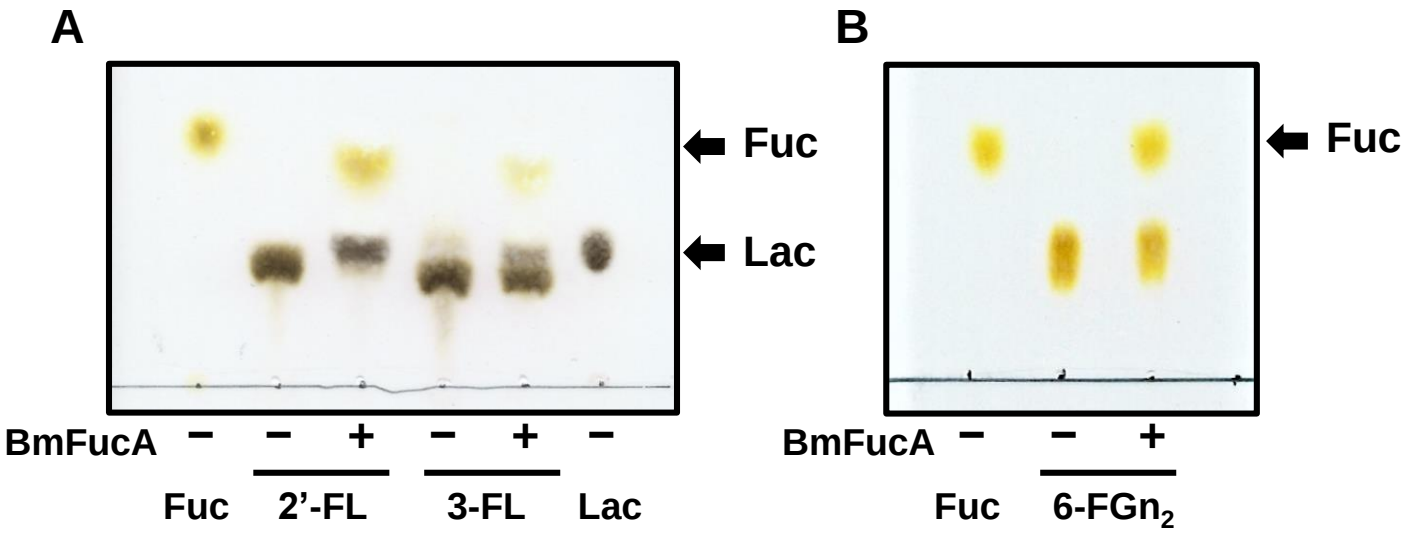
Table 2. Hydrolytic activity of GST-BmFucA and other α-L-fucosidases for fucosylated substrates.

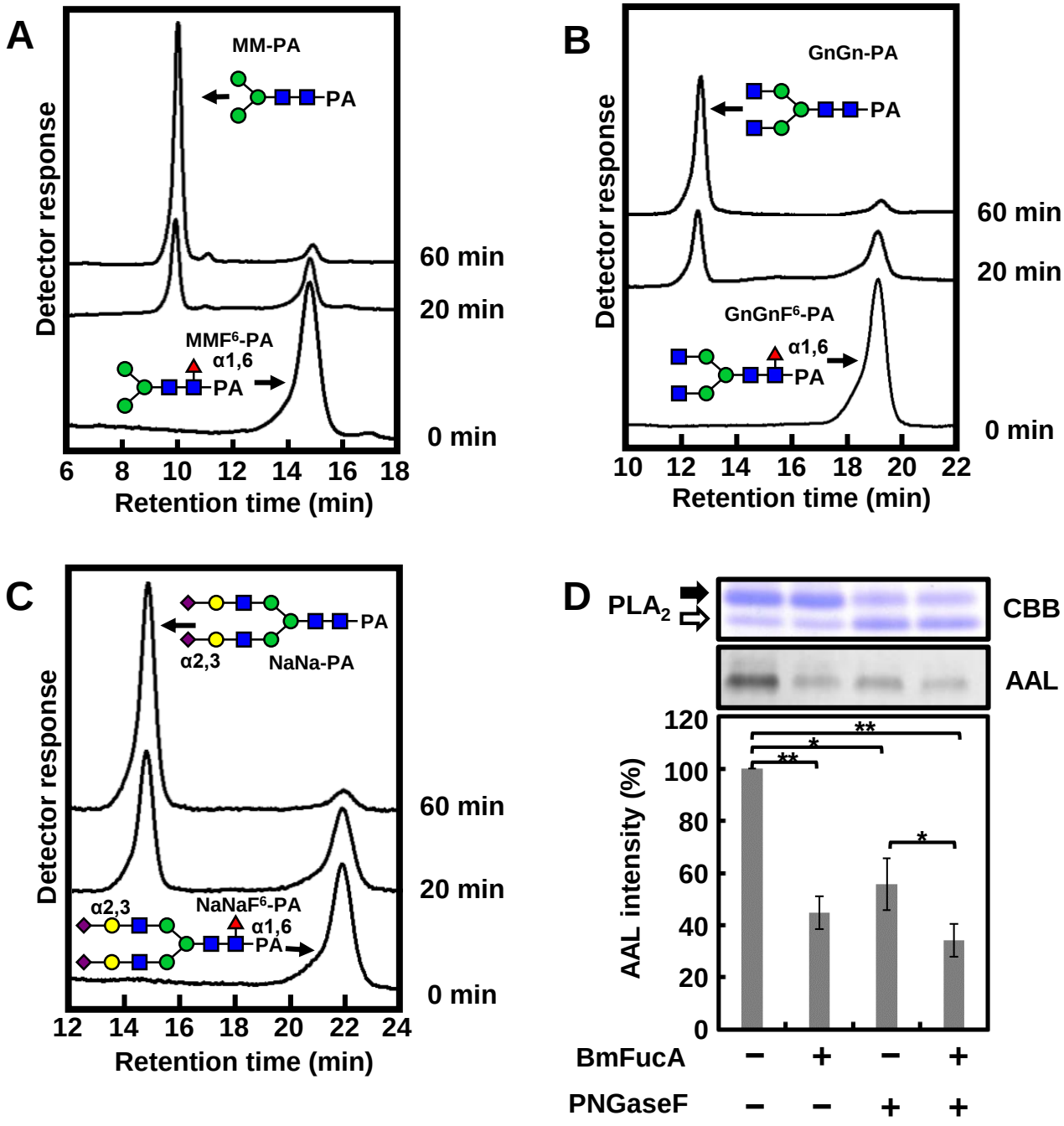
Substrate	Relative activity (%)									
	GH29-A							GH29-B		GH95
	BmFucA	HsFucA1[a]	Blon_0426[b]	AlfB[c]	AlfC[c]	BT_2970[d]	cFaseI[e]	BT_2192[d]	*Sp*GH29[f]	AfcA[g]
pNP-α-L-Fuc	100	–	100	100	100	100	7.53	0.025	–	–
4MUαFuc	–	100	–	–	–	–	–	–	–	–
2'-FL	10.5	28.8	0.56	1.2	N.D.	0	N.D.	0.067	N.D.	100
3-FL	2.2	1	–	N.D.	N.D.	0	100	100	+	<1
6-FGn_2	7.9	–	–	–	–	–	N.D.	–	–	N.D.
Fucα1-6GlcNAc	–	6.0	37.8	N.D.	61.3	0	N.D.	0	N.D.	–
3-FGn_2	1.2	–	–	–	–	–	–	–	–	–
Fucα1-3GlcNAc	–	–	–	458.3	0.0018	5.3	–	0	N.D.	–

[a]Dawaon and Tsay. (1977). [b]Ashida et al., (2020). [c]Rodríguez-Díaz et al. (2011). [d]Sakurama et al. (2012). [e]Li et al. (2018). [f]Ashida et al. (2009). [g]Katayama et al. (2004). +, detected on TLC; –, not tested; N.D., not detected. The highest activity was defined as 100% for each enzyme.


46
92
98
99
99
100
37
99
37
48
Nephilingis crucentata (NcFuc)
Bombyx mori (BmFucA)
Drosophila melanogaster (DmFucA)
Homo sapiens (HsFucA1)
Omnitrophica bacterium (Fucosidase O)
Xanthomonas campestris pv. campestris (NixE)
Fusarium graminearum (FgFCO1)
Thermotoga maritima (TmFuc)
Bifidobacterium longum subsp. infantis (Blon_0426)
Bacteroides thetaiotaomicron (BT2970)
Lactobacillus casei (AlfB)
Lactobacillus casei (AlfC)
Elizabethkingia anophelis (cFase I)
Bacteroides thetaiotaomicron (BT2192)
Arabidopsis thaliana (AtFUC1)
Streptococcus pneumoniae (SpGH29)
GH29-A
GH29-B
100
87
0.2


A
(kDa)
1
2
3
240
180
140
100
72
60
45
B
Relative activity (%)
120
100
80
60
40
20
0
2
3
4
5
6
7
8
9
pH
C
Relative activity (%)
120
100
80
60
40
20
0
20
40
60
80
Temperature (ºC)


A
Fuc
Lac
BmFucA − − + − + −
Fuc
2'-FL
3-FL
Lac
B
Fuc
BmFucA − − +
Fuc
6-FGn2


A
MM-PA
PA
Detector response
60 min
20 min
MMF6-PA
α1,6
PA
0 min
6 8 10 12 14 16 18
Retention time (min)
B
GnGn-PA
PA
Detector response
60 min
20 min
GnGnF6-PA
α1,6
PA
0 min
10 12 14 16 18 20 22
Retention time (min)
C
PA
α2,3
NaNa-PA
Detector response
60 min
20 min
α2,3
NaNaF6-PA
α1,6
PA
0 min
12 14 16 18 20 22 24
Retention time (min)
D
PLA2
CBB
AAL
AAL intensity (%)
120
100
80
60
40
20
0
**
*
**
*
BmFucA − + − +
PNGaseF − − + +