Supporting Information

Prospecting for α-N-acetylgalactosaminidases yields a new class of GH31 O-glycanase

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Table S1. Accession numbers of proteins characterized in this study.

Name	GH family	Database hit	Accession #	Hit #	Organism
*Bc*GH31	GH31	DUF5110 domain-containing protein	WP_005682123.1	A2	*Bacteroides caccae*
*Bp*GH31	GH31	DUF5110 domain-containing protein	WP_007559952.1	O1	*Bacteroides plebeius*
*Bv*GH109_1	GH109	gfo/Idh/MocA family oxidoreductase	WP_005848863.1	A1	*Bacteroides vulgatus*
*Cp*GH31	GH31	DUF4968 domain-containing protein	WP_011590658.1		*Clostridium perfringens*

Table S2. Kinetic parameters for the hydrolysis of GalNAc-α-MU by truncated and full-length BcGH31, BpGH31 and BvGH109_1. Reactions were carried out in 50 mM HEPES pH 7.0 at 37°C and observed via fluorescence (ex 360 nm/ em 450 nm). Kinetic parameters were obtained by fitting initial velocities obtained from three replicate assays to the Michalis-Menten equation using GraFit. Values indicate averages ± standard error (n=3).

Enzyme	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} mM^{-1})
t*Bc* GH31	2.08 ± 0.03	236 ± 8	8.8 ± 0.3
***Bc* GH31**	1.09 ± 0.01	154 ± 5	7.08 ± 0.2
t*Bp* GH31	4.4 ± 0.2	160 ± 20	27 ± 3
***Bp* GH31**	1.42 ± 0.02	144 ± 6	9.9 ± 0.4
***Bv* GH109_1**	1.25 ± 0.04	1.1 ± 0.2	1100 ± 200

Table S3. Primers used in this study.

Primer Name	Sequence	Purpose
tBcGH31woSig_Eco31I_fwd	ATGGTCTC G CCAT GTC ATT GTC GAT ACT GGT AGA AAT G	Cloning of tBcGH31 into pET28a
tBcGH31_Eco31I_fwd	ATGGTCTC G ATTC TTA CTC GAG GGG ATC GGA CTT GG	
tBpGH31woSig_Eco31I_fwd	ATGGTCTC G CCAT GCA CAG CAG ACT GAG ATA ACT ATC	Cloning of tBpGH31 into pET28a
tBpGH31_Eco31I_fwd	ATGGTCTC G ATTC TTA TTC CAA CGG ATC TGA TTT AGT AGT AGC	
pET28_Eco31I_fwd	ATGGTCTC C GAAT TCGA GCT CCG TCG AC	Amplification of pET28a for insertion of tBcGH31 and tBpGH31
pET28_Eco31I_rev	ATGGTCTC G ATGG CT GCC GCG CGG CAC	
Bc_c_term_PIPE_fwd	AAGTCCGATCCCCTCGA ATTTGCTATCCGTGGTATCAGG	Attachment of synthesised C-terminus of BcGH31
Bc_c_term_PIPE_rev	TA GGTCTC G TGAT AT TTACTGAACACCCGATCTACG	
Bp_c_term_PIPE_fwd	CTAGATCCGTTGGAA TTCGCCATTCATGGAAT	Attachment of synthesised C-terminus of BpGH31
Bp_c_term_PIPE_rev	TA GGTCTC G TGAT AT TCAAAACTTCACTGTATTCAGATT	
Bc_N_term_PIPE_rev	TACCACGGATAGCAAAT TCGAGGGGATCGGACTTGGTC	Amplification of pET28A tBcGH31 for attachment of C-terminus
Bp_N_term_PIPE_rev	ATTCCATGAATGGCGAA TTCCAACGGATCTGATTTAGTAGTAGC	Amplification of pET28A tBpGH31 for attachment of C-terminus
Bcp_Nterm_PIPE_fwd	AT ATCA CGAGACCTA TCGA GCT CCG TCG ACAAGC	Amplification of pET28a tBc/tBpGH31 and attachment of C-termini of BcGH31 and BpGH31
BpGH31_D465A_fwd	C TGG TCG GGC GCC CAG ACT GGA GGT GAA	For site-directed mutagenesis of putative catalytic acid/base of BpGH31
BpGH31_D465G_fwd	C TGG TCG GGC GGC CAG ACT GGA GGT GAA	
BpGH31_D465S_fwd	C TGG TCG GGC TCC CAG ACT GGA GGT GAA	
BpGH31_D465A_rev	C TCC AGT CTG GGC GCC CGA CCA GAT ACC	
BpGH31_D465G_rev	C TCC AGT CTG GCC GCC CGA CCA GAT ACC	
BpGH31_D465S_rev	C TCC AGT CTG GGA GCC CGA CCA GAT ACC	
BpGH31_D413A_fwd	G TTG AAA ACC GCC GTA GCC TGG GTA GGA G	For site-directed mutagenesis of putative catalytic nucleophile of BpGH31
BpGH31_D413A_rev	C CCA GGC TAC GGC GGT TTT CAA CAC ACG	
BpGH31_D413G_fwd	G TTG AAA ACC GGC GTA GCC TGG GTA GGA G	
BpGH31_D413G_rev	C CCA GGC TAC GCC GGT TTT CAA CAC ACG	
BpGH31_D413S_fwd	G TTG AAA ACC AGC GTA GCC TGG GTA GGA G	
BpGH31_D413S_rev	C CCA GGC TAC GCT GGT TTT CAA CAC ACG	
CpGH31_NoSignalPeptide_33_fwd	CCGCGCGGCAGCCAT TTTCCAACAGAAGGGATAAA	Cloning of CpGH31 33-933 from C. perfringens DSM 798 genomic DNA into pET28a
CpGH31_NoCBM_933_rev	GTCGACGGAGCTCGA TCTTACACGATAACTATATTC	
pET28A_CpGH31_PIPE_fwd	TAGTTATCGTGTAAGA TCGAGCTCCGTCGACAAGCT	Amplification of pET28a for insertion of CpGH31
pET28A_CpGH31_PIPE_rev	CCCTTCTGTTGGAAA ATGGCTGCCGCGCGGCACCA	
BvGH109_NdeI_F_27aaNtermDel	GAG CTC GAG TTA TTT TGC TTC TTT AGC CCA TTC TTT CGC	Cloning of BvGH109_1 into pET16b
BvGh109_OEPCR_XhoI_R	GAG CTC GAG TTT TGC TTC TTT AGC CCA TTC TTT CGC	

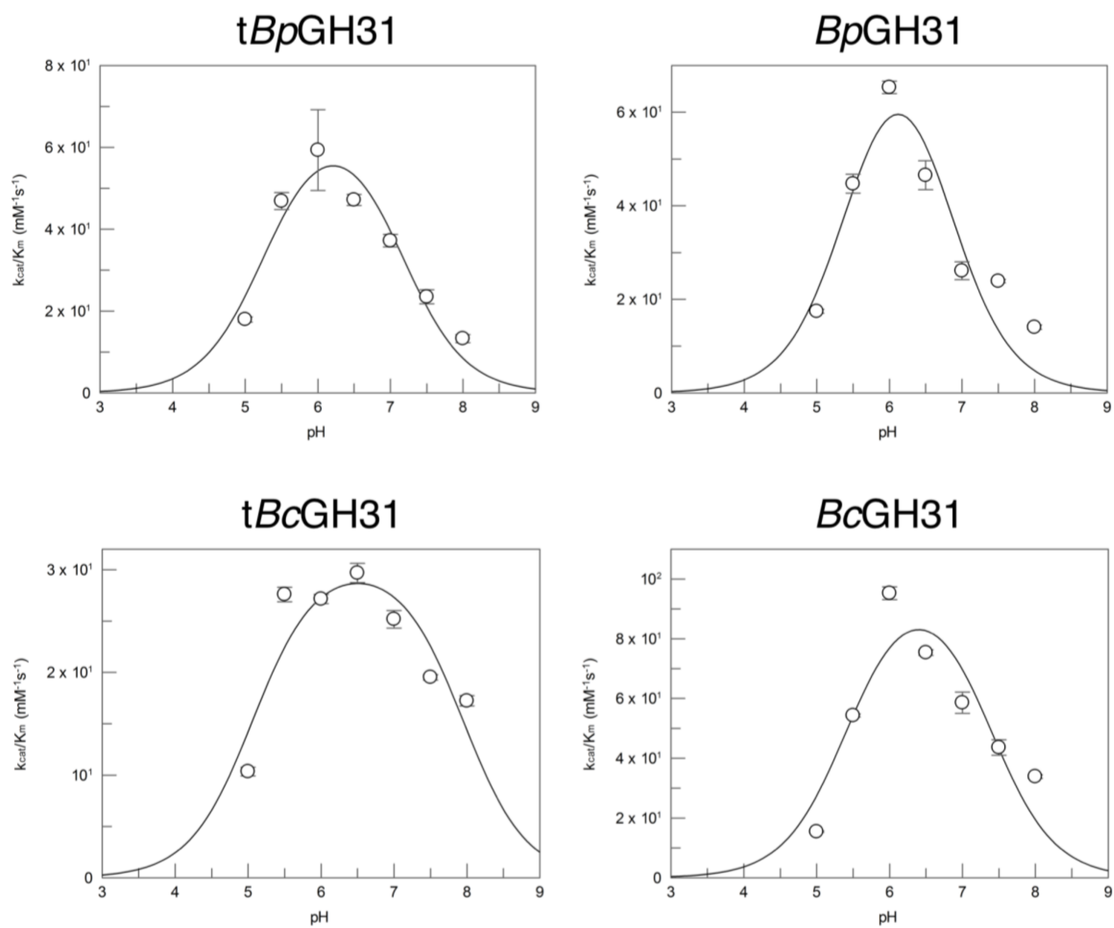
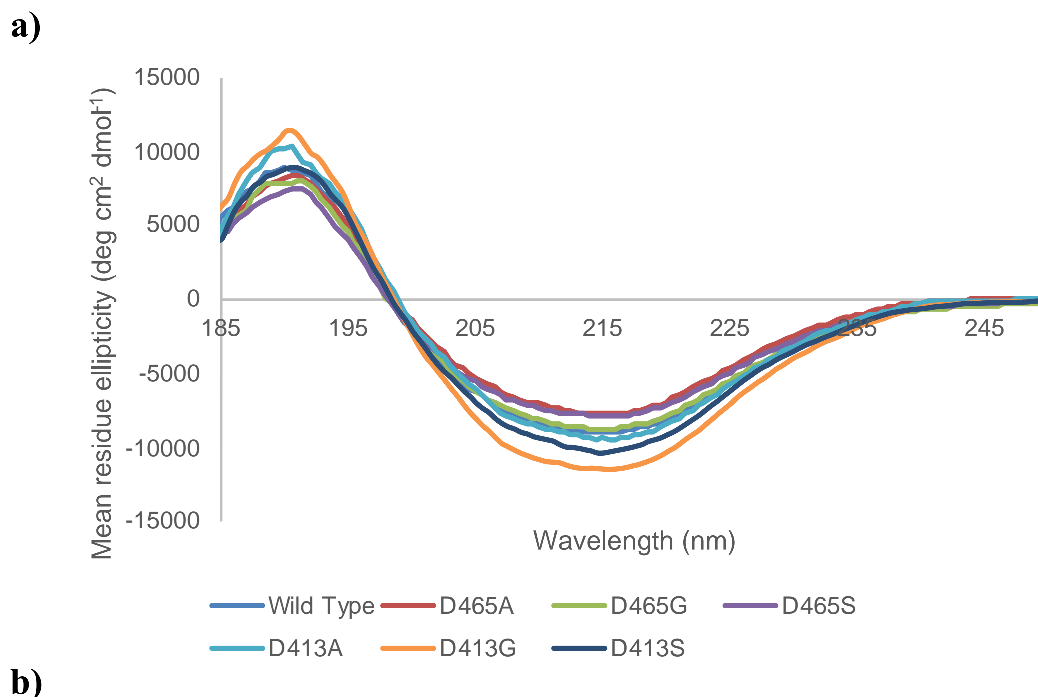


Figure S1. pH activity profiles of GH31 enzymes. Michaelis-Menten kinetic parameters (k_{cat}/K_M) values were extracted substrate depletion assays using GraFit 7.0 and fit to an expression for the pH dependence of an enzyme with two essential ionizations using GraFit 7.0. All enzymes tested had similar ionizations with pK_a values at approximately 5.3 and 7.1, with the exception being the slightly broader profile of t*Bc*GH31 with pK_a values of 5.0 and 7.9. Error bars indicate standard error (n=3-6).

a)



b)

Enzyme	%Helix	%Beta	%Turn	%Random	Total
t*Bp* GH31 WT	25.9	27.4	12.5	32.1	97.9
t*Bp* GH31 D413A	19.7	31	12.5	31.6	94.8
t*Bp* GH31 D413G	17.4	35.3	12.5	32.9	98.1
t*Bp* GH31 D413S	19.5	30.2	12.5	33	95.2
t*Bp* GH31 D465A	28.5	23.8	12.5	31.3	96.1
t*Bp* GH31 D465G	25.5	23.7	12.5	33.5	95.2
t*Bp* GH31 D465S	23.8	28.9	12.5	32.7	97.9

Figure S2. t*Bp*GH31 catalytic mutants have similar secondary structure as determined by far-UV CD-spectroscopy. a) Far-UV CD-spectroscopy of the t*Bp*GH31 catalytic mutants. Each curve represents a total of 20 accumulations of CD-spectra of 0.075 mg/mL mutant or wild-type t*Bp*GH31 in 5 mM phosphate pH 7 after smoothing. **b**) Data from far-UV CD experiments were analyzed using the method described by Raussens *et al.* (1) through the available web server.

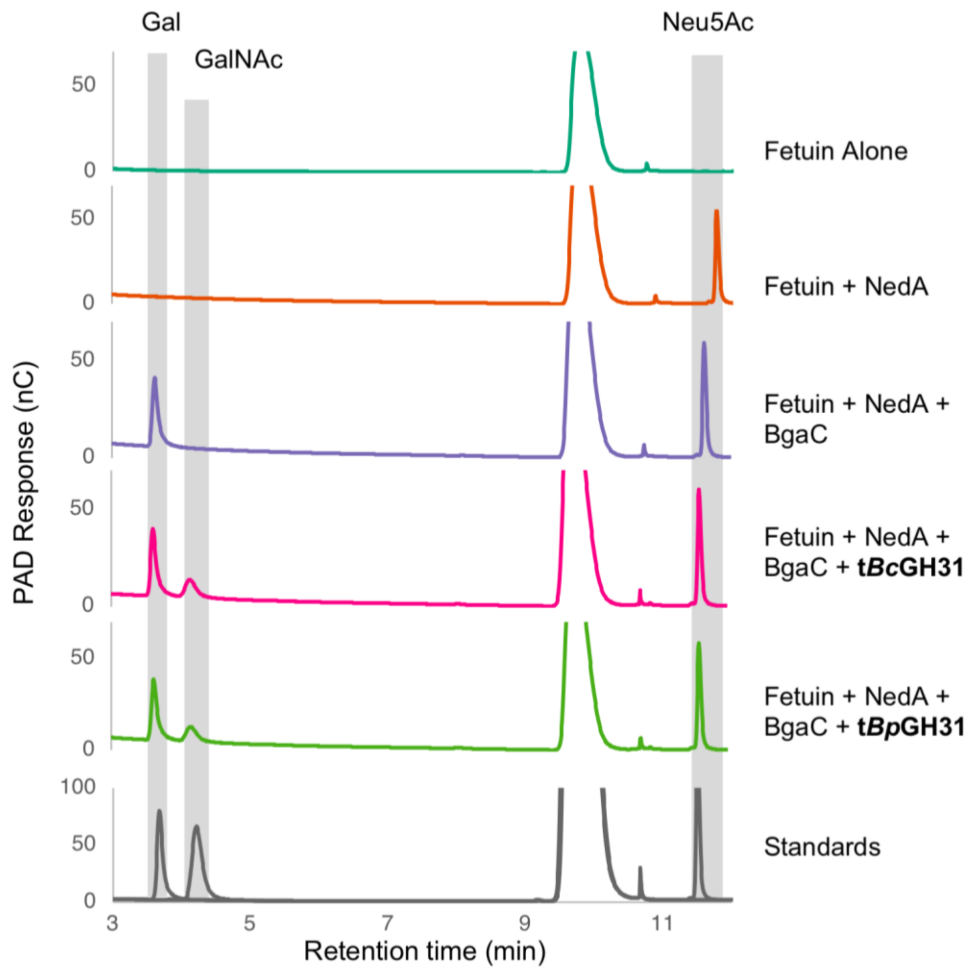


Figure S3. Truncated α-GalNAcase GH31 enzymes show exo-cleavage of O-glycans from fetuin. Native fetuin was subjected to overnight enzymatic cleavage of glycans by sequential activity of a neuraminidase (NedA), β-1,3-galactosidase (BgaC), and exo-α-GalNAcases (t*Bc*GH31 or t*Bp*GH31). Cleavage products were then separated and analyzed via HPAEC-PAD. Sugars were identified based on buffer (20 mM HEPES pH 7, 150 mM NaCl) spiked with free Neu5Ac, Gal and GalNAc. Some variations in the elution time accrued during the HPAEC-PAD runs, and so the identity of the released sugars have been assigned with allowance for these variations.

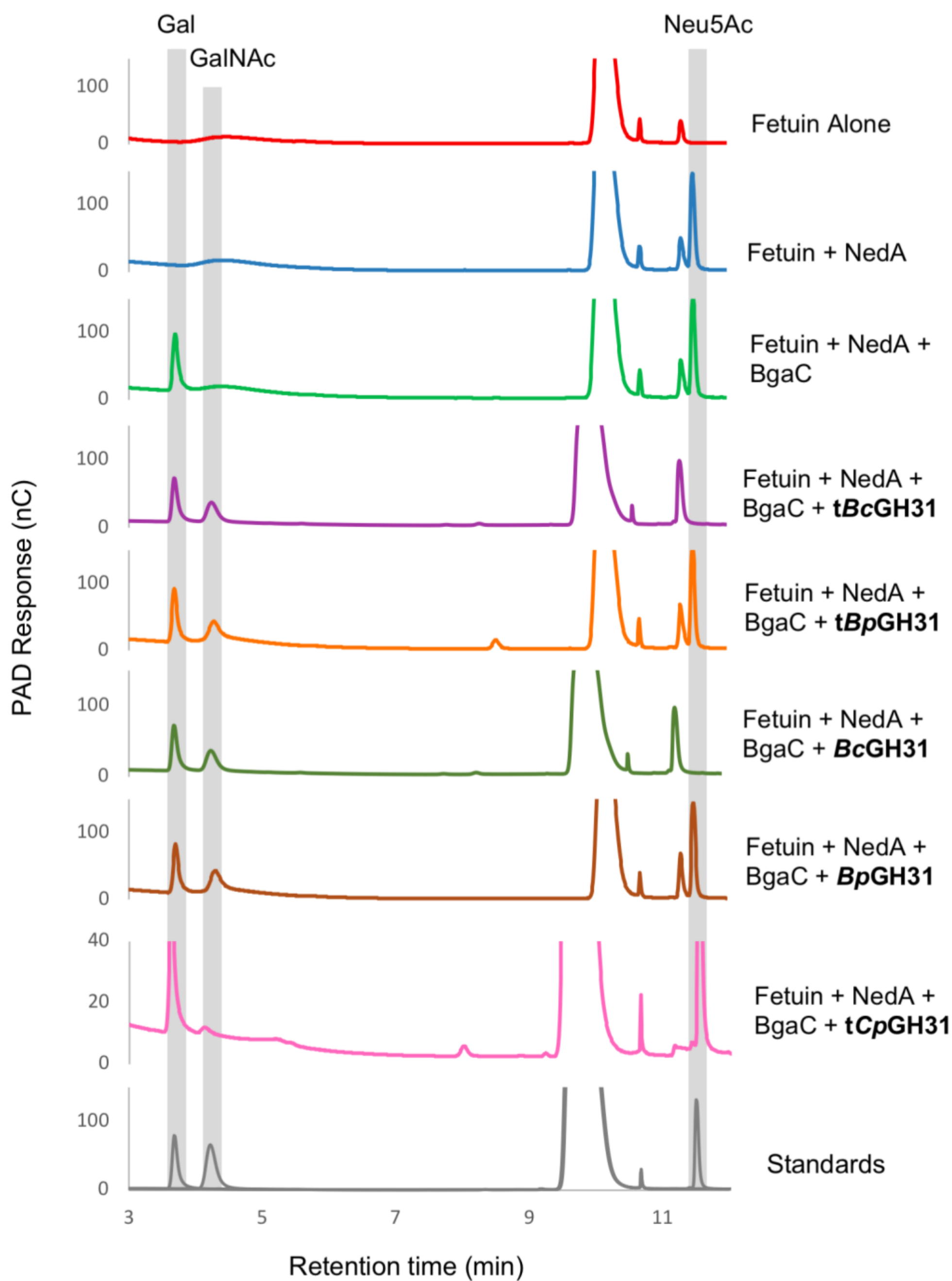


Figure S4. Exo-α-GalNAcase GH31s are active on denatured fetuin. Fetuin was denatured as described in the text and then subjected to overnight enzymatic cleavage of glycans by sequential activity of a neuraminidase (NedA), β-galactosidase (BgaC), and truncated and full-length exo-α-GalNAcase *Bp*GH31 and *Bc*GH31, as well as the GH domain of *Cp*GH31 (t*Cp*GH31). Cleavage products were then separated and analyzed via HPAEC-PAD. All tested GH31s were able to cleave GalNAc from denatured fetuin. Sugars were identified based on buffer spiked with Neu5Ac, Gal and GalNAc.

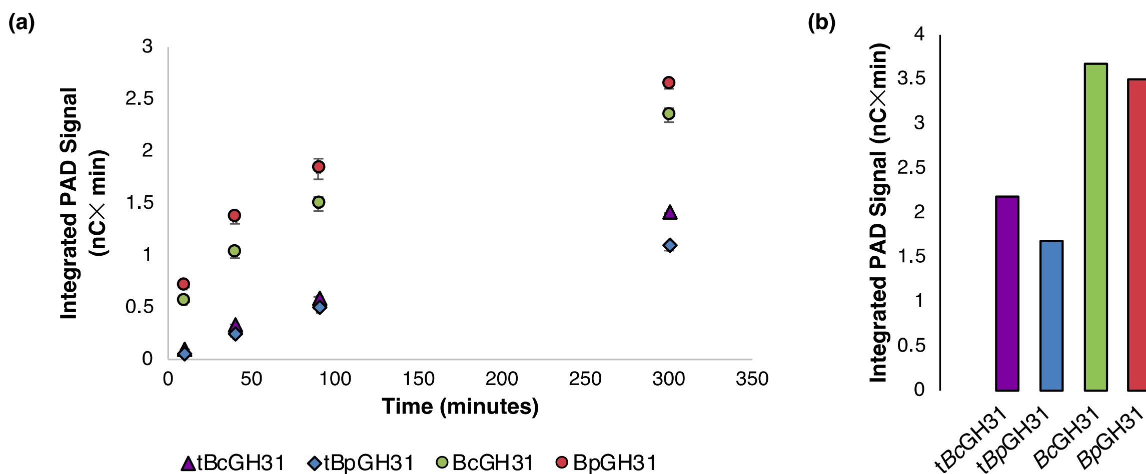


Figure S5. Time-dependent cleavage of α-GalNAc from fetuin. (a) Fetuin pre-treated with NedA and BgaC was treated with equimolar amounts of truncated and full-length *Bc*GH31 and *Bp*GH31. At different time points, cleavage products were then separated and analyzed via HPAEC-PAD. Error bars indicate standard error (n=2). (b) A similar procedure was carried out as in Panel A, however native fetuin was used and measurements were only conducted after four hours.

References:

1. Raussens, V., Ruysschaert, J.-M., and Goormaghtigh, E. (2003) Protein concentration is not an absolute prerequisite for the determination of secondary structure from circular dichroism spectra: a new scaling method. *Anal. Biochem.* **319**, 114–121