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Engineering *Bifidobacterium longum* Endo-α-N-acetylβ-galactosaminidase for Neu5Acα2-3Galβ1-3GalNAc reactivity on Fetuin

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**Abstract**

Endo-α-N-acetylβ-galactosaminidase from *Bifidobacterium longum* (EngBF) belongs to the glycoside hydrolase family GH101 and has a strict preference towards the mucin type glycan, Galβ1-3GalNAc, which is O-linked to serine or threonine residues on glycopeptides and -proteins. While other enzymes of the GH101 family exhibit a wider substrate spectrum, no GH101 member has until recently been reported to process the α2-3 sialidated mucin glycan, Neu5Acα2-3Galβ1-3GalNAc. However, work published by others (ACS Chem Biol 2021, 16, 2004–2015) during the preparation of the present manuscript demonstrated that the enzymes from several bacteria are able to hydrolyze this glycan from the fluorophore, methylumbelliferyl. Based on molecular docking using the EngBF homolog, EngSP from Streptococcus pneumoniae, substitution of active site amino acid residues with the potential to allow for accommodation of Neu5Acα2-3Galβ1-3GalNAc were identified. Based on this analysis, the mutant EngBF variants W750A, Q894A, K1199A, E1294A and D1295A were prepared and tested, for activity towards the Neu5Acα2-3Galβ1-3GalNAc O-linked glycan present on bovine fetuin. Among the mutant EngBF variants listed above, only E1294A was shown to release Neu5Acα2-3Galβ1-3GalNAc from fetuin, which subsequently was also demonstrated for the substitutions: E1294 M, E1294H and E1294K. In addition, the *kcat/ Km* of the EngBF variants for cleavage of the Neu5Acα2-3Galβ1-3GalNAc glycan increased between 5 and 70 times from pH 4.5 to pH 6.0.

**1. Introduction**

Endo-α-N-acetylβ-galactosaminidase from *Bifidobacterium longum* (EngBF), has been shown to be quite specific for the O-linked Galβ1-3GalNAc glycan [1–3] (Fig. 1A) as have other members of the CAZy GH101 [4] family. However, to a varying extent some enzyme family members do release alternative glycans including GalNAc, Galβ1-3(GlcNAcβ1-6)GalNAc, GlcNAcβ1-3GalNAc from para-nitrophenol in synthetic substrates [1–5]. The main species of O-glycosylation on bovine fetuin is the sialylated glycan, Neu5Acα2-3Galβ1-3GalNAc (Fig. 1B), linked to serine or threonine residues [5]. Until recently, no GH101 family member has been shown to release glycans from fetuin without prior sialidase treatment to convert these to Galβ1-3GalNAc [1–3]. However, during preparation for the present manuscript, activity towards the extended glycan, Neu5Acα2-3Galβ1-3GalNAc, was demonstrated for four endo-α-N-acetylβ-galactosaminidases: EngSP (Streptococcus pneumoniae), EngCP (Clostridium perfringens), EngSN (Streptomyces natalensis) and EngCS (Collinsella stercoris) utilizing a

**Abbreviations:** PmST1, *Pasteurella multocida* strain P-1059 Sialyltransferase; NanI, NanI sialidase from *Clostridium perfringens*; Asialo-fetuin, fetuin treated with NanI; DMAB, para-dimethylaminobenzaldehyde; EngBF, EngSP; EngCP and EngSN and EngCS, endo-α-N-acetylβ-galactosaminidase from *Bifidobacterium longum; Streptococcus pneumoniae*, *Clostridium perfringens*; *Streptomyces natalensis* and *Collinsella stercoris*, respectively; Gal, galactose; GalNAc, N-acetylgalactosamine; Neu5Ac, N-acetylneuraminic acid; GlcNAc, N-acetylglucosamine; TLC, thin-layer chromatography; HPEC-PAD, High-performance Anion Exchange Chromatography coupled to Pulsed Amperometric Detection.

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In addition, EngSN and EngSP wild-type enzyme and the EngSP variants, Q868T and Q868G, were also able to process fetuin with the EngSP variants showing up to four-fold increased release of the Neu5Ac ligand. In an analogy for O-glycans and their release from glycoproteins and -peptides, endo-β-2-3GalNAc. A) Galβ1-3GalNAc. B) Neu5AcO2-3Galβ1-3GalNAc. C. PmST1 was then removed by filtering as described previously [15]. The ligand Neu5Acβ1-2-3GalNAc was built using the carbohydrate builder at Glycam.org [22] and prepared for docking in the Maestro LigPrep wizard with charges calculated for pH 6. Ligand docking used the Glide SP algorithm [19,20] with flexible ligand sampling, in which hydrogen bond formation is rewarded. The initial output was set to a maximum of 10,000 poses, which were subsequently energy minimized in a full force-field to optimize bond length, angles and torsional angles before scoring of the poses using the modified Glide Energy score [18,19].

Surface electrostatic potentials were calculated using the Adaptive Poisson-Boltzmann Solver package [23] in PyMol (Shrödinger Inc.) and displayed using the electrostatic potential from ±5 kT/e. Analysis of enzymatically released saccharides. The identity of the glycan released from fetuin by the EngBF variants was analyzed and established in two ways. The first method using HPEC-PAD was performed as follows, 1 mL of 10 mg/mL fetuin was incubated with 1.5 μM of the E1294K variant at 37 °C for 72 h in 50 mM Acetic acid, 100 mM NaCl, pH 6.0. After incubation, the released saccharides were isolated from enzyme and fetuin, by filtering through an Amicon Ultra-0.5 mL 3 K MWCO centrifugal filter (Merck, Germany). The pH of 250 μL of the filtrate was adjusted to 5.0 by addition of HCl, and subsequently added 2 μL of 200 mM CMP and 36 μg of purified PmST1 sialytransferase and incubated for 2 h at 37 °C. PmST1 was then removed by filtering as above. The filtrate of fetuin reacted with E1294K and the sample additionally treated with PmST1 as well as the standard markers to be used for anion chromatography, were all diluted to 50 μM in 10 mM NaOH by the two-reaction site directed mutagenesis procedure [14] as described previously [15] using the following oligo-deoxyribonucleotides: EngBF E1294K Fw: CGGAAGGCTTAAAGGACAGCGGATCC.

EngBF E1294K Rv: GGTTCGAGGCGCTCTGCAGGCTTCGG.

EngBF E1294A Fw: GGTTCGAGGCGCTCTGCAGGCTTCGG.

EngBF E1294A Rv: GGTTCGAGGCGCTCTGCAGGCTTCGG.

EngBF E1294H Fw: GGTTCGAGGCGCTCTGCAGGCTTCGG.

EngBF E1294H Rv: GGTTCGAGGCGCTCTGCAGGCTTCGG.

EngBF E1294 M Fw: GGTTCGAGGCGCTCTGCAGGCTTCGG.

EngBF E1294 M Rv: GGTTCGAGGCGCTCTGCAGGCTTCGG.

The ligand Neu5Acβ1-2-3GalNAc was built using the carbohydrate builder at Glycam.org [22] and prepared for docking in the Maestro LigPrep wizard with charges calculated for pH 6. Ligand docking used the Glide SP algorithm [19,20] with flexible ligand sampling, in which hydrogen bond formation is rewarded. The initial output was set to a maximum of 10,000 poses, which were subsequently energy minimized in a full force-field to optimize bond length, angles and torsional angles before scoring of the poses using the modified Glide Energy score [18,19].

The RMSD values between the co-crystallized ligand, Galβ1-3GalNAc, and this moiety of the docked trisaccharide Neu5AcO2-3Galβ1-3GalNAc in the poses were calculated and plotted against the Glide Energy score. The poses were evaluated for lowest values of RMSD for the docked ligand compared to the co-crystallized disaccharide and the Glide Energy Score.

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EngBF E1294K Rv: GGTTCGAGGCGCTCTGCAGGCTTCGG.

EngBF E1294A Fw: GGTTCGAGGCGCTCTGCAGGCTTCGG.

EngBF E1294A Rv: GGTTCGAGGCGCTCTGCAGGCTTCGG.

EngBF E1294H Fw: GGTTCGAGGCGCTCTGCAGGCTTCGG.

EngBF E1294H Rv: GGTTCGAGGCGCTCTGCAGGCTTCGG.

EngBF E1294 M Fw: GGTTCGAGGCGCTCTGCAGGCTTCGG.

EngBF E1294 M Rv: GGTTCGAGGCGCTCTGCAGGCTTCGG.

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The RMSD values between the co-crystallized ligand, Galβ1-3GalNAc, and this moiety of the docked trisaccharide Neu5AcO2-3Galβ1-3GalNAc in the poses were calculated and plotted against the Glide Energy score. The poses were evaluated for lowest values of RMSD for the docked ligand compared to the co-crystallized disaccharide and the Glide Energy Score.

Surface electrostatic potentials were calculated using the Adaptive Poisson-Boltzmann Solver package [23] in PyMol (Shrödinger Inc.) and displayed using the electrostatic potential from ±5 kT/e. Analysis of enzymatically released saccharides. The identity of the glycan released from fetuin by the EngBF variants was analyzed and established in two ways. The first method using HPEC-PAD was performed as follows, 1 mL of 10 mg/mL fetuin was incubated with 1.5 μM of the E1294K variant at 37 °C for 72 h in 50 mM Acetic acid, 100 mM NaCl, pH 6.0. After incubation, the released saccharides were isolated from enzyme and fetuin, by filtering through an Amicon Ultra-0.5 mL 3 K MWCO centrifugal filter (Merck, Germany). The pH of 250 μL of the filtrate was adjusted to 5.0 by addition of HCl, and subsequently added 2 μL of 200 mM CMP and 36 μg of purified PmST1 sialytransferase and incubated for 2 h at 37 °C. PmST1 was then removed by filtering as above. The filtrate of fetuin reacted with E1294K and the sample additionally treated with PmST1 as well as the standard markers to be used for anion chromatography, were all diluted to 50 μM in 10 mM NaOH.
based on the concentration determined by the Morgan Elson colorimetric assay [24,25] for the glycans (see above) or the known stock concentrations for small-molecule markers. The samples and standards were then analyzed on a ICS-3000 (Dionex) chromatography system which was also used for visualization.

The second method using TLC of products from incubation of fetuin and asialo-fetuin with E1294K was performed as follows: Fetuin (10 mg/mL) was incubated with E1294K for 24 h at 37 °C. Upon completion, a fraction of this incubation was added NanI sialidase, prepared as described above, to a final concentration of 2.6 μM/mL and incubated for 24 h to remove the Neu5Ac moiety from the released fetuin glycans. The saccharides from samples of enzymatically treated fetuin were isolated by filtering through an Amicon Ultra-0.5 mL 3 K MWCO centrifugal filter (Merck, Germany). The filtrate was loaded on a Silica gel 60 (Merck, Germany) stationary phase by pipetting 5 times 1 mL and allowing the spot to dry between loadings. The chromatogram was developed in a closed chamber with 1-propanol:28% ammonia:water (15:1:6, v/v) mobile phase and subsequently dried before the migrated glycan moieties were visualized by submerging in a solution composed of 2 mL aniline, 10 mL phosphoric acid, 2 g diphenylamine, and acetic acid to a final volume of 100 mL, followed by heating at 100°C for 10–15 min.

**Steady-state kinetic analysis.** Fetuin substrate was prepared as follows: 50 mg/mL of fetuin dissolved in 50 mM sodium acetate, 250 mM NaCl, pH 6.0 was dialyzed against the same buffer and centrifuged to recover the soluble fraction resulting in a solution with a concentration ranging from 30 to 35 mg/mL. For preparation of asialo-fetuin, the concentration of 2.6 μM/mL fetuin solution from above was added NanI sialidase to a final concentration of 2.6 μg/mL, and incubated for 24 h at 37 °C, after which no further increase in Galβ1-3GalNAc release upon treatment with EngBF was detected. Subsequently, the asialo-fetuin preparation was dialyzed against several changes of 50 mM sodium acetate, 50 mM NaCl, pH 4.5.

Assays of glycan hydrolysis on fetuin and asialo-fetuin based on quantification of released Neu5Acα2-3Galβ1-3GalNAc or Galβ1-3GalNAc using the Morgan-Elson colorimetric reaction [24,25] were performed as described previously [15], except for changing the incubation temperature to 30 °C. Examples of data sets including progress-curves used in calculations of initial rates and glycan concentrations for the processing of fetuin and asialo-fetuin by the EngBF variant E1294K could be found in Supplementary Materials. Depending on the kinetic parameters of EngBF or the studied variants the concentration of fetuin or asialo-fetuin was varied up to 30 mg/mL, corresponding to approximately 1.8 mM cleavable Galβ1-3GalNAc or Neu5Acα2-3Galβ1-3GalNAc in the assay incubation. The exact concentration range used for wild-type EngBF and its variants are listed in Tables 1 and 2. As stated in results, assays were performed either at pH 4.5 (50 mM sodium acetate, 50 mM NaCl) or pH 6.0 (100 mM sodium acetate, 50 mM NaCl). Assay time and enzyme concentrations were adjusted accordingly to obtain full progress-curves as described previously [15]. Fits to obtain initial velocities and substrate concentration from progress-curves, as well as steady-state kinetic parameters and their reported errors from the derived saturation curves, were performed as previously described [15].

### Table 1

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_M$ (μM)</th>
<th>$k_{cat}/K_M$ (mM$^{-1}$ s$^{-1}$)</th>
<th>Substrate range (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>111 ± 5</td>
<td>163 ± 21</td>
<td>679 ± 64</td>
<td>800–35</td>
</tr>
<tr>
<td>E1294K</td>
<td>108 ± 6</td>
<td>128 ± 23</td>
<td>846 ± 109</td>
<td>700–30</td>
</tr>
<tr>
<td>E1294A</td>
<td>118 ± 3</td>
<td>133 ± 11</td>
<td>883 ± 52</td>
<td>970–35</td>
</tr>
<tr>
<td>E1294 M</td>
<td>151 ± 7</td>
<td>218 ± 28</td>
<td>693 ± 63</td>
<td>870–35</td>
</tr>
<tr>
<td>E1294A</td>
<td>119 ± 3</td>
<td>178 ± 14</td>
<td>669 ± 36</td>
<td>910–35</td>
</tr>
<tr>
<td>E1294K:Q894A</td>
<td>119 ± 8</td>
<td>477 ± 70</td>
<td>250 ± 26</td>
<td>1400–30</td>
</tr>
<tr>
<td>E1294K:K1199A</td>
<td>109 ± 3</td>
<td>201 ± 16</td>
<td>539 ± 32</td>
<td>1130–30</td>
</tr>
</tbody>
</table>

$^a$ Experiments were performed as described in Experimental procedures.

$^b$ The concentration range of the substrate calculated as released GalNAc residues determined with the Morgan-Elson colorimetric assay and obtained from end points fitted to the progress curves of substrate turnover (see Supplementary Materials).

### Table 2

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>pH</th>
<th>$k_{cat}/K_M$ (mM$^{-1}$ s$^{-1}$)</th>
<th>Substrate range (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>4.5</td>
<td>NA$^b$</td>
<td></td>
</tr>
<tr>
<td>E1294K</td>
<td>4.5</td>
<td>0.095 ± 0.001</td>
<td>1120–160</td>
</tr>
<tr>
<td>E1294A</td>
<td>4.5</td>
<td>0.030 ± 0.001</td>
<td>1350–160</td>
</tr>
<tr>
<td>E1294 M</td>
<td>4.5</td>
<td>0.0176 ± 0.0008</td>
<td>850–110</td>
</tr>
<tr>
<td>E1294H</td>
<td>4.5</td>
<td>0.26 ± 0.01</td>
<td>710–150</td>
</tr>
<tr>
<td>E1294K:Q894A</td>
<td>4.5</td>
<td>0.144 ± 0.005</td>
<td>1240–140</td>
</tr>
<tr>
<td>E1294K:K1199A</td>
<td>4.5</td>
<td>0.031 ± 0.001</td>
<td>1200–200</td>
</tr>
<tr>
<td>Wild-type</td>
<td>6.0</td>
<td>NA$^d$</td>
<td></td>
</tr>
<tr>
<td>E1294K</td>
<td>6.0</td>
<td>1.14 ± 0.03</td>
<td>490–10</td>
</tr>
<tr>
<td>E1294A</td>
<td>6.0</td>
<td>1.47 ± 0.06</td>
<td>570–20</td>
</tr>
<tr>
<td>E1294 M</td>
<td>6.0</td>
<td>0.78 ± 0.06</td>
<td>470–60</td>
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<tr>
<td>E1294H</td>
<td>6.0</td>
<td>1.26 ± 0.09</td>
<td>370–20</td>
</tr>
<tr>
<td>E1294K:Q894A</td>
<td>6.0</td>
<td>4.4 ± 0.13</td>
<td>350–30</td>
</tr>
<tr>
<td>E1294K:K1199A</td>
<td>6.0</td>
<td>0.76 ± 0.04</td>
<td>350–20</td>
</tr>
</tbody>
</table>

$^a$ Experiments were performed as described in Experimental procedures.

$^b$ NA, no detectable activity.

$^c$ The concentration range of the substrate calculated as released GalNAc residues determined with the Morgan-Elson colorimetric assay and obtained from end points fitted to the progress curves of substrate turnover (see Supplementary Materials).

$^d$ $k_{cat}/K_M$ was derived as the slope from a linear fit of catalytic rate versus substrate concentration.

3. Results and discussion

**Molecular ligand docking in the endo-α-N-acetylgalactosaminidase active site.** To probe the accommodation of the Neu5Acα2-3Galβ1-3GalNAc glycan in the endo-α-N-acetylgalactosaminidase active site, molecular dockings with this ligand were performed using the software Glide as described in Experimental procedures. The high-resolution structure of the EngBF homolog, EngSP, in complex with Galβ1-3GalNAc (PDB ID: 5A59) was used as a template for the docking. For each docking experiment the RMSD obtained from comparing the docked Galβ1-3GalNAc moiety of the extended glycan to that of the co-crystallized disaccharide Galβ1-3GalNAc in the EngSP structure were plotted against the Glide energy score (Fig. 2A). Intriguingly, the extended glycan could readily be docked into the active site to position itself close to the subsites of the Galβ1-3GalNAc glycan. Analyzing the best scoring poses for the extended glycan, demonstrated a marked favorable decrease in the Glide energy score concomitant with lowered RMSD values. In the docked complexes, the galactose C6-hydroxyl in the extended glycan was either within hydrogen bonding
distance of the Asp-658 carboxylate, common to the crystal structure of the EngSP Galβ1-3GalNAc complex, or rotated towards the Asp-1254 carboxylate (Fig. 2B). Inspection of the best poses, judged by lowest values of Glide energy score and RMSD, showed that the Neu5Ac C1 carboxylate was either pointing towards the enzyme surface (Fig. 2C) or the solvent (Fig. 2D). Not surprisingly, the docking experiments with the Neu5Acα2-3Galβ1-3GalNAc ligand in the EngSP active site show that binding of this glycan is restricted by accommodation of the Neu5Ac moiety. And perhaps more important, the docking experiments indicate that the Neu5Ac residue exerts restrictions on correctly locating the galactose residue to its subsite as defined in the binding of the Galβ1-3GalNAc glycan. Thus, a simple metric for a rational substitution of side chains in the active site of the enzyme that facilitate docking of Neu5Acα2-3Galβ1-3GalNAc, seemed to be the aim for a further concomitant decrease in the Glide energy score and RMSD.

To provide some guidance for the engineering of the EngBF active site, we tested both wild-type enzyme and different EngBF variants with alanine substituted amino acid residues for the ability to cleave the Neu5Acα2-3Galβ1-3GalNAc glycan of fetuin. Most variants are previously described [7,15] and form part of the active site lid, W750A, or comprise the binding site for the galactose moiety, Q894A, K1199A and D1295A (Fig. 3A). To complement these, we also constructed the E1294A variant, as Glu-1294 is also potentially blocking an extended binding site for Neu5Acα2-3Galβ1-3GalNAc (Fig. 3A). Despite recent findings with endo-α-N-acetylgalactosaminidases from other sources [6], no activity on fetuin was detected for the wild-type EngBF or the variants W750A, Q894A, K1199A and D1295A under the reaction conditions and assay method used in the present work. Nevertheless, the side-chain substitutions at the positions corresponding to Gln-894 and Lys-1199 in EngBF were recently shown to improve the activity of EngSP.
Fig. 4. Molecular docking of Neu5Acα2-3Galβ1-3GalNAc in EngSP(TIGR4) (PDB ID: 5A59) and variants substituted at the position of Glu-1253 (Glu-1294 in EngBF). A) The Glide energy score plotted as a function of the RMSD calculated for the superimposition of the Galβ1-3GalNAc moiety of the docked glycan, Neu5Acα2-3Galβ1-3GalNAc, and that of Galβ1-3GalNAc co-crystallized with the EngSP enzyme. The inset shows all the data using an extended x-axis. The data points in the square of the main plot represents the poses with lowest RMSD and Glide energy score for the model EngSP E1253K variant (homologous to the E1294K variant of EngBF). B) Superimposition of the Galβ1-3GalNAc co-crystallized with the EngSP enzyme (dark grey) and the best poses (green) from dockings of Neu5Acα2-3Galβ1-3GalNAc into the model EngSP E1253K structure. Side chains interacting with the Neu5Ac moiety are displayed as sticks (dark grey). The side-chain numbering is according to EngSP and numbering for the corresponding residues in EngBF is shown in parentheses. The figure was created using PyMol (Shr¨odinger Inc.). C) The Glide energy score plotted as a function of the RMSD calculated for the superimposition of the Galβ1-3GalNAc moiety of the docked glycan, Neu5Acα2-3Galβ1-3GalNAc, and that of Galβ1-3GalNAc co-crystallized with the EngSP enzyme. The inset shows all the data using an extended x-axis. The main plot shows the poses with lowest RMSD and Glide energy score for the model EngSP E1253K variant and the double variants, E1253K:Q868A and E1253K:K1156A (homologous to the E1294K, E1294K:Q894A and E1294K:K1199 variants of EngBF). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
on fetuin up to four-fold [6]. EngBF is almost identical to EngSP in the region of the active site (Fig. 3B) suggesting that our assay is not sensitive enough to detect activity at the level reported for EngSP under the reaction conditions that we have chosen. However, the E1294A variant was found to release the Neu5Ac reaction conditions that we have chosen. However, the E1294A variant sensitive enough to detect activity at the level reported for EngSP under the... 

Fig. 5. Products from fetuin incubation with E1294K analyzed by HPEAC-PAD and TLC. A) HPEAC-PAD glycan analysis of E1294K treated fetuin (black line) before and (red line) after treatment with PmST1 sialyltransferase. Labeled peaks were identified from chromatography of standards. B) TLC of samples containing 1) asialo-fetuin, 2) and 3) fetuin and 4) Galβ1-3GalNAcα1-para-nitrophenol. The chromatogram shows glycan release after incubation in the (−) absence or (−) presence of E1294K. In (3), the E1294K treated sample from 2) was subsequently incubated with Nani sialidase to release Neu5Ac. The composition of the released glycans and carbohydrates are indicated above the chromatogram. Experiments were performed as described in Experimental procedures. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 6. Progress-curves and end-point determination of glycan product formation from processing of fetuin and asialo-fetuin. Data points are the mean of three determinations and error bars are visible where they extend beyond the data point symbol. A) Progress-curves from fetuin (10 mg/mL) incubated up to 24 h with a final concentration of (filled squares) 3.4 μM EngBF and (filled circles) 3.9 μM EngBF E1294K. B) Progress-curves from asialo-fetuin (10 mg/mL) incubated 90 min with a final concentration of (filled squares) 6.7 nM EngBF and (filled circles) 6.7 nM EngBF E1294K.

In docking experiments, we also tested the influence of substituting the side chains of Gln-868 and Lys-1156 in EngSP, corresponding to Gln-894 and Lys-1199 in EngBF, when combined with the E1253K substitution (E1294K in EngBF). The E1253K/Q868A variant showed poses of docking with Neu5Acα2-3Galβ1-3GalNAc having a markedly lower RMSD and Glide energy score compared to wild type enzyme and that of other side-chain substitutions at the homologous position, Glu-1253, in the EngSP structure used for the docking experiments. Here, the E1253K variant was found to produce docking poses with the Neu5Acα2-3Galβ1-3GalNAc glycans having a markedly lower RMSD and Glide energy score compared to wild type enzyme and that of other side-chain substitutions at this position (Fig. 4A). Dissection of the best poses from docking of the extended glycan in the E1253K variant (Fig. 4B) shows the hydroxyl groups of C5 and C6 of the galactose moiety to be within hydrogen bond distance of the Asp-658 and Asp-1254 carboxylates. Thus, the interactions of the wild-type enzyme and the Galβ1-3GalNAc ligand found in the crystal structure of the EngSP complex and those of this moiety of the Neu5Acα2-3Galβ1-3GalNAc glycans in dockings performed on the E1253K variant, are in better agreement than what is found with the latter glycan in the dockings with the wild-type EngSP enzyme. In addition, the positioning of the Neu5Ac moiety is identical for all the dockings in this group of best poses for the E1253K variant with a solvent exposed positioning of the Neu5Ac carboxylate (Fig. 4B).
We also performed a TLC analysis (Fig. 5B) of product from incubation of fetuin with E1294K. The application of standards and the treatment of the product with NanI sialidase indicates that the released glycan is Neu5Acα2-3Galβ1-3GalNAc.

Progress curves of fetuin processed by E1294K showed a maximal release of two glycans per fetuin molecule (Fig. 6A), compared to 2-2.5 glycans obtained with wild-type EngBF or E1294K upon incubation with asialo-fetuin (Fig. 6B). This stoichiometry of Neu5Acα2-3Galβ1-3GalNAc glycans, or Galβ1-3GalNAc after NanI sialidase treatment, per fetuin molecule is in agreement with an average of 2-2.5 O-glycosylations per fetuin molecule with the majority being the Neu5Acα2-3Galβ1-3GalNAc-Ser/Thr as previously determined [5].

Steady-state kinetic analysis of wild-type EngBF and variants using asialo-fetuin or fetuin as a substrate. The EngBF variants altered at position Glu-1294 were subjected to a steady-state kinetic analysis on asialo-fetuin to evaluate the impact of the substituted side chains with respect to the native activity of EngBF of processing Galβ1-3GalNAc O-glycosylations (Table 1). The steady-state kinetic parameters for all variants were close to those of wild-type EngBF (Table 1) and Glu-1294 appears not in any way critical for activity. In addition, this position seems functionally very tolerant to side-chain substitutions, both in terms of size and charge. With fetuin as a substrate, all the EngBF variants altered at Glu-1294 were active, but showed a linear increase in activity with increasing fetuin concentrations and no sign of substrate saturation at up to 30 mg/mL fetuin. This only allowed for the determination of $k_{cat}$ for release of glycans by the EngBF variants active on both asialo-fetuin and fetuin, the values of $k_{cat}/K_M$ for Neu5Acα2-3Galβ1-3GalNAc (Table 2) indicate a $K_M$ for fetuin of the EngBF variants to be in the molar range at pH 4.5 and in the millimolar range at pH 6.0. Also, it may be that the greatly improved catalytic efficiency displayed by the variants at pH 6.0 compared to pH 4.5, points to a titration of the Neu5Acα2-3Galβ1-3GalNAc glycan resulting in a favorable uniform charge distribution at the higher pH. In the future, docking experiments with Neu5Acα2-3Galβ1-3GalNAc in the active site of EngBF, evaluated as here by superimposition of the glycan moiety and the native substrate, could be combined with e.g. possible neutralization of electrostatic repulsion (Fig. 7A and B). This approach may pave the way for engineering endo-α-N-acetylgalactosaminidase variants with much improved activity towards this extended and negatively charged glycan. As for now, the enzyme variants described here may serve as a tool for identification of O-glycosylations and for determination of stoichiometry of the Neu5Acα2-3Galβ1-3GalNAc glycan in glycoproteins, as suggested previously [6]. But perhaps more interestingly, is the further development of a variant with similar activity levels on Neu5Acrα2-3Galβ1-3GalNAc as for wild-type enzyme on Galβ1-3GalNAc, which may find its use in applications involving transglycosylation, which the wild-type endo-α-N-acetylgalactosaminidase performs readily [2,3,26]. Such EngBF variants could show to be important tools in the synthesis of O-linked glycoconjugates.

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Appendix A. Supplementary data

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References


