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In an effort to identify novel endo-\(\alpha\)-\(N\)-acetylgalactosaminidases (endo-\(\alpha\)-GalNAcases), four potential genes were cloned. Three of the expressed proteins EngEF from Enterococcus faecalis, EngPA from Propionibacterium acnes, and EngCP from Clostridium perfringens were purified and characterized. Their substrate specificity was investigated and compared to the commercially available endo-\(\alpha\)-GalNAcases from Streptococcus pneumoniae (EngSP) and Alcaligenes sp. (EngAL). All enzymes were incubated with various synthetic substrates, and natural glycoproteins and the released sugars were detected by colorimetric assay and thin layer chromatography analysis. The Core 1 disaccharide Gal\(\beta\)1,3GalNAc\(\alpha\)1pNP was the most rapidly hydrolyzed substrate by all enzymes tested. EngEF exhibited the highest \(k_{cat}\) for this substrate. EngEF and EngPA were also able to fully hydroyze the Core 3 disaccharide GlcN\(\beta\)1,3GalNAc\(\alpha\)1pNP. This is the first report of endo-\(\alpha\)-GalNAcases EngEF and EngPA acting on Core 3 in addition to Core 1 O-glycans. Interestingly, there were no significant differences in transglycosylation activities when Gal\(\beta\)1,3GalNAc\(\alpha\)1pNP or GlcN\(\beta\)1,3GalNAc\(\alpha\)1pNP was incubated with various 1-alkanols in the presence of the endo-\(\alpha\)-GalNAcases tested in this work.

Keywords: deglycosylation/endo-\(\alpha\)-\(N\)-acetylgalactosaminidases/\(\alpha\)-glycosylation

Introduction

Glycosylation is a common posttranslational modification of proteins. Glycans are implicated in a wide range of biological events such as cell–cell interactions and recognition, inflammation, and autoimmune diseases (Varki 1993; Ohtsubo and Marth 2006). Detailed knowledge of the glycan structure helps to correlate them to their respective function. To do so, tools are required for highly sensitive analysis of glycan chains. For structural analysis of asparagine-linked carbohydrates (N-linked glycans), sugars are released from the protein backbone by enzymes such as PNGase F (Tarentine and Plummer 1994). The O-linked glycans are most commonly attached to serine or threonine residues through the GalNAc residue at the reducing end. Presently, there is no enzymatic way of releasing of O-glycans intact. This is achieved by chemical methods, typically by \(\beta\)-elimination with mild alkali (Kakehi et al. 1994) or mild hydrazinolysis (Royle et al. 2002). These chemical methods are limited because after this treatment the protein is no longer functional.

Endo-\(\alpha\)-\(N\)-acetylgalactosaminidase (endo-\(\alpha\)-GalNAcase, EC 3.2.1.97) catalyzes the hydrolysis of an \(\alpha\)-glycosidic \(\alpha\)-linkage between galactosyl \(\beta\)1,3 \(N\)-acetyl-D-galactosamine (Gal\(\beta\)1,3GalNAc) and the serine or threonine residue in mucins and mucin-type glycoproteins from various animal sources. This \(O\)-linked disaccharide (Core 1 type \(O\)-glycan) is one of the most abundant core structures found in mucin glycoproteins. It is known as the Thomsen–Friedenreich antigen (T antigen) immunodeterminant group and is used as a specific marker of carcinoma (Varki 1993; Ohtsubo and Marth 2006).

Endo-\(\alpha\)-GalNAcases have been purified from Clostridium perfringens (Huang and Aminoff 1972), Streptococcus pneumoniae (Glasgow et al. 1977; Umemoto et al. 1977; Brooks and Savage 1997), Alcaligenes sp. (Fan et al. 1990), Bacillus sp. (Ashida et al. 2000), and Bifidobacterium longum (Fujita et al. 2005). All of these enzymes have a strict substrate specificity, acting only on the \(\alpha\)-linked disaccharide, Gal\(\beta\)1,3GalNAc. An enzyme isolated from Streptomyces sp., has been reported to release longer sugar chains than the disaccharide from porcine mucin (Ishii-Karakasa et al. 1992, 1997) although further studies are needed to confirm this activity. These endo-\(\alpha\)-GalNAcases liberate \(O\)-linked oligosaccharides from glycoproteins without damaging the protein backbone. This makes these enzymes powerful tools for the investigation of the structure and function of \(O\)-glycans.

The first gene encoding endo-\(\alpha\)-GalNAcase was isolated from B. longum JCM1217 (engBF) (Fujita et al. 2005). In an effort to identify endo-\(\alpha\)-GalNAcases with broader substrate specificity a BLAST search was done (Altschul et al. 1997) using the EngBF protein sequence as a template. Four potential endo-\(\alpha\)-GalNAcases were selected from the BLAST results and their genes were cloned; the expressed proteins were purified and characterized. Two of these enzymes can release Core 1 and Core 3 type \(O\)-glycans. This is the first report of these enzymes exhibiting this broader substrate specificity.

Results

Identification and selection of the putative endo-\(\alpha\)-\(N\)-acetylgalactosaminidases

In order to identify putative endo-\(\alpha\)-GalNAcases, a BLAST (Altschul et al. 1997) search was run using the endo-\(\alpha\)-GalNAcase protein sequence EngBF from B. longum JCM1217. The top nine hits were from bacteria and had 28% and higher top ten hits were from bacteria and had 28% and higher identities of their respective proteins.

The top nine hits were from bacteria and had 28% and higher identities of their respective proteins.
Fig. 1. Schematic representation of the putative endo-α-GalNAcase. Alignment of the putative endo-α-GalNAcases that were revealed by BLAST search against the protein sequence of EngBF and the identity results are shown. The gray shaded boxes correspond to the centrally located conserved region, while the black and patterned boxes indicate different types of SBDs. CBM_4_9, carbohydrate binding module (Pfam 02018); NPCBM, novel putative carbohydrate binding module (Pfam 08305); FIV AR, uncharacterized sugar-binding domain (Pfam 07554); F5-F8 type C, member of the galactose-binding domain-like superfamily (Pfam 00754). The protein accession numbers are B. longum (ABY836679), R. torques (ZP_01966813), B. capillosus (ZP_02035456), S. pneumoniae (YP_873926.1), E. faecalis (NP_815498.1), Janibacter sp. (ZP_00995766.1), A. aurescens (YP_947259.1), S. coelicolor (NP_630440.1), P. acnes (YP_056270.1), and C. perfringens (YP_695137.1).

Cloning and expression of the target endo-α-GalNAcase genes

Oligonucleotide primers (Table I) were designed to amplify the genes engEF, engCP, and engAA from the genomic DNA of E. faecalis ATCC 700802, C. perfringens ATCC 13124, and A. aurescens TCI. Following polymerase chain reaction (PCR), the amplified genes were cloned in frame into the pET21a expression vector. Unlike engEF and engCP, expression of engAA was very low. The very weak endo-α-GalNAcase activity of EngAA could be detected using Galβ1,3GalNAcα1pNP as a substrate but only after an extended incubation. For this reason, EngAA was dropped from this study. The gene coding the putative endo-α-GalNAcase protein engPA from P. acnes could not be amplified by PCR from the genomic DNA of P. acnes ATCC25746. To clone the ORF coding for protein EngPA, it was necessary to chemically synthesize the gene using codons optimized for gene expression in Escherichia coli. Once synthesized the gene was cloned into the expression vector pNEB206A. All three recombinant proteins were purified to apparent homogeneity by the chromatographic steps described in the experimental procedures section. The purified proteins migrated as single bands and the apparent molecular weights were in agreement with the
Identification of novel endo-α-N-acetylgalactosaminidases

Table I. Primers used for the cloning of the endo-α-N-acetylgalactosaminidase genes

| Primer       | Sequence                                      |
|--------------|-----------------------------------------------|
| AAfor-NdeI   | 5′-CCCATATGCCCCCGCTTGTCATCCC-3′              |
| AArev-HindIII| 5′-CCCAAGCTTCCCGCCAGTGAACCTGGAATTC-3′       |
| EFor-NdeI    | 5′-CCCATATGAAATGAAAATAAAAAAGCTTGAC-3′       |
| EFrev-XhoI   | 5′-CCCTCGAGTTTTTGGATTTCCCACTGGAACGGATT-3′   |
| CPfor-NdeI   | 5′-CCCATATGGTGAAATAAGCTGAAATAAACG-3′        |
| CPrev-XhoI   | 5′-CCCTCGAGTTTTTGGATTTCCCACTGGAACGGATT-3′   |
| PAfor        | 5′-GGGAAAGUTTAACGACCTTGACGATCC-3′           |
| PArev        | 5′-GGGAAAGUTTAACGACCTTGACGATCC-3′           |

Underlined are the restriction sites, while in bold are the uracil residues that are excised from the amplified DNA product by using the USER™ Friendly Cloning Kit (Bitinaite et al. 2007).

predicted molecular masses (188,000 Da for EngCP, 147,000 Da for EngEF, 142,000 Da for EngPA) (Figure 2).

Substrate specificity
The substrate specificities of the purified enzymes EngCP, EngEF, and EngPA and the commercially available EngSP and EngAL were determined. Each enzyme was incubated with various synthetic substrates. The released sugars were detected by colorimetric assay and thin layer chromatography (TLC) analysis (Table II, Figure 3). Galβ1,3GalNAcα1pNP was the most rapidly hydrolyzed substrate by all the enzymes tested. After 16 h of incubation, only EngEF and EngPA were capable of fully hydrolyzing the Core 3 disaccharide (GlcNAcβ1,

Table II. Substrate specificity using pNP substrates

| Substrate                      | EngCP | EngEF | EngPA | EngSP | EngAL |
|--------------------------------|-------|-------|-------|-------|-------|
| Galβ1,3GalNAcα1pNP (Core 1)    | 100   | 100   | 100   | 100   | 100   |
| Galβ1,3(GlcNAcβ1,6)GalNAcα1pNP (Core 2) | 2.5   | 2     | 0     | 0.6   | 0     |
| GlcNAcβ1,3GalNAcα1pNP (Core 3) | 6     | 100   | 100   | 3     | 27    |
| Galβ1,3GlcNAcα1pNP             | 0     | 0     | 0     | 0     | 0     |
| GalNAcα1pNP                    | 4.4   | 2.2   | 1.8   | 1.2   | 30    |

Reaction mixtures were incubated with different endo-α-GalNAcases at 25°C for 16 h. Product release was measured at 405 nm.
Gal\(\alpha\) with a k addition to hydrolysis activity. To test for this activity, Several endoglycosidases have transglycosylation activity in Transglycosylation activity. The released sugars migrated at the same Rf on the TLC analysis, sugars were only released when asialofetuin and mucin shown). Extra spot at the same Rf as GalNAc was observed (data not extra). The released sugars migrated at the same Rf on the TLC plate as the Core 1 and 3 disaccharides (Figure 3). As expected where used as substrates (Figure 3). Coincubating the substrates with neuraminidase enabled the enzymes to release the core sugars. The released sugars migrated at the same Rf on the TLC plate as the Core 1 and 3 disaccharides (Figure 3).

The enzymes were also tested for their ability to release sugars from natural glycoproteins. Calf \(\kappa\)-casein, human glycoporphin A, porcine mucin, calf fetuin, and calf asialofetuin were incubated with different endo-\(\alpha\)-GalNAcases. Based on the TLC analysis, sugars were only released when asialofetuin and mucin where used as substrates (Figure 3). Coincubating the substrates with neuraminidase enabled the enzymes to release the core sugars. The released sugars migrated at the same Rf on the TLC plate as the Core 1 and 3 disaccharides (Figure 3).

The \(k_{cat}\) of all enzymes was determined on Gal\(\beta\)\(1,3\)GalNAc\(\alpha\)\(1\)pNP and GlcNAc\(\beta\)\(1,3\)GalNAc\(\alpha\)\(1\)pNP (Table III). When Gal\(\beta\)\(1,3\)GalNAc\(\alpha\)\(1\)pNP was used as a substrate EngEF exhibited the highest \(k_{cat}\). EngPA had the lowest activity on that substrate with a \(k_{cat}\) about 25 times lower than EngEF. In the case of GlcNAc\(\beta\)\(1,3\)GalNAc\(\alpha\)\(1\)pNP the kinetic parameters were determined only for EngPA and EngEF. Interestingly, EngPA had a 3-fold higher \(k_{cat}\) than EngEF.

### Table III. Kinetic parameters of endo-\(\alpha\)-GalNAcases using Gal\(\beta\)\(1,3\)GalNAc\(\alpha\)\(1\)pNP (Core 1) and GlcNAc\(\beta\)\(1,3\)GalNAc\(\alpha\)\(1\)pNP (Core 3) as substrates

|       | \(k_{cat}\) (1/s) | \(K_m\) (\(\mu\)M) |
|-------|------------------|-------------------|
| Core 1 |                  |                   |
| EngCP | 19.9             | 70.93             |
| EngEF | 51.17            | 47.85             |
| EngPA | 2.099            | 3.781             |
| EngSP | 10.51            | 40.37             |
| EngAL | 25.89            | 33.87             |
| EngBF*| 17.8             | 21.8              |
| Core 3 |                  |                   |
| EngEF | 9.434            | 20.03             |
| EngPA | 28.9             | 11.15             |

*a*Data from Fujita et al. (2005).

3GalNAc\(\alpha\)\(1\)pNP, EngAL could only partially hydrolyze the Core 3 disaccharide (27%) after 16 h of incubation (Table II). EngAL could also partially release GalNAc while the rest of the enzymes released only traces of the monosaccharide (Table II). None of the enzymes could act on Gal\(\beta\)\(1,3\)GlcNAc\(\alpha\)\(1\)pNP and low or no activity was detected when Core 2 trisaccharide (Gal\(\beta\)\(1,3\)GlcNAc\(\beta\)\(1,6\)GalNAc\(\alpha\)\(1\)pNP) was used as a substrate (Table II).

The enzymes were also tested for their ability to release sugars from natural glycoproteins. Calf \(\kappa\)-casein, human glycoporphin A, porcine mucin, calf fetuin, and calf asialofetuin were incubated with different endo-\(\alpha\)-GalNAcases. Based on the TLC analysis, sugars were only released when asialofetuin and mucin where used as substrates (Figure 3). Coincubating the substrates with neuraminidase enabled the enzymes to release the core sugars. The released sugars migrated at the same Rf on the TLC plate as the Core 1 and 3 disaccharides (Figure 3). As expected from the pNP data, when EngAL was incubated with mucin an extra spot at the same Rf as GalNAc was observed (data not shown).

The \(k_{cat}\) of all enzymes was determined on Gal \(\beta\)\(1,3\)GalNAc\(\alpha\)\(1\)pNP and GlcNAc\(\beta\)\(1,3\)GalNAc\(\alpha\)\(1\)pNP (Table III). When Gal\(\beta\)\(1,3\)GalNAc\(\alpha\)\(1\)pNP was used as a substrate EngEF exhibited the highest \(k_{cat}\). EngPA had the lowest activity on that substrate with a \(k_{cat}\) about 25 times lower than EngEF. In the case of GlcNAc\(\beta\)\(1,3\)GalNAc\(\alpha\)\(1\)pNP the kinetic parameters were determined only for EngPA and EngEF. Interestingly, EngPA had a 3-fold higher \(k_{cat}\) than EngEF.

Transglycosylation activity

Several endoglycosidases have transglycosylation activity in addition to hydrolysis activity. To test for this activity, Gal\(\beta\)\(1,3\)GalNAc\(\alpha\)\(1\)pNP was incubated with various 1-alkanols in the presence of EngCP, EngEF, EngPA, EngSP, and EngAL. Reaction products were analyzed on TLC plates (Figure 4). All of the enzymes tested exhibited similar transglycosylation activity. The longest 1-alkanol successfully incorporated in these transglycosylation reactions was 1-pentanol though the level of product was very low (Figure 4A and B). EngEF, EngPA, and EngSP were also tested for transglycosylation activity using the disaccharide GlcNAc\(\beta\)\(1,3\)GalNAc\(\alpha\)\(1\)pNP as the donor and 1-alkanols again as the acceptors. Since only EngEF and EngPA were capable of fully hydrolyzing GlcNAc\(\beta\)\(1,3\)GalNAc\(\alpha\)\(1\)pNP compared to the rest of the endo-\(\alpha\)-GalNAcases which had very low activity on this substrate (Table II), it was surprising to find no significant difference in the amount of transglycosylation products produced by all three enzymes when observed on a TLC (Figure 4C).

**Discussion**

This is the first report presenting data about the substrate specificity of all available endo-\(\alpha\)-GalNAcases. Three novel endo-\(\alpha\)-GalNAcases: EngEF from *E. faecalis*, EngPA from *P. acnes*, and EngCP from *C. perfringens* have been cloned, purified, and characterized. The commercially available endo-\(\alpha\)-GalNAcases from *S. pneumoniae* (EngSP) and *Alcaligenes* sp. (EngAL) have also been tested. Data from a previous report on EngBF from *B. longum* (Fujita et al. 2005) have also been included. All six of these enzymes were able to completely hydrolyze Core 1 disaccharide Gal\(\beta\)\(1,3\)GalNAc\(\alpha\)\(1\)pNP. None of them could act on Gal\(\beta\)\(1,3\)GlcNAc\(\alpha\)\(1\)pNP showing that there is a strict preference for GalNAc as the sugar participating in the O-glycosidic linkage. EngEF exhibited the highest \(k_{cat}\) on Gal\(\beta\)\(1,3\)GalNAc\(\alpha\)\(1\)pNP while EngPA had the lowest.
EngEF and EngPA were the only enzymes in this study able to fully hydrolyze the Core 3 disaccharide (GlcNAcβ1,3GalNAca1pNP). EngAL could partially hydrolyze it (Table II) while EngCP, EngSP, and EngBL had even lower activity on this substrate (less than 6%). Interestingly, EngPA was more active on the Core 3 substrate than EngEF contrary to the results on the Core 1 disaccharide (Table III). A detailed mutagenesis study will help in identifying the functional residues responsible for the ability of EngEF and EngPA to act on Core 3 disaccharides.

Unfortunately, none of the enzymes could act on natural glycoproteins prior to treatment with neuraminidase. A major challenge would be to design an endo-α-GalNAcase that could act on sialylated O-glycans.

All the enzymes were tested for their transglycosylation activity. When the enzymes were incubated in the presence of Galβ1,3GalNAca1pNP and several 1-alkanols, we observed no major differences in the amount of transglycosylation products produced (Figure 4A and B). EngEF, EngPA, and EngSP were also tested for transglycosylation activity using GlcNAcβ1,3GalNAca1pNP as a donor. Interestingly, the same transglycosylation activity profile for these three enzymes was observed with this donor (Figure 4C) even though only EngEF and EngPA are capable of fully hydrolyzing GlcNAcβ1,3GalNAca1pNP in contrast to EngSP which is significantly less active on this substrate.

After submission of this manuscript, a characterization of EngBF from B. longum and EngCP from C. perfringens was published (Ashida et al. 2008). Their results for the substrate specificity of EngCP agreed closely with the substrate specificity we had observed for EngCP.

In this study, three endo-α-GalNAcases were cloned, purified, and characterized. This is the first report of these endo-α-GalNAcase genes. EngEF and EngPA acting on Core 3 in addition to Core 1 O-glycans. This property could make these enzymes a powerful tool for the release of O-glycan sugars from glycoproteins. They can also be used as templates in future protein engineering experiments toward the creation of endo-α-GalNAcases capable of acting on O-linked glycans regardless of their sugar composition.

Materials and methods

Cloning and expression of the endo-α-GalNAcase genes

Based on the DNA sequence of AAO81568 from E. faecalis,YP_695137.1 from C. perfringens, and YP_947239.1 from A. aurescens, oligonucleotide primer pairs EFfor-Ndel/EFrevc-Xhol,CPfor-Ndel/CPrev-Xhol, and AArev-Ndel/AArev-HindIII were designed (Table I). The putative endo-α-GalNAcase genes were amplified by PCR using these primer pairs from the genomic DNA of E. faecalis ATCC 700802, C. perfringens ATCC 13124, and A. aurescens TCI, respectively. The amplified genes (engEF, engCP, and engAA) were digested with the appropriate restriction enzymes (New England Biolabs, Inc., Ipswich, MA) and inserted into the corresponding sites of pET-21a (Novagen, Madison, WI). The resulting plasmids (pET21a-engEF, pET21a-engCP, and pET21a-engAA) were transformed into E. coli T7 Express lysY (New England Biolabs, Inc., Ipswich, MA). The transformed cells were grown overnight at 30°C in a 20 mL LB medium containing 100 μg/mL ampicillin. These transformations were used to inoculate 1000 mL of fresh medium and antibiotics and grown at 25°C. After these cultures reached an A600 of 0.6–0.7, 0.3 mM isopropyl thio-β-D-galactopyranoside was added and the cultures were shifted to 20°C. Incubation was continued for 12–14 h. The cells were harvested by centrifugation. The cell pellet was suspended in a final volume of 15 mL of 20 mM Tris–HCl, pH 7.6, 200 mM NaCl, 1 mM dithiothreitol. The resuspended cells were sonicated for eight 20-s bursts at 50% duty cycle using a Sonicator Ultrasonic processor model-375 (Misonix, Farmingdale, NY). Samples were subsequently centrifuged and the supernatant was collected for further purification.

All purification steps were carried out at 4°C. All columns used for protein purification were purchased from GE Healthcare (Piscataway, NJ).

Based on the DNA sequence of YP_056270.1 from P. aerocolon, the putative endo-α-GalNAcase gene was chemically synthesized. The codons were optimized for gene expression in E. coli using DNAWorks software (Hoover and Lukbowski 2002). The optimized sequence was divided into six building blocks and synthesized (Hoover and Lukbowski 2002). After the sequence of each block was verified, the full sized gene was assembled using the USER method (Bitinaite et al. 2007). Once assembled the synthesized engPA gene was amplified by PCR using the primer pair PAfor/PArev (Table I) and inserted into the corresponding sites of pNEB206A using the USER Friendly Cloning Kit (New England Biolabs, Inc., Ipswich, MA) (Bitinaite et al. 2007). The resulting plasmid pNEB206A-engPA was transformed, expressed, and lysed as previously described for other endo-α-GalNAcase genes.

EngEF purification

The cell extract was diluted 3-fold with a buffer containing 20 mM Tris–HCl, pH 7.6 (buffer A) and loaded onto a HiTrap Q HP column that had been previously equilibrated in buffer A. After the column was washed with five column volumes of buffer A, the enzyme was eluted with a linear gradient of 0–1 M NaCl in buffer A. Fractions with the enzyme eluted from 0.2 to 0.75 M NaCl. This was pooled and loaded onto a HiTrap HP column previously equilibrated with buffer B (20 mM Tris–HCl, pH 7.6, 500 mM NaCl). After the column was washed with five column volumes of buffer B, the enzyme was eluted with a linear gradient of 0–0.5 M imidazole in buffer B. The peak of enzyme activity eluted at an imidazole concentration range of 0.06–0.27 M. These fractions were combined, dialyzed overnight against buffer A, and loaded onto a Source 15Q column elutiated in buffer A. After the column was washed with five column volumes of buffer A, the proteins were eluted with a linear gradient of 0–0.23 M NaCl in buffer A. Active EngEF eluted from 0.14 to 0.18 M NaCl. These fractions were combined and concentrated with a Centricon Concentrator 10 (Millipore, Billerica, MA) to about 5 mL. Concentrated samples were loaded onto a Superdex 75 column previously equilibrated with buffer C (20 mM Tris–HCl, pH 7.6, 200 mM NaCl) and washed with buffer C. Fractions with EngEF activity were pooled and concentrated. After the addition of glycerol at 50%, the purified enzyme preparation was stored at −20°C. Enzyme purity was judged by gradient polyacrylamide gel electrophoresis under denaturing conditions (Laemmli 1970). Protein concentration was determined using Bradford’s dye binding assay (Bio-Rad,
The hydrolytic activity of the enzymes was assayed using Galβ1,3GlcNAc1pNP (CAS 59837-14-8), GlcNAcβ1,3GalNAc1pNP (CAS 125455-64-3), Galβ1,3GlcNAc1pNP (CAS 57467-13-7), GalNAc1pNP (CAS 23646-68-6), calf κ-casein, human glycoporphin A, porcine mucin, calf fetuin, and calf asialofetuin were purchased from Sigma. Galβ1,3(GlcNAcβ1,6)GalNAc1pNP (CAS 139459-55-5) was from Toronto Research Chemicals Inc. (North York, Ontario). For TLC analysis, a Silica Gel 60 plate (Merck, Whitehouse Station, NJ) was developed in a solvent system of chloroform/methanol/water, 3/3/1 (v/v/v), and the sugars were visualized by spraying a diphenylamine/aniline/phosphate reagent (Bailey and Bourne 1960).

**Transglycosylation assays**

The 15 μL transglycosylation reaction mixture contained a 50 mM sodium phosphate buffer, pH 7.5, 5 mM MgCl₂, 0.8 mM GlcNAcβ1,3GlcNAc1pNP or 1.6 mM Galβ1,3GlcNAc1pNP as donors, 0.8 μg of endo-α-GalNAcase and various 1-alcanols as acceptors (13%, v/v). The reactions were incubated at room temperature for 16 h. Methanol, ethanol, 1-propanol, 1-butanol, 1-pentanol, 1-hexanol, 1-heptanol, 1-octanol, and 1-nonanol were purchased from Sigma (St. Louis, MO). The transglycosylation reaction mixtures were analyzed on a Silica Gel 60 TLC plate using chloroform/methanol/water 65/35/8 as the developing solvent and the sugars were visualized by spraying a diphenylamine/aniline/phosphate reagent.

**Optimum pH**

The pH optimum for each enzyme was determined in a pH range of 2.0–9.0 using the following buffers (50 mM): glycine–HCl (2.0–4.0), sodium acetate (3.5–6.0), sodium phosphate (5.5–8.0), and Tris–HCl (7.0–9.0).

**Enzyme kinetics**

Steady-state enzyme kinetics were performed at 25°C. The program HYPER v 1.01 was used to determine V$_{\text{max}}$ and K$_{\text{m}}$ values. The k$_{\text{cat}}$ values were calculated from V$_{\text{max}}$ using a molecular mass of 188,000 Da for EngCP, 147,000 Da for EngEF, 142,000 Da for EngPA, 190,000 Da for EngSP, and 160,000 Da for EngAL. Reported values are the average of three measurements. The standard deviations do not exceed 5%.

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**Conflict of interest statement**

This work was supported by New England Biolabs, Inc., a company that may profit from the sale of enzymes described herein.
The authors are (DL, EPG) or were (DK) employees of New England Biolabs, Inc.

**Abbreviations**

Endo-α-GalNAcase, endo-α-N-acetylgalactosaminidase; Gal β1,3GalNAc, D-galactopyranosyl-β1,3N-acetyl-D-galactosamine pyranoside; GlcNAcβ1,3GalNAc, N-acetyl-D-glucosaminepyranosyl-β1,3N-acetyl-D-galactosamine pyranoside; ORF, open reading frame; PCR, polymerase chain reaction; pNP, p-nitrophenol; SBD, sugar binding domain; TLC, thin layer chromatography.

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