# Differential release of high mannose structural isoforms by fungal and bacterial *endo*-β-*N*-acetylglucosaminidases.

**Jared Q. Gerlach,[a] Michelle Kilcoyne,[a] Mark P. Farrell,[a] Marian Kane,[a] and Lokesh Joshi[a] ***

*Endo*-β-*N*-acetylglucosaminidases (ENGases) are widely used to remove *N*-linked oligosaccharides from glycoproteins for glycomic and proteomic studies and biopharmaceutical processes. Although several ENGases are widely available and their main oligosaccharide structural preferences are generally known (i.e. high mannose, hybrid or complex), the preferences of ENGases from different kingdoms for individual structural isoforms within the major classes of *N*-linked oligosaccharides have previously not been compared. In this work, a fungal ENGase (Endo Tv) was purified for the first time from a commercial *Trichoderma viride* chitinase mixture by sequential anion exchange and size exclusion chromatography, a commonly used strategy for purification of chitinases and endo enzymes. Oligosaccharides released from substrate glycoproteins by Endo Tv were identified and quantified by high pH anion exchange chromatography with pulsed amperometric detection and verified by mass spectrometric analysis. Unlike the widely-used bacterial ENGases Endo H and Endo F1, Endo Tv released exclusively high mannose *N*-linked oligosaccharides from RNase B, ovalbumin, and yeast invertase. Endo Tv did not hydrolyze fucosylated, hybrid, complex type or bisecting *N*-acetylglucosamine-containing structures from bovine fetuin, ovalbumin and IgG. When compared to the bacterial ENGase, Endo H, the relative ratio of high-mannose oligosaccharide structural isoforms released from RNase B by Endo Tv was found to differ, with Endo Tv releasing more $Man_5GlcNAc$ and $Man_7GlcNAc$ isoform I and less $Man_9GlcNAc$ from RNase B. Based on these data, it is suggested that use of ENGases from multiple sources may serve to balance an introduced bias in quantitative analysis of released structural isoforms and may further prove valuable in biochemical structure-function studies.

## 1. Introduction

Enzymatic release is often the preferred method of preparing both intact oligosaccharides and their source polypeptides for glycomic and proteomic studies [1-3]. Of the enzymes most commonly used for this purpose, amidases (*N*-glycopeptidase, PNGase, EC 3.5.1.52), cleave the amide bond of *N*-acetylglucosamine (GlcNAc) β-linked to asparagine (Asn) in the protein backbone, while endoglycosidases (*endo*-β-*N*-acetylglucosaminidase, ENGase, EC 3.2.1.96), hydrolyze the glycosidic bond between the two GlcNAc residues (di-*N*-acetylchitobiose core, GlcNAc-β-(1,4)-GlcNAc) proximal to the polypeptide, leaving one GlcNAc residue remaining on the protein backbone [4-6] (see Table 1 for examples). While amidases typically require the denaturation of the protein to allow the enzyme access to the amide bond, ENGases are often used on native glycoproteins with good efficiency and are thus preferred for some applications requiring native protein for downstream analysis, e.g. enzyme characterization, or in determining the glycosylation site. ENGases have a high degree of selectivity depending upon the particular enzyme [5-8] and the substitution of constituent residues within the oligosaccharide.

[a] *Glycoscience Group, National Centre for Biomedical Engineering Science, National University of Ireland, Galway, Ireland.*
** Orbsen Building, National University of Ireland, Galway, Ireland. Tel.: +353-91-49-5768; Email: lokesh.joshi@nuigalway.ie*

For example, the bacterial ENGases, Endo H and Endo F1, have similar specificities except for the tolerance of Endo H for fucose (Fuc) core modifications [5] (see Table 1). Because of these attributes, ENGases are in high demand for glycobiological research and industrial processes.

Quantitative glycomic analyses depend on the release of a representative distribution of intact glycoforms and/or a reliable method of normalization to compensate for a bias in glycoform or structural isoform release introduced by the enzyme used. Inference of relative percentages of released products can only be accurate if the characteristics of the chosen enzyme are well known and the physical characteristics (e.g. steric arrangements) of the substrate are conducive to full-access and activity of the deglycosylating enzyme. High mannose *N*-linked oligosaccharides are found in fungi, plants and animals, although there are differences in the occurrence of certain structural isoforms [9]. The preference of ENGases from rice and tomato for certain high-mannose structural isoforms typical of plants has been reported [10-12]. Therefore, the characterization and comparison of a range of ENGases from different kingdoms with distinct preferences for certain oligosaccharide structural isoforms would be useful in the fields of glycomics, proteomics, structure-function elucidation and industrial processes.

Filamentous fungi of the *Trichoderma* genus are a known source of a variety of potent enzymes which facilitate the breakdown of carbohydrate polymers [13-16]. Recently, a novel, secreted, high-mannose-specific ENGase from *T. reesei* (*Hypocrea* jecorina) was reported (Endo T) [17] which is of dissimilar sequence than currently known ENGases from bacteria. ENGases characterised

**Table 1.** Specificities of select enzymes which cleave *N*-linked oligosaccharides from glycoproteins.

| Enzyme | E.C. No. | Source organism | Kingdom | Activity | Specificity | References |
|---|---|---|---|---|---|---|
| PNGase F | 3.5.1.52 | *Elizabethkingia meningosepticum* | Eubacteria | Amidase | Complex, hybrid, high mannose, tolerates α-(1→4,6) fucosylation only | 24,25 |
| PNGase A | 3.5.1.52 | *Prunus dulcis* | Plantae | Amidase | Complex, hybrid, high mannose, tolerates α-(1→3, 4, 6) fucosylation | 26 |
| Endo D | 3.2.1.96 | *Streptococcus pneumoniae* | Eubacteria | Endoglycosidase | Trimannosyl, non-sialylated complex | 8 |
| Endo H | 3.2.1.96 | *Streptomyces plicatus* | Eubacteria | Endoglycosidase | High mannose, hybrid, tolerates α-(1→3, 4, 6) fucosylation | 4 |
| Endo S | 3.2.1.96 | *Streptococcus pyogenes* | Eubacteria | Endoglycosidase | Complex biantennary, α-(1→6) fucosylation | 7 |
| Endo T | 3.2.1.96 | *Trichoderma reesei* | Fungi | Endoglycosidase | High mannose | 17 |
| Endo F1 | 3.2.1.96 | *Elizabethkingia meningosepticum* | Eubacteria | Endoglycosidase | Hybrid, high mannose | 27 |
| Endo F2 | 3.2.1.96 | *Elizabethkingia meningosepticum* | Eubacteria | Endoglycosidase | High mannose, biantennary complex | 28 |
| Endo F3 | 3.2.1.96 | *Elizabethkingia meningosepticum* | Eubacteria | Endoglycosidase | Trimannosyl, biantennary and triantennary complex, fucose position dependent | 9, 29 |

fungal sources, including examples isolated from *Mucor hiemalis* [18], *Aspergillus oryzae* [19], *Flammulina velutipes* [20] and *Sporotrichum dimorphosporum* [21]. We have previously observed ENGase activity in a commercial preparation of chitinase from *T. viride* (laboratory observation). Chitin, a component of insect and crustacean exoskeletons as well as the cell walls of fungi, is a polymer of β-1,4-linked GlcNAc. Under specific conditions, certain *T. viride* enzymes have displayed additional activities e.g. in addition to producing a distinct β-xylosidase [22], at least one *T. viride* cellulase also cleaves the xylosyl-serine linkage between a glycosaminoglycan chain and its core protein [23]. However, although the di-*N*-acetylchitobiose core of *N*-linked oligosaccharides is similar in structure to chitin, there have been no reports describing the cleavage of this core using chitinases. Therefore, we targeted the chitinase preparation to identify the ENGase responsible for this activity and to assess its structural isoform release preferences in comparison to those of a bacterial ENGase.

In this work, we report the isolation of a previously unreported ENGase from a *T. viride* preparation, which we propose to call Endo Tv. We describe the ability of Endo Tv to exclusively cleave high-mannose *N*-linked oligosaccharides, and the preference for different structural isoforms of high mannose-type oligosaccharides in comparison to a bacterially-derived ENGase, Endo H. To our knowledge, this is the first direct comparison of high-mannose structural isoform preferences of ENGases across different kingdoms (Fungi and Eubacteria).

## 2. Methods

### 2.1 Materials

Whole *T. viride* chitinase preparation (WChTv, catalogue C8241, lot 108K4005 and WChTv1, catalog C6242, lot 127K4076), chitinase from *Streptomyces griseus* (WChSg, catalogue C6137) and fungal protease inhibitor cocktail were purchased from Sigma-Aldrich Co. (Poole, United Kingdom). GlycoClean H cartridges were from Prozyme, Inc. (Hayward, CA). Lectins were from EY Laboratories (San Mateo, CA). All other reagents were from Sigma-Aldrich Co. unless otherwise noted and were of the highest grade available.

### 2.2 Enzymatic digestion of glycoproteins

Overnight (18 h) incubations of 1 mg portions of bovine fetuin from fetal calf serum and ribonuclease B (RNase B) from bovine pancreas were performed using 6 mU (10 μL) of WChTv, prepared in 50 mM sodium phosphate, pH 5.0, at 37 °C. Enzymatic digestions included 1 mM (final concentration) phenylmethylsufonyl fluoride (PMSF) or 1 μL/mL (final concentration) fungal protease inhibitor cocktail. Digestions using WChSg and Endo H were essentially as described above except with 6 and 2 mU of enzyme, respectively. Concurrent negative control blanks (without enzyme) were carried out and subsequently purified and analysed in the same manner as enzymatic digestions (see below). Enzymatic digests which were directly compared with one another were also carried out at the same time, i.e. separate Endo H digests were carried out for comparison to WChTv and Endo Tv digests. Digests of yeast invertase, bovine immunoglobulin G (IgG) and chicken egg ovalbumin were performed under similar conditions.

### 2.3 Electrophoresis and lectin blotting

Enzymatically digested and non-digested protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the method of Laemmli [30]. Samples were mixed with loading buffer containing 25% β-mercaptoethanol and denatured at 100 °C for 5 min. Samples (2 μg) were loaded and electrophoresed in NuPAGE 4-12% Bis-Tris gels using MES running buffer (Invitrogen, Carlsbad, CA) at 150 V constant for approximately 1 h. Gels were stained with 0.05% (w/v) Coomassie G-250 in a fixative solution containing 30% ethanol and 10% acetic acid, and partially destained with distilled deionised water (dd$H_2O$) or stained with silver (Pierce SilverSNAP Kit, Thermo-Fisher Scientific, UK). Gels were imaged using a ChemiDoc imaging system (Bio-Rad, Hercules, CA).

For blotting experiments, after SDS-PAGE, proteins were transferred to polyvinylidene fluoride (PVDF) membrane in a semi-dry transfer apparatus (Bio-Rad) at 1.5 mA/cm$^2$ for 2 h at a

maximum of 15 V. Prior to transfer, sequencing grade, 0.22 μm PVDF membrane was washed twice in methanol and rinsed in transfer buffer. Membranes were probed directly after transfer.

Membranes were blocked with 1% bovine serum albumin (BSA, >99% purity) in 20 mM Tris-HCl, 100 mM NaCl, 0.05% Tween 20, pH 7.4 (TBST) for 1 h. Membranes were washed with TBST three times prior to probing with wheat germ agglutinin (WGA) conjugated to alkaline phosphatase (WGA-AP) essentially as previously described [31]. WGA-AP was used at a concentration of 1.5 μg/mL lectin buffer (TBST supplemented with 1 mM each of $MgCl_2$, $MnCl_2$, and $CaCl_2$). A control membrane, to validate that lectin binding was carbohydrate-mediated [32], was carried out in parallel. This membrane was incubated in an identical manner to the lectin blotting experiment except that the lectin was pre-incubated in 100 mM GlcNAc for 1 h prior to, and in 10 mM GlcNAc during, the incubation step. Lectin binding was visualised colorimetrically by reaction of bromo-chloro-indolyl phosphate/nitroblue tetrazoleum substrate (BCIP/NBT) and was stopped by rinsing the membranes in water. The membranes were air dried at room temperature in the dark, imaged with white light and stored digitally.

### 2.4 Fluorescently-labelled lectin assay (FLLA)

WChTv-treated and untreated samples of RNase B were diluted to 100 μg/mL in alkaline Tris-HCl buffer (50 mM Tris-HCl, pH 10.1, 21 °C) in a 96-well microtitre plate and 50 μL was used to coat each well bottom for 12 h at room temperature with gentle shaking. Protein solution was then removed and the wells were washed and blocked with 150 μL 0.5% BSA in TBST at room temperature for 2 h. All washes were done three times with TBST. The wells were washed again, incubated with 2 μg/mL of fluorescein isothiocyanate (FITC)-labelled concanvalin A (Con A-FITC) for 1 h at room temperature, washed again and read dry at 485 nm excitation and 535 nm emission on a Spectra Fluor microplate reader (Tecan, Männedorf, Switzerland). All steps were performed in the dark and each assay was performed in quadruplicate.

### 2.5 Purification of ENGase (Endo Tv) from whole chitinase (WChTv)

All chromatography was performed on an ÄKTA™ Purifier FPLC (GE Healthcare, Uppsala, Sweden). Anion exchange chromatography (AEC) was done on a Mono Q 5/50 column (GE Healthcare, USA) which was converted to acetate form by sequential washes with 1 M sodium hydroxide (NaOH) for 20 column volumes (CV), 5 CV of deionised water ($dH_2O$) and 5 CV of 1 N acetic acid. The column was then equilibrated with 5 CV of 5 mM sodium acetate (NaOAc), pH 5.0. WChTv (5-8 mg) was suspended in 500 μL 5 mM NaOAc, pH 5.0, vortexed extensively, filtered through a 0.2 μm spin filter and then loaded onto the column. A linear gradient from 5 mM to 1 M NaOAc, pH 5.0 was applied over 5 CV at 1 mL/min. Protein elution was monitored at 280 nm and 500 μL fractions were collected and assayed for chitinase activity (see below) and for ENGase activity by incubation with RNase B as follows: 15 μL of each fraction was added to 20 μg of RNase B dissolved in 25 μL 100 mM NaOAc, pH 5.0 (final volume 40 μL) for 2 h at 37 °C. Any resulting mass shift was visualized by SDS-PAGE and Coomassie staining as described above.

The AEC fraction of interest was further purified by size exclusion chromatography (SEC) on a Superdex 200 10/300 GL column (GE Healthcare) isocratically eluted with 50 mM NaOAc, pH 5.0 at 250 μL/min. The eluate was monitored at 280 nm, collected in 500 μL fractions and the fractions were assayed for activity as above. Fractions were pooled based on ENGase activity and concentrated in a 5 kDa molecular weight cut off (MWCO) spin filter (Millipore Billerica, MA) and stored at 4 °C. The SEC column was calibrated with IgG (150 kDa), BSA (64 kDa), ovalbumin (45 kDa) and RNase B (17 kDa).

### 2.6 Colourimetric glycosidase activity assays

Assays for *endo*- and *exo*-chitinase activity were carried out using *para*-nitrophenyl (*p*NP) derivatives of β-D-GlcNAc, chitobiose, and chitotriose. 2 μL of each AEC or SEC fraction was added to 30 μL *p*NP carbohydrate derivative (1 mM final concentration) in 50 mM NaOAc, pH 5.0 and incubated for 2 h at 37 °C. The reaction was quenched by the addition of 30 μL of saturated sodium carbonate ($Na_2CO_3$), briefly vortexed and the entire volume transferred to a 96 well microtitre plate. Absorbance was measured at 405 nm against a blank of reaction mixture with 2 μL of $ddH_2O$ substituted for enzyme. 1 mU of WChTv and WChSg were used for positive controls. Assays for α-*N*-acetylgalactosaminidase, β-galactosidase and α-mannosidase activity were conducted similarly using *p*NP derivatives of α-D-acetylgalactosamine (α-D-GalNAc), β-D-galactose (β-D-Gal) and α-D-mannose (α-D-Man), respectively.

### 2.7 Oligosaccharide analysis

Oligosaccharides released by enzymatic digests of glycoproteins were purified on GlycoClean H cartridges according to manufacturer's instructions. The purified oligosaccharides were filtered through a 0.22 μm membrane, dried and reconstituted in 100 μL 18.2 MΩ water. The equivalent of 1 μL of the reconstituted oligosaccharide was analysed by high-pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on an ICS-3000 system (Dionex, Sunnyvale, CA) equipped with a PA-100 column (4 x 250 mm, Dionex) and PA-100 guard column (4 x 50 mm, Dionex). Oligosaccharides were eluted using a binary gradient, composed of 100 mM NaOH and 1 M NaOAc in 100 mM NaOH, at a flow rate of 1 mL/min over 80 min at a column temperature of 25 °C. The gradient was started at 5 mM NaOAc, increased linearly to 50 mM by 10 min and further increased to 230 mM NaOAc by 50 min. This molarity was held for 10 min and at 60.1 min the column was washed with 450-500 mM NaOAc for 7 min. The column was then re-equilibrated at starting conditions for 13 min. The average of the quantititative relative data from two replicates (WChTv compared with Endo H) and four replicates (Endo Tv compared with Endo H) is reported. For the comparison between Endo Tv and Endo H, significance was established by paired, two-tailed t-test of the unaveraged quadruplicate data.

Liquid chromatography electrospray inonization mass spectrometry (LC-ESI-MS) was used to verify released carbohydrate identity. The purified oligosaccharides were evaporated to dryness and taken up in 100 μL of HPLC-grade water. 5 μL of each sample was injected onto a 1100 Series HPLC (Agilent Technologies, USA) equipped with a Hypercarb

porous graphitised carbon column (3 µm, 0.32 x 100 mm) (Thermo Fisher Scientific) and eluted at 7 µL/min using a binary gradient of (A) 10 mM ammonium carbonate ($NH_4HCO_3$) and (B) 80% acetonitrile (ACN) in 10 mM $NH_4HCO_3$. The gradient was linear 0 to 25% B over 45 min, increased to 100% B by 57 min and held for 3 min, decreased to 0% at 61 min and then re-equilibrated at 0% B for 20 min before the next injection. Eluate was sprayed into an Agilent LC/MSD Trap XCT mass spectrometer (Agilent) in negative ion mode at a source temperature of 350 °C and needle voltage set at -4300 V.

### 2.8 Protein identification

A Coomassie-stained gel band was excised, cut into 1 mm cubes, destained with ACN and reduced and alkylated prior to in-gel trypsin digestion at 37 °C based on Shevchenko *et al* [33]. A 10% formic acid extraction of peptides was followed by concentration on a SpeedVac (Thermo Fisher Scientific). NanoLC separation was performed with a PepMap C18 trap and column with the following gradient: 5-35% ACN with 0.1% formic acid over 18 min, 35-50% over 7 min, followed by 95% ACN. Eluate was analysed on a Q-Star Pulsar XL tandem mass spectrometer (Applied Biosystems, Foster City, CA) in Information Dependent Acquisition (IDA) mode. Non-smoothed centroid MS/MS data for doubly and triply charged precursor ions (settings: tolerances of 0.2 Da for precursor and fragment ions, trypsin cleavage, one missed cleavage, fixed carbamidomethyl cysteine modification, methionine oxidation) using Mascot 2.2 (Matrix Science, London, UK) against UniProt (Swiss-Prot and TREMBL combined) April 2009, without species restriction.

## 3. Results

### 3.1 ENGase activity in *T. viride* whole chitinase preparation (WChTv)

RNase B is a model glycoprotein with exclusively high-mannose type *N*-linked oligosaccharides [34]. Whole chitinase (WChTv) treatment of RNase B produced a mass shift comparable to that resulting from treatment of RNase B with the bacterial ENGases Endo H and Endo F1 (Fig. 1A), both of which cleave high-mannose type *N*-linked oligosaccharides from glycoproteins in an *endo* manner [27].

Con A binds to α-linked mannose residues and is commonly used to demonstrate the presence or absence of mannose-containing *N*-linked oligosaccharides [35, 36]. Con A binding to RNase B was reduced by approximately 80% after treatment with WChTv (Fig. 1B), which verified that the *N*-linked oligosaccharides were removed from the glycoprotein. Similarly, WGA lectin is known to bind GlcNAc, [37] and was used to probe PVDF membrane-bound electrophoresed RNase B with and without WChTv treatment. Two different preparations of WChTv (WChTv, as above, and WChTv1) were used to determine whether the observed enzyme activity was batch-specific. WGA bound to the untreated RNase B (Fig. 1C, lane 1) and to RNase B treated by both WChTv batches (Fig. 1C, lanes 2 and 3), which indicated that release action was most likely *endo*, as a GlcNAc residue remains linked to the protein backbone by this mechanism [38], and that the oligosaccharide release activity was not limited to a single batch.

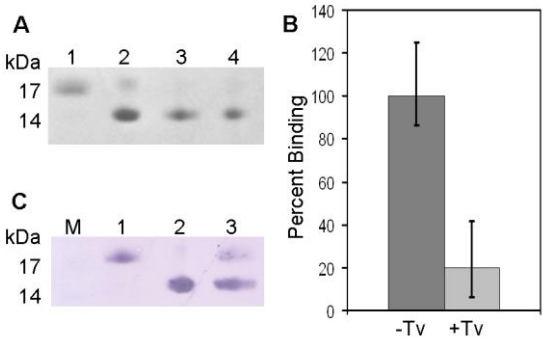


**Fig. 1** (A) Coomassie G-250-stained 4-12% SDS-PAGE of (1) untreated RNase B and RNase B treated with (2) WChTv, (3) Endo H, and (4) Endo F1. (B) Bar chart of Con A-FITC binding to RNase B when untreated (-Tv) and treated (+Tv) with WChTv. Untreated sample mean binding = 100%, error bars indicate the average deviation from four replicates. (C) WGA staining of PVDF-bound untreated RNase B (1) and RNase B after treatment with (2) WChTv, and (3) WChTv1.

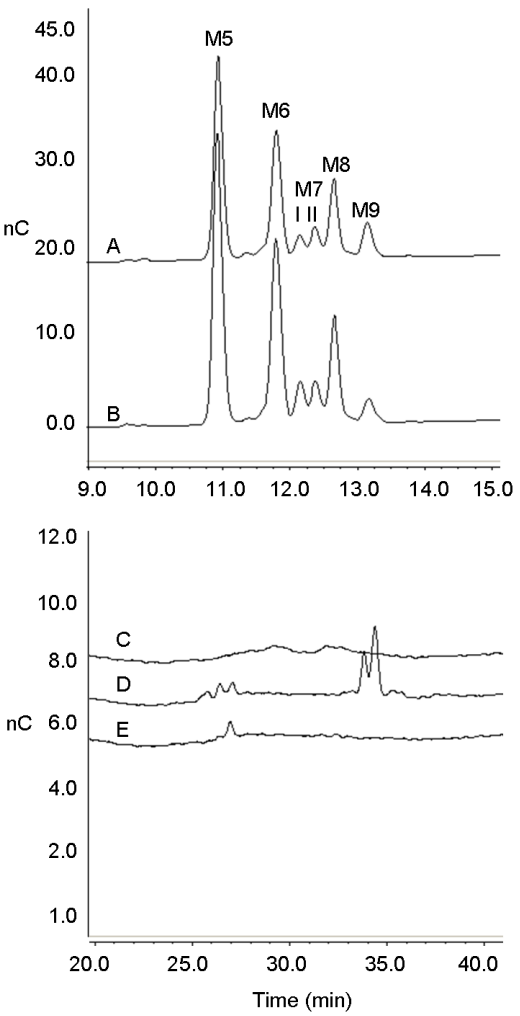


**Fig 2.** HPAEC-PAD chromatograms of oligosaccharides from RNase B released by (A) Endo H and (B) WChTv, and oligosaccharides released from fetuin by (C) WChTv (none), (D) Endo F3, and (E) Endo F2. M5 to M9 denote $Man_5GlcNAc$ to $Man_9GlcNAc$,

HPAEC-PAD analysis of the purified products cleaved from RNase B by WChTv confirmed that high mannose *N*-linked oligosaccharides were released. A comparison of retention times of high mannose structures released from RNase B by WChTv and Endo H confirmed that the series $Man_5GlcNAc$-$Man_9GlcNAc$ (Fig. 2A and B) was released by WChTv, and not $Man_5GlcNAc_2$-$Man_9GlcNAc_2$, thus corroborating *endo* activity.

The identity of the series $Man_5GlcNAc$-$Man_9GlcNAc$ was verified by LC-ESI-MS. For native high mannose structures released from RNase B by PNGase F, deprotonated molecular $[M-H]^-$ ions were observed for only $Man_5GlcNAc_2$ and $Man_6GlcNAc_2$ with ESI-LC-MS analysis in the negative mode. However, the doubly charged $[M-2H]^{2-}$ ions of all released structures were seen (see Table S-1, supplementary data). The mass spectra of the native high mannose structures released by WChTv were very similar to the analysis of those released by the bacterial ENGase, further confirming *endo* release of the series $Man_5GlcNAc$ to $Man_9GlcNAc$. The only molecular ion detected of the native ENGase-released structures was at m/z 1030.4 for $Man_5GlcNAc$, and ionisation under these conditions produced glycosidic bond and cross ring cleavages for this molecule and the rest of the structural series (see Table S-2 for expected and observed m/z list with predicted structures, supplementary data).

In addition, the purified products from WChTv, Endo F2 and Endo F3 digestions of fetuin were compared by HPAEC-PAD (Fig. 2C through E). No complex-type oligosaccharide release was observed from the WChTv digest of fetuin, although a small peak eluting at the $Man_5GlcNAc$ position was observed (see section 3.3).

A chitinase preparation from the Gram-negative bacterium *Streptomyces griseus* (WChSg) was also tested for ENGase activity using RNase B and ovalbumin as substrates. No release of oligosaccharides from RNase B was observed with WChSg (not shown). Ovalbumin has been reported to contain high mannose structures [39], and no oligosaccharide peaks were observed by HPAEC-PAD analysis (Fig. 3B) of released components in comparison to those released from ovalbumin by WChTv (Fig. 3C) or Endo H (Fig. 3D). These results indicated that ENGase activity observed with the *T. viride* preparation was unlikely to be due to the action of the chitinase components and therefore was attributable to a separate ENGase enzyme component of the WChTv preparation. This component was targeted for purification and comparison to bacterial ENGase.

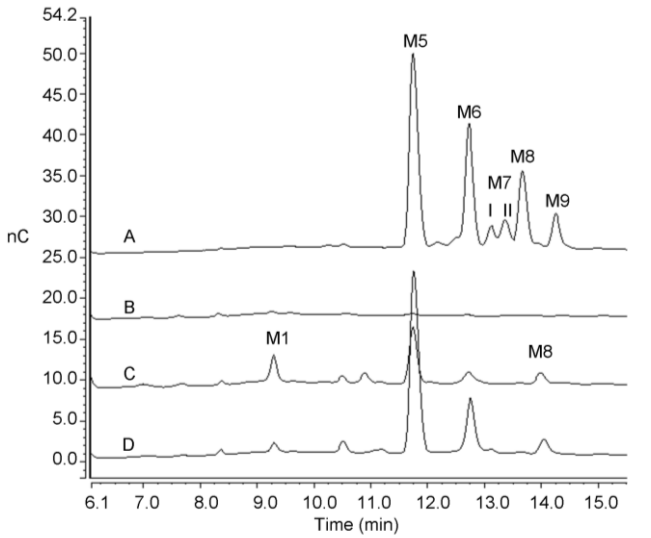


**Fig. 3** HPAEC-PAD chromatograms of released products from RNase B by (A) Endo H and products released from ovalbumin by (B) WChSg, (C) WChTv and (D) Endo H. M1 denotes ManGlcNAc and M5 to M9 denote $Man_5GlcNAc$ to $Man_9GlcNAc$ oligosaccharides,

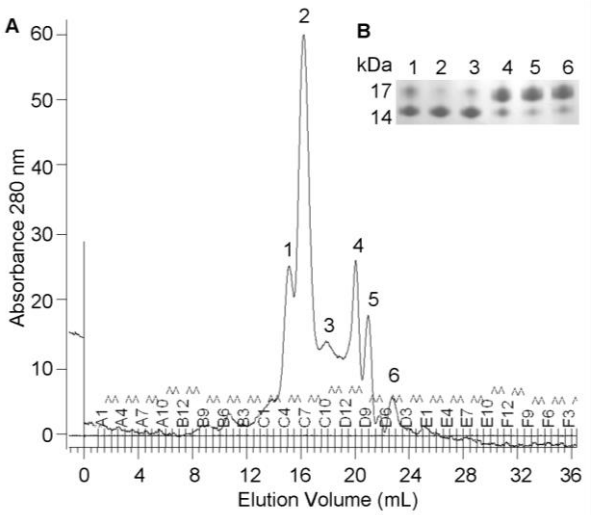


**Fig 4.** (A) SEC chromatogram of the ENGase-active peak which eluted at approximately 292 mM NaOAc by AEC. Peaks numbered 1-6 indicate fractions screened for ENGase activity. (B) ENGase assays using RNase B as substrate and numbered fractions from SEC as the enzyme source.

### 3.2 Purification of fungal ENGase, Endo Tv

The chromatographic strategy pursued followed that commonly used for purification of chitinases and endo enzymes involving sequential anion exchange and size exclusion chromatography [7, 18]. This purification method was found to be very reproducible and similar chromatographic profiles and activities were achieved on four different occasions with different batches of WChTv. In the initial step, WChTv was fractionated by AEC and only those fractions which eluted between 195 to 295 mM NaOAc contained ENGase activity, which indicated the presence of either multiple ENGases or multiple isoforms of one ENGase. A well defined peak at approximately 295 mM NaOAc (not shown) contained the highest ENGase activity and was further purified by isocratic SEC (Fig. 4). SEC peaks 1 to 3 (Fig. 4A), corresponding to estimated molecular masses of 50 kDa (peak 1), 35 kDa (peak 2)

and 20 kDa (peak 3), demonstrated ENGase activity (Fig. 4B). The greatest ENGase activity was from peak 2, with the lower activity present in peaks 1 and 3 attributed to the significant overlap of elution with peak 2. Peaks 4-6 did not have any ENGase activity (Fig. 4B). ENGase-active SEC fractions 1-3 were also assayed for *endo*- and *exo*-chitinase activity using *p*NP derivatives of β-D-GlcNAc, chitobiose, and chitotriose. While some hydrolysis of β-D-GlcNAc and chitobiose substrates was observed with the SEC peak 1 fraction, no hydrolysis was observed with the peak 2 and peak 3 fractions (data not shown), confirming that the enzyme responsible for ENGase activity was distinct from chitinase.

SDS-PAGE of peaks 1, 2 and 3 (Fig. 5A) was performed under reducing (R) and non-reducing (NR) conditions. The apparent masses for the major bands of peaks 1 and 2 in the R-SDS-PAGE gel (approximately 45 and 35 kDa, respectively) corresponded well with those estimated by SEC (see above). In the NR-SDS-PAGE lanes (Fig 5A), some higher-mass protein complexes typical of dimer formation were apparent with protein from peaks 1 and 2. No protein was observed from peak 3 under either R or NR conditions, which indicated that the quantity of protein present was below the sensitivity threshold of Coomassie G250 staining. The total protein content of peak 2 was estimated as 0.31% of the original WChTv protein content.

To identify the enzyme responsible for ENGase activity, the 35 kDa band from SEC peak 2 (Fig. 5A, arrow) was excised and trypsinized, and the resulting peptides were analyzed by nanoLC-MS/MS. Three peptide sequences were acquired and BLAST search of non-redundant protein sequence database from the National Centre for Biological Information (NCBI) resulted in a single direct match (Fig 5B) for the peptide AEPTDLPR (observed mass 449.73 Da) with Endo T, the ENGase reported previously from *T. reesei* [17]. The search also produced two homologous matches for Endo T with additional peptides EPARLIAR (observed mass 463.32 Da), and LLAVVLSTLLVFGFAPVAK (observed mass 979.70 Da). However, the 979.70 Da ENGase peptide from *T. viride* was split when aligned with Endo T (Fig. 5B). The mass reported for Endo T (33 kDa) is very close to that observed by both chromatographic and SDS-PAGE estimation (approximately 35 kDa) for the intact ENGase purified from WChTv, which we propose to name Endo Tv. These data taken together suggest that Endo Tv is a homolog of Endo T [17], rather than identical match.

### 3.3 Comparison of fungal and bacterial ENGase and further Endo Tv characterization

Although multiple structural isoforms of each oligosaccharide exist [34], high-mannose *N*-linked oligosaccharides with the same mannose number mainly eluted in the same peak, except in the case of $Man_7GlcNAc$ and $Man_8GlcNAc$ where oligosaccharides eluted in two separate peaks ( [40] and Fig. 6A). We were unable to attribute a small peak between those attributed to $Man_5GlcNAc$ and $Man_6GlcNAc$ and hence this was designated M5/6 (Fig. 6A and Table 2).

After purification, the proportions of high-mannose *N*-linked oligosaccharides released from RNase B by Endo Tv were altered


**Fig 5.** (A) Coomassie-stained SDS-PAGE of 50 µL (containing approximately 2 µg of material) of SEC fractions 1, 2 and 3 under reducing (R) and non-reducing (NR) conditions. Arrow indicates Endo Tv and source for nLC-MS/MS analysis. (B) ClustalW alignment of resulting peptides from nLC-MS/MS analysis of Endo Tv (bold) and the reported sequence for *T. reesei* ENGase. Asterisks (*****) under sequences indicate fully conserved residues while colons (**:**) indicate conserved functional substitutions and single dots (**.**) indicate semi-conserved substitutions.

**Table 2** Relative percentage area of each high mannose oligosaccharide peak (where M5 means $Man_5GlcNAc$, etc.) released from RNase B by Endo H, WChTv and Endo Tv treatment. '% Diff' column is the percentage increase or decrease of structural isoform released by WChTv or Endo Tv in comparison to that released by Endo H based on relative percentage values. Digests for comparison were performed, purified and analysed in parallel, WChTv, n=2; Endo Tv, n=4.

| Structure | Endo H | WChTv | % Diff. | Endo H | Endo Tv | % Diff. | *p* value |
|---|---|---|---|---|---|---|---|
| M5 | 36.7 | 39.8 | (8.45) | 37.6 | 45.2 | (20.21) | 0.00089 |
| M5/6 | 1.3 | 0.8 | 38.46 | 1.3 | 2 | (53.85) | 0.00015 |
| M6 | 26.5 | 27.2 | (2.64) | 26.4 | 24.7 | 6.44 | 0.00055 |
| M7 I | 4.6 | 5.9 | (28.26) | 4.3 | 7.6 | (76.74) | 0.00004 |
| M7 II | 6.5 | 5.5 | 15.38 | 6.2 | 6 | 3.23 | 0.01462 |
| M8 I | 15.3 | 15.1 | 1.31 | 15.7 | 10.5 | 33.12 | 0.00013 |
| M8 II | 1.1 | 0.8 | 27.27 | 0.9 | 0.8 | 11.11 | 0.54101 |
| M9 | 8.1 | 4.9 | 39.51 | 7.7 | 3.3 | 57.14 | 0.00003 |
| Total | 100.1 | 100 | | 100.1 | 100.1 | | |

as compared to those released by WChTv (Fig. 6B and Table 2). The change in the relative ratio of high-mannose oligosaccharides released from RNase B (Table 2) after purification of Endo Tv from WChTv further indicated that additional ENGase(s) and/or exo-glycosidases were present in WChTv. There was an increase in the relative percentage release of $Man_5GlcNAc$ and $Man_7GlcNAc$ isomer I and a decrease in the proportion of $Man_8GlcNAc$ isomer I (see Table 2 and Fig. S-1, supplementary data).

In comparison to oligosaccharides released from RNase B by the bacterial ENGase Endo H, the fungal ENGase Endo Tv released proportionately more $Man_5GlcNAc$ and $Man_7GlcNAc$ isomer I, and less of $Man_8GlcNAc$ isomer I and $Man_9GlcNAc$ (Table 2 and Fig. S-1, supplementary data).

To determine whether the structural preference of Endo Tv was exclusively high mannose, the well-characterized glycoproteins bovine fetuin, IgG, invertase and ovalbumin were also tested as substrates for Endo Tv. In the case of ovalbumin, high-mannose, hybrid and bisecting GlcNAc *N*-linked structures from purified ovalbumin itself, as well as from co-purified glycoprotein contaminants, have been reported [39]. Of interest to this work, the high-mannose type structures $ManGlcNAc_2$, and $Man_2GlcNAc_2$, to $Man_8GlcNAc_2$ [39, 41-43] have all been reported in purified or commercial preparations of ovalbumin. Endo Tv released only high-mannose structures from ovalbumin including ManGlcNAc (assigned after α-mannosidase digestion of oligosaccharides released from RNase B by Endo H, not shown), $Man_3GlcNAc$, $Man_5GlcNAc$, $Man_6GlcNAc$ and $Man_8GlcNAc$ (Fig. 6D). When fetuin was used as a substrate, a small amount of oligosaccharide corresponding to the elution time of $Man_5GlcNAc$ was observed upon treatment with Endo Tv (Fig. 6E). This may have indicated the presence of a small percentage of fetuin containing incompletely processed *N*-linked oligosaccharides in the total glycoform population. No oligosaccharides were released from bovine IgG showing no activity on fucosylated complex *N*-linked oligosaccharides [44] (see Table S-2, supplementary data).

*N*-linked oligosaccharides of yeast glycoproteins are exclusively of the high-mannose type. Invertase from *Saccharomyces cerevisiae* has two main size distributions: $Man_8GlcNAc_2$ to $Man_{14}GlcNAc_2$ (referred to here as high-mannose structures) and $Man_{>20}GlcNAc_2$ (referred to here as oligomannose structures) [46, 47]. Similar HPAEC-PAD profiles were observed for oligosaccharides released from yeast invertase by Endo H, which has previously been reported to hydrolyze all oligosaccharides on yeast invertase, and Endo Tv (Fig. 7C and D), with several additional peaks eluted before $Man_8GlcNAc$ from the Endo Tv-treated invertase digest population. A different profile was observed when invertase was treated with WChTv (Fig. 7B), supporting the presence of multiple enzymatic components within the WChTv extract acting in concert in this digestion e.g. multiple ENGases and exoglycosidases, including mannosidase, in addition to the expected chitinase.

## 4. Discussion

The purified fungal ENGase, Endo Tv, released $Man_5GlcNAc$ to $Man_9GlcNAc$ oligosaccharides from RNase B, ManGlcNAc, $Man_3GlcNAc$, $Man_5GlcNAc$, $Man_6GlcNAc$ and $Man_8GlcNAc$ from ovalbumin, and high-mannose and oligomannose structures from yeast invertase. Furthermore, the enzyme did not act on fucosylated, hybrid, complex-type or bisecting *N*-acetylglucosamine-containing *N*-linked oligosaccharide structures from ovalbumin, IgG and fetuin. This exclusive selection of the fungal ENGase for high mannose type oligosaccharides without substitutions is in contrast to the bacterial ENGases, Endo H and Endo F1, which additionally cleave hybrid structures, and also tolerate fucosylation in the case of Endo H (Table 1).

To our knowledge, this is the first report of an ENGase purified from a *T. viride* source. Fungi are known to produce high-mannose and oligomannose structures [46, 48] and the secretion of an enzyme capable of releasing high-mannose and oligomannose *N*-linked structures often found in fungal species is consistent with reported *Trichoderma* mycoparasitism [49]. Based on the homologous sequences from Endo Tv and full-length sequence data provided for Endo T [17], these fungal ENGases can be assigned to glycosyl-hydrolase family 18 (GH18), which includes both ENGases (EC 3.2.1.96) and chitinases (EC 3.2.1.14) from many diverse organisms. Interestingly, unlike the bacterial ENGases in common use, Endo F1 and Endo H [4, 5], which share high degrees of conserved sequence with each other, BLAST comparisons suggested that the fungal ENGases Endo T

and Endo Tv are more closely related to chitinases rather than to the bacterial ENGases.

The relatively-high homology between fungal chitinases and fungal ENGases raises questions about the evolutionary origins of these enzymes and their relationship to bacterial ENGases. For example, the ENGase from the fungus *Flammulina velutipes* shares 37-39% homology to chitnases from *Aspergillus* species and the catalytic domains of these enzymes are identical [20]. In contrast, these conserved fungal ENGase and chitinase catalytic domains differ significantly from those of the bacterial ENGases, Endo F1 and Endo H. However, while only 6-10% of homology (6-10%) exists between full-length sequences for the fungal and bacterial ENGases, folding models indicate a common tertiary structure among these enzymes [20]. Conservation between fungal ENGases and chitinases within GH18 suggests divergence from strict recognition of chitin to acceptance of a wider variety of substrates by an ancestral fungal chitinase. This ancestral precursor to fungal ENGases could have potentially hydrolyzed GlcNAc-β-(1,4)-GlcNAc linkages within both chitin and *N*-linked oligosaccharides, but no chitinolytic activity has been reported for known ENGases to date. From this, it may be inferred that pressure for substrate selectivity must have been an important part of ENGase evolution..

This comparison of oligosaccharide structural isoforms released by Endo Tv and Endo H is the first direct comparative analysis of ENGase release products of enzymes from the kingdoms Fungi and Eubacteria. We found the preference of Endo Tv to release certain $Man_5GlcNAc$ to $Man_9GlcNAc$ structural isoforms from RNase B was different than that of Endo H (approximately 45, 25, 14, 11 and 3% compared to 37, 27, 11, 16 and 8%, respectively, for $Man_5GlcNAc$ to $Man_9GlcNAc$ (see Table 2)). Previously, Endo T was described as being similar in activity to Endo H and Endo F1, but whether Endo T was able to act on hybrid *N*-glycans was not established nor were the differences in proportional structural isoform release of high mannose oligosaccharides noted [17].

Based on these data, it is suggested that the source of the enzyme chosen, if not the individual enzyme itself, has an influence on the distribution of structural isoforms of oligosaccharides released from glycoproteins. Hence, the released population analyzed may be inadvertantly biased compared to the actual population present on the protein. Therefore, a comparison of the isoform population released by an ENGase with that released by another ENGase with the same function but from another kingdom would be beneficial in establishing the true quantities and distribution of structural isoforms actually present on the glycoprotein under investigation. Furthermore, this potential increase in fidelity may help to identify particular isoforms which have defined roles in inter-cellular carbohydrate recognition and signalling.

In addition, apart from the obvious utility of Endo Tv in a variety of applications, including glycomic characterizations [38] or the removal of high mannose and oligomannose-type glycosylation from recombinant products produced in yeast [50], a structure-function study of the exclusively high-mannose releasing fungal ENGase compared to the less discriminating bacterial ENGases should shed light on targeting enzyme structures/sequences to the required specific oligosaccharide isoforms or for enzyme engineering.

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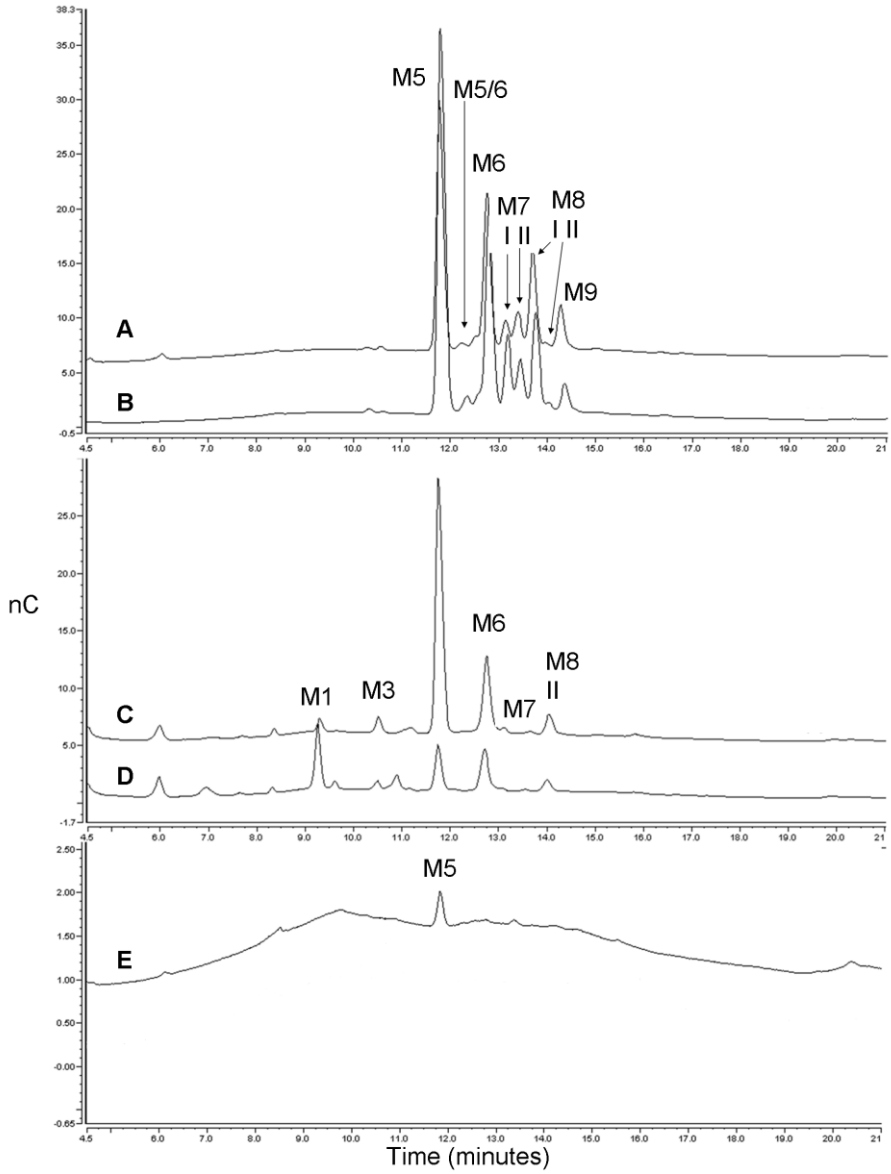


**Fig 6.** HPAEC-PAD chromatograms of released products purified from RNase B treated with (A) Endo H and (B) Endo Tv, and digestions of ovalbumin with (C) Endo H and (D) Endo Tv. Chromatogram (E) shows a peak from Endo Tv-digested fetuin. , M1 denotes ManGlcNac, M3 $Man_3GlcNAc$ and M5 to M9 denote $Man_5GlcNAc$ to $Man_9GlcNAc$ oligosaccharides,

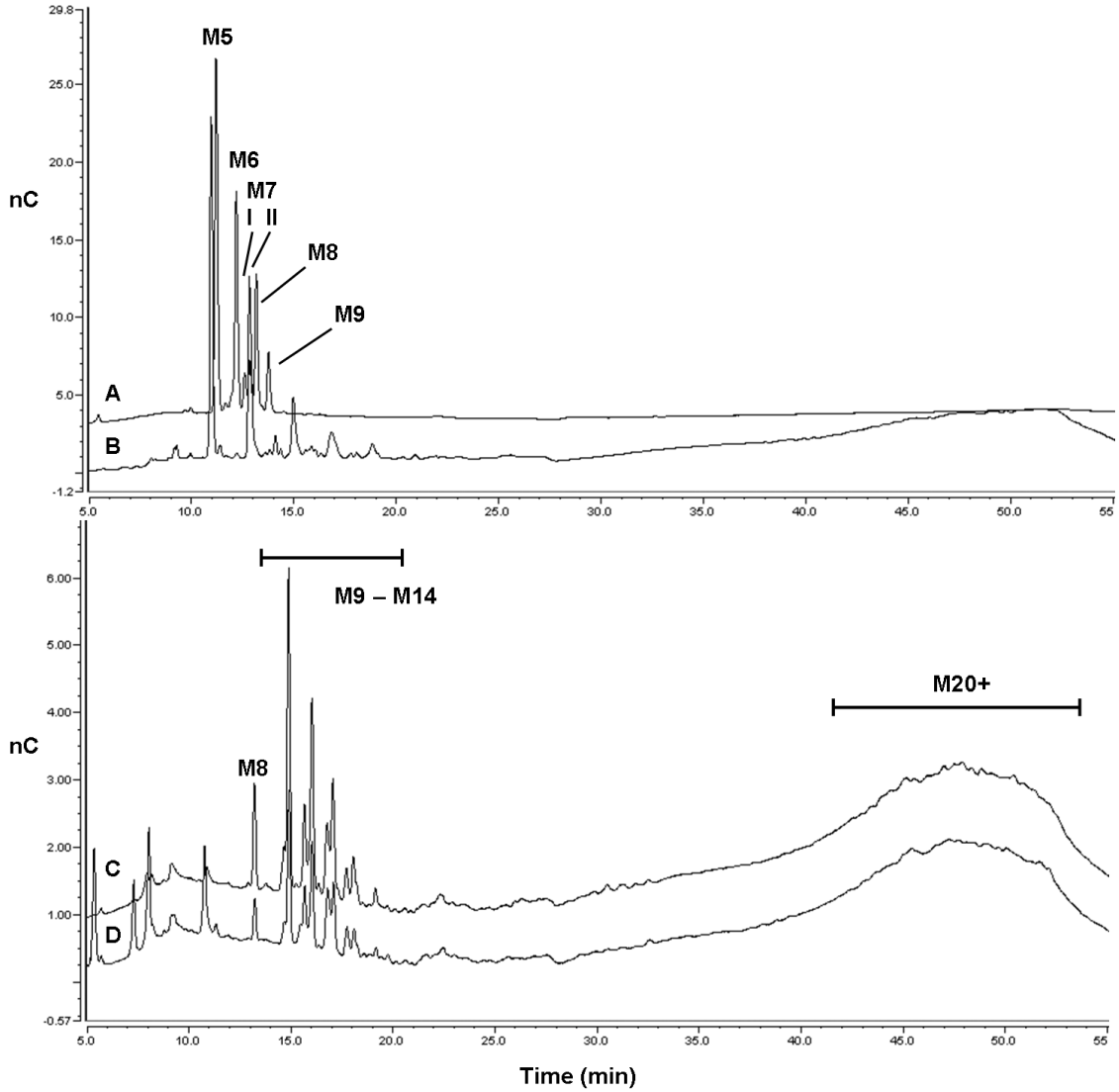


**Fig 7.** HPAEC-PAD chromatograms of released products purified from (A) Endo H-digested RNase B and from yeast invertase digested with (B) WChTv, (C) Endo H, and (D) Endo Tv. M5 to M9 denote $Man_5GlcNAc$ to $Man_9GlcNAc$ oligosaccharides. M9–M14 and M20+ in the lower panel were assigned based on the known oligosaccharide structures of invertase [42, 43] and likely retention times of these *N*-linked oligosaccharides on HPAEC-PAD [51].

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**Supplementary data**

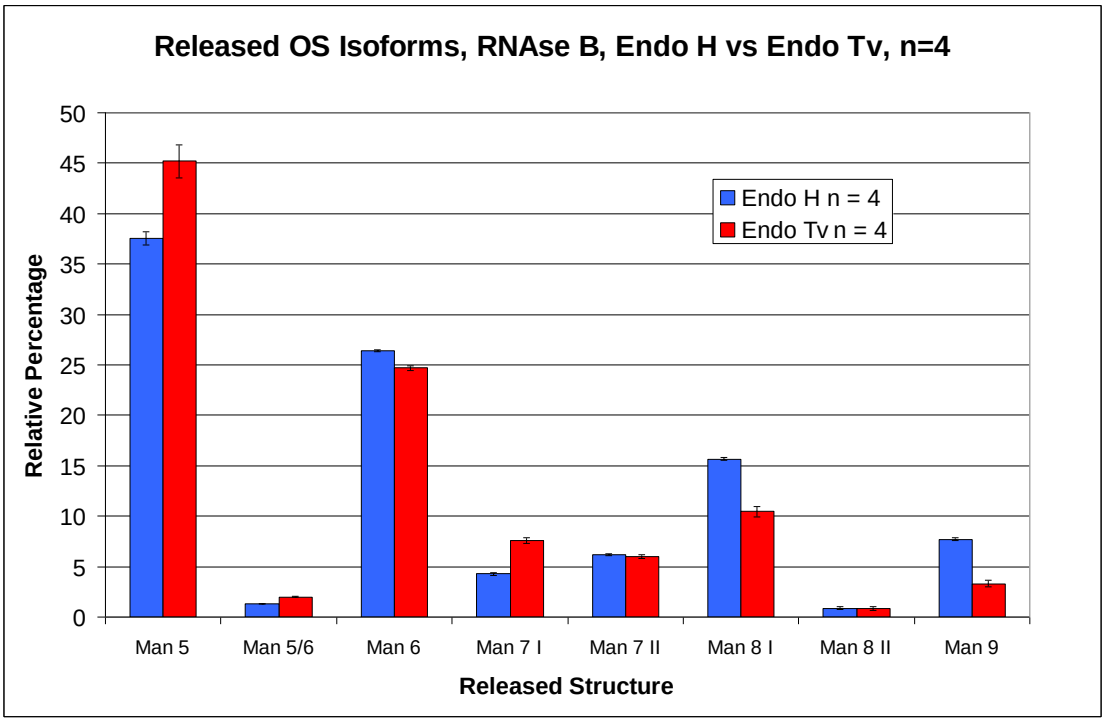


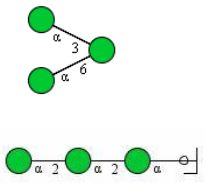
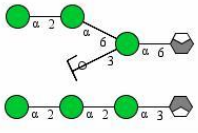
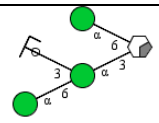
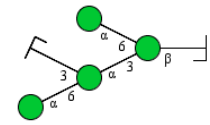
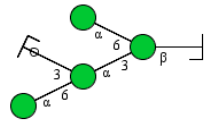
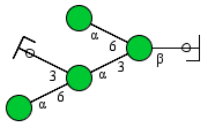
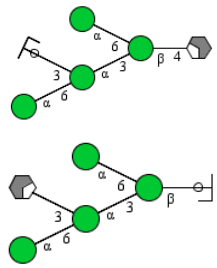
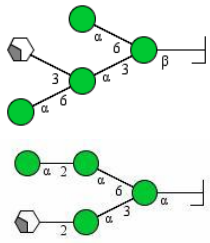
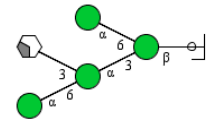

**Fig. S-1** Relative percentages of individual high mannose glycoforms ($Man_5GlcNAc$ to $Man_9GlcNAc$) released by purified Endo Tv and Endo H as quantified by HPAEC-PAD. Data is mean of four replicates, error bars represent standard deviation.

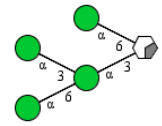
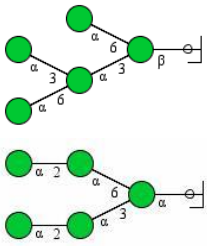
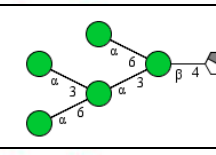
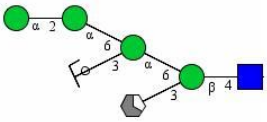
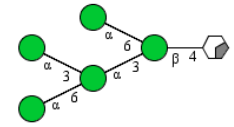
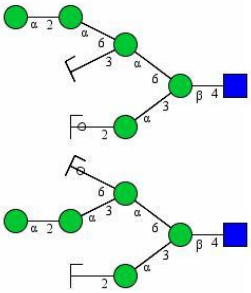
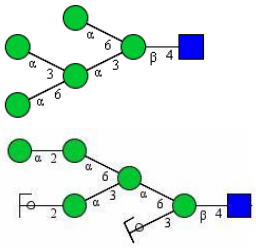
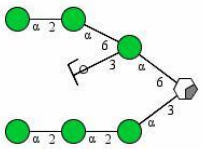
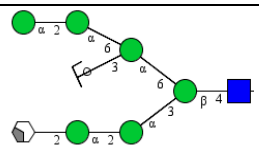
**Table S-1** Expected and observed m/z values of PNGase F released structures from RNase B by ESI-MS in negative ion mode (+/- 0.5 Da calibration).

| | **Expected m/z** | | **Observed m/z** | |
|---|---|---|---|---|
| **Structures** | **$[M-H]^-$** | **$[M-2H]^{2-}$** | **$[M-H]^-$** | **$[M-2H]^{2-}$** |
| $Man_5GlcNAc_2$ | 1233.43 | 616.215 | 1233.6 | 616.8 |
| $Man_6GlcNAc_2$ | 1397.48 | 698.24 | 1395.7 | 698 |
| $Man_7GlcNAc_2$ | 1557.53 | 778.265 | - | 778.9 |
| $Man_8GlcNAc_2$ | 1719.58 | 859.29 | - | 859.7 |
| $Man_9GlcNAc_2$ | 1881.63 | 940.315 | - | 940.5 |

- not observed.

**Table S-2** Deprotonated fragment ions from ESI-LC-MS of bacterial ENGase, Endo F1, and fungal WChTv-released native high mannose structures from RNase B. Fragment structures were in GlycoWorkbench v2.1.

| Fragment ion | Type | Expected m/z | Observed m/z | Bacterial | Fungal |
|---|---|---|---|---|---|
|  | C | 503.16 | 503.0 | Y | Y |
|  | $^{0,4}A_{Man}Y$<br>$^{1,3}A_{Man}$ | 545.17 | 545.1 | Y | Y |
|  | $^{0,2}A_{Man}Y$ | 605.19 | 605.1 | Y | Y |
|  | BZ | 629.19 | 629.1 | Y | Y |
|  | BY | 647.2 | 647.1 | Y | Y |
|  | CY | 665.21 | 665.2 | Y | Y |
|  | $^{2,4}A_{GlcNAc}Y$<br>$C^{0,2}X_{Man}$ | 707.22 | 707.2 | Y | Y |
|  | $B^{2,4}X_{Man}$ | 749.24 | 749.2 | Y | Y |
|  | $C^{2,4}X_{Man}$ | 767.25 | 767.2 | Y | Y |

| | | | | | |
|---|---|---|---|---|---|
|  | $^{0,2}A_{Man}$ | | | | |
|  | C | 827.27 | 827.2 | Y | Y |
|  | $^{2,4}A_{GlcNAc}$ | 869.28 | 869.3 | Y | Y |
|  | $^{1,5}X_{Man}Y$ | 896.29 | 896.3 | N | Y |
|  | $^{0,2}A_{GlcNAc}$ | 929.3 | 929.3 | Y | Y |
|  | YZ<br><br>ZY | 1012.34 | 1012.3 | Y | Y |
|  | $[M-H]^-$<br><br>YY | 1030.35 | 1030.4 | Y | Y |
|  | $^{0,2}A_{Man}Y$ | 1091.35 | 1091.4 | Y | N |
|  | $^{2,4}X_{Man}Y$<br><br>$Y^{1,3}X_{Man}$ | 1294.43 | 1294.6 | Y | Y |

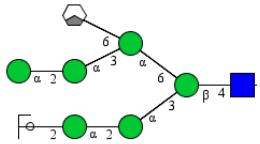
| | | | | | |
|---|---|---|---|---|---|
|  | | | | | |

**Table S-2.** Glycoproteins tested with WChTv and their *N*-linked oligosaccharide structures.

| **Glycoprotein** | **Source Organism** | **Most Prevalent *N*-linked Oligosaccharides** |
|---|---|---|
| Fetuin | *B. taurus* | Bi-, tri- and tetra-antennary complex |
| RNase B | *B. taurus* | High mannose |
| Ovalbumin | *G. gallus* | High mannose, hybrid, bisecting GlcNAc |
| IgG | *B. taurus* | Biantennary complex, bisecting GlcNAc, α(1,6)-linked fucose |
| Invertase | *S. cerevisiae* | High mannose, yeast-type oligomannose |