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Mutational studies on endo-β-*N*-acetylglucosaminidase D which hydrolyzes core portion of asparagine-linked complex type oligosaccharides

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Endo-β-*N*-acetylglucosaminidase D (Endo D) produced by *Streptococcus pneumoniae* hydrolyzes the di-*N*-acetylchitobiose structure in the core of complex-type asparagine-linked oligosaccharides, and has a molecular weight of 180 kDa. A truncated Endo D of 102 kDa in which 134 N-terminal amino acids and 599 C-terminal amino acids were deleted, still retained the enzymatic activity. The truncated Endo D has specificity indistinguishable from the intact enzyme, and also acted on the core structure of asparagine-linked oligosaccharides attached to intact IgG. Because of its lower molecular weight, the truncated enzyme may be useful as a tool for protein deglycosylation. The entire region of the truncated Endo D had 32% sequence identity to endo-β-*N*-acetylglucosaminidase BH (Endo BH) from *Bacillus halodurans*, which acted on high-mannose type oligosaccharides. Chimeric constructs of the truncated Endo D and Endo BH showed no activity. Glutamic acid 324 (E 324) in Endo D is conserved in Endo BH and Endo M, and is an essential amino acid in Endo M. Mutation of E324 abolished Endo D activity. The specificity of Endo D for complex type oligosaccharides is probably defined by multiple domains in the Endo D structure.
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Abbreviations: **DE: delayed extraction; Endo: endo-β-*N*-acetylglucosaminidase; LC: liquid chromatography; MS: mass spectrometry; MALDI-TOF: matrix-assisted laser desorption ionization-time of flight; PBS: Dulbecco's phosphate buffered saline.**

Introduction

Asparagine-linked oligosaccharides are classified into three types, complex type with terminal sialyl-galactosyl-*N*-acetylglucosamine chains, high-mannose type with exposed larger clusters of mannose residues, and hybrid type [1]. Endo-β-*N*-acetylglucosaminidases, which cleave the di-*N*-acetylchitobiose structure in the core of asparagine-linked oligosaccharides, are important tools in glycoprotein research [2,3]. Endo-β-*N*-acetylglucosaminidases have different substrate specificities especially with respect to the core structure of asparagine-linked oligosaccharides. Endo-β-*N*-acetylglucosaminidase D (Endo D) from *Streptococcus pneumoniae* hydrolyzes the core of complex type oligosaccharides, but does not act on most high-mannose oligosaccharides [4,5]. On the other hand, endo-β-*N*-acetylglucosaminidase H from *Streptomyces plicatus* (Endo H) [6] and endo-β-*N*-acetylglucosaminidase A from *Arthrobacter protophormiae* (Endo A) [7] hydrolyze high-mannose but not complex-type oligosaccharides.

Structurally, endo-β-*N*-acetylglucosaminidases are classified into two groups. Endo D and Endo A belong to one group, while Endo H belongs to the other [5,7–9]. We have recently cloned Endo D and expressed it in *Escherichia coli* [5]. Endo D can remove complex type oligosaccharides from intact glycoproteins in collaboration with neuraminidase, β-galactosidase and β-*N*-acetylglucosaminidase in the absence of detergent

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[10,11]. Although *N*-glycanase is effective in removing most asparagine-linked oligosaccharides, it frequently requires the presence of detergent for efficient action [12,13]. The aim of the present investigation is to reveal the active domain of Endo D. Since Endo D is the first cloned enzyme with the above-mentioned specificity [5], such information is expected to be valuable in using the enzyme for glycoprotein research: an enzyme with altered specificity or a lower molecular weight will be especially helpful.

Materials and methods

Materials

$(Man)_5(GlcNAc)_2Asn$ and $(Man)_6(GlcNAc)_2Asn$, components of ovalbumin glycopeptides [14], were provided by Seikagaku Kogyo (Tokyo, Japan). Bovine IgG glycopeptides were prepared as reported [4]. [^{14}C]-acetylation of the glycopeptides was performed as reported [4] using [^{14}C] acetic anhydride (4.37 GBq/mmol, Amersham Biosciences, Piscataway, NJ, USA or 266.4 MBq/mmol, NEN ™ Life Science Products, Boston, MA, USA). β-Galactosidase [15] and β-*N*-acetylglucosaminidase [16] from *S. pneumoniae* were purchased from PROzyme (San Leandro, CA, USA). Bovine IgG was from Sigma-Aldrich (Milwaukee, WI, USA).

Assay of enzymatic activity

Enzyme preparations were incubated with [^{14}C]-acetylated glycopeptides, and the amount of [^{14}C]-acetylated Asn-GlcNAc or [^{14}C]-acetylated FucGlcNAc-peptide released was measured as the unabsorbed fraction upon concanavalin A-Sepharose column chromatography as described previously [17]. One unit of the enzyme is defined as the amount of the enzyme which releases 1 μmole of the product per minute.

Analysis of glycopeptides by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF/MS)

Glycopeptides were dissolved in pure H_2O (18.2 MΩ/cm) at a concentration of 100–1000 μM, and 1 μl of the solution was mixed with 1 μl of pure H_2O saturated with 2,5-dihydroxybenzoic acid. Then, 1 μl of the mixture was applied onto the sample plate and allowed to evaporate in air. Next, glycopeptides were subjected to MALDI-TOF/MS with Voyager-DE™ Elite (Applied Biosystems, Foster City, CA, USA). In the positive ion selection mode with delayed extraction (DE) and a reflector, the accelerating voltage, DE time and laser intensity were set at 20 kV, 70 nsec and 2500, respectively. In the negative ion selection mode with DE and a reflector, the accelerating voltage, DE time and laser intensity were set at 20 kV, 70 nsec and 2500, respectively.

Liquid chromatography/mass spectrometry (LC/MS) of oligosaccharides and glycopeptides

The mixture of oligosaccharides and glycopeptides was separated and analyzed quantitatively with LC/MS using a MAGIC 2002™ HPLC system (Michrom Bioresources, Inc., Auburn, CA, USA) coupled to an API 300 LC/MS/MS system (Applied Biosystems) equipped with an ESI source. The solution containing oligosaccharides and glycopeptides (10 μl) was subjected to reversed-phase HPLC with a C4 column (Michrom Bioresources). The glycopeptides and oligosaccharides were separated with a linear gradient of 2–60% acetonitrile containing 0.1% trifluoroacetic acid, over 30 min at a flow rate of 50 μl/min, with monitoring at 214 nm. Then oligosaccharides and glycopeptides were detected with the API 300 LC/MS/MS system in the positive ion selection mode. The sprayer voltage, deflector voltage, and multiplier voltage were set at 5000 V, −250 V, and 2100 V, respectively.

Preparation and identification of the core portion of bovine IgG glycopeptides

Purified bovine IgG glycopeptides (100 μg) were digested with 2.38 mU of β-galactosidase from *S. pneumoniae* in 50 μl of 25 mM Tris-HCl buffer, pH 6.8, at 37°C for 15 h. Then the digest was treated with 22.5 mU of β-*N*-acetylglucosaminidase at 37°C for 15 h. The product was purified by Sephadex G-50 column chromatography using 25 mM Tris –HCl buffer, PH 6.8 as the eluate. MALDI-TOF/MS analysis of intact glycopeptides (Figure 1A) and those treated with glycosidases (Figure 1B and C) was performed. The analysis identified the final product as $(Man)_3(GlcNAc)_2$Fuc-Asn-Ser-Thr, molecular weight of which is 1358 Da: in positive mode two peaks, $[M + Na]^+$ and $[M + 2Na]^+$ were observed, and in the negative mode a peak of $[M–H]^-$ was found (Figure 1C). In support of this conclusion, the intact glycopeptide gave spectra indicating a mixture of $Gal(GlcNAc)_2(Man)_3(GlcNAc)_2$Fuc-Asn-Ser-Thr (molecular weight,1926 Da) and $(Gal)_2(GlcNAc)_2(Man)_3(GlcNAc)_2$Fuc-Asn-Ser-Thr (2088 Da) (Figure 1A). Upon Endo D treatment, the core glycopeptide, $(Man)_3(GlcNAc)_2$Fuc-Asn-Ser-Thr, gave peaks corresponding to $(Man)_3$GlcNAc (707 Da) and GlcNAc-Fuc-Asn-Ser-Thr (669 Da) (Figure 1D). The Former peak was observed only in the positive mode.

Cloning of Endo BH gene from *Bacillus halodurans*

Endo BH gene was amplified using a PCR method with Pyrobest® DNA Polymerase (TaKaRa Biochemical, Berkeley, CA, USA) and the following conditions and primers: 30 cycles of 98°C for 10 s, 55°C for 5 min and 72°C for 3 min, followed by 2 min at 98°C, 5′ primer 5′GCACTCGA GATGAAAAAAGGTAGTGTC3′, 3′ primer 5′TGTTCTAG ATTCGTGCCGCTTGATCG3′. Genomic DNA of *B. halodurans* strain C-125 (Japan Collection of Microorganisms, The Institute of Physical and Chemical Research, Saitama,

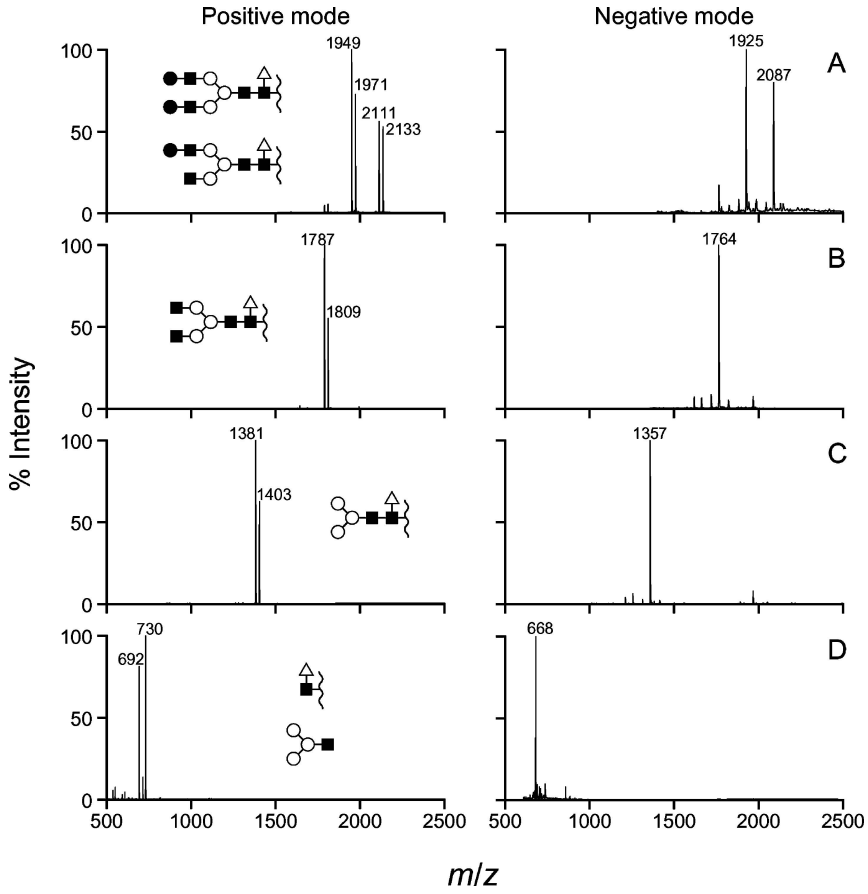

Figure 1. Characterization of bovine IgG glycopeptides by MALDI-TOF/MS in positive ion selection mode (left) and negative ion selection mode (right). A, intact glycopeptides; B, glycopeptides after β-galactosidase digestion; C, glycopeptides after digestion with β-galactosidase and β-*N*-acetylglucosaminidase; D, Endo D digestion products of IgG glycopeptides treated with β-galactosidase and β-*N*-acetylglucosaminidase. Estimated structures were schematically shown: closed square, *N*-acetylglucosamine; open triangle, fucose; closed circle, galactose; open circle, mannose; wavy line, Asn-Ser-Thr.

Japan) was used as a template. The amplified DNA was digested with the restriction endonucleases *Xho*I and *Xba*I and was subcloned into the expression vector pBAD/gIII A (Invitrogen, Carlsbad, CA, USA), which was designed for regulated, secreted expression of recombinant proteins containing C-terminal *Myc* epitope and 6 × histidine Tags. The sequences were verified with an ABI PRISM® 377 DNA Sequencer or ABI PRISM®310 Genetic Analyzer (Applied Biosystems) after subcloning.

Mutagenesis and production of mutated or chimeric proteins of Endo D

Nested deletion sets of Endo D were created with the PCR method, utilizing Pyrobest®DNA Polymerase and a full-length Endo D construct as the template under the following conditions; 30 cycles of 98°C for 10 s, 55°C for 5 s, and 72°C for optimal length (1 min per kbp) followed by 2 min at 98°C. Point mutations were introduced with a modified version of the heterodimeric PCR method. The same method was applied to generate the chimeric proteins. First, the former and latter fragments of the coding region were amplified with the mutagenic primers under the conditions described above. Then two fragments were annealed and a full-length version of the coding region was constructed with 10 cycles of 98°C for 10 s, 55°C for 5 s, and 72°C for 2 min followed by 2 min at 98°C. Finally, the entire coding region was amplified under the same conditions. Then, all the mutants were digested with suitable endonucleases and subcloned into the expression vector pBAD/gIII A. The sequences were confirmed by DNA sequencing.

Overexpression and purification of Endo BH and Endo D mutants

After subcloning into the vector, the recombinant clones were transformed into *E. coli* strain TOP10 (*ara*). The bacteria were cultured at 37°C in 200 ml of LB containing 50 mg/l of ampicillin until the optical density at 600 nm reached 0.5. Expression of the recombinant enzymes was induced by adding 0.002% of *L*-arabinose to the medium. After 4 to 5 h, cells were collected by centrifugation at 1,400 × *g* for 30 min at 4°C, resuspended in Dulbecco's phosphate-buffered saline

(PBS) containing 1% polyethylene glycol p-isooctylphenyl ether, and disrupted by sonication. After centrifugation at 20,000 × *g* for 30 min at 4°C, the supernatant was applied to a HiTrap ™ Chelating column conjugated with Ni^{++}(Amersham Biosciences AB). The recombinant enzymes were eluted with 300 mM of imidazole. All the mutants were detected as single or double bands upon SDS-PAGE followed by staining with Coomassie brilliant blue R-250.

Action of Endo D or its derivative on IgG

Bovine IgG (3 mg) was digested with 23.8 mU of β-galactosidase and 22.5 mU of β-*N*-acetylglucosaminidase in 100 μl of 25 mM Tris-HCl buffer, pH 6.8, at 37°C for 15 h. A 10 μl aliquot of the reaction mixture was incubated with Endo D or its derivative at 37°C for 1 h. After inactivation of Endo D by heating at 60°C, the product was analyzed by SDS-PAGE or trypsin digestion followed by LC/MS. For the latter, 3 μg of trypsin and 90 μl of 25 mM Tris-HCl, pH 6.8 containing 1 mM $CaCl_2$was added to the reaction mixture. After incubation at 37°C for 15 h, the product was analyzed by LC/MS.

The amounts of an oligosaccharide and peptides were determined from the area of the mass chromatogram. For calibration, purified $(Man)_3(GlcNAc)_2$was used as a standard for the oligosaccharide product. The peak derived from the trypsin digest of IgG treated with β-galactosidase and

	Man3	Man5	Man6	GlcNAc2-Man3	IgGGP
Native Endo D	6.5	5.4	N.D.	N.D.	N.D.
Native Endo BH	N.D.	2.2	1.4	N.D.	N.D.
3' nested deletion mutants					
-603 (201)	6.9	6.6	N.D.	N.D.	N.D.
-1203 (401)	5.7	5.8	N.D.	N.D.	N.D.
-1797 (599)	4.1	2.6	N.D.	N.D.	N.D.
-1950 (650)	N.D.	N.D.	N.D.	N.D.	N.D.
-2247 (749)	N.D.	N.D.	N.D.	N.D.	N.D.
-2400 (800)	N.D.	N.D.	N.D.	N.D.	N.D.
-2700 (900)	N.D.	N.D.	N.D.	N.D.	N.D.
-2721 (907)	N.D.	N.D.	N.D.	N.D.	N.D.
-3000(1000)	N.D.	N.D.	N.D.	N.D.	N.D.
5' nested deletion mutants					
-204 (68)	5.6	5.5	N.D.	N.D.	N.D.
-402 (134)	5.7	2.8	N.D.	N.D.	N.D.
-600 (200)	N.D.	N.D.	N.D.	N.D.	N.D.
-750 (250)	N.D.	N.D.	N.D.	N.D.	N.D.
-900 (300)	N.D.	N.D.	N.D.	N.D.	N.D.
Chimeric mutants					
D 1-719 + BH 315-2637	N.D.	N.D.	N.D.	N.D.	N.D.
D 1-773 + BH 390-2637	N.D.	N.D.	N.D.	N.D.	N.D.
BH 1-326 + D 733-773 + BH 315-2639	N.D.	N.D.	N.D.	N.D.	N.D.
Partial deletion mutants					
Δ304-326	N.D.	N.D.	N.D.	N.D.	N.D.
Δ304-361	N.D.	N.D.	N.D.	N.D.	N.D.
Point mutants					
V260A	0.67	0.55	N.D.	N.D.	N.D.
V269,271A	0.069	0.048	N.D.	N.D.	N.D.
Y316,319N, F320S	0.11	<0.001	N.D.	N.D.	N.D.
W359G, Y360N	N.D.	N.D.	N.D.	N.D.	N.D.
Y314N	Insoluble				
G318A	Insoluble				
Y319N	Insoluble				
F320S	Insoluble				
N322D	1.1	2.9	N.D.	N.D.	N.D.
E324A	N.D.	N.D.	N.D.	N.D.	N.D.
T325I	1.0	2.7	N.D.	N.D.	N.D.
W359G	Insoluble				
D361A	Insoluble				

Figure 2. Activities of mutated Endo D. Results are shown by units/ mg protein. N.D., less than 0.005 units/ mg protein. In deletion mutants, numbers represent the DNA base pairs deleted and numbers in parentheses represent the amino acids deleted. 5′-deletion mutants were formed based on a mutant devoid of 599 C-terminal amino acids. Schematic drawing for truncated mutants of Endo D are shown beside the name of each variant. In chimeric mutants, numbers indicate nucleotide number. In partial deletion and point mutants numbers indicate amino acids. In point mutants, amino acids in the left were mutated to those in the right. Insoluble indicates that derivatives were not soluble after extraction. $(Man)_3$, $(Man)_3$ Fuc $(GlcNAc)_2$-peptide; $(Man)_5$, $(Man)_5(GlcNAc)_2$Asn; $(Man)_6$, $(Man)_6(GlcNAc)_2$Asn; $(GlcNAc)_2$ $(Man)_3$; $(GlcNAc)_2$ $(Man)_3$Fuc$(GlcNAc)_2$-peptide; IgGGP, IgG glycopeptides. Structure of each substrate is shown on the first row. Closed square, *N*-acetylglucosamine; open triangle, fucose; closed circle, galactose; open circle, mannose; wavy line, peptide or Asn.

β-*N*-acetylglucosaminidase was used as the standard for the $(Man)_3$Fuc $(GlcNAc)_2$-peptide. The amount of FucGlcNAc-peptide in the fully digested sample was assumed to be identical to the amount of $(Man)_3$GlcNAc released and was utilized as the standard for the estimation of FucGlcNAc-peptide.

Results

Truncated Endo D

We deleted the C-terminal portion of Endo D in a stepwise fashion and found that even after deletion of 599 amino acids, substantial activity of Endo D was preserved (Figure 2). The removal of 650 C-terminal amino acids resulted in a loss of activity. Then, the *N*-terminal region was deleted from the C-terminal truncated enzyme; the deletion of 134 amino acids did not affect the activity, while the deletion of 200 amino acids inactivated the enzyme. Therefore, truncated Endo D of molecular weight 102 kDa was found to be active. The truncated Endo D acted on both $(Man)_5(GlcNAc)_2$Asn and $(Man)_3(GlcNAc)_2$Asn as in the case of the full-length Endo D. The specific activity of the truncated enzyme was comparable to that of the full-length enzyme (Figure 2).

Specificity of recombinant Endo BH and attempts to produce Endo D/BH chimeric enzyme

We isolated a genomic clone of Endo BH from *B. halodurans*, since the protein sequence predicted from its genome sequence showed high homology to large segments of Endo D. This point has been noted previously [5,18]. Genomic sequence of Endo BH was identical to that in the database. The protein sequence of Endo BH deduced from the DNA sequence had 32% homology to Endo D over 878 amino acids. Endo BH with a polyhistidine tag was expressed in *E. coli* and purified to homogeneity (Figure 3). Its apparent molecular weight (99 kDa) was in good agreement with the predicted value. The expressed Endo BH hydrolyzed $(Man)_6(GlcNAc)_2$Asn and $(Man)_5(GlcNAc)_2$Asn, but not IgG glycopeptides or its core glycopeptide, $(Man)_3$ Fuc $(GlcNAc)_2$-peptide (Figure 2). Therefore its specificity was identical to that of Endo A from *A. protophormiae* [7]. Indeed, Endo BH and Endo A have 50% identity over the whole sequence of Endo A [18]. Because of the presence of extensive homology between Endo D and Endo BH and distinct specificity of each enzyme we tried to produce a chimeric enzyme comprising Endo D and Endo BH (Figure 2). However, the effort was unsuccessful, and the whole chimeric proteins were inactive.

Identification of amino acids required for activity of Endo D

Comparing Endo D with Endo A and Endo BH, many amino acids were found to be conserved and some of the amino acids are essential for Endo A activity [18] (Figure 4). We deleted some of them to clarify their role in the enzymatic activity (Figure 2). Point mutation of E324 resulted in loss of the enzymatic activity. Double mutation of W359 and Y360 also resulted in a loss of activity; each single mutation caused the formation of insoluble protein. Other mutations resulted in a partial inactivation of the enzyme. Interestingly, triple mutation of Y316, Y319 and F320 completely removed the activity for $(Man)_5(GlcNAc)_2$Asn, while a low level of activity towards $(Man)_3(GlcNAc)_2$Asn remained (Figure 2).

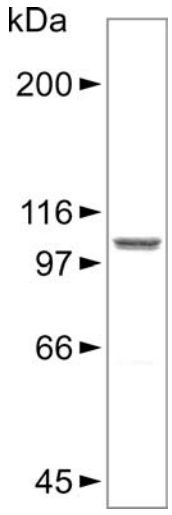

Figure 3. SDS-PAGE of purified Endo BH. Endo BH was electrophoresed in 6% denaturing polyacrylamide gel with molecular weight markers and stained with Coomassie brilliant blue.

Action of the truncated Endo D on IgG

Since the truncated Endo D of 102 kDa might be easier to produce and purify than the full-length 180 kDa enzyme, we examined whether the truncated form acts on IgG treated with β-galactosidase and β-*N*-acetylglucosaminidase. The action of Endo D was stopped by heating at 60°C for 5 min, since Endo D is a heat labile enzyme (Figure 5). To determine the degree of action of Endo D, IgG was digested with trypsin, and the product was analyzed by LC/MS. Among many peaks detected, 3 represent the released oligosaccharide and peptide containing the N-glycan binding site (Figure 6). The tetrasaccharide $(Man)_3$ GlcNAc (707 Da) was eluted with 5% acetonitrile and detected as a peak at 730 m/z $[M + Na]^+$. The peptide containing the N-glycan binding site was eluted separately, depending on the structure of N-glycans. The peptide (EEQFNSTYR) conjugated with the $(Man)_3$ Fuc $(GlcNAc)_2$ structure (2209 Da) was eluted with 55% acetonitrile and detected as a peak at 1105.5 m/z $[M + 2H]^{2+}$, while the peptide with the FucGlcNAc structure (1520 Da) was eluted with 40% acetonitrile and detected as the peak at 761 m/z $[M + 2H]^{2+}$.

We found that Endo D and truncated Endo D acted with similar activity against IgG treated with β-galactosidase and β-*N*-acetylglucosaminidase by measuring either the release of $(Man)_3$GlcNAc, production of FucGlcNAc-peptide or decrease of $(Man)_3$Fuc $(GlcNAc)_2$-peptide (Figure 7).

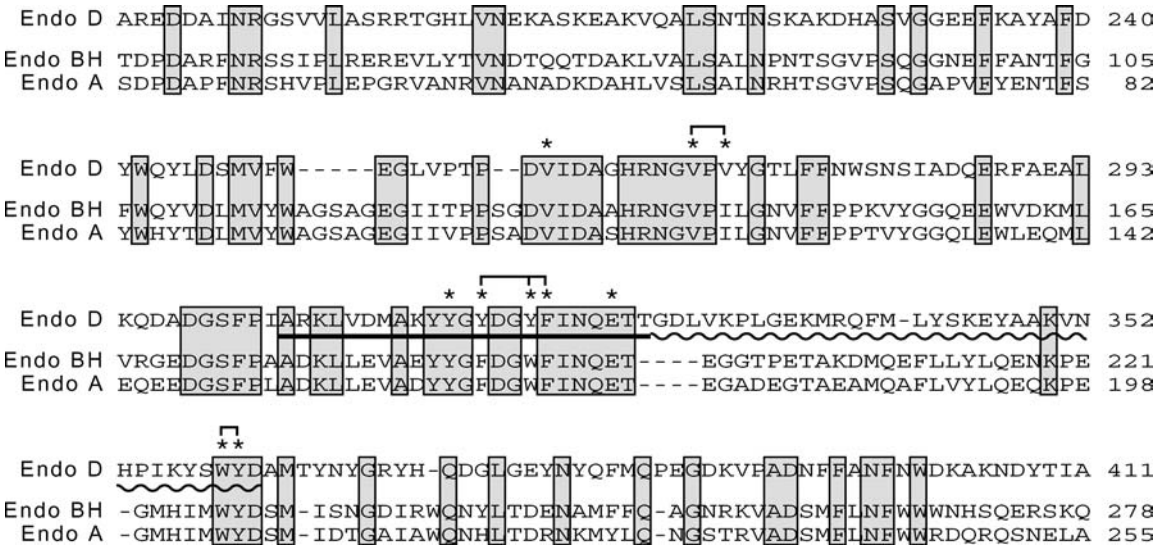

Figure 4. Multiple alignment of Endo D amino acids. The amino acid sequence of Endo D is compared with other endo-β-*N*-acetylglucosaminidases. Conserved residues are shaded. Asterisks indicate amino acids selected for site-directed mutagenesis. Sequences underlined by a continuous line and by a wavy line indicate amino acids deleted in partial deletion mutant Δ304–326 and in partial deletion mutant Δ304–361 (Figure 2), respectively.

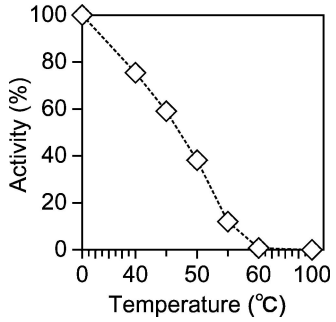

Figure 5. Heat stability of Endo D. Endo D was treated at the indicated temperature for 5 min. The activity is indicated as the percentage of the activity remained after the treatment.

Discussion

The truncated Endo D of 102 kDa was found to retain the activity to act on the $(Man)_3$ Fuc $(GlcNAc)_2$structure. Therefore the other portion of the enzyme, especially the C-terminally located long segment of 60 kDa, was not essential for the enzymatic activity and is expected to play other roles. Indeed, the LPXTG motif near the C-terminal end of the enzyme is also present in β-galactosidase and β-*N*-acetylglucosaminidase produced by the bacteria [19,20] and is likely to be involved in anchoring the enzymes to the surface of the bacteria.

The truncated Endo D is comparable in size to other structurally related enzymes such as Endo A and Endo BH. However, Endo A and Endo BH act on high-mannose oligosaccharides, but not on the core portion of complex-type oligosaccharides. We tried to pinpoint the structure that is essential for determining the substrate specificity. However, none of the chimeric constructs of Endo D and Endo BH was active, and point mutation did not convert Endo D to an enzyme with specificity similar to that of Endo A. The three dimensional conformation of the truncated Endo D may need to be deduced to reveal the structure involved in the specificity. On the other hand, E324

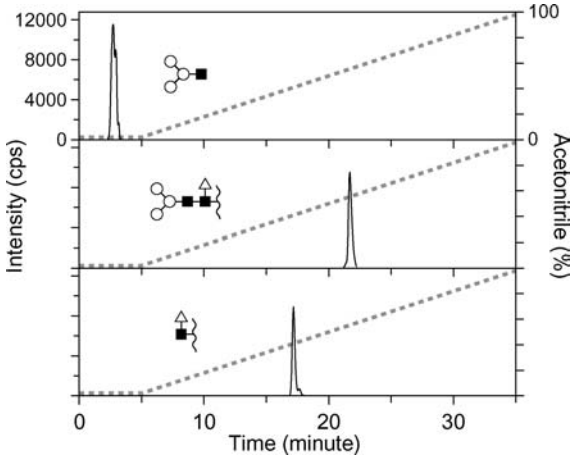

Figure 6. Elution profiles of tetrasaccharides and glycopeptides produced by Endo D digestion of exoglycosidase-treated IgG. Tetrasaccharide $(Man)_3$-GlcNAc (upper column); peptide conjugated with $(Man)_3$-Fuc$(GlcNAc)_2$ (middle column); peptide conjugated with FucGlcNAc (lower column). The products were digested with trypsin, applied to HPLC and analyzed by MS as described in Materials and Methods, except that 2–98 % gradient of acetonitrile was used to show the overall picture of separation. Estimated structures were schematically shown: closed square, *N*-acetylglucosamine; open triangle, fucose; open circle, mannose; wavy line, peptide.

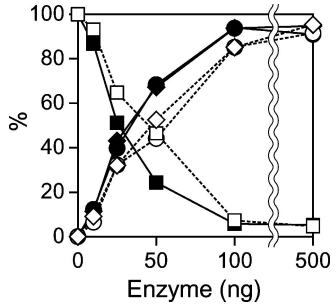

Figure 7. Action of intact (solid line with closed marks) and truncated (dashed line with open marks) Endo D to 2nM of IgG treated with β-galactosidase and β-*N*-acetylglucosaminidase. Circles, amount of released $(Man)_3$ GlcNAc; diamonds, amount of FucGlcNAc bound to protein; squares, amount of $(Man)_3$ $Fuc(GlcNAc)_2$ bound to protein. The amounts of oligosaccharides and those bound to protein were determined by LC/MS after trypsin digestion. Results are shown by percent to an asparagine-linked oligosaccharide chain in IgG.

was found to be essential for the activity of Endo D; this residue is conserved in Endo A and BH, and is involved in the activity of Endo A [18]. However, mutation of V260, which corresponds to V109 necessary for the activity of Endo A, only reduced the activity of Endo D.

The mutation of Y316, 319N,F320S is an interesting one, since the activity toward $(Man)_3Fuc$ $(GlcNAc)_2$peptide was reduced but still significant, but the activity directed at $(Man)_5$ $(GlcNAc)_2$Asn was almost completely lost. Possibly, the structure around the substrate binding site was mutated, and the bulky $(Man)_5$cluster cannot be contained in the space.

Finally, the truncated Endo D was effective in removing oligosaccharides from intact IgG pretreated with β-galactosidase and β-*N*-acetylglucosaminidase. Since the truncated enzyme was easier to produce and purify, it will be an effective tool for the functional modification of glycoproteins.

Acknowledgments

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