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Note

Mucor hiemalis endo-β-*N*-acetylglucosaminidase can transglycosylate a bisecting hybrid-type oligosaccharide from an ovalbumin glycopeptide

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Abstract—We found that the recombinant *endo*-β-*N*-acetylglucosaminidase of *Mucor hiemalis* (Endo-M) expressed in *Candida boidinii* had the transglycosylation activity of transferring a bisecting hybrid-type oligosaccharide from an ovalbumin glycopeptide to the acceptor (*p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside) in a good yield of 43%.

Keywords: *endo*-β-*N*-Acetylglucosaminidase; Endo-M; Transglycosylation; Bisecting hybrid-type oligosaccharide

Although the addition of an oligosaccharide to a substance is one of the important objectives in glycotechnology, no effective procedure is available. Recently, a practical method for adding an oligosaccharide to a peptide to produce a glycopeptide has been developed using the transglycosylation activity of *endo*-β-*N*-acetylglucosaminidases from *Mucor hiemalis* (Endo-M)[1] and from *Arthrobacter protophormiae* (Endo-A).[2] Haneda et al. have successfully prepared several biologically important neoglycopeptides by adding an oligosaccharide from glycopeptides or glycoproteins to chemically synthesized peptides using the transglycosylation activity of Endo-M.[3–5] This innovative chemoenzymatic method improved the synthetic procedure of novel neoglycopeptides. Following these results, our recent studies showed that the recombinant Endo-M expressed in *Candida boidinii* was stable in the presence of organic solvents such as acetone, methanol, and dimethyl sulfoxide, and it did not lose its transglycosylation activity. Therefore, any compounds, which are sparingly soluble in water, could be utilized as glycosyl acceptors.[6] We also found that a variety of sugar derivatives modified at C-1 or C-2 could be used as the transglycosylation acceptors of Endo-M.[7] These findings should permit the syntheses of many novel neoglycoconjugates.

Endo-M hydrolyzes the glycosidic bond in the *N*,*N*′-diacetylchitobiose moiety of asparagine-linked oligosaccharides bound to peptides or proteins. Its substrate specificity for hydrolysis has been clarified in detail.[8] The enzyme can hydrolyze various kinds of high-mannose type, complex-type, and hybrid-type oligosaccharides. The hydrolysis activity for the high-mannose type oligosaccharides is significantly higher than that for the complex- and hybrid-type oligosaccharides. On the other hand, the substrate specificity of the enzyme for transglycosylation has not been completely elucidated. It is only known that the enzyme can transfer sialo and asialo biantennary complex-type and high-mannose type

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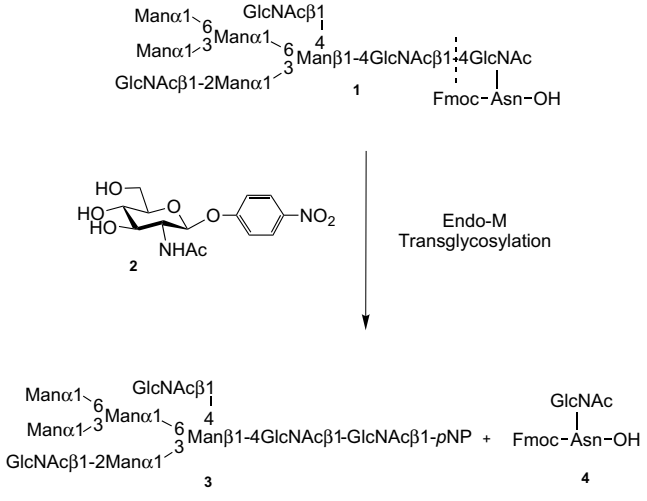
oligosaccharides, and these biantennary complex types are more effective as the oligosaccharide donor than the high-mannose type, contrary to its hydrolysis activity.[9] The transglycosylation activity of Endo-M for the hybrid-type oligosaccharides has not yet been determined.

Hybrid-type oligosaccharides are known to participate in several biological phenomena. For example, Fukuda reported that the accumulation of hybrid-type oligosaccharides caused by an *N*-glycan processing deficiency was observed in the erythrocyte membranes of HEMPAS (hereditary erythroblastic multinuclearity with positive acidified serum lysis test).[10] Eskhardt et al. indicated that hybrid-type oligosaccharides worked as the receptors of *Clostridium botulinum* C2 toxin.[11] These findings may increase attention to the biological roles of hybrid-type oligosaccharides and the importance of developing an efficient chemoenzymatic method for synthesizing bioactive molecules containing hybrid-type oligosaccharides. However, no *endo*-β-*N*-acetylglucosaminidases are known to transglycosylate hybrid-type oligosaccharides.

In this study, in order to clarify the substrate specificity of Endo-M for oligosaccharide donors in transglycosylation reactions and to utilize transglycosylation in glycotechnology, we investigated the transglycosylation of Endo-M for a bisecting hybrid-type oligosaccharide from an ovalbumin glycopeptide.

We used the 9-fluorenylmethyloxycarbonylated (Fmoc) glycosylasparagine **1**[12] having a bisecting hybrid-type oligosaccharide as the glycosyl donor (the consumption of **1** could be easily monitored by HPLC analysis upon absorption at 254 nm), and *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (*p*NP-GlcNAc, **2**) as the glycosyl acceptor (Scheme 1). The transglycosylation reaction was carried out in a reaction



Scheme 1.

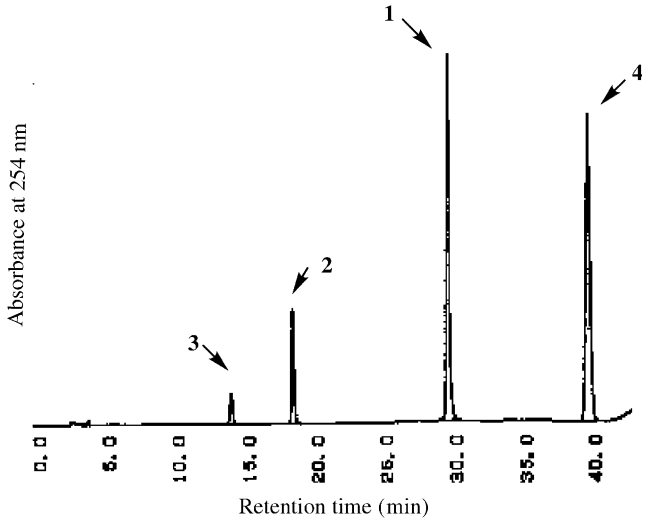


Figure 1. The HPLC profile of the reaction mixture of **1** and **2** incubated with Endo-M at 25 °C for 2 h. The reaction mixture was analyzed by HPLC on a reversed-phase column (Inertsil ODS-3, 4.6 × 250 mm). Elution was carried out with a linear gradient of 10–30% acetonitrile containing 0.1% aqueous TFA in 20 min, and subsequently with 30% acetonitrile containing 0.1% aq TFA at a flow rate of 1 mL/min.

mixture composed of 0.2 μmol of **1**, 0.1 μmol of **2**, and 1.48 mU of recombinant Endo-M in a total volume of 10 μL of 60 mM potassium phosphate buffer (pH 6.25). After incubation for 2 h at 25 °C, the reaction was terminated by the addition of 490 μL of 0.2% trifluoroacetic acid solution. HPLC analysis of the reaction mixture is shown in Figure 1. MALDI-TOF mass spectrometric analysis of the peak at a retention time of 14 min indicated the desired transglycosylation product **3** (**3**; found *m*/*z* [M+Na] 1784.2; calcd for $C_{68}H_{107}N_5O_{48}$ [M+Na] 1784.6). The peak indicating the hydrolysis product of **1** (Fmoc-Asn-GlcNAc, **4**) was also observed. Moreover, we found that the maximum yield of **3** reached about 40% after a 1 h incubation by HPLC analysis (data not shown).

We then attempted to obtain a large amount of **3** to measure its ^{1}H NMR spectra and compare it with the reported ^{1}H NMR data for hybrid-type asparagine-linked oligosaccharides.[13] After the reaction mixture of **1** (43.3 mg, 22 μmol) and **2** (3.7 mg, 11 μmol) was incubated with Endo-M (128 mU) in a total volume of 1 mL of 6 M potassium phosphate buffer (pH 6.25) at 25 °C for 1 h, **3** (8.1 mg, 43% yield) was isolated by HPLC. In the ^{1}H NMR spectrum of **3**, the anomeric protons of five mannosyl residues were observed at 4.89, 4.88, 4.76, 4.75, 4.58 ppm with singlet peaks, and the anomeric protons of four GlcNAc residues were observed at 5.15 (*J* 8.2 Hz), 4.44 (*J* 7.6 Hz), 4.36 (*J* 8.2 Hz), 4.24 (*J* 7.6 Hz) ppm as doublets, as shown in Figure 2. The values of the coupling constant of the GlcNAc residues indicated that the newly formed glycosidic bond by this enzymatic transglycosylation was β.

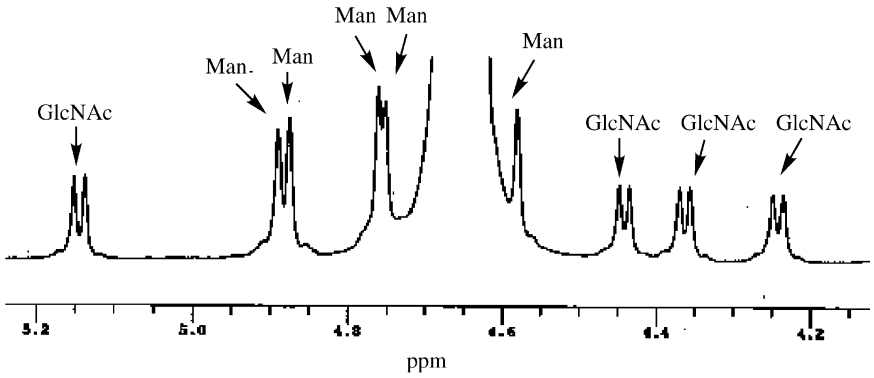


Figure 2. ^{1}H NMR spectrum of the H-1 signals of **3** in the range of 4.2–5.2 ppm. The ^{1}H NMR spectrum of **3** was measured at 600 MHz in D_2O at 24 °C. Water suppression was not used.

This is the first report that microbial *endo*-β-*N*-acetylglucosaminidase can transglycosylate a hybrid-type oligosaccharide of asparagine-linked sugar chains to an acceptor. This finding will increasingly improve the utilization of Endo-M for the syntheses of neoglycoconjugates.

1. Experimental

1.1. General methods

Recombinant Endo-M was obtained from the cell extract of *C. boidinii* (protease-deficient (pep4) strain).[14] A bisecting hybrid-type asparagine-linked oligosaccharide (**1**) was prepared from hen egg albumin according to the method described in ref. 12. *p*-Nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**2**) was purchased from the Seikagaku Corporation. All other chemicals were obtained from commercial sources and were of the highest grade available. Analyses or isolation of the transglycosylation product were done using HPLC (Shimadzu LC-10AT chromatograph equipped with a SPD-10A ultraviolet spectrophotometer) on a reversed-phase column (analysis: 4.6 × 250 mm; isolation: 20 × 250 mm, Inertsil ODS-3, GL Sciences Inc.). Elution was carried out with a linear gradient of acetonitrile (analysis: 10–30%; isolation: 20–30%) containing 0.1% aq trifluoromethanesulfonic acid (TFA) in 20 min (analysis) or 10 min (isolation), and subsequently with 30% acetonitrile containing 0.1% aq TFA at a flow rate of 1 mL/min (analysis) or 10 mL/min (isolation). The reaction products were monitored by absorption at 254 nm. Matrix-associated laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was performed in the positive-ion mode using 2,4,6-trihydroxyacetophenone as the matrix on a Voyager Biospectrometry Work-station (PerSeptive Biosystems, USA). The ^{1}H NMR spectrum was recorded in D_2O using a JEOL ACE-600.

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