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Note

Transglycosylation reaction of *Mucor hiemalis* endo-β-*N*-acetylglucosaminidase using sugar derivatives modified at C-1 or C-2 as oligosaccharide acceptors

Takashi Yamanoi,[a,*] Maki Tsutsumida,[a] Yoshiki Oda,[a] Eri Akaike,[a] Kenji Osumi,[a] Kenji Yamamoto[b] and Kiyotaka Fujita[b]

[a]*The Noguchi Institute, 1–8–1 Kaga, Itabashi-ku, Tokyo 173-0003, Japan*
[b]*Graduate School of Biostudies, Kyoto University, Kitashirakawa, Sakyo-ku, Kyoto 606-8502, Japan*

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Abstract—We investigated the transglycosylation reaction of the recombinant endo-β-*N*-acetylglucosaminidase from *Mucor hiemalis* (Endo-M) expressed in *Candida boidinii* using such sugar derivatives as N-acylated D-glucosamines, *C*-glucosyl derivatives, and a 2-O-glycosylated disaccharide as acceptors. We found that a variety of sugar derivatives modified at C-1 or C-2 could be used as acceptors for transglycosylation by Endo-M to create novel oligosaccharides.

Keywords: Endo-β-*N*-acetylglucosaminidase; Endo-M; Transglycosylation; N-acylated D-glucosamine; *C*-glucosyl derivative

1. Introduction

Although the addition of an oligosaccharide to an acceptor is important 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*-acetylglucosaminidase. Endo-β-*N*-acetylglucosaminidases hydrolyze the glycosidic bond in the *N,N′*-diacetylchitobiose moiety of N-linked oligosaccharides in glycoproteins and glycopeptides. In addition to hydrolysic activity, the enzyme of *Mucor hiemalis* (Endo-M) has transglycosylation activity, transferring both complex-type oligosaccharides and high mannose-type oligosaccharides of N-linked sugar chains from the glycopeptide to suitable acceptors having an *N*-acetylglucosamine residue.[1] Using the transglycosylation activity of Endo-M, Haneda et al. succeeded in adding oligosaccharides to chemically synthesized peptides having *N*-acetylglucosaminyl–asparagine or –glutamine residues. They chemoenzymatically synthesized biologically important neoglycopeptides having natural N-linked oligosaccharides, such as the glycopeptide derivatives of calcitonin,[2] substance P,[3] and peptide T.[4] This is the only effective method for synthesizing the glycopeptide, and Endo-M has proved an efficient tool for the construction of oligosaccharides.

Our recent studies showed that an enzymatic reaction using the recombinant Endo-M expressed in *Candida boidinii* proceeded in media containing 30% of such organic solvents as dimethyl sulfoxide (Me_2SO), methanol, and acetone.[5] This means that the recombinant Endo-M is stable in the presence of these organic solvents without losing its transglycosylation activity, and is applicable with compounds sparingly soluble in water, thus improving the utilization of Endo-M for syntheses of glycoconjugates. We then investigated the acceptor specificity of the transglycosylation activity of Endo-M, and attempted the possible synthesis of novel oligosaccharides and glycoconjugates.

* Corresponding author. Tel.: +81-3-5944-3213; fax: +81-3-5944-3213/3217; e-mail: tyama@noguchi.or.jp

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Several naturally occurring hexopyranoses and cyclohexanol derivatives have been investigated as transglycosylation acceptors. It was found that Endo-M transferred a sialo-complex-type oligosaccharide from the glycopeptide to D-glucopyranose and D-mannopyranose, as well as *N*-acetyl-D-glucosamine. Moreover, not only the β but also the α-anomers of these sugars served as good acceptors for the transglycosylation. However, the transglycosylation activity of Endo-M on D-xylopyranose was very low, and it did not transfer the oligosaccharide to such sugars as D-galactopyranose, L-fucopyranose, D-glucopyranosyluronic acid, cyclohexanol, and cyclohexane-1,2-diol. These results suggested that Endo-M strictly recognized the hydroxyl groups and their configurations at C-4 and C-6 of the sugar acceptors (C-4 having OH equatorial and C-6 being CH_2OH), while the configurations at C-1 and C-2 were scarcely recognized by Endo-M. Based on the assumption that Endo-M would have a transglycosylation activity for sugar derivatives of D-glucopyranose and D-glucosamine modified at C-1 or C-2, we investigated transglycosylation by the recombinant Endo-M on such artificial sugar derivatives as the N-acylated D-glucosamine and *C*-glucosyl derivatives, and on 2-O-glycosylated disaccharides (Scheme 1).

2. Results and discussion

We first examined the transglycosylation activity of Endo-M for various N-acylated D-glucosamine derivatives as acceptors for the oligosaccharide. 2-Benzamido-2-deoxy-D-glucopyranose (**1**), 2-deoxy-2-2′-naphthoamido-D-glucopyranose (**2**), 2-deoxy-2-phenoxyacetamido-D-glucopyranose (**3**), 2-benzyloxycarbonylamino-2-deoxy-D-glucopyranose (**4**), and 2-deoxy-2-phthalimido-D-glucopyranose (**5**) were used as the acceptors. The hen egg-yolk glycopeptide, H-Lys-Val-Ala-Asn[(NeuAc-Gal-GlcNAc-Man)$_2$-Man-GlcNAc$_2$]-Lys-Thr-OH (SGP), was used as the oligosaccharide donor. As shown in Table 1, the enzymatic reaction with 10 mM of **1** and 20 mM of SGP at 37 °C for 30 min gave the desired transglycosylation product in 24% yield, based on the total amount of **1** (entry 1). This corresponds closely to the yield (28%) of the reaction with *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (*p*NP-GlcNAc) as an acceptor under the same transglycosylation conditions, suggesting that the benzoyl group of **1** exerted little steric hindrance during the transglycosylation reaction. Next, we examined the transglycosylation reaction for compound **2**, having the more bulky *N*-2′-naphthoyl group. As compound **2** was poorly soluble in water, the reaction mixture was incubated in 30% Me_2SO solution (v/v). The yield of the transglycosylation product was 17%, showing that Endo-M had a significant transglycosylation activity for a sugar derivative having a naphthoyl group at C-2 (entry 2). We also investigated the transglycosylation activity of Endo-M for the N-acylated glucosamine derivatives (**3**–**5**) having the benzyloxycarbonyl and phthaloyl protective groups. The yields of the transglycosylation products for **3**, **4**, and **5** as the acceptors were 20%, 24%, and 28%, respectively, under incubation in water or 30% Me_2SO solution (v/v). Compounds **3**–**5** were thus suitable transglycosylation acceptors (entries 3–5).

To investigate the transglycosylation activity of Endo-M for sugar acceptors modified at C-2, we used the 2-O-glycosylated disaccharide 2-*O*-α-L-rhamnopyranosyl-β-D-gluopyranoside naringin, **6** as an acceptor. Although **6** was poorly soluble in water, the yield of the transglycosylation product reached 38% after 3 h of incubation in 30% Me_2SO solution (v/v) (entry 6). These results indicated that Endo-M had low recognition ability for functional groups at C-2 of the sugar acceptors, and the enzyme could transfer an oligosaccharide to a sugar acceptor irrespective of functional groups at C-2 of the acceptor.

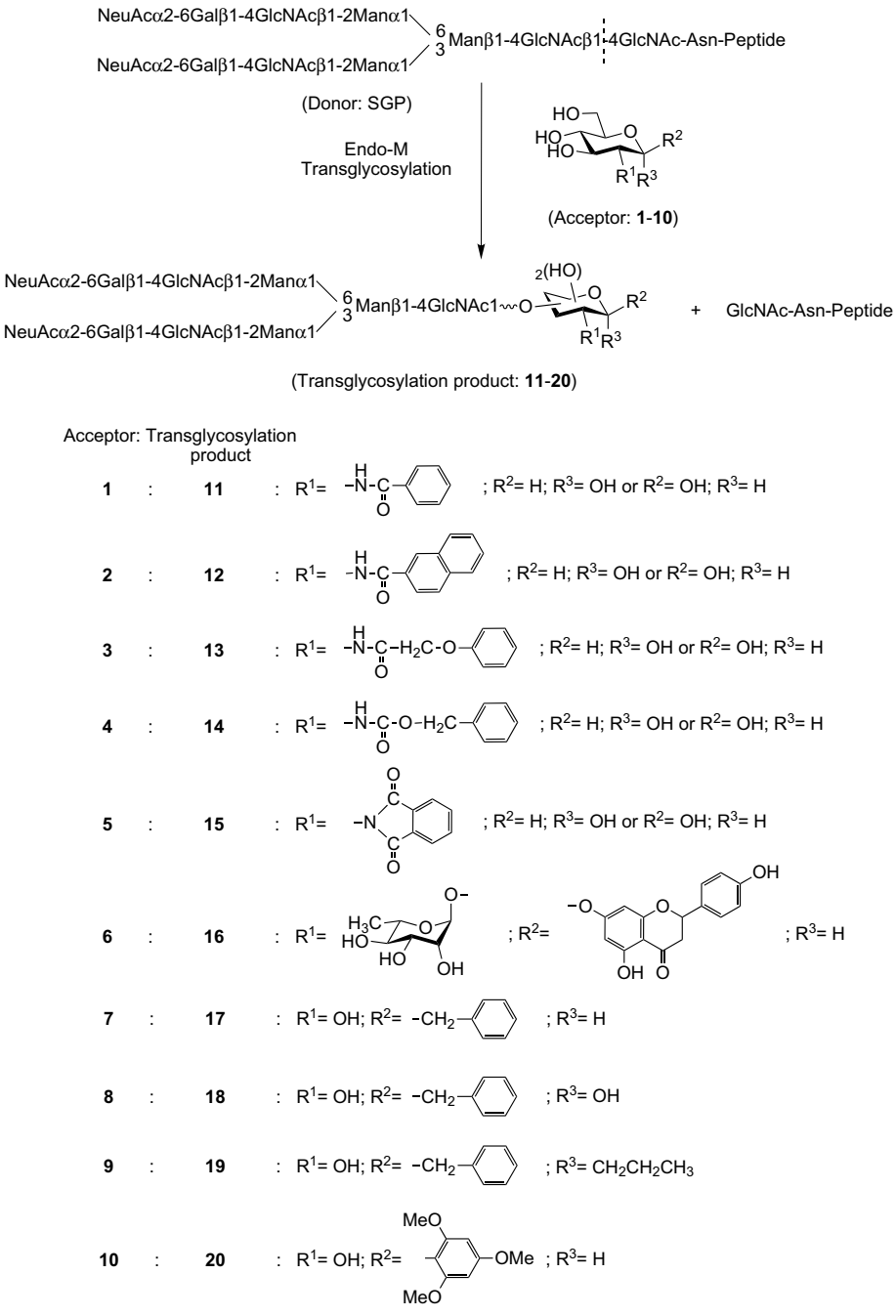
Finally, we investigated the transglycosylation reaction by Endo-M for *C*-glycosyl derivatives as acceptors, to determine whether Endo-M could transfer an oligosaccharide to sugar derivatives wherein the oxygen atom at C-1 is replaced by carbon. β-D-Glucopyranosyl-1-phenylmethane (**7**), 1-deoxy-1-phenyl-α-D-*gluco*-2-heptulopyranose (**8**), a *C,C*-dialkyl glucopyranose derivative (**9**), and 1-β-D-glucopyranosyl-2,3,4-trimethoxy benzene (**10**) were used as the acceptors. These *C*-glycosyl derivatives are mimetics of O-glycosides and are useful for monitoring the enzymatic reaction. The enzymatic reaction with 10 mM of **7** and 20 mM of SGP at 37 °C for 30 min gave the desired transglycosylation product in good yield (36%, entry 7). Although the transglycosylation reactions using the glucopyranosyl derivatives **8** and **10** as acceptors afforded the transglycosylation products in 13% yields, Endo-M could not transfer the oligosaccharide to the *C,C*-dialkyl glucopyranoside **9** (entries 8–10). These results showed that Endo-M had a transglycosylation activity for the mono-*C*-alkyl β-glucopyranosyl derivatives as acceptors. We confirmed the mass values of all the transglycosylation products **11**–**20** by MALDI-TOF mass spectrometric analysis, although we did not determine the positions of glycosylation.

Thus we found that a variety of useful sugar derivatives modified at C-1 or C-2 could be used as transglycosylation acceptors by Endo-M. These findings should permit chemo-enzymatic synthesis of many novel neoglycocojugates.

3. Experimental

3.1. Preparation of the enzyme

Recombinant Endo-M was obtained as previously reported from a cell extract of *Candida boidinii* (protease deficient (pep4) strain).[6]



Scheme 1.

3.2. Materials

The glycosyl donor, a sialoglycopeptide having a disialo biantennary complex-type oligosaccharide: H-Lys-Val-Ala-Asn[(NeuAc-Gal-GlcNAc-Man)$_2$-Man-GlcNAc$_2$]-Lys-Thr-OH, named SGP, was prepared from hen egg yolk according to the reported method.[7] *p*-Nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside and naringin (**6**) were purchased from the Seikagaku Corporation and Tokyo Kasei Kogyo Co., Ltd., respectively. 2-Benzamido-2-deoxy-D-glucopyranose (**1**),[8] 2-deoxy-2-phthalimido-D-glucopyranose (**5**),[9] 2-benzyloxycarbonylamino-2-deoxy-D-glucopyranose (**4**),[10] β-D-glucopyranosyl-1-phenylmethane (**7**),[11] 1-deoxy-1-phenyl-α-D-*gluco*-2-heptulopyranose (**8**),[12] 1-β-benzyl-1-deoxy-1-α-propyl-D-glucopyranoside (**9**),[13] and 1-β-D-glucopyranosyl-2,3,4-trimethoxybenzene (**10**)[14] were prepared by the reported methods. 2-Deoxy-2-2′-naphthoamido-D-glucopyranose (**2**) and 2-deoxy-2-phenoxyacetamido-D-glucopyranose (**3**) were prepared according to the method described in Ref. 11. All other chemicals were obtained from commercial sources and were of the highest grade available.

Table 1. Transglycosylation reaction by Endo-M using various sugar derivatives modified at C-1 or C-2 and the mass values of the transglycosylation products obtained

Entry[a]	Sugar acceptor	Incubation time (h)	Transglycosylation product	Transglycosylation yield (%)[b]	Found m/z $[M-H]^-$	Calcd for $[M-H]^-$
1	**1**	0.5	**11**	24	2282.86	2283.80
2[c]	**2**	3	**12**	17	2332.19	2333.81
3	**3**	0.5	**13**	20	2312.17	2313.81
4[c]	**4**	3	**14**	24	2312.02	2313.81
5	**5**	1	**15**	28	2307.65	2309.78
6[c]	**6**	3	**16**	38	2579.24	2580.87
7	**7**	0.5	**17**	36	2251.62	2254.81
8	**8**	2	**18**	13	2270.10	2270.80
9	**9**	3	**19**	0	—	—
10	**10**	1	**20**	13	2328.62	2330.82

[a]The incubation with the sugar derivative (10 mM) and SGP (20 mM) was carried out at 37 °C in potassium phosphate buffer (pH 6.25).
[b]Determined by HPLC.
[c]The enzymatic reaction was carried out in 30% Me_2SO solution (v/v) due to the poor water-solubility of the sugar acceptors.

3.3. Transglycosylation reaction with Endo-M

The transglycosylation reaction (at an analytical level) was performed with a mixture composed of 0.1 μmol of a sugar acceptor, 0.2 μmol of SGP, and 1.48 mU of Endo-M in a total volume of 10 μL of 60 mM potassium phosphate buffer (pH 6.25) or in 10 μL of the same buffer containing 30% of Me_2SO (v/v). After incubation for 0.5–3 h at 37 °C, the reaction was terminated by the addition of 490 μL of 0.2% trifluoroacetic acid (TFA) solution.

3.4. HPLC analysis

Analyses of the transglycosylation products were done using an HPLC (Shimadzu LC-10AT chromatograph equipped with a SPD-10A ultraviolet spectrophotometer) on a reversed-phase column (4.6 × 250 mm, Inertsil ODS-3, GL Sciences Inc.). Elution was carried out with a linear gradient of acetonitrile (entries 1 and 5: 5–25%; entries 3, 4, and 7–10: 10–30%; entry 2: 15–35%; entry 6: 20–40%) containing 0.1% aqueous TFA in 20 min at a flow rate of 1 mL/min. The reaction products were monitored by absorption at 254 nm (entries 1, 2, 5, 6, and 10), 220 nm (entry 3) and 214 nm (entries 4 and 7–9). The yields of the transglycosylation products were calculated from the ratio (%) of their peaks to the initial ones of the oligosaccharide acceptors. This was based on the assumption that the absorptivity of the transglycosylation products and that of the acceptors are approximately the same. Transglycosylation products were purified by HPLC.

3.5. Mass spectrometry

Matrix-associated laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was performed in the negative-ion mode using 2,4,6-trihydroxyacetophenone (THAP) or 2,5-dihydroxybenzoic acid, as the matrix on a Voyager Biospectrometry Work-station (PerSeptive Biosystems, USA).

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