Carbohydrate Research 344 (2009) 592–598

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Endo-β-*N*-acetylglucosaminidase-catalyzed polymerization of β-Glc*p*-(1→4)-Glc*p*NAc oxazoline: a revisit to enzymatic transglycosylation

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ARTICLE INFO

Article history:
Received 15 November 2008
Received in revised form 9 January 2009
Accepted 14 January 2009
Available online 19 January 2009

Keywords:
Transglycosylation
Enzymatic polymerization
Endoglycosidase
Carbohydrate oxazoline derivative
Glycopeptides

ABSTRACT

An alternative synthesis of β-Glc*p*-(1→4)-Glc*p*NAc oxazoline is described, and its enzymatic reaction with the endo-β-*N*-acetylglucosaminidase from *Arthrobacter protophormiae* (Endo-A) was re-investigated. Under normal transglycosylation conditions with a catalytic amount of enzyme, Endo-A showed only marginal activity for transglycosylation with the disaccharide oxazoline, consistent with our previous observations. However, when used in a relatively large quantity, Endo-A could promote the transglycosylation of the disaccharide oxazoline to a Glc*p*NAc-Asn acceptor. In addition to the initial transglycosylation product, a series of large oligosaccharides were also formed due to the tandem transglycosylation to the terminal glucose residues in the intermediate products. In the absence of an external acceptor, Endo-A could polymerize the disaccharide oxazoline to form oligo- and polysaccharides having the -4-β-(Glc*p*-(1→4)-β –Glc*p*NAc)-1—repeating units. This is the first example of an endo-β-*N*-acetylglucosaminidase-promoted polymerization of activated oligosaccharide substrates. This enzymatic polymerization may find useful applications for the synthesis of novel artificial polysaccharides.

1. Introduction

Enzymatic glycosylation has become a powerful method for the synthesis of polysaccharides and glycoconjugates. It has been demonstrated that some endoglycosidases can take activated glycosyl donors as substrates for transglycosylation and polymerization under kinetically controlled conditions.[1,2] For example, it has been shown that some family 18 chitinases and family 56 hyaluronidases are able to polymerize synthetic disaccharide oxazolines to form artificial chitin, hyaluronan, and related polysaccharides.[3–9] Endo-β-*N*-acetylglucosaminidases (ENGases) are a class of family 85 glycoside hydrolases that release N-glycans from N-glycoproteins by hydrolyzing the β-(1→4)-glycosidic bond in the *N*,*N*′-diacetylchitobiose core. In addition to hydrolytic activity, some members of this class possess transglycosylation activity, that is, the ability to transfer the released N-glycan to an acceptor to form a new glycosidic linkage. Among others, the Endo-A from *Arthrobactor protophormiae*[10,11] and Endo-M from *Mucor hiemalis*[12] have found useful applications for the synthesis of oligosaccharides and glycopeptides.[13,14] Similar to the reactions by family 18 chitinases, the ENGase-catalyzed hydrolysis is also proposed to proceed by a mechanism of substrate-assistant catalysis via an oxazolinium ion intermediate. On the basis of this assumption, we and others have explored synthetic sugar oxazolines as activated substrates for Endo-A- and Endo-M-catalyzed transglycosylation for a highly efficient synthesis of N-glycopeptides.[15–22] This chemoenzymatic strategy was also efficiently extended to glycoprotein synthesis and glycosylation engineering, including the preparation of various glycoforms of ribonuclease B and human IgG1-Fc.[23–25]

Our initial studies on the substrate structural requirement have suggested that the β-Man*p*-(1→4)-Glc*p*NAc oxazoline moiety was the minimum substrate structure required for an efficient Endo-A-catalyzed transglycosylation.[18] While β-Man*p*-(1→4)-Glc*p*NAc oxazoline was an efficient substrate for Endo-A-catalyzed transglycosylation, our experimental data showed that the other oxazolines of disaccharide β-Glc*p*-(1→4)-Glc*p*NAc, β-Gal*p*-(1→4)-Glc*p*NAc, and β-Glc*p*NAc-(1→4)-Glc*p*NAc did not demonstrate substrate activity toward Endo-A under the same enzymatic reaction conditions. However, Fairbanks and co-workers recently reported that Endo-A could take the β-Glc*p*-(1→4)-Glc*p*NAc disaccharide oxazoline for transglycosylation, leading to the formation of a trisaccharide product in 48% yield when the Glc*p*NAc-Asn-Cbz was used as the acceptor substrate.[26]

We were puzzled by these apparently discrepant results. Particularly, if the β-Glc*p*-(1→4)-Glc*p*NAc oxazoline was an efficient donor substrate for Endo-A under certain conditions, then tandem transglycosylation might occur with the glucose-terminal species,

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doi:10.1016/j.carres.2009.01.016

as it was previously reported that glucose, like GlcNAc, acted as a good acceptor substrate for Endo-A-catalyzed transglycosylation.[27,28] Indeed, our recent work has demonstrated that terminal glucose in various natural products could serve as excellent acceptor for Endo-A-catalyzed transglycosylation with sugar oxazoline, providing a highly efficient approach to introducing N-glycans into natural products.[29] These observations prompted us to revisit the Endo-A-catalyzed reaction with β-Glc*p*-(1→4)-Glc*p*NAc oxazoline.

We have found that under normal transglycosylation conditions with a catalytic amount of enzyme and a short reaction time (1–3 h), Endo-A showed only marginal activity for transglycosylation with the disaccharide oxazoline, which confirms our previous observations.[18] However, when used in a relatively large quantity for a prolonged incubation time, Endo-A could promote the transglycosylation of the disaccharide oxazoline to a Glc*p*NAc-Asn-Fmoc acceptor to form a trisaccharide derivative, which is consistent with the observation of Fairbanks and co-workers.[26] Nevertheless, the reaction did not stop at the first transglycosylation. We have found that the glucose-terminated species did serve as the acceptor substrates for tandem transglycosylation. Moreover, Endo-A promoted self-condensation of the disaccharide oxazoline was observed, leading to the formation of oligo- and polysaccharides with disaccharide repeating units.

2. Results and discussion

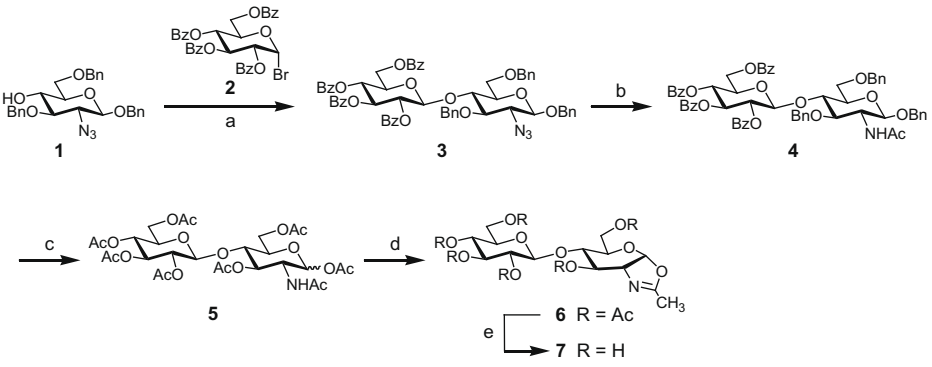
2.1. Endo-A-catalyzed transfer of β-Glc*p*-(1→4)-Glc*p*NAc-oxazoline to an acceptor

β-Glc*p*-(1→4)-Glc*p*NAc oxazoline was synthesized previously by different approaches.[9,18,20] We describe here an alternative synthesis of this disaccharide oxazoline, as outlined in Scheme 1. Glycosylation of acceptor **1**[30] with glycosyl donor **2** using silver triflate (AgOTf) as the promoter gave the known disaccharide **3** in 69% yield.[31] Treatment of **3** with thioacetic acid gave the N-acetylated derivative **4** in 89% yield. Sequential removal of *O*-benzoyl and *O*-benzyl groups followed by acetylation gave the fully acetylated derivative **5**. Oxazoline ring formation was achieved by treatment of **5** with TMSBr, $BF_3{\cdot}OEt_2$, and 2,4,6-collidine to give **6** in 64% yield. Finally, de-O-acetylation with NaOMe–MeOH afforded the oxazoline **7** in quantitative yield.

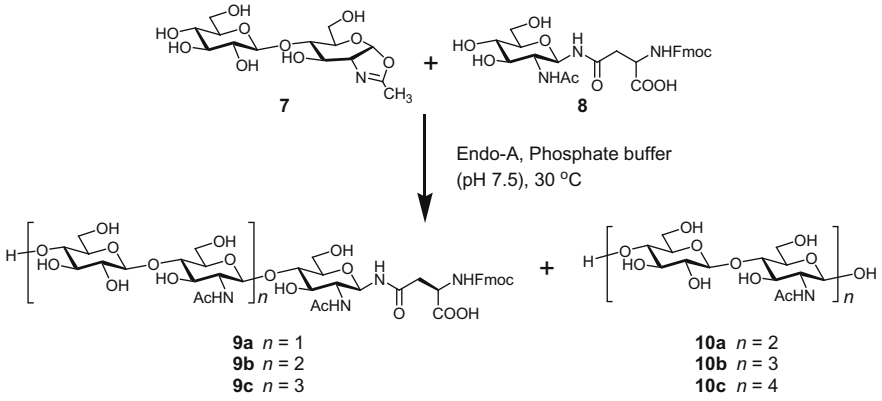
With the disaccharide sugar oxazoline **7** in hand, we first repeated its Endo-A-catalyzed reaction using a Glc*p*NAc-Asn-Fmoc derivative **8** as the acceptor substrate, following our previously reported procedure.[18] The enzymatic reaction was performed using a catalytic amount of Endo-A (25 mU) in phosphate buffer (50 mM, pH 6.5) at 30 °C (molar ratio of donor–acceptor, 3:1) (Scheme 2). The reaction was monitored by RP-HPLC (UV detection at 254 nm) to track the formation of transglycosylation products. It was found that under these conditions after 5 h, the peak for starting material **8** changed almost not at all, and only a very small new peak (less than 2%, compared to the peak of the acceptor **8**) was generated, which appeared slightly earlier than that of the acceptor **8**. In contrast, the Endo-A-catalyzed transglycosylation with β-Man*p*-(1→4)-Glc*p*NAc oxazoline and Manp_3Glc*p*NAc oxazoline gave 65% and 86% yield of transglycosylation products, respectively, under the same reaction conditions.

These results confirm our previous experimental observation that no apparent enzymatic transglycosylation was found with the β-Glc*p*-(1→4)-Glc*p*NAc oxazoline in the presence of a catalytic amount of Endo-A.[18] Nevertheless, the appearance of a tiny new peak in front of the acceptor **8** does implicate the possibility of a very slow enzymatic reaction with the disaccharide oxazoline to form transglycosylation products. Indeed, a prolonged reaction time (24 h) resulted in a moderate increase (ca. 5%) for this new peak (Fig. 1A, peak 2). By increasing the pH value to 7.5, the area of peak 2 was slightly increased to about 8%. This might be attributed to the increased stability of the sugar oxazoline at a relatively high pH, as non-enzymatic hydrolysis of the sugar oxazoline was observed under slightly acidic conditions (pH 6.5) (data not shown).

To obtain enough quantity of products for characterization, we have run the enzymatic reaction with a relatively large amount of Endo-A (500 mU). Indeed, the reaction with high Endo-A activity resulted in a dramatic increase of the transglycosylation product as revealed by the increased ratio of the new peak (peak 2) vs. the acceptor substrate peak (peak 1) (Fig. 1B). Integration of the peak areas gave a 71% transformation of the acceptor **8**. The new peak was collected and analyzed by MALDI-TOF mass spectrometry. Surprisingly, the peak was not derived from a single product. In addition to the formation of an initial transglycosylation product, the trisaccharide derivative **9a** ($n = 1$), which was the major product, extension of the trisaccharide **9a** by further transglycosylation was observed, resulting in the formation of pentasaccharide **9b** ($n = 2$) and heptasaccharide **9c** ($n = 3$) (Fig. 2A). This result indicates that the non-reducing terminal Glc*p* residue of the first transglycosylation product **9a** could be recognized by Endo-A as an acceptor substrate to participate in the second round of Endo-A-catalyzed transglycosylation and so on, leading to the formation of the oligosaccharide derivatives **9b** and **9c** (Fig. 2A). However, these oligosaccharide derivatives appeared as a broad peak under the RP-HPLC conditions (Fig. 1B). An attempt to isolate pure oligosaccharide forms was not successful. To investigate the possibility of self-condensation of the disaccharide oxazoline itself, the products of which might not be detected by UV monitoring because of their weak UV absorbance, we performed direct MALDI-TOF MS analysis of the reaction mixture. As shown in Figure 2B, some oligosaccharides (**10a**, **10b**, and **10c**) resulted in self-condensation



Scheme 1. Synthesis of oligosaccharide oxazoline **1**. Reagents and conditions: (a) AgOTf, 2,6-di-*tert*-butylpyridine, MS 4 Å, CH_2Cl_2, 69%; (b) AcSH, pyridine, $CHCl_3$, 89%; (c) (i) NaOMe, MeOH; (ii) $Pd(OH)_2$–C, H_2, MeOH, AcOH; (iii) Ac_2O, pyridine, 95% (3 steps); (d) TMSBr, $BF_3{\cdot}OEt_2$, 2,4,6-collidine, dichloroethane, 64%; (e) NaOMe, MeOH, quant.



Scheme 2. Endo-A-catalyzed transfer of β-Glc*p*-(1→4)-Glc*p*NAc oxazoline to a GlcNAc acceptor.

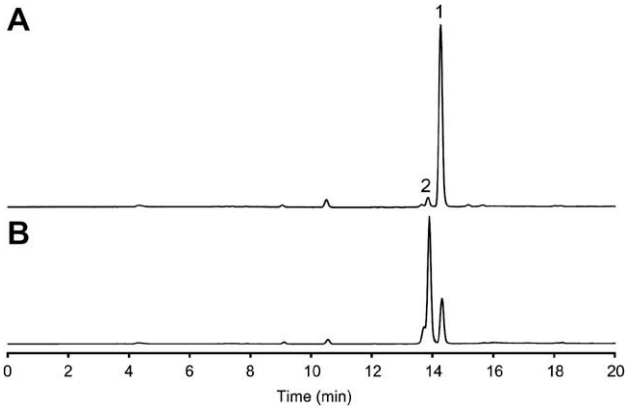


Figure 1. HPLC monitoring of the enzymatic reactions: (A) with catalytic amount (25 mU) of Endo-A for 24 h; (B) with a large amount (500 mU) of Endo-A for 48 h. Peak 1, acceptor **8**; peak 2, transglycosylation products. The HPLC was monitored by a UV detector at 254 nm.

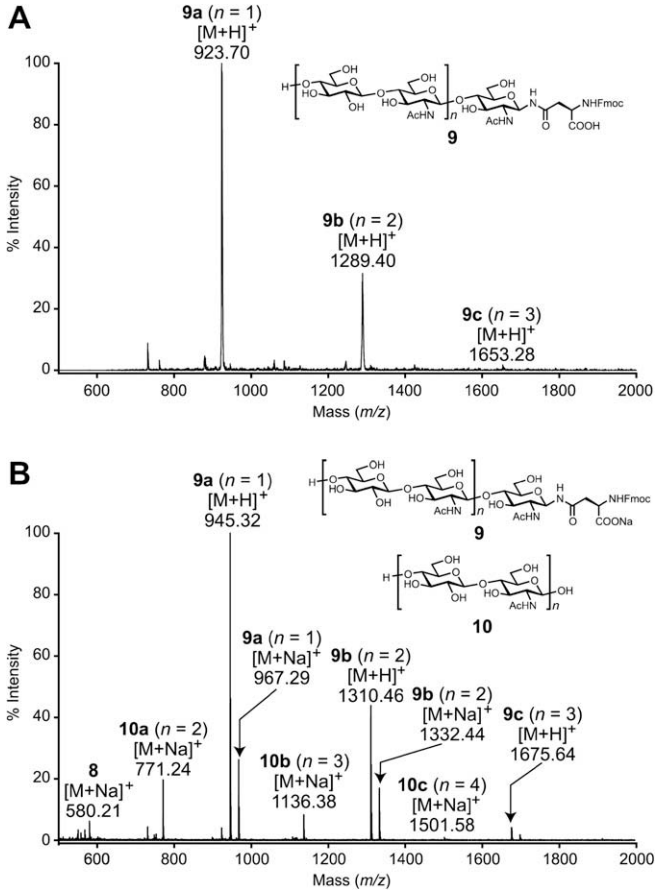


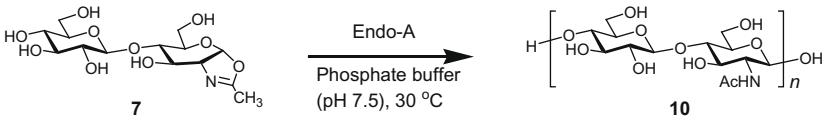
Figure 2. MALDI-TOF MS profiles of the products from the Endo-A-catalyzed reactions between the disaccharide oxazoline **7** and the acceptor **8**. Panel A, the MS profile of the HPLC-isolated product (peak 2 in Fig. 1); panel B, the MS profile of the reaction mixture.

were detected, together with the Glc*p*NAc-Asn primed transglycosylation products (**9a**, **9b**, and **9c**).

These results help explain the seemingly contradictory results previously reported by our group and Fairbanks' group.[18,26] The present study has shown that the disaccharide oxazoline β-Glc*p*-(1→4)-Glc*p*NAc oxazoline was a poor substrate with a very low reactivity toward Endo-A. This explains why no apparent transglycosylation was observed when a catalytic amount of enzyme was used.[18] Nevertheless, the use of large amount of Endo-A for a long incubation time could force the transglycosylation between the disaccharide oxazoline and an Glc*p*NAc-Asn derivative to form a trisaccharide derivative, consistent with the previous report of Fairbanks and co-workers.[26] Indeed, a personal communication with Dr. Fairbanks confirmed that a relatively large amount of Endo-A and a prolonged incubation time (up to 4000 min) were applied to reach the observed 48% yield of the trisaccharide product. Moreover, our present study also revealed an interesting new reaction with the disaccharide oxazoline, the Endo-A promoted tandem transglycosylation. Perhaps due to the weak UV absorbance of the self-condensation products, this new self-condensation reaction might escape the attention in the previous report[26] (Fairbanks, personal communications).

2.2. Endo-A promoted polymerization of β-Glc*p*-(1→4)-Glc*p*NAc-oxazoline

Despite its low activity toward Endo-A, our experimental results indicate that β-Glc*p*-(1→4)-Glc*p*NAc-oxazoline could serve as both donor and acceptor substrates. Therefore, it would be interesting to examine whether Endo-A could effectively promote self-condensation of the disaccharide oxazoline in the absence of any external acceptor. For the enzymatic polymerization, a solution of disaccharide oxazoline **7** (200 mM) in a phosphate buffer (50 mM, pH 7.5) was incubated with a sufficient amount of Endo-A at 30 °C (Scheme 3). After 48 h, precipitation was observed indicating the formation of polysaccharides with high-molecular weight. The water-soluble portions of the products were subject to Dionex HPAEC-PED analysis[32,33] to determine the degree of



Scheme 3. Endo-A-catalyzed polymerization of β-Glc*p*-(1→4)-Glc*p*NAc oxazoline.

polymerization for the low molecular weight fractions. As shown in Figure 3A, a series of oligosaccharides were formed in the enzymatic reaction.

It had been previously reported that the chitinase from *Bacillus* sp. was able to polymerize disaccharide oxazoline **7** to form a hybrid cellulose–chitin polymer.[9] Therefore, we repeated the *Bacillus* chitinase-catalyzed reaction. Similar to the Endo-A-catalyzed reaction, the chitinase-catalyzed condensation of the disaccharide oxazoline also gave two portions of products, the high-molecular weight (insoluble precipitate) polysaccharide and the water-soluble polysaccharide. The Dionex HPAEC analysis of the water-soluble products is shown in Figure 3B. A comparison of the results (Fig. 3A vs Fig. 3B) indicated that the two enzymatic reactions gave a similar pattern of product formation, but the Endo-A-catalyzed reaction resulted in less hydrolysis of the disaccharide oxazoline than the chitinase-catalyzed reaction (peak a in Fig. 3 was identified as the hydrolytic product, β-Glc*p*-(1→4)-Glc*p*NAc). For further characterization, the polymerization products (soluble fractions) were subjected to analysis by MALDI-TOF mass spectrometry. As shown in Figure 4, the peaks derived from oligosaccharides up to a dodecasaccharide ($n = 6$) with 12 sugar residues were detected. Therefore, the insoluble polysaccharides (the high-molecular weight portions) should be at least larger than 12 sugar residues.

It was observed that the oligomer products, once formed, were resistant to Endo-A-catalyzed hydrolysis, thus allowing accumulation of the products. The yields of the water-insoluble product (high-molecular weight portions) and the water-soluble product (low molecular weight portions) were 18% and 61%, respectively. The overall yield (79%) of polymerization was comparable to the yield in the chitinase-catalyzed polymerization.[9] In comparison, we also examined Endo-M, another endo-β-*N*-acetyl-glucosaminidase, for the enzymatic reaction. In contrast to Endo-A, it was found that Endo-M could not catalyze the polymerization of the sugar oxazoline effectively. Incubation of similar amount of Endo-M with the oxazoline **7** under the same reaction conditions as for Endo-A gave only trace amount of polymerization product. After 48 h, less than 3% of the polymerization products, up to only hexasaccharide ($n = 3$), were detected by DIONEX and MALDI-TOF/MS analysis (data not shown).

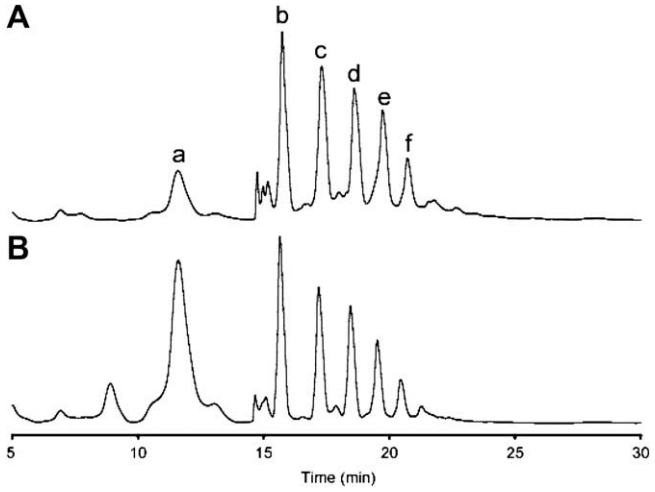


Figure 3. HPAEC profiles of the soluble products obtained from enzyme-catalyzed polymerization of the disaccharide oxazoline (**7**). Panel A, the Endo-A-catalyzed reaction; panel B, the *Bacillus* chitinase-catalyzed reaction. Peak a was identified as the monomer ($n = 1$) as confirmed by co-injection with the standard disaccharide β-Glc*p*-(1→4)-Glc*p*NAc.

To our knowledge, this is the first example of endo-β-*N*-acetylglucosaminidase-catalyzed polymerization of activated oligosaccharide substrates. Enzymatic polymerization by polysaccharide-hydrolyzing enzymes such as cellulase, chitinase, and hyaluronidase had previously been reported, using activated glycosyl donors such as glycosyl fluoride and/or sugar oxazolines as the substrates.[1,2,34] But a major problem for using these polysaccharide-hydrolyzing enzymes for synthesis is their ability to also hydrolyze the synthetic polysaccharides thus formed. The natural roles of the endo-β-*N*-acetylglucosaminidases are to cleave N-glycans from glycoproteins. The present studies revealed a new property of the endo-β-*N*-acetylglucosaminidase for promoting polymerization of activated oligosaccharides. Moreover, the resistance of the product hydrolysis in the Endo-A-catalyzed polymerization might be potentially superior to the chitinase-catalyzed polymerization, in which the chitinase could potentially hydrolyze the polysaccharides thus formed. Nevertheless, a major problem of the wild type Endo-A is its low activity toward the artificial sugar oxazoline substrate. A large amount of enzyme is required for an efficient polymerization. This problem may be addressed by mutagenesis of Endo-A with subsequent screening of Endo-A mutant library to discover novel mutants that possess enhanced activity and broader substrate specificity for synthetic purpose.

2.3. Endo-A-catalyzed transfer of α-Man*p*-(1→3)-β-Glc*p*-(1→4)-Glc*p*NAc-oxazoline to an acceptor

For comparison, we also examined the Endo-A-catalyzed reaction with a trisaccharide oxazoline, α-Man*p*-(1→3)-β-Glc*p*-(1→4)-Glc*p*NAc-oxazoline (**11**) in which an α-linked mannose residue was attached to the 3-position of the terminal glucose moiety. This trisaccharide oxazoline was previously shown to be a substrate for Endo-A and Endo-M.[26] When oxazoline **11** and acceptor **8** were incubated with a catalytic amount of Endo-A in a phosphate buffer for 24 h at 30 °C, the expected transglycosylation product, the Asn-linked trisaccharide derivative **12**, was formed in 43% yield (Scheme 4). In contrast to the β-Glc*p*-(1→4)-Glc*p*NAc oxazoline, which gave much less transglycosylation product (less than 5%) when a catalytic amount of Endo-A was used, the trisaccharide oxazoline was much more active toward the Endo-A-catalyzed reaction. The data suggest that the attachment of an α-linked mannose at the 3-position of the glucose significantly enhanced the substrate activity, probably by enhancing the binding of the substrate to Endo-A. On the other hand, MALDI-TOF mass spectrometry of the product did not show any other oligosaccharide products derived from sugar chain elongation. These results suggest that the attachment of the mannose residue at the terminal glucose moiety could effectively block the self-condensation. We have recently reported that this trisaccharide oxazoline was also a substrate for *Bacillus* chitinase for transglycosylation to form the tetrasaccharide product **12**.[35] However, the yield (27%) was lower than that of the Endo-A-catalyzed transglycosylation, mainly because of the simultaneous hydrolysis of the sugar oxazoline by the chitinase.

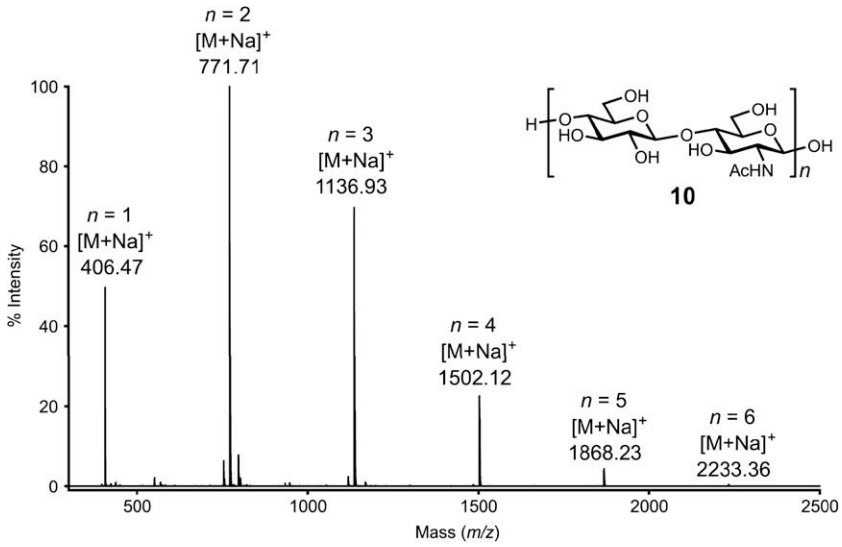
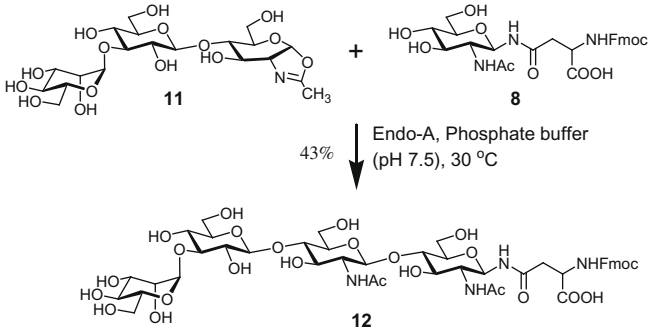


Figure 4. MALDI-TOF MS profile of the soluble products obtained from the Endo-A-catalyzed polymerization of disaccharide oxazoline (**7**).



Scheme 4. Endo-A-catalyzed transfer of α-Man-(1→3)-β-Glc*p*-(1→4)-Glc*p*NAc oxazoline to an Glc*p*NAc acceptor.

3. Conclusion

We have re-investigated the Endo-A-catalyzed glycosylation with the β-Glc*p*-(1→4)-Glc*p*NAc oxazoline. Our experimental results confirm that the disaccharide oxazoline had a low activity toward Endo-A in comparison with β-Man*p*-(1→4)-Glc*p*NAc oxazoline and other efficient substrates. Interestingly, we have also found that the use of a large amount of Endo-A can promote the transglycosylation as well as self-condensation of the otherwise low reactivity substrate. In the absence of any external acceptor, Endo-A can polymerize the disaccharide oxazoline to form oligo- and polysaccharides with the β-Glc*p*-(1→4)-Glc*p*NAc repeating units. This is the first example of an endo-β-*N*-acetylglucosaminidase-promoted polymerization of activated oligosaccharide. This enzymatic polymerization may find useful applications for the synthesis of novel artificial polysaccharides.

4. Experimental

4.1. Materials

2,3,4,6-Tetra-*O*-benzoyl-α-D-glucopyranosyl bromide (**2**) was purchased from Sigma–Aldrich, and was used as received. The α-Man-(1→3)-β-Glc-(1→4)-GlcNAc-oxazoline (**11**) was prepared according to the previously reported procedures.[20] Endo-β-*N*-acetylglucosaminidase from *Arthrobacter protophormiae* (Endo-A) was overproduced in *Escherichia coli* following the reported procedure.[36] One unit of Endo-A was defined as the amount of the enzyme that hydrolyzes 1 μmol of Man*p*$_9$Glc*p*NAc$_2$Asn (5 mM) in 1 min at 30 °C. Chitinase from *Bacillus* sp. was purchased from Wako Pure Chemicals Inc. (Osaka, Japan).

4.2. Analytical methods

TLC was performed using silica gel on aluminum plates (Sigma-Aldrich). Flash column chromatography was performed on silica gel 60 (230–400 mesh). NMR spectra were recorded on JEOL ECX 400 MHz spectrometer. The chemical shifts were assigned in ppm. Analytical RP-HPLC was performed on a Waters 626 HPLC instrument with a Symmetry300^{TM} C18 column (5.0 μm, 4.6 × 250 mm) at 40 °C. The column was eluted with a linear gradient of 0–90% MeCN containing 0.1% TFA for 20 min at the flow rate of 1.0 mL/min. Preparative HPLC was performed with a Waters 600 HPLC instrument of a Waters C18 column (Symmetry 300, 19 × 300 mm). The column was eluted with a suitable gradient of MeCN–H_2O containing 0.1% TFA at a flow rate of 12 mL/mi. MALDI-TOF/MS spectra were recorded on an Autoflex MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA) by using 2,5-dihydroxybenzoic acid as a matrix under positive ion conditions. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was performed on a Dionex DX600 chromatography system (Dionex Corporation, Sunnyvale, CA) equipped with an electrochemical detector (ED50, Dionex Corporation, Sunnyvale, CA). Separation was achieved by an anion exchange column (CarboPac PA1 (4 × 250 mm), Dionex Corporation, Sunnyvale, CA). The mobile phase (flow rate, 1.0 mL/mi) was composed of deionized water (eluent A), 1 M NaOAc (eluent B), and 0.2 M NaOH (eluent C). The gradient used was as follows: 0 min, 50% eluent A, 0% eluent B, and 50% eluent C; 5.0 min, 50% eluent A, 0% eluent B, and 50% eluent C; and 25.0 min, 35% eluent A, 15% eluent B, and 50% eluent C.

4.3. Benzyl 2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranosyl-(1→4)-2-azido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside (3)

To a suspension of benzyl 2-azido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside **1**[30] (200 mg 0.42 mmol), 2,3,4,6-tetra-*O*-benzoyl-α-D-glucopyranosyl bromide **2** (554 mg 0.84 mmol), 2,6-di-*tert*-butylpyridine (404 μL, 1.8 mmol) and activated 4 Å

molecular sieves (905 mg) in CH_2Cl_2 (7.5 mL) was added AgOTf (255 mg 0.99 mmol) at 0 °C. After stirring at room temperature overnight, the mixture was filtered through a Celite pad and the filtrate was concentrated. The resulting residue was subjected to silica gel column chromatography (hexanes–EtOAc 5:1) to afford **3** (304 mg, 69%) as a white amorphous powder. ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 7.96–7.94 (2H, m, Ar), 7.90–7.86 (4H, m, Ar), 7.81–7.78 (2H, m, Ar), 7.56–7.18 (27H, m, Ar), 5.68 (1H, t, J = 9.6 Hz, H-3^{II}), 5.55 (1H, t, J = 9.6 Hz, H-4^{II}), 5.48 (1H, dd, J = 9.7, 8.3 Hz, H-2^{II}), 5.04 (1H, d, J = 11.0 Hz, CH_2Ph), 4.90 (1H, d, J = 8.2 Hz, H-1^{II}), 4.85 (1H, d, J = 12.4 Hz, CH_2Ph), 4.76–4.73 (2H, m, CH_2Ph × 2), 4.58 (1H, d, J = 11.9 Hz, CH_2Ph), 4.42 (1H, dd, J = 12.4, 3.2 Hz, H-6^{II}a), 4.40 (1H, d, J = 11.9 Hz, CH_2Ph), 4.25 (1H, dd, J = 12.1, 5.3 Hz, H-6^{II}b), 4.19 (1H, d, J = 7.8 Hz, H-1^{I}), 4.06 (1H, t, J = 9.2 Hz, H-4^{I}), 3.75 (1H, m, H-5^{II}), 3.69 (1H, dd, J = 11.5, 3.2 Hz, H-6^{I}a), 3.54 (1H, m, H-6^{I}b), 3.42–3.34 (2H, m, H-2^{I}, H-3^{I}), 3.14 (1H, m, H-5^{I}); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.90, 165.61, 165.03 and 164.72 (*C*OPh), 138.18, 137.84, 136.66, 133.37, 133.14, 132.98 and 129.66–127.45 (Ar), 100.31 (C-1^{I}), 100.26 (C-1^{II}), 80.66 (C-3^{I}), 76.69 (C-4^{I}), 75.08 (*C*H$_2$Ph), 74.38 (C-5^{I}), 73.52 (*C*H$_2$Ph), 72.91 (C-3^{II}), 72.09 (C-2^{II}), 71.92 (C-5^{II}), 70.77 (*C*H$_2$Ph), 69.72 (C-4^{II}), 67.31 (C-6^{I}), 65.85 (C-2^{I}), 62.96 (C-6^{II}).

4.4. Benzyl 2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranosyl-(1→4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside (4)

To a solution of **3** (294 mg, 0.28 mmol) in $CHCl_3$ (3.0 mL) were added pyridine (3.0 mL) and thioacetic acid (3.0 mL) at room temperature. After stirring for 48 h, the mixture was concentrated and purified by silica gel column chromatography (hexanes–EtOAc 1:1) to afford **4** (265 mg, 89%) as a white amorphous powder. ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 7.99–7.97 (2H, m, Ar), 7.93–7.88 (4H, m, Ar), 7.83–7.81 (2H, m, Ar), 7.56–7.19 (27H, m, Ar), 5.78 (1H, t, J = 9.6 Hz, H-3^{II}), 5.68 (1H, d, J = 8.7 Hz, N*H*^{I}), 5.60 (1H, t, J = 9.9 Hz, H-4^{II}), 5.49 (1H, dd, J = 9.9, 8.1 Hz, H-2^{II}), 4.88 (1H, d, J = 8.2 Hz, H-1^{II}), 4.83 (1H, d, J = 11.9 Hz, CH_2Ph), 4.73 (1H, d, J = 11.9 Hz, CH_2Ph), 4.67–4.63 (3H, m, CH_2Ph × 2, H-1^{I}), 4.51 (1H, dd, J = 12.3, 3.2 Hz, H-6^{II}a), 4.39 (1H, d, J = 11.9 Hz, CH_2Ph), 4.37–4.29 (2H, m, H-6^{II}b, CH_2Ph), 4.11 (1H, t, J = 6.9 Hz, H-4^{I}), 3.90 (1H, t, J = 7.1 Hz, H-3^{I}), 3.82 (1H, m, H-5^{II}), 3.74–3.68 (2H, m, H-2^{I}, H-6^{I}a), 3.63 (1H, dd, J = 10.1, 4.1 Hz, H-6^{I}b), 3.44 (1H, m, H-5^{I}), 1.86 (3H, s, COC*H*$_3$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.04 (*C*OCH$_3$), 165.97, 165.63, 165.19 and 165.03 (*C*OPh), 138.52, 137.97, 137.43, 133.44, 133.39 and 133.19–127.35 (Ar), 99.78 (C-1^{II}), 99.62 (C-1^{I}), 77.78 (C-3^{I}), 76.02 (C-4^{I}), 74.40 (C-5^{I}), 73.44 (*C*H$_2$Ph), 72.65 (C-3^{II}), 72.06 (C-2^{II}), 71.99 (C-5^{II}), 70.58 (*C*H$_2$Ph), 69.57 (C-4^{II}), 68.33 (C-6^{I}), 62.84 (C-6^{II}), 54.18 (C-2^{I}), 23.28 (CO*C*H$_3$).

4.5. 2-Methyl-4,5-dihydro-[4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-3,6-di-*O*-acetyl-1,2-dideoxy-α-D-glucopyranoso][2,1-d]-1,3-oxazole (6)

Compound **4** (255 mg, 0.24 μmol) was dissolved in 1:1 (v/v) CH_2Cl_2–MeOH (16 mL), and a solution of 0.5 M NaOMe in MeOH (0.7 mL, 0.35 mmol) was added. After being stirred at room temperature overnight, the solution was neutralized with Dowex 50W (H^+), filtered, and concentrated. To a solution of the residue in 100:1 (v/v) MeOH–AcOH (8.1 mL) was added 20% palladium(II) hydroxide on activated carbon (80 mg), and the reaction mixture was vigorously stirred at room temperature under hydrogen atmosphere for 4 h. The mixture was filtered through a Celite pad and the filtrate was concentrated in vacuo. Pyridine (10 mL) and Ac_2O (10 mL) were added, and the mixture was stirred at room temperature overnight. The solution was concentrated, diluted with CH_2Cl_2, and washed sequentially with 1 M HCl, saturated $NaHCO_3$, and brine. The organic layer was dried over $MgSO_4$, filtered, and concentrated. Silica gel column chromatography (CH_2Cl_2–MeOH 40:1) of the residue afforded 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl-(1→4)-2-acetamido-1,3,6-tri-*O*-acetyl-2-deoxy-D-glucopyranose **5** (152 mg, 95% from **4**) as a white amorphous powder.

To a solution of this disaccharide (72 mg, 0.11 mmol) in dichloroethane (11 mL) containing activated 4 Å molecular sieves (1.3 g) were added 2,4,6-collidine (70 μL, 0.53 mmol), TMSBr (206 μL, 1.6 mmol), and $BF_3{\cdot}OEt_2$ (200 μL, 1.6 mmol). The reaction mixture was stirred at room temperature overnight. The mixture was diluted with CH_2Cl_2, filtered through a Celite pad, washed sequentially with saturated $NaHCO_3$ and brine, dried over $MgSO_4$, filtered, and concentrated. The residue was chromatographed (CH_2Cl_2–MeOH 90:1) to give **6** (42 mg, 64%) as a white amorphous powder. ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 5.92 (1H, d, J = 7.3 Hz, H-1^{I}), 5.66 (1H, d, J = 2.3 Hz, H-3^{I}), 5.19 (1H, t, J = 9.4 Hz, H-3^{II}), 5.10 (1H, t, J = 9.6 Hz, H-4^{II}), 5.00 (1H, t, J = 9.0 Hz, H-2^{II}), 4.72 (1H, d, J = 7.8 Hz, H-1^{II}), 4.29 (1H, dd, J = 12.2, 4.4 Hz, H-6^{II}a), 4.20 (1H, dd, J = 11.9, 2.3 Hz, H-6^{I}a), 4.14 (1H, dd, J = 12.3, 2.3 Hz, H-6^{II}b), 4.12 (1H, m, H-2^{I}), 4.04 (1H, dd, J = 12.2, 5.7 Hz, H-6^{I}b), 3.79 (1H, ddd, J = 9.9, 4.3, 2.5 Hz, H-5^{II}), 3.64 (1H, d, J = 9.2 Hz, H-4^{I}), 3.48 (1H, m, H-5^{I}), 2.11–2.00 (21H, m, COC*H*$_3$, C*H*$_3$ of oxazoline); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.68, 170.54, 170.24 and 169.34 (*C*OCH$_3$), 166.82 (O–*C*=N of oxazoline), 102.05 (C-1^{II}), 98.99 (C-1^{I}), 78.12 (C-4^{I}), 72.99 (C-3^{II}),71.97 (C-5^{II}), 71.27 (C-2^{II}), 70.14 (C-3^{I}), 68.05 (C-4^{II}), 67.44 (C-5^{I}), 64.81 (C-2^{I}), 63.50 (C-6^{I}), 61.80 (C-6^{II}), 20.96, 20.75, 20.69, 20.55 and 20.50 (CO*C*H$_3$), 13.92 (*C*H$_3$ of oxazoline).

4.6. 2-Methyl-4,5-dihydro-[4-*O*-(β-D-glucopyranosyl)-1,2-dideoxy-α-D-glucopyranoso][2,1-d]-1,3-oxazole (7)

To a solution of **6** (37 mg, 59 μmol) in MeOH (3 mL) was added NaOMe in MeOH (0.5 M, 11.8 μL, 5.9 μmol). After stirring at room temperature for 2 h, the reaction mixture was concentrated to dryness. The residue was dissolved in water and lyophilized to give the oxazoline **7** (22 mg, quantitative). ^{1}H NMR (400 MHz, D_2O) δ 5.97 (1H, d, J = 7.3 Hz, H-1^{I}), 4.37 (1H, d, J = 7.8 Hz, H-1^{II}), 4.28 (1H, m, H-3^{I}), 4.08 (1H, m, H-2^{I}), 3.80 (1H, m, H-6^{I}a), 3.70 (1H, m, H-6a^{II}), 3.64–3.53 (3H, m, H-6^{I}b, H-4^{I}, H-6b^{II}), 3.37–3.22 (4H, m, H-3^{II}, H-5^{II}, H-5, H-4^{II}), 3.16 (1H, dd, J = 8.5 Hz, H-2^{II}), 1.94 (3H, s, C*H*$_3$ of oxazoline); ^{13}C NMR (100 MHz, D_2O) δ 168.33 (O–*C*=N of oxazoline), 104.10 (C-1^{II}), 99.83 (C-1^{I}), 78.38 (C-4^{I}), 75.99 (C-5^{II}), 75.55 (C-3^{II}), 73.16 (C-2^{II}), 70.90 (C-3^{I}), 69.50 (C-5^{I}), 69.07 (C-4^{II}) 65.23 (C-2^{I}), 61.66 (C-6^{II}), 60.65 (C-6^{I}), 12.96 (*C*H$_3$ of oxazoline).

4.7. Endo-A-catalyzed transglycosylation with Glc-β-(1→4)-GlcNAc oxazoline (7)

A solution of the disaccharide oxazoline **7** (625 μg, 1.7 μmol) and the GlcNAc-Asn-Fmoc **8** (318 μg, 0.57 μmol) in a phosphate buffer (50 mM, pH 7.5, 125 μL) was incubated at 30 °C with Endo-A (500 mU). After 48 h, the reaction was terminated by 10% TFA and the mixture was analyzed by RP-HPLC. The new peak was isolated and subject to MS analysis. MALDI-TOF MS of the products; *m/z* 923.70 [M+H]$^+$ (**9a**), 1289.40 [M+H]$^+$ (**9b**), 1653.28 [M+H]$^+$ (**9c**).

4.8. A typical procedure for the Endo-A-catalyzed polymerization of Glc-β-(1→4)-GlcNAc oxazoline (7)

A solution of oxazoline **7** (2.28 mg, 6.24 μmol) in a phosphate buffer (50 mM, pH 7.5, 31.2 μL) was incubated at 30 °C with Endo-A (624 mU) for 48 h. A white precipitate that was formed during the reaction was separated by centrifugation and washed with distilled water (200 μL) three times to give the high-molecular

weight polysaccharide **10** (0.41 mg, 18%). The water-soluble portion (the low-molecular weight polysaccharide or oligosaccharide) of the reaction mixture was analyzed by HPAEC-PED and the yield of the low molecular weight portion (61%) was estimated by the integration of the hydrolyzed product (peak a) and the oligomers. MALDI-TOF MS of the products; *m/z* 406.47 $[M+Na]^+$ ($n = 1$), 771.71 $[M+Na]^+$ ($n = 2$), 1136.93 $[M+Na]^+$ ($n = 3$), 1502.12 $[M+Na]^+$ ($n = 4$), 1868.23 $[M+Na]^+$ ($n = 5$), and 2233.36 $[M+Na]^+$ ($n = 6$).

4.9. Synthesis of glyco-asparagine derivative through the Endo-A-catalyzed transglycosylation

A solution of glycosyl acceptor **8** (3.0 mg, 5.4 μmol) and glycosyl donor **11** (9.0 mg, 17 μmol) in a phosphate buffer (50 mM, pH 7.5, 3.0 mL) containing Endo-A (100 mU) was incubated at 30 °C for 24 h. Preparative HPLC purification gave the product **12** (2.5 mg, 43%). ^{1}H NMR (500 MHz, DMSO-d_6 + 5% D_2O, selected signals): δ 7.80 (2H, d, $J = 8.0$ Hz, Ar), 7.65 (2H, m, Ar), 7.38 (2H, t, $J = 7.5$ Hz, Ar), 7.30 (2H, m, Ar), 5.01 (1H, s, H-1^{IV}), 4.82 (1H, d, $J = 7.5$ Hz, H-1^{I}), 4.38 (1H, d, $J = 7.5$ Hz, H-1^{II}), 4.26 (1H, d, $J = 8.0$ Hz, H-1^{III}), 4.18 (2H, m, CH_2 of Fmoc), 1.83 (3H, s, $COCH_3$), 1.75 (3H, s, $COCH_3$); MS (MALDI-TOF): calcd for $C_{47}H_{65}N_4O_{25}$, $M = 1085.39$; found 1086.54 $[M+H]^+$.

Acknowledgments

We thank Professor Kaoru Takegawa for providing the pGEX-2T/Endo-A plasmid that was used for the expression of Endo-A, and Dr. Cishan Li for helpful discussions. We thank Dr. Antony Fairbanks for kindly sharing his experimental details that allowed a comparison of the experimental results from our two laboratories. This work was supported by the National Institutes of Health (NIH Grant R01 GM080374).

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