An Investigation of Enzymatic Phosphorolysis of α(1→4)-Linked Oligo-D-glucosaminides by Thermostable α-Glucan Phosphorylase Catalysis

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Abstract
This paper reports an investigation of enzymatic phosphorolysis of α(1→4)-linked oligo-D-glucosaminide substrates by thermostable α-glucan phosphorylase (from *Aquifex aeolicus* VF5) catalysis. α-Glucan phosphorylase catalyzes both phosphorolysis and glucosylation/polymerization at the nonreducing end of α(1→4)-linked glucose substrates depending on conditions. The authors also found that α(1→4)-linked D-glucosaminide polymer (chitosan stereoisomer) was obtained by the thermostable α-glucan phosphorylase-catalyzed enzymatic polymerization of α-D-glucosamine 1-phosphate from a maltotriosaccharide primer. In the present study, we intend to reveal whether the enzyme catalyzes phosphorolysis of substrates containing such D-glucosaminide chains. The α(1→4)-linked oligo-D-glucosaminide substrates elongated from maltotriose were first prepared by the thermostable α-glucan phosphorylase-catalyzed enzymatic polymerization of α-D-glucosamine 1-phosphate from the maltotriose primer and subsequent purification by preparative HPLC. The phosphorolysis of the resulting substrates are conducted in the presence of thermostable α-glucan phosphorylase in phosphate buffer. The analytical results of the products fully supported the occurrence of phosphorolysis at the nonreducing end of both the chains of D-glucosamine-α(1→4)-D-glucosamine and D-glucosamine-α(1→4)-D-glucose sequences.

Keywords: α-glucan phosphorylase; α(1→4)-linked D-glucosaminides; Phosphorolysis

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Introduction
Enzymatic polymerization has been identified as a powerful approach to synthesize polysaccharides composed of stereo- and regio-controlled glycosidic linkages [1-6]. α-Glucan phosphorylase is one of the enzymes that have practically been used as a catalyst for enzymatic polymerization to produce well-defined polysaccharides. α-Glucan phosphorylase catalyzes in vivo phosphorolysis of α(1→4)-glucans such as amylase and glycogen at the nonreducing end in the presence of inorganic phosphate (Pi) to produce α-D-glucose 1-phosphate (Glc-1-P) [7-9]. Because of the reversibility of such phosphorylolytic reaction, this enzyme also catalyzes in vitro glucosylation of α(1→4)-glucan as a glycosyl acceptor such as maltotriosaccharide using Glc-1-P as a glycosyl donor to produce α(1→4)-glycosidic linkage with liberating Pi [10]. The reversible reaction by α-glucan phosphorylase catalysis is summarized as follows; [α (1→4)-Glc]ₙ⁺Pi ⇄ [α (1→4)-Glc]ₙ + Glc-1-P. Under the conditions in the large donor/acceptor ratios, successive glucosylations at the elongating nonreducing end from maltotriosaccharide as a primer take place by α-glucan...
phosphorylase catalysis, leading to the enzymatic polymerization to produce (1→4)-linked polysaccharide, that is, amylase [11-13]. The degree of polymerization (DP) of amylase can be controlled by the donor/acceptor feed ratio.

α-Glucan phosphorylases have exhibited the different specificity for the recognition of substrates, depending on their sources. For example, the smallest substrates recognized by the most common α-glucan phosphorylase isolated from potato (potato α-glucan phosphorylase) in phosphorolysis and glucosylation reactions are typically maltpentaose (Glc5) and maltotetraose (Glc4), respectively. On the other hand, Glc, and maltotriose (Glc3) are the smallest substrates recognized by α-glucan phosphorylase isolated from thermophilic bacterial sources (thermostable α-glucan phosphorylase) in phosphorolysis and glucosylation reactions, respectively [13-16]. Moreover, α-glucan phosphorylases are known to show weak specificity for the recognition of substrates, which has also been found to be depended on their sources [17-19]. Potato α-glucan phosphorylase catalyzes enzymatic glucosaminylations using α-D-glucosamine 1-phosphate (GlcN-1-P) and its derivative, N-formyl-α-D-glucosamine 1-phosphate (GlcNF-1-P), as non-native glycosyl donors in acetate buffer to produce oligosaccharides having one GlcN(F) unit at the nonreducing end [20,21], whereas successive glucosaminylations using GlcN-1-P occur by thermostable α-glucan phosphorylase (from Aquifex aeolicus VF5) catalysis in acetate buffer, giving rise to α(1→4)-linked oligo-GlcN chains at the nonreducing end of glycosyl acceptors, e.g., Glc, the smallest glycosyl acceptor for this enzyme. With increasing the donor/acceptor feed ratio, however, the DP values of the α(1→4)-GlcN chains retained at most five. This result indicated that further chain-elongation was inhibited by Pi produced from GlcN-1-P, because which is a native substrate for phosphorolysis by α-glucan phosphorylase catalysis. An attempt was then made to remove Pi as a precipitate from the reaction mixture by the addition of ammonium hydrochloride salt of the product. The resulting crude product was then maintained at 100 °C for 1 h in order to deactivate the enzyme, the precipitate was removed by centrifugation and the supernatant was lyophilized. Glucoamylase (GA, 20 U) was added to a solution of the resulting product in water (1.0 mL) and the mixture was incubated at 40°C for 1 h. The reaction mixture was then maintained at 100°C for 1 h in order to deactivate the enzyme. The deactivated enzyme was removed by centrifugation and the supernatant was lyophilized and subjected to MALDI-TOF measurement. To a dispersion of the resulting product (0.1658 g) in acetone (5.0 mL), 0.5 M methanolic hydrochloric acid (1.0 mL) was added dropwise and the mixture was stirred at room temperature for 15 min. The filtration of the dispersion gave the hydrochloride salt of the product. The resulting crude product (0.1650 g) was dissolved in water (1.0 mL) and subjected to

**Experimental**

**Materials**

A substrate, GlcN-1-P, was synthesized according to previously reported procedure [25]. Thermostable α-glucan phosphorylase from *Aquifex aeolicus* VF5 [26] was kindly supplied by Ezaki Glico Co. Ltd, Osaka, Japan. Other reagents and solvents were used as commercially received.

**Synthesis of substrates for phosphorolysis, GlcN\textsubscript{m} - Glc\textsubscript{3}**

A mixture of GlcN-1-P (0.1145 g, 0.44 mmol) and Glc\textsubscript{3} (0.0222 g, 0.044 mmol) in 0.1 M ammonia buffer (pH 8.5, 3.0 mL) dissolving MgCl\textsubscript{2} was incubated in the presence of thermostable α-glucan phosphorylase (21 U) at 40°C for 48 hrs. After the reaction mixture was maintained at 100 °C for 1 h in order to deactivate the enzyme, the precipitate was removed by centrifugation and the supernatant was lyophilized. Glucoamylase (GA, 20 U) was added to a solution of the resulting product in water (1.0 mL) and the mixture was incubated at 40°C for 1 h. The reaction mixture was then maintained at 100°C for 1 h in order to deactivate the enzyme. The deactivated enzyme was removed by centrifugation and the supernatant was lyophilized and subjected to MALDI-TOF measurement. To a dispersion of the resulting product (0.1658 g) in acetone (5.0 mL), 0.5 M methanolic hydrochloric acid (1.0 mL) was added dropwise and the mixture was stirred at room temperature for 15 min. The filtration of the dispersion gave the hydrochloride salt of the product. The resulting crude product (0.1650 g) was dissolved in water (1.0 mL) and subjected to

**Figure 1** Thermostable α-glucan phosphorylase-catalyzed enzymatic polymerization of GlcN-1- P from Glc\textsubscript{3} primer to produce α(1→4)-linked D-glucosaminide polymer (chitosan stereoisomer).
preparative HPLC with Wakosil 5NH column (10 × 250 mm) with acetonitrile/water (3:1, v/v) as the eluent (flow rate: 2.0 mL/min) to isolate a tetrascaroside (GlcN-Glc₃) and a mixture of penta- and hexascarosides (GlcN₃-Glc₄, GlcN₄-Glc₅, and GlcN₅-Glc₆) by the thermostable α-glucan phosphorylase-catalyzed enzymatic polymerization of GlcN-1-P according to the literature procedure (Figure 1) [23]. The smallest substrate, Glc₋, was used as the primer for the polymerization to avoid the occurrence of phosphorolysis of the primer, because the enzyme does not catalyze its phosphorolysis. The reaction was performed in the GlcN-1-P/Glc₋ feed ratio of 10:1 in the presence of thermostable α-glucan phosphorylase in ammonia buffer (pH 8.5) containing Mg²⁺ ion at 40°C for 48 h. The shorter reaction time was employed in this study than the literature condition (7 days) to produce GlcN₃₋Glc₆ with small DP values. The MALDI-TOF MS of the crude product after GA-treatment observes several peaks corresponding to the molecular masses of oligosaccharides containing one-five GlcN units (Figures 2a), supporting the production of the shorter GlcN₃₋Glc₆ than the literature products (m~ ~12). Moreover, the MALDI-TOF MS result also indicates no occurrence of the exo-type hydrolysis at the Glc₃ segment in the produced oligosaccharides by GA catalysis, suggesting the presence of the Glc₃N chains at the nonreducing end of Glc₋. The HPLC chromatogram after neutralization of the product with hydrochloric acid shows the plural peaks corresponding to oligosaccharides with different DPs (Figure 2b). Accordingly, the fractions of a peak A corresponding to a tetrascaroside and peaks B and C corresponding to penta- and hexascarosides were collected by preparative HPLC. The MALDI-TOF MS of the former and latter fractions shows peaks corresponding to molecular masses of GlcN₋Glc₃ and GlcN₋Glc₄(GlcN₋Glc₅), respectively (Figures 3a and 4a), indicating that the tetrascaroside and a mixture of the penta- and hexascarosides were successfully isolated.

The obtained two samples were then subjected to the thermostable α-glucan phosphorylase-catalyzed phosphorolysis reaction (Figure 5). The tetrascaroside, GlcN₋Glc₃, after dehydrochlorination by NaHCO₃ was incubated in the presence of thermostable α-glucan phosphorylase in phosphate buffer (pH 6.2, source of Pi) at 40°C for 48 h. After deactivation of the enzyme, the reaction mixture was lyophilized and subjected to the MALDI-TOF MS measurement. The MALDI-TOF MS of the product (Figure 3b) observes a peak corresponding to the molecular mass of Glc₋, but does not show the molecular mass peak of GlcN₋Glc₃. In the MALDI-TOF MS of the product (Figure 4b) by the same procedure using the GlcN₋Glc₃/GlcN₋Glc₄ sample, a molecular mass peak of Glc₋ is also detected, but the molecular mass peaks of GlcN₋Glc₃/GlcN₋Glc₄ are not observed. These results strongly suggested the occurrence of the thermostable α-glucan phosphorylase-catalyzed phosphorolysis of all the substrates, GlcN₋Glc₃, GlcN₋Glc₄, and GlcN₋Glc₅ in the presence of Pi. Both the ¹H NMR spectra (D₂O) of the products by the above two reactions indicated the production of GlcN₋P, which also supported the occurrence of the phosphorolysis by thermostable α-glucan phosphorylase catalysis. For example, the ¹H NMR spectrum (D₂O) of the products from the GlcN₋Glc₃/GlcN₋Glc₄ sample (Figure 6) observes the characteristic signal due to H-1 proton of GlcN₋P at around 5.7 ppm (multiplet). All the above results revealed that thermostable α-glucan phosphorylase...
Figure 2  (a) MALDI-TOF MS of crude product by thermostable α-glucan phosphorylase-catalyzed enzymatic polymerization of GlcN-1-P, followed by treatment with GA and (b) HPLC chromatogram after its neutralization with hydrochloric acid.

Figure 3  MALDI-TOF MS of (a) GlcN-Glc₃ and (b) its phosphorolyzed product by thermostable α-glucan phosphorylase catalysis.
catalyzed phosphorolysis at the nonreducing ends of both the chains of GlcN-α(1→4)-GlcN and GlcN-α(1→4)-Glc sequences in the presence of Pi.

**Conclusion**

In this paper, we revealed that thermostable α-glucan phosphorylase from *Aquifex aeolicus* VF5 catalyzed phosphorolysis at the nonreducing ends of both the chains of GlcN-α(1→4)-GlcN and GlcN-α(1→4)-Glc sequences in the presence of Pi. The substrates, GlcN$_m$-Glc$_3$ (m=1~3), were first prepared by the thermostable α-glucan phosphorylase-catalyzed enzymatic polymerization of GlcN-1-P from the Glc$_3$ primer in ammonia buffer containing Mg$^{2+}$ ion and isolated by preparative HPLC. The thermostable α-glucan phosphorylase-catalyzed phosphorolysis of the resulting substrates was then conducted in phosphate buffer. The MALDI-TOF MS of the products suggested that the enzymatic phosphorolysis of the substrates were fully progressed.

**Figure 4** MALDI-TOF MS of (a) mixture of GlcN$_2$-Glc$_3$ and GlcN$_3$-Glc$_3$ and (b) its phosphorolyzed product by thermostable α-glucan phosphorylase catalysis.

**Figure 5** Thermostable α-glucan phosphorylase-catalyzed phosphorolysis of GlcN$_m$-Glc$_3$ in phosphate buffer.

**Figure 6** $^1$H NMR spectrum (D$_2$O) of phosphorolyzed products from mixture of GlcN$_2$-Glc$_3$ and GlcN$_3$-Glc$_3$ by thermostable α-glucan phosphorylase catalysis.
to produce Glc$_3$. The production of GlcN-1-P was observed by the $^1$H NMR spectra of the products, which also supported the progress of phosphorolysis. The findings in the present study will contribute to further development of the precise enzymatic reactions by α-glucan phosphorylase catalysis to produce well-defined saccharide materials.

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