

An Investigation of Enzymatic Phosphorolysis of $\alpha(1\rightarrow4)$ -Linked Oligo-D-glucosaminides by Thermostable α -Glucan Phosphorylase Catalysis

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Abstract

This paper reports an investigation of enzymatic phosphorolysis of $\alpha(1\rightarrow4)$ -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 $\alpha(1\rightarrow4)$ -linked glucose substrates depending on conditions. The authors also found that $\alpha(1\rightarrow4)$ -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 maltooligosaccharide primer. In the present study, we intend to reveal whether the enzyme catalyzes phosphorolysis of substrates containing such D-glucosaminide chains. The $\alpha(1\rightarrow4)$ -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- $\alpha(1\rightarrow4)$ -D-glucosamine and D-glucosamine- $\alpha(1\rightarrow4)$ -D-glucose sequences.

Keywords: α -glucan phosphorylase; $\alpha(1\rightarrow4)$ -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 $\alpha(1\rightarrow4)$ -glucans such as amylose 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 phosphorolytic reaction, this enzyme also catalyzes in vitro glucosylation of $\alpha(1\rightarrow4)$ -glucan as a glycosyl acceptor such as maltooligosaccharide using Glc-1-P as a glycosyl donor to produce $\alpha(1\rightarrow4)$ -glucosidic linkage with liberating Pi [10]. The reversible reaction by α -glucan phosphorylase catalysis is summarized as follows; $[\alpha(1\rightarrow4)\text{-Glc}]_{n+1} + \text{Pi} \rightleftharpoons [\alpha(1\rightarrow4)\text{-Glc}]_n + \text{Glc-1-P}$. Under the conditions in the large donor/acceptor ratios, successive glucosylations at the elongating nonreducing end from maltooligosaccharide as a primer take place by α -glucan

phosphorylase catalysis, leading to the enzymatic polymerization to produce (1→4)-linked polysaccharide, that is, amylose [11-13]. The degree of polymerization (DP) of amylose 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 maltopentaose (Glc₅) and maltotetraose (Glc₄), respectively. On the other hand, Glc₄ 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 $\alpha(1\rightarrow4)$ -linked oligo-GlcN chains at the nonreducing end of glycosyl acceptors, e.g., Glc₃ the smallest glycosyl acceptor for this enzyme. With increasing the donor/acceptor feed ratio, however, the DP values of the $\alpha(1\rightarrow4)$ -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 enzymatic glucosaminylation field by performing the reaction in an ammonium buffer (0.5 M, pH 8.5) containing MgCl₂, because the previous publication has reported that Pi forms an insoluble salt with ammonium and magnesium ions [22]. Consequently, the successive enzymatic glucosaminylations were accelerated in the buffer system to occur polymerization of GlcN-1-P from the Glc₃ primer, giving rise to the $\alpha(1\rightarrow4)$ -linked D-glucosaminide polymer, which corresponded to the structure of a chitosan stereoisomer (**Figure 1**) [23,24]. On the basis of the above results, we have speculated that thermostable α -glucan phosphorylase

catalyzes not only the glucosaminylation using GlcN-1-P, but also the phosphorolysis at the nonreducing end of the $\alpha(1\rightarrow4)$ -linked glucosaminide chain, depending on the concentration of Pi. Moreover, it was reported that thermostable α -glucan phosphorylase (from *Escherichia coli*) exhibited the different catalytic behaviors toward phosphorolysis and glucosylation depending on reaction conditions [15]. In the present paper, we report the precise investigation to reveal whether thermostable α -glucan phosphorylase catalyzes phosphorolysis of $\alpha(1\rightarrow4)$ -linked oligo-D-glucosaminides-maltotriose (GlcN_m-Glc₃) substrates.

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_m-Glc₃

A mixture of GlcN-1-P (0.1145 g, 0.44 mmol) and Glc₃ (0.0222 g, 0.044 mmol) in 0.1 M ammonia buffer (pH 8.5, 3.0 mL) dissolving MgCl₂ 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

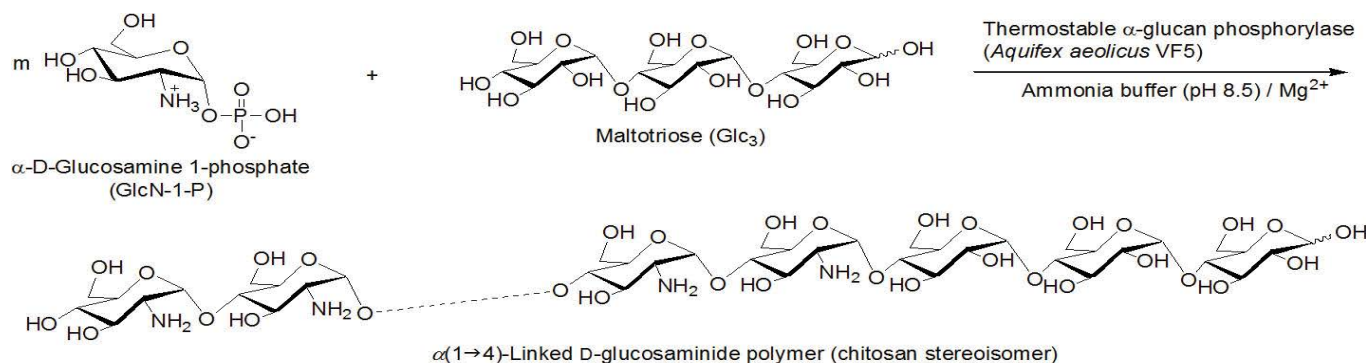


Figure 1 Thermostable α -glucan phosphorylase-catalyzed enzymatic polymerization of GlcN-1-P from Glc₃ primer to produce $\alpha(1\rightarrow4)$ -linked D-glucosaminide polymer (chitosan stereoisomer).

preparative HPLC with Wakosil 5NH₂ column (10 × 250 mm) with acetonitrile/water (3:1, v/v) as the eluent (flow rate; 2.0 mL/min) to isolate a tetrasaccharide (GlcN-Glc₃) and a mixture of penta- and hexasaccharides (GlcN₂-Glc₃, GlcN₃-Glc₃).

MALDI-TOF MS: [GlcN₂-Glc₃+Na]⁺: Calcd *m/z* 688.5511, Found *m/z* 688.3399, [GlcN₂-Glc₃+Na]⁺, [GlcN₃-Glc₃+Na]⁺: Calcd *m/z* 849.5557, 1010.5670, Found *m/z* 849.4399, 1010.4151.

Phosphorolysis of GlcN-Glc₃

The isolated GlcN-Glc₃ (1.7 mg) was treated with aqueous NaHCO₃ (0.22 mg, 2.62 μmol) at room temperature for 15 min for dehydrochlorination. After the resulting solution was diluted with 0.2 M phosphate buffer (pH 6.2, 2.0 mL), the mixture was incubated in the presence of thermostable α-glucan phosphorylase (21 U) at 40 °C for 2 days. 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 to give the phosphorolyzed product.

MALDI-TOF MS: [Glc₃+Na]⁺: Calcd *m/z* 527.4268, Found *m/z* 528.3082

Phosphorolysis of mixture of GlcN₂-Glc₃ and GlcN₃-Glc₃

The mixture of GlcN₂-Glc₃ and GlcN₃-Glc₃ (1.3 mg) was treated with aqueous NaHCO₃ (0.14 mg, 1.66 μmol) at room temperature for 15 min for dehydrochlorination. After the resulting solution was diluted with 0.2 M phosphate buffer (pH 6.2, 2.0 mL), the mixture was incubated in the presence of thermostable α-glucan phosphorylase (21 U) at 40 °C for 2 days. 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 to give the phosphorolyzed product.

MALDI-TOF MS: [Glc₃+Na]⁺: Calcd *m/z* 527.4268, Found *m/z* 527.1282

Measurements

¹H NMR spectra were recorded on JEOL ECA 600 and ECX 400 spectrometers. MALDI-TOF MS measurements were conducted by using SHIMADZU Voyager Biospectrometry Workstation Ver. 5.1 with 2,5-dihydroxybenzoic acid as a matrix containing 0.05% trifluoroacetic acid under positive ion mode. Analytical HPLC was performed on a PU-2080 apparatus (JASCO) using RI-2031 RI detector (JASCO) with MERCK LiChrospher 100 NH₂ column (10 μm, 4.0 × 250 mm) with acetonitrile/water (3:2, v/v) as the eluent (flow rate; 0.5 mL/min). Preparative HPLC was performed on a NPL apparatus (Nihon Seimitsu Kagaku Co., LTD) using YRD-833 RI detector (Shimadurtech) with Wakosil 5NH₂ column (10 × 250 mm) with acetonitrile/water (3:1, v/v) as the eluent (flow rate; 2.0 mL/min).

Results and Discussion

We first prepared the α(1→4)-linked oligo-D-glucosaminides-maltotriose substrates for the phosphorolysis, that is, tetra-

hexasaccharides (GlcN-Glc₃, GlcN₂-Glc₃, and GlcN₃-Glc₃) by the thermostable α-glucan phosphorylase-catalyzed enzymatic polymerization of GlcN-1-P according to the literature procedure (**Figure 1**) [23]. The smallest substrate, Glc₃, 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/Glc₃ 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 GlcN_m-Glc₃ 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 GlcN_m-Glc₃ 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 GlcN chains at the nonreducing end of Glc₃. 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 tetrasaccharide and peaks B and C corresponding to penta- and hexasaccharides 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 GlcN₂-Glc₃/GlcN₃-Glc₃, respectively (**Figures 3a and 4a**), indicating that the tetrasaccharide and a mixture of the penta- and hexasaccharides were successfully isolated.

The obtained two samples were then subjected to the thermostable α-glucan phosphorylase-catalyzed phosphorolysis reaction (**Figure 5**). The tetrasaccharide, 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 Glc₃, 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 GlcN₂-Glc₃/GlcN₃-Glc₃ sample, a molecular mass peak of Glc₃ is also detected, but the molecular mass peaks of GlcN₂-Glc₃/GlcN₃-Glc₃ are not observed. These results strongly suggested the occurrence of the thermostable α-glucan phosphorylase-catalyzed phosphorolysis of all the substrates, GlcN-Glc₃, GlcN₂-Glc₃, and GlcN₃-Glc₃ in the presence of Pi. Both the ¹H NMR spectra (D₂O) of the products by the above two reactions indicated the production of GlcN-1-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 GlcN₂-Glc₃/GlcN₃-Glc₃ sample (**Figure 6**) observes the characteristic signal due to H-1 proton of GlcN-1-P at around 5.7 ppm (multiplet). All the above results revealed that thermostable α-glucan phosphorylase

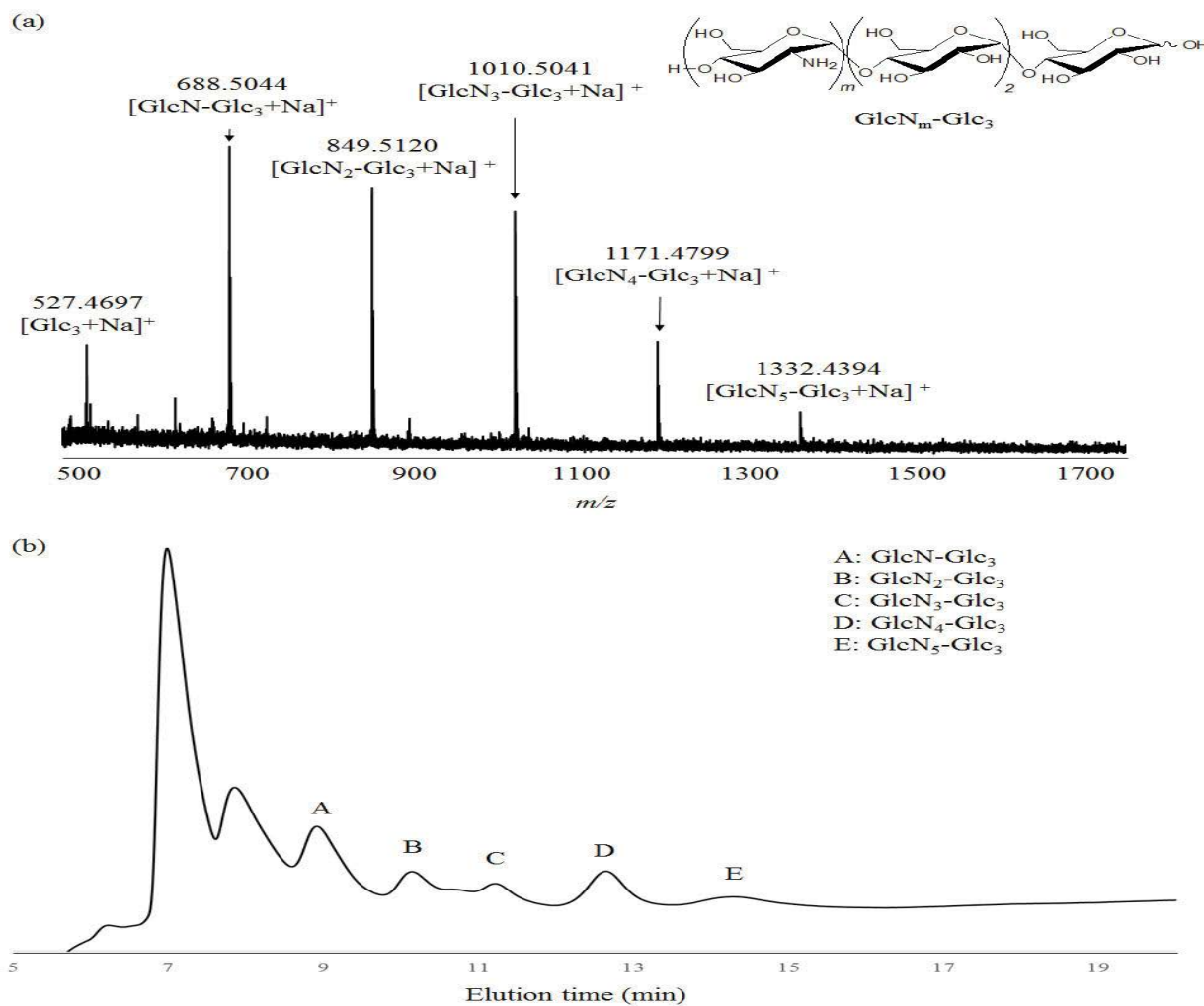


Figure 2 (a) MALDI-TOF MS of crude product by thermostable α -glucan phosphorylase-catalyzed enzymatic polymerization of GlcN-1-P , followed by treatment with GA and (b) HPLC chromatogram after its neutralization with hydrochloric acid.

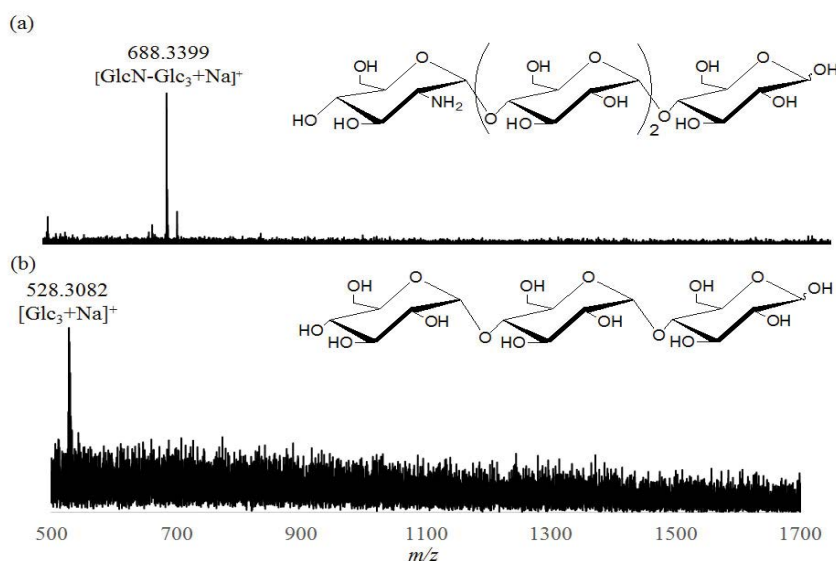


Figure 3 MALDI-TOF MS of (a) GlcN-Glc_3 and (b) its phosphorylated product by thermostable α -glucan phosphorylase catalysis.

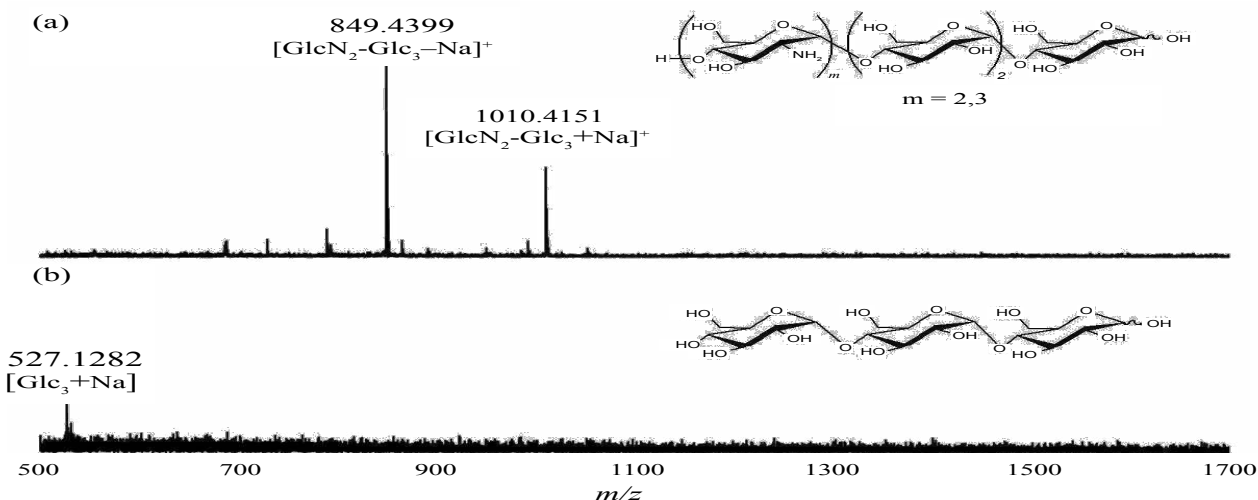


Figure 4 MALDI-TOF MS of (a) mixture of $\text{GlcN}_2\text{-Glc}_3$ and $\text{GlcN}_3\text{-Glc}_3$ and (b) its phosphorylated product by thermostable α -glucan phosphorylase catalysis.

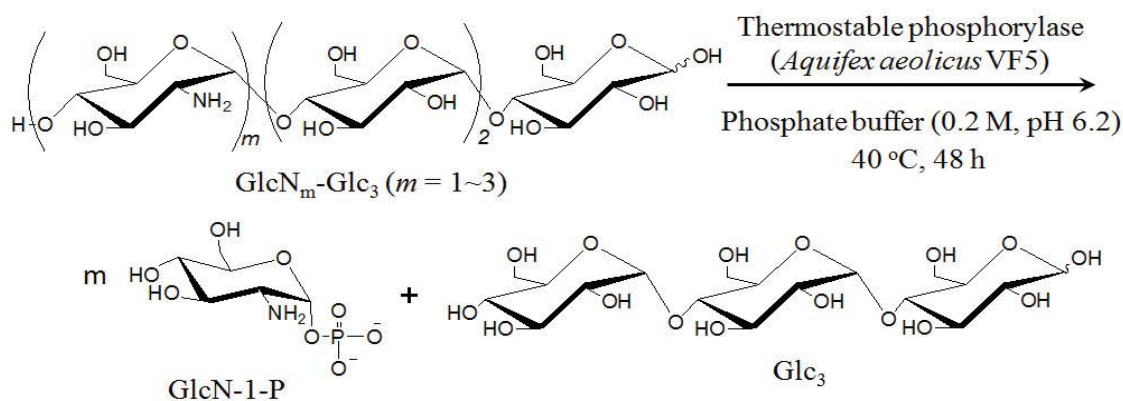


Figure 5 Thermostable α -glucan phosphorylase-catalyzed phosphorolysis of $\text{GlcN}_m\text{-Glc}_3$ in phosphate buffer.

catalyzed phosphorolysis at the nonreducing ends of both the chains of $\text{GlcN-}\alpha(1\rightarrow 4)\text{-GlcN}$ and $\text{GlcN-}\alpha(1\rightarrow 4)\text{-Glc}$ sequences in the presence of P_i .

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 $\text{GlcN-}\alpha(1\rightarrow 4)\text{-GlcN}$ and $\text{GlcN-}\alpha(1\rightarrow 4)\text{-Glc}$ sequences in the presence of P_i . The substrates, $\text{GlcN}_m\text{-Glc}_3$ ($m=1\sim 3$), 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

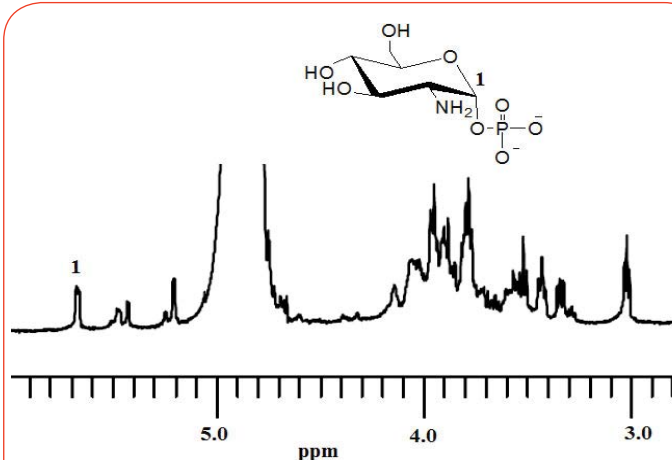


Figure 6 ^1H NMR spectrum (D_2O) of phosphorylated products from mixture of $\text{GlcN}_2\text{-Glc}_3$ and $\text{GlcN}_3\text{-Glc}_3$ by thermostable α -glucan phosphorylase catalysis.

to produce Glc₃. The production of GlcN-1-P was observed by the ¹H 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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