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Synthesis of Chitin and Chitosan Stereoisomers by Thermostable α-Glucan Phosphorylase-Catalyzed Enzymatic Polymerization of α-D-Glucosamine 1-Phosphate

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The relationship between two aminopolysaccharide stereoisomers, namely α-(1→4)- and β-(1→4)-linked (*N*-acetyl)-D-glucosamine polymers, is of significant interest within the field of polysaccharide science, as they correspond to amino analogs of the representative abundant natural polysaccharides, viz. amylose and cellulose. While the latter glucosamine polymer is the basis of well-known natural polysaccharides, chitin and chitosan (linear polysaccharides composed of β-(1→4)-linked *N*-acetyl-D-glucosamine and D-glucosamine), to the best of our knowledge, the former (α-(1→4)-linked) has not been observed in nature. For the purpose of these studies, the synthesis of such non-natural aminopolysaccharides was performed by the thermostable α-glucan phosphorylase (from *Aquifex aeolicus* VF5)-catalyzed enzymatic polymerization of α-D-glucosamine 1-phosphate (GlcN-1-P), via successive α-glucosaminylations, in ammonia buffer containing Mg^{2+} ions, resulting in the production of the α-(1→4)-linked D-glucosamine polymers, corresponding to the structure of the chitosan stereoisomer. Subsequent *N*-acetylation of the products gave the aminopolysaccharides, corresponding to the chitin stereoisomer.

Introduction

Natural polysaccharides play a vital role in a number of important *in vivo* functions. They function as both energy sources and as structural materials, for example, amylose (a component of starch), and cellulose (a structural component in plant cell walls).[1] Both of these polysaccharides are glucose (Glc) polymers, so-called glucans, but differ in their linkage, with amylose being linked through α-(1→4)-glucosidic bonds and cellulose through β-(1→4)-glucosidic bonds (Fig. 1). Due to the opposite stereoregularity between the glucose units in (1→4)-linkages, the roles of such stereoisomers in nature are very different as aforementioned.[2,3] Besides their native roles, they have also been used in applications as functional materials. Amylose, for example, has been employed as a host in host-guest chemistry for the construction of supramolecular inclusion complexes, thanks to its helical conformation.[4,5] Another abundant natural polysaccharide is chitin, an aminopolysaccharide composed of β-(1→4)-linked *N*-acetyl-D-glucosamine (GlcNAc) residues (Fig. 1).[6-8] Accordingly, chitin could be considered to be an amino analog of cellulose, as it also acts as a structural material in nature. For example, chitin can be found in the exoskeletons of crustaceans, shellfish, and insects. Chitosan is the *N*-deacetylated derivative of chitin, composed of D-glucosamine (GlcN) repeating units as shown in Fig. 1. Chitosan has been extensively studied in drug and gene delivery formulations because of its ability to electrostatically bind and encapsulate anionic biopolymers.[9] Because these properties are owing to the presence of amino groups, amine-functionalized polysaccharides have attracted attention as advantageous materials in biomedical applications.[10] Stereoisomeric aminopolysaccharides of chitin/chitosan, the amino analogs of amylose (Fig. 1), should therefore have interesting properties, and could be considered as new high performance functional polysaccharide materials. However, to the best of our knowledge, such aminopolysaccharides are not known in nature and have not yet been chemically synthesized.

In this study, we describe the enzymatic glycosylation approach for the synthesis of the chitin/chitosan stereoisomers, as it is a powerful and efficient technique for the connection of two unprotected glycosyl substrates (one donor and one acceptor), by a stereo- and regio-controlled glycosidic linkage.[11,12] Furthermore, enzymatic glycosylations have been employed for enzymatic polymerization according to the successive reaction manner for the production of polysaccharides,[13-17] such as the first report of the synthesis of cellulose *via* cellulase-catalyzed enzymatic polymerization by Kobayashi *et al*.[18] By means of such reverse reactions catalyzed by glycosyl hydrolases such as cellulase, the enzymatic

polymerization of monomers composed of both the glycosyl donor and acceptor structures in a molecule, gave not only cellulose and its derivatives,[19] but also aminopolysaccharides. These aminopolysaccharides include chitin, glycosaminoglycans, and other related non-natural analogues,[20-23] where the structures are composed mainly of β-glycosidic linkages. Another enzyme that has been applied as a catalyst in enzymatic polymerization is α-glucan phosphorylase (EC 2.4.1.1, also known as starch phosphorylase or glycogen phosphorylase). This enzyme catalyzes the reversible phosphorolysis of an α-(1→4)-glucan chain at the nonreducing end, in the presence of inorganic phosphate (Pi), to produce an *n*-1 chain and α-D-glucose 1-phosphate (Glc-1-P), as follows: $[\alpha\text{-}(1\rightarrow4)\text{-Glc}]_n + \text{Pi} \rightleftarrows [\alpha\text{-}(1\rightarrow4)\text{-Glc}]_{n-1} + \text{Glc-1-P}$.[24-26] Due to the reversible nature of the reaction, this enzyme has also been used as a catalyst for α-glucosylation, using α-D-glucose 1-phosphate (Glc-1-P) and maltooligosaccharides as the glycosyl donor and acceptor, respectively, where the degree of polymerization (DP) of the acceptor is higher than the smallest one recognized by the enzyme.[27] The reaction proceeds *via* transfer of a Glc residue from the donor to the nonreducing end of the acceptor. This results in the formation of an α-(1→4)-glucosidic linkage simultaneously with the production of inorganic phosphate. By using an excess of the donor molecule, α-glucan phosphorylase catalyzes the enzymatic polymerization reaction *via* successive α-glucosylations, to produce the α-(1→4)-linked glucose polymer, amylose.[28-31] α-Glucan phosphorylase displays strict specificity in the reaction, with the formation of only α-(1→4)-glucosidic linkages, but exhibits some loss of specificity in the recognition of glycosyl donors. Therefore, analogous substrates of Glc-1-P have also been used for α-glucan phosphorylase-catalyzed enzymatic glycosylations.[32-39] For example, the authors found that α-glucan phosphorylase from potatoes recognized α-D-glucosamine 1-phosphate (GlcN-1-P). Thus, it is capable of catalyzing the α-glucosaminylation of maltotetraose (Glc_4), the smallest glycosyl acceptor recognized by potato α-glucan phosphorylase, to produce a pentasaccharide having a GlcN residue at the nonreducing end.[40] It has also been found that α-glucan phosphorylase from thermostable bacteria (thermostable α-glucan phosphorylase) displays a different substrate specificity to the potato phosphorylase. The smallest acceptor for potato α-glucan phosphorylase catalysis is Glc_4 as mentioned previously, whereas thermostable α-glucan phosphorylase is capable of recognizing maltotriose (Glc_3), which consists of one less Glc unit.[30,41,42] Taking this into account, we found that thermostable α-glucan phosphorylase catalyzes the α-glucuronylation of Glc_3 as a glycosyl acceptor, using α-D-glucuronic acid 1-phosphate as a glycosyl donor, which is not recognized by potato α-glucan phosphorylase.[43,44] Moreover, we have also reported successive α-mannosylations catalyzed by thermostable α-glucan phosphorylase, using an excess of α-D-mannose 1-phosphate as a glycosyl donor, and Glc_3 as the acceptor, to give oligo α-(1→4)-mannan chains composed of up to five mannose units.[45]

Based on these interesting observations, we report the finding that thermostable α-glucan phosphorylase (from *Aquifex aeolicus* VF5)[46] catalyzes successive α-glucosaminylations using GlcN-1-P[47] as a glycosyl donor, for the enzymatic synthesis of the α-(1→4)-linked GlcN polymer, which is the chitosan stereoisomer. This polymer can be further converted into the α-(1→4)-linked GlcNAc polymer, the chitin stereoisomer. To obtain the chitin and chitosan stereoisomers with high DPs, furthermore, the enzymatic reactions were conducted in an ammonia buffer solution containing magnesium ions, leading to precipitation of the phosphate salt produced by α-glucosaminylation. This reaction system was successfully implemented into further α-glucosaminylations, namely enzymatic polymerizations, and subsequent *N*-acetylations, to produce the chitin/chitosan stereoisomers with higher DPs, corresponding to (Fig. 2). The present materials, *i.e.*, the chitin/chitosan stereoisomers, could be classified as new functional polysaccharides, as they are the amino analogs of amylose, a well-known high performance functional natural polysaccharide with a well-defined helical conformation.

Results and Discussion

We first demonstrated the thermostable α-glucan phosphorylase-catalyzed α-glucosaminylation of Glc_3 as the glycosyl acceptor (the smallest substrate recognized by the enzyme), with GlcN-1-P in a feed ratio of 1:10, in sodium acetate buffer (0.2 M, pH 6.2) at 40 °C. The MALDI-TOF MS of the crude product mixture after 7 days showed signals corresponding to the masses of tetra-octasaccharides containing one – five GlcN residues separated by *m/z* 161 (Fig. 3a). This result indicated that thermostable α-glucan phosphorylase catalyzed successive α-glucosaminylations to the chain-elongating nonreducing ends to produce chains of α-GlcN. However, the mass spectrometry results also suggested the formation of short oligosaccharides having at most five GlcN units. We assumed that the inorganic phosphate produced in the α-glucosaminylations likely inhibited the progress of the reaction by interaction with the active site of the enzyme, due to the fact that it is a native substrate for the phosphorolysis reaction of α-glucan phosphorylase. Therefore, an attempt was made to remove inorganic phosphate from the reaction system by precipitation. For this purpose, we noted the previous publication, which reported that inorganic phosphate formed an insoluble salt with ammonium and magnesium.[48] Thus, the thermostable α-glucan phosphorylase-catalyzed α-glucosaminylation of Glc_3 with GlcN-1-P (1:10) was carried out in an ammonium buffer (0.5 M, pH 8.6) in the presence of $MgCl_2$ at 40 °C, where we adjusted the quantities of ammonium and magnesium ions were approximately equimolar with GlcN-1-P. The precipitate formed during the course of the reaction was therefore removed from the reaction mixture by centrifugation after 7 days, and the filtrate was lyophilized to give the crude products. The ^{31}P and ^{1}H NMR spectra of the precipitate, which was solubilized in DCl/D_2O, confirmed it to be a phosphate salt, which did not contain any sugar moieties (Figs. S1a and b). From the weight of the precipitated ammonium magnesium phosphate, the α-glucosaminylation ratio (the conversion of GlcN-1-P) was calculated to be 95.3%. The MALDI-TOF MS of the crude product mixture (Fig. 3b) showed signals separated by *m/z* 161, corresponding to products of larger molecular masses, composed of up to twelve GlcN units, more than in the previous reaction (Fig. 3a). This result strongly supports our hypothesis that the removal of inorganic phosphate from the reaction media successfully induces further α-glucosaminylations, according to the reaction manner of the enzymatic polymerization, to produce larger polysaccharides composed of a greater number of GlcN units. After treatment of the crude products with glucoamylase (EC 3.2.1.3) to enzymatically hydrolyze any unreacted Glc_3 into Glc, the MALDI-TOF MS analysis was repeated once more. It

was expected that if the GlcN units were positioned at the nonreducing site in the products, they would not be hydrolyzed by glucoamylase due to its exo-type catalytic mechanism for the hydrolysis of α-(1→4)-glucan into Glc. This was confirmed by MALDI-TOF MS, where signals corresponding to the glucoamylase-treated products remained intact (Fig. S2), thus confirming the presence of the GlcN units at the nonreducing site in the products. In order to further confirm the identity of the products by MALDI-TOF MS, *N*-acetylation of the treated products was carried out, using acetic anhydride in aqueous Na_2CO_3. As the difference in molecular mass between the anhydroglucose and anhydroglucosamine units is only 1 amu (162 and 161, respectively), we chose to carry out *N*-acetylation of the latter unit in order to amplify the mass difference and confirm the structure of the products. The MALDI-TOF MS of the *N*-acetylated sample (Fig. 3c), which was obtained as described in the supporting information, displayed a number of signals separated by *m/z* 203, corresponding to the molecular masses of polysaccharides containing up to ten GlcNAc units. This data strongly support the presence of a glucosamine chain structure in the enzymatic polymerization products.

The enzymatic polymerization of GlcN-1-P, initiated from Glc_3 through successive α-glucosaminylations, was then conducted using various GlcN-1-P/Glc_3 feed ratios under the same conditions. After removal of the precipitated phosphates by centrifugation, the reaction solutions were treated with an anion-exchange resin, and the products were isolated as fractions insoluble in methanol. The monomer conversions determined by weight of the precipitates were calculated to be greater than 70%, with the isolated yields being around 50-65 % in all cases (Table 1). The ^{1}H NMR spectrum of the isolated material (run 3, GlcN-1-P/Glc_3 feed ratio = 30) in D_2O (Fig. 4a) showed a signal assignable to the H-2 proton of the GlcN units (2.91 ppm), anomeric signals assignable to the protons at the nonreducing end of the polymer (4.65 and 5.23 ppm, β and α protons, respectively), an anomeric signal ascribable to the α-glycoside protons (5.41 ppm), and a range of signals due to the saccharide backbone (3.42-3.94 ppm). In particular, detection of a characteristic signal at higher magnetic field (2.91 ppm) corresponding to the protons at C-2 position linked to the amino group, confirmed the presence of the GlcN units in the products. Because the anomeric signal at 5.41 ppm was potentially assignable to protons on both the α-glucoside and α-glucosaminide residues, ^{1}H NMR measurements were carried out in a mixed solvent of D_2O and acetic acid-d_4 (Fig. 4b). Consequently, two different anomeric signals were observed and could be assigned to the α-glucoside and α-glucosaminide (5.39 and 5.71 ppm, respectively), owing to a shift of the latter to lower magnetic field, due to protonation of the C-2 amino group in the GlcN units under acidic conditions. A shift in the signal corresponding to the H-2 protons of the GlcN units was also observed (2.91→3.45 ppm, Figs. 4a and b). From the integrated ratio of the reducing anomeric signals to the α-glucosaminide signal, the average DP of the α-GlcN chain in the polysaccharide product was calculated to be 20.2. Based on this DP value, the M_n value of the produced polysaccharide ($GlcN_n$-Glc_3) was calculated to be 3760. These combined results suggested the successful production of non-natural α-(1→4)-linked GlcN chains, corresponding to the chitosan stereoisomer, by the thermostable phosphorylase-catalyzed enzymatic polymerization of GlcN-1-P. Similarly, the DP values of the chitosan stereoisomers produced by a range of GlcN-1-P/Glc_3 feed ratios were estimated by integration of the anomeric signals in the ^{1}H NMR spectra, as shown in Table 1. It was observed that increasing the GlcN-1-P/Glc_3 feed ratios resulted in an increase in the DP value.

The isolated chitosan stereoisomers were then converted to chitin stereoisomers by *N*-acetylation, using acetic anhydride in aqueous Na_2CO_3. After successive treatment of the reaction mixtures with both cation- and anion-exchange resins, the products were isolated as fractions insoluble in ethanol. Fig. 4c shows the ^{1}H NMR spectrum of the *N*-acetylated polysaccharide obtained by the enzymatic polymerization using a GlcN-1-P/Glc_3 feed ratio of 30 (run 3) in D_2O. In addition to the signals corresponding to the sugar protons at 3.75-3.99 ppm (H-2,3,4,5,6), 4.66 ppm and 5.24 ppm (H-1α and β of the reducing end, respectively), and 5.40 ppm (H-1 of α-glucosaminide and α-glucoside), a signal assignable to the methyl protons of the acetamido group was detected at 2.07 ppm. Furthermore, the integrated ratio of the methyl signal to the anomeric signal at 5.40 ppm was calculated to be ~3, suggesting that the amino groups in the polysaccharide were fully acetylated to give the desired GlcNAc units. We can therefore conclude based on the ^{1}H NMR and MALDI-TOF MS data (Fig. 3c) that the α-(1→4)-linked GlcNAc chains, corresponding to the chitin stereoisomer, were successfully prepared and isolated.

The gel permeation chromatographic (GPC) measurements of the *N*-acetylated polysaccharides, *i.e.*, the chitin stereoisomers, were conducted using DMSO as the eluent, to further evaluate the molecular weights. From the GPC chromatogram of the *N*-acetylated polysaccharide obtained using a GlcN-1-P/Glc_3 feed ratio of 30 (run 3), the M_n value was estimated to be 2240, using a calibration curve of the standard amylose samples, maltoheptaose, and Glc_3. This M_n value was smaller than that estimated from the ^{1}H NMR spectrum, most likely due to the difference in the eluting times of the chitin stereoisomer and the amylose/maltooligosaccharide standards in the GPC measurement, owing to the different GlcN and Glc repeating unit structures. The GPC results also show an increase in the M_n values depending on the GlcN-1-P/Glc_3 feed ratios (Table 1), with the trend in good agreement with that determined by the ^{1}H NMR analysis. Because α-glucan phosphorylase from *Aquifex aeolicus* VF5 retains ca. 80-66% of its original activity at ~90-100 °C, which indicates to tolerate the reactions at higher temperatures,[46] the production of the chitin/chitosan stereoisomers under additional reaction conditions, *e.g.*, at higher temperatures, will be demonstrated.

In order to further confirm the structures of α-(1→4)-linked GlcN(Ac) chains by COSY NMR analysis, the hydroxy groups of the *N*-acetylated aminopolysaccharide from run 3 were fully acetylated using acetic anhydride in pyridine, in the presence of *N*,*N*-dimethylaminopyridine as catalyst. Successful *O*-acetylation was confirmed by ^{1}H NMR spectroscopy with the detection of seven sugar signals (H-1, 2, 3, 4, 5, 6a, 6b) at 3.87-5.14 ppm (DMSO-d_6, Fig. 4d). From the integrated ratio of the acetyl signals to the saccharide backbone signals, moreover, the degree of acetylation was calculated to be *ca.* 3, also supporting successful *O*-acetylation. ^{1}H-^{1}H COSY NMR analysis was then carried out in order to determine the exact chemical shift of each signal and in order to assign each signal to its corresponding proton in the structure (Fig. S3). It was observed that the signals corresponding to protons H-2, 3, and 6 shifted to lower magnetic fields from those in Fig. 4c, whereas the chemical shifts of protons H-1, 4, and 5 remained essentially unaltered. Furthermore, the chemical shifts of these signals were comparable to those of amylose tri-*O*-acetate,[49] with the

exception of the signal corresponding to H-2, due to the difference between the acetamido and acetate groups at C-2 position. These data indicated that hydroxy groups at the former three positions were acetylated, whereas the acetylation at the latter three positions did not occur because of the absence of hydroxy groups. It could therefore be concluded that formation of the glycosidic linkages between C-1 and C-4 in the GlcN residues was successful using α-glucan phosphorylase, to give an α-(1→4)-linked GlcN chain corresponding to the chitosan stereoisomer, which was successively converted to the chitin stereoisomer by *N*-acetylation.

Conclusions

In the present paper, we reported that the thermostable α-glucan phosphorylase-catalyzed enzymatic polymerization of GlcN-1-P as a monomer with Glc_3 took place under the conditions of the removal of inorganic phosphate as the precipitate to produce the aminopolysaccharides corresponding to the chitosan stereoisomer. Furthermore, the products were successfully converted to the corresponding chitin stereoisomers by *N*-acetylation. Following *O*-acetylation, the structures of these non-natural aminopolysaccharides were confirmed by ^{1}H NMR and ^{1}H-^{1}H COSY NMR spectroscopy. We also observed that the DP (M_n) values of the enzymatic polymerization products increased with increasing GlcN-1-P/Glc_3 feed ratios. The present materials can be expected to exhibit new functions, not only as the stereoisomeric materials of chitin/chitosan, but also as the amino analogs of amylose. We are therefore now carrying out additional studies on these materials, such as investigation into their higher-ordered structures and biomedical applications. We expect to report these new studies in forthcoming publications in the near future.

Experimental Section

Instruments and methods

^{1}H NMR spectra were recorded on JEOL ECA600 and ECX400 spectrometers. MALDI-TOF MS measurements were carried out by using a SHIMADZU Voyager Biospectrometry Workstation Ver.5.1 with 2.5-dihydroxybenzoic acid as matrix containing 0.05% trifluoroacetic acid under positive ion mode. UV–vis measurements were conducted using a Jasco V-650Q1 spectrometer. Gel permeation chromatography (GPC) analysis was performed using an NS NPC-5010 pump and TOSOH RI-8020 RI detector under the following conditions: Shodex GPC LF-804 column with DMSO as the eluent at a flow rate of 0.3 mL/min at 60 ° C.

Materials

α-D-Glucosamine 1-phosphate (GlcN-1-P) was synthesized according to the literature procedure.[47] Maltoheptaose was prepared by selective cleavage of one glycosidic bond of β-cyclodextrin under acidic conditions.[50] Thermostable α-glucan phosphorylase from *Aquifex aeolicus* VF5 was kindly supplied from Ezaki Glico Co. Ltd., Osaka, Japan.[30,42,46] Other reagents and solvents were used as commercially received.

General procedure for the synthesis of chitosan stereoisomers by enzymatic polymerization

A typical experimental procedure was as follows (run 3). A mixture of GlcN-1-P (37.6 mg, 0.145 mmol) and Glc_3 (2.4 mg, 4.76 μmol) in 0.5 M ammonia buffer (pH 8.6, 300 μL, 0.150 mmol dissolving $MgCl_2$ (14.5 mg, 0.155 mmol) was incubated in the presence of thermostable α-glucan phosphorylase (20 U) at 40 °C for 7 days. The precipitate was then removed by centrifugation, and the supernatant was 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. After the produced crude products were dissolved in water (1.0 mL), the resulting solution was treated with an anion-exchange resin (Amberlite IRA-400J Cl) for 1 h, filtered, and concentrated. Subsequent addition of methanol to the concentrated solution resulted in precipitation of the product, which was isolated by filtration to give the chitosan stereoisomer (15.7 mg) in 61.8% yield based on the total GlcN and Glc residues present in the reaction system. ^{1}H NMR (D_2O + acetic acid-d_4, ppm) δ 3.45-4.14 (H-2, 3, 4, 5, 6), 4.64 (H-1β), 5.22 (H-1α), 5.39 (Glc-H-1), and 5.71 (GlcN-H-1).

N-Acetylation of chitosan stereoisomers

To the chitosan stereoisomer prepared above (run 3, 15.7 mg) and Na_2CO_3 (9.50 mg, 0.0896 mmol) in water (1.0 mL) was added acetic anhydride (0.0086 mL, 0.0860 mmol), and the mixture stirred at room temperature for 10 min. The reaction mixture was then treated successively with cation-exchange (Amberlite IR-120(plus) (H)) and anion-exchange resins (Amberlite IRA-400J Cl) for 5 min each, and the resulting solution was concentrated. To the concentrated solution, was added ethanol. The resulting precipitate was isolated by filtration, and lyophilized to give the chitin stereoisomer (15.7 mg) in 81.6% yield. ^{1}H NMR (D_2O, ppm) δ 2.07 (CH_3), 3.75-3.99 (H-2, 3, 4, 5, 6), 4.66 (H-1β), 5.24 (H-1α), and 5.40 (Glc-H-1 and GlcN-H-1).

O-Acetylation of chitin stereoisomers

A mixture of the chitin stereoisomer (run 3, 8.5 mg), acetic anhydride (0.50 mL, 5.00 mmol), and *N*,*N*-dimethylaminopyridine (0.50 mg, 4.09 μmol) in pyridine (1.0 mL) was stirred at 80 °C for 6 h. After concentration of the reaction mixture, diethyl ether was added to the residue. The resulting precipitate was isolated by filtration, and dried under reduced pressure to give the *O*-acetylated aminopolysaccharide (7.50 mg) in 60.1% yield. ^{1}H NMR (DMSO-d_6, ppm) δ 1.80-2.13 (CH_3), 3.87 (H-5), 3.93 (H-4), 4.13 (H-2), 4.30, 4.36 (H-6a, 6b), 5.07 (H-1), 5.14 (H-3), and 6.56 (NH).

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Notes and references

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† Electronic Supplementary Information (ESI) available: Detailed experimental procedures for MALDI-TOF MS and standard amylose samples, ^{31}P and ^{1}H NMR spectra of precipitated phosphate salt, MALDI-TOF MS of glucoamylase-treated materials, and ^{1}H-^{1}H COSY NMR spectrum of *O*-acetylated aminopolysaccharide. See DOI: 10.1039/b000000x/

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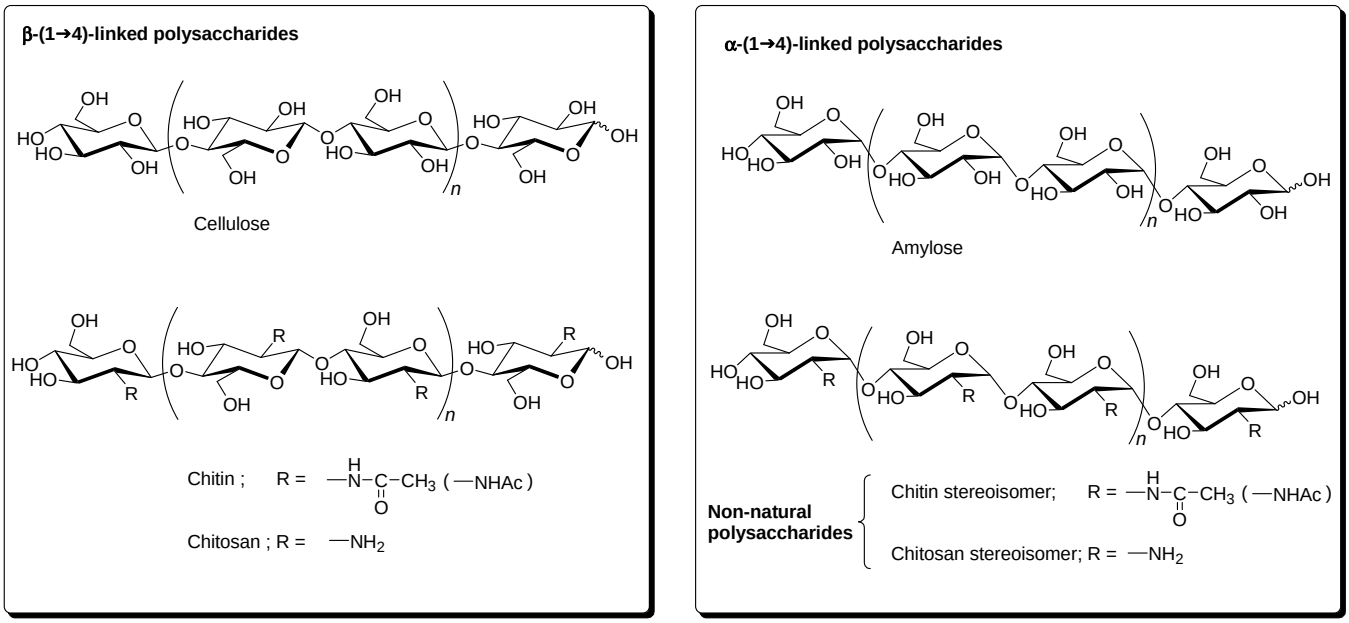

Fig. 1 Chemical structures of cellulose, amylose, chitin/chitosan and chitin/chitosan stereoisomers (target polysaccharides).

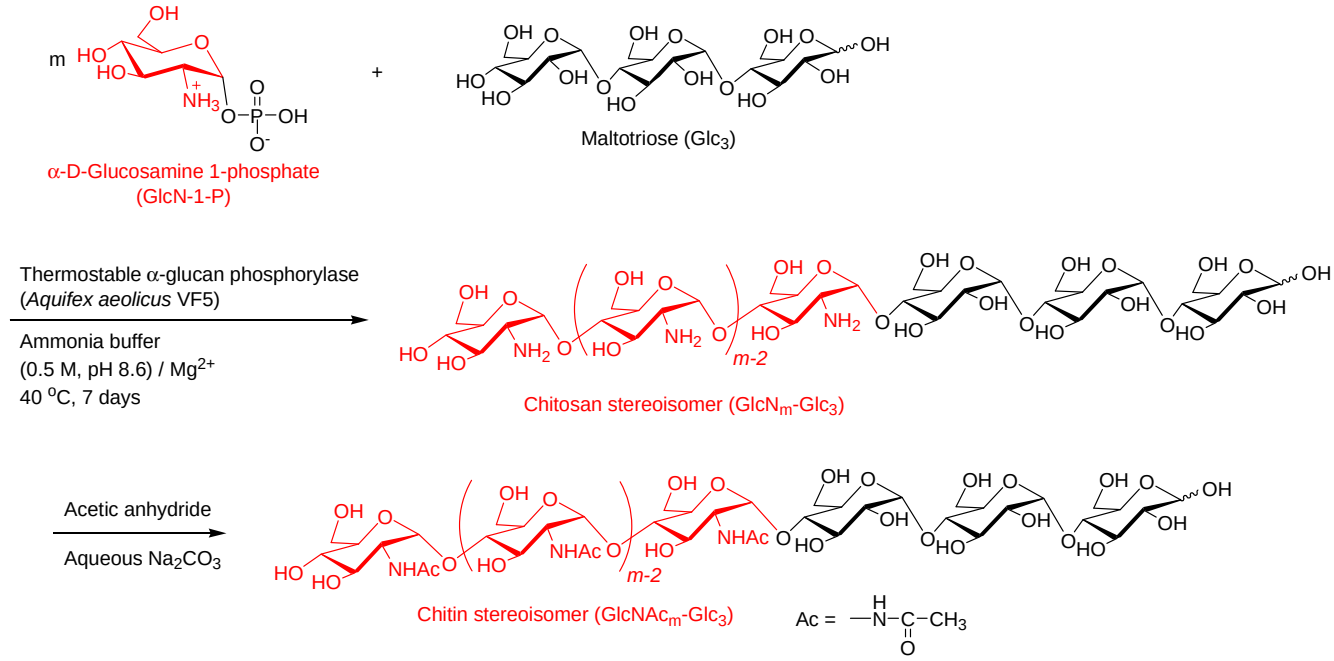

Fig. 2 Thermostable α-glucan phosphorylase-catalyzed enzymatic polymerization of GlcN-1-P with Glc_3 to produce the chitosan stereoisomer and its *N*-acetylation to give the chitin stereoisomer.

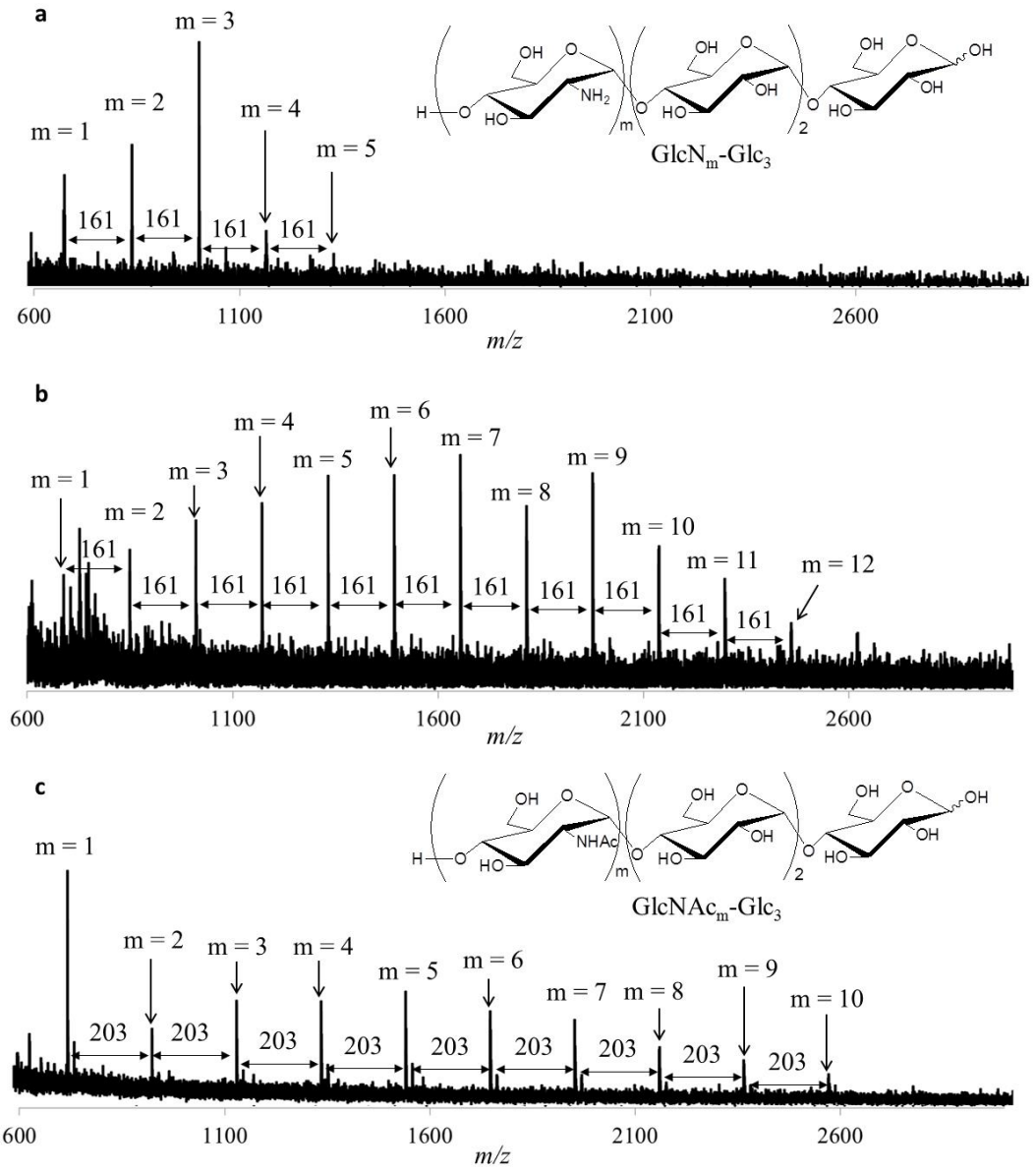

Fig. 3 MALDI-TOF MS of crude products from thermostable α-glucan phosphorylase-catalyzed glucosaminylations using GlcN-1-P in acetate buffer (a) and ammonium buffer containing Mg^{2+} (b) (GlcN-1-P/Glc_3 feed ratio = 10) and MALDI-TOF MS of *N*-acetylated materials from enzymatic reaction products obtained in ammonium buffer containing Mg^{2+} (c).

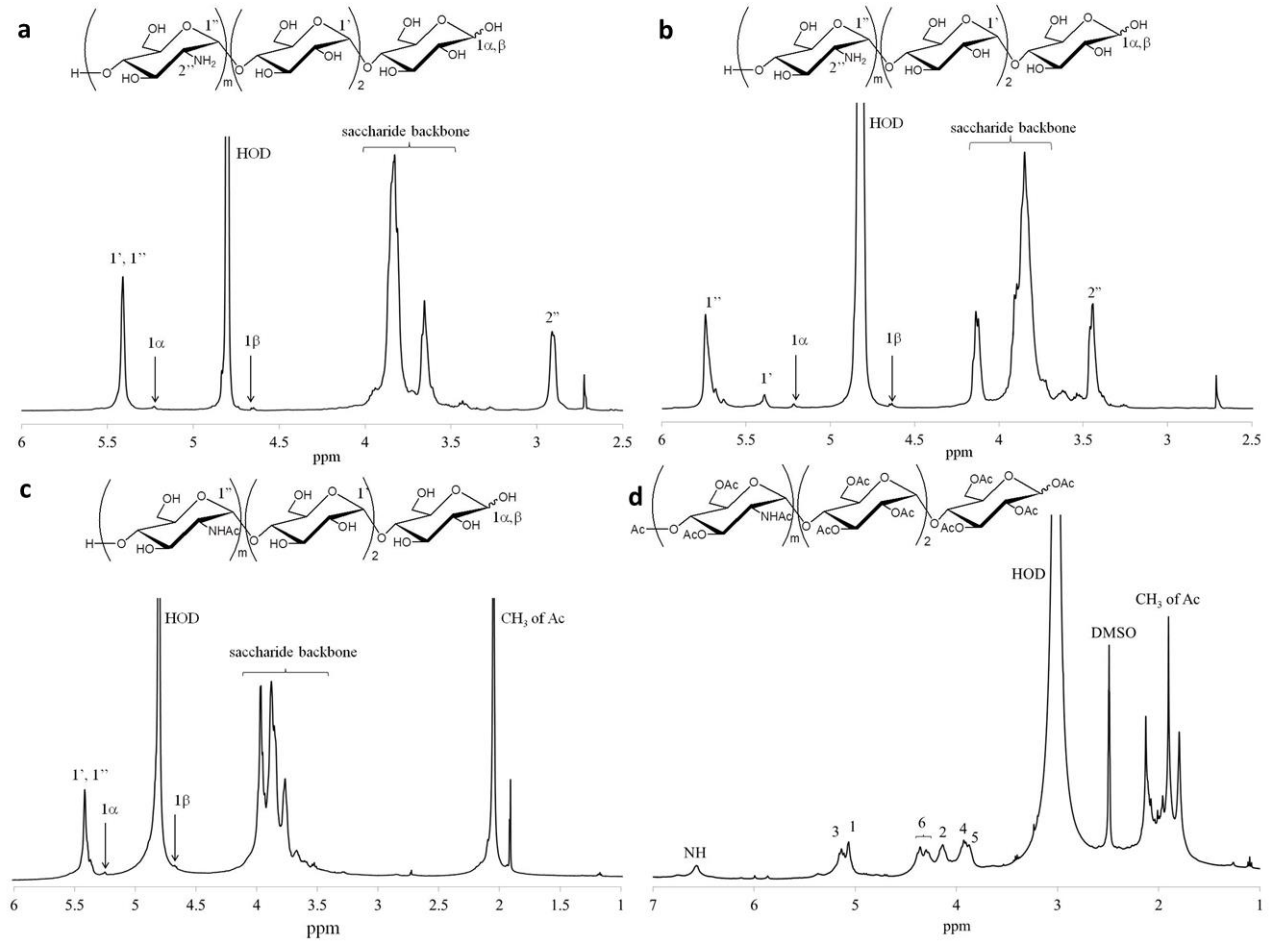

Fig. 4 ^{1}H NMR spectra of the isolated chitosan stereoisomer (run 3) in D_2O (a), D_2O/acetic acid-d_4 (b), the chitin stereoisomer obtained *via* *N*-acetylation in D_2O (c) and its fully *O*-acetylated aminopolysaccharide in DMSO-d_6 (d).

Table 1 Thermostable α-glucan phosphorylase-catalyzed enzymatic polymerization of GlcN-1-P with Glc_3[a] and *N*-acetylation of products

Run	Polymerization				*N*-Acetylation	
	GlcN-1-P/Glc_3	Conv. (%)[b]	Yield (%)[c]	DP / M_n[d]	Yield (%)[e]	M_n[f]
1	10	95.3	60.6	10.1 / 2130	57.2	1480
2	20	97.2	59.2	16.5 / 3160	71.7	1930
3	30	86.9	61.8	20.2 / 3760	81.6	2240
4	40	70.5	50.8	24.8 / 4500	71.6	2500
5	50	71.1	63.9	28.0 / 5010	61.4	3320

[a] Reaction was carried out using Glc_3 (4.76 μmol) in the presence of thermostable α-glucan phosphorylase (20U) in ammonia buffer containing Mg^{2+} ion (equimolar amounts of ammonia and Mg^{2+} for GlcN-1-P) at 40 °C for 7 days. [b] Determined by weight of ammonium magnesium phosphate precipitate from reaction mixture. [c] Fraction insoluble in methanol. [d] DP = the number of GlcN units, M_n = number-average molecular weight of produced polysaccharide ($GlcN_m$-Glc_3); Determined by ^{1}H NMR spectra. [e] Fraction insoluble in ethanol. [f] Estimated by GPC measurement with DMSO as eluent at 60 °C using enzymatically synthesized amylose and maltooligosaccharide standards.