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Review

α-Glucan Phosphorylase-Catalyzed Enzymatic Reactions Using Analog Substrates to Synthesize Non-Natural Oligo- and Polysaccharides

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Abstract: As natural oligo- and polysaccharides are important biomass resources and exhibit vital biological functions, non-natural oligo- and polysaccharides with a well-defined structure can be expected to act as new functional materials with specific natures and properties. α-Glucan phosphorylase (GP) is one of the enzymes that have been used as catalysts for practical synthesis of oligo- and polysaccharides. By means of weak specificity for the recognition of substrates by GP, non-natural oligo- and polysaccharides has precisely been synthesized. GP-catalyzed enzymatic glycosylations using several analog substrates as glycosyl donors have been carried out to produce oligosaccharides having different monosaccharide residues at the non-reducing end. Glycogen, a highly branched natural polysaccharide, has been used as the polymeric glycosyl acceptor and primer for the GP-catalyzed glycosylation and polymerization to obtain glycogen-based non-natural polysaccharide materials. Under the conditions of removal of inorganic phosphate, thermostable GP-catalyzed enzymatic polymerization of analog monomers occurred to give amylose analog polysaccharides.

Keywords: analog substrate; α-glucan phosphorylase; non-natural oligo- and polysaccharides

1. Introduction

Oligo- and polysaccharides are widely distributed in nature and enact specific important biological functions in accordance with their chemical structures [1]. Owing to a variety of sugar unit structures and various types of linkages among them in accordance with regio- and stereoarrangements, so-called glycosidic linkages, oligo- and polysaccharides typically have very complicated structures [2]. Accordingly, it has been well accepted that a profound effect on their properties and functions is often produced by a subtle change in the type of glycosidic linkage and the unit structure. The precise synthesis of structurally well-defined oligo- and polysaccharides through stereo- and regio-controlled glycosidic linkages among desired sugar units has therefore attracted much attention in the development of new functional carbohydrate-related materials. However, common organic reactions do not easily provide well-defined oligo- and polysaccharides under perfect control conditions in regio- and stereoarrangements [3–5]. Compared to such common organic reactions, enzymatic reactions exhibit significant advantages in terms of stereo- and regioselectivities. Enzymatic reaction, furthermore, can be operated under mild conditions, eliminating undesired side reactions [6,7]. Of the six main classes of enzymes, transferase (glycosyl transferase, GT) and hydrolase (glycosyl hydrolase, GH) have been used successfully as catalysts in practical synthesis of well-defined saccharide chains via the formation of glycosidic linkages [8,9]. In the enzymatically formation of glycosidic linkages, so-called 'enzymatic glycosylation', two substrates, that is, a glycosyl donor and

a glycosyl acceptor are used, generally, in their unprotected forms in aqueous media. A glycosidic linkage is enzymatically formed by the reaction of a glycosyl donor at the anomeric position with the hydroxy group of a glycosyl acceptor in regio- and stereocontrolled fashion. From the viewpoint of polymerization chemistry, moreover, oligo- and polysaccharides are theoretically produced by repeated enzymatic formation of glycosidic linkages (enzymatic polymerization).

Phosphorylases are one of GTs, which have been used as catalysts for practical synthesis of oligo- and polysaccharides [10–12]. Phosphorylases catalyze the in vivo phosphorolysis (phosphorolytic cleavage in the presence of inorganic phosphate (Pi)) of glycosidic linkages at the non-reducing end of each specific saccharide chain, to produce 1-phosphate of a monosaccharide residue and to simultaneously generate the saccharide chain with one lower degree of polymerization (DP). Phosphorylases exert strict regio- and stereospecificities, which catalyze the phosphorolysis of a specific glycosidic linkage under ether retention or inversion manner to form monosaccharide 1-phosphates with the same, or opposite, anomeric stereoarrangement as the glycosidic linkage (Figure 1).

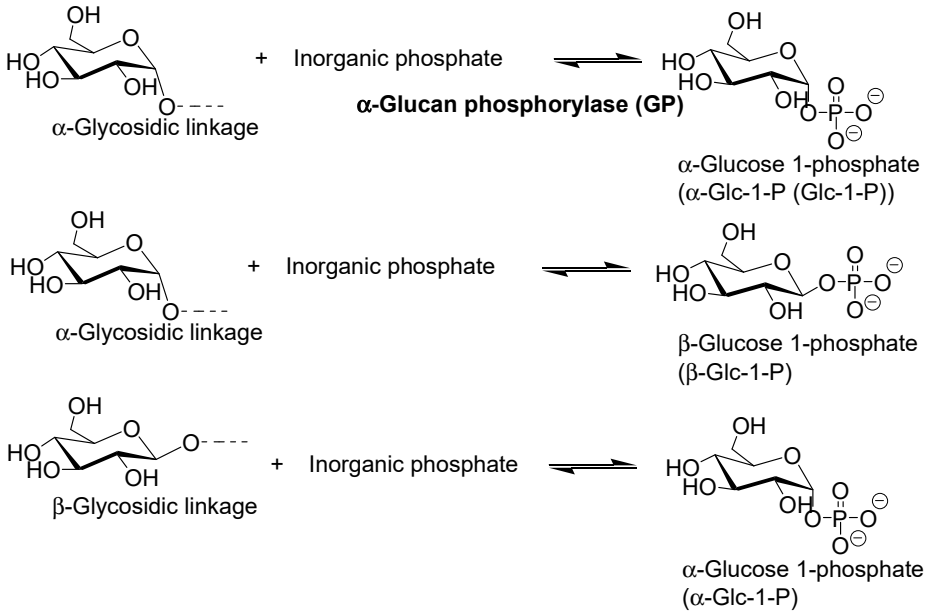

Figure 1. Typical reversible reactions catalyzed by phosphorylases.

Because bond energy of a glycosidic linkage in the starting saccharide chain is comparable to that of a phosphate ester at the anomeric position of the phosphorolysis product, phosphorylase-catalyzed reactions show a reversible nature. With respect to reversibility, phosphorylases catalyze reactions for the manner of glycosidic bond formation under adapted conditions [13–17]. In these reactions, a monosaccharide residue is transferred from monosaccharide 1-phosphate to the non-reducing end of a specific saccharide chain to form a glycosidic linkage with strictly controlled regio- and stereoarrangements, accompanied by liberating Pi. Among a variety of phosphorylases found in nature, α-glucan phosphorylase (GP, also called glycogen phosphorylase, or starch phosphorylase) belonging to the GT35 family (EC 2.4.1.1) has been widely investigated and practically employed for the synthesis of oligo- and polysaccharides under the retention manner (Figure 1) [13,14,16,18]. Furthermore, as a result of the weak specificity for the recognition of non-native (analog) substrates by GP, non-natural oligo- and polysaccharides have been synthesized by catalysis of this enzyme [19–21]. On the basis of the above background, this review article presents GP-catalyzed enzymatic reactions using analog substrates to precisely synthesize well-defined non-natural oligo- and polysaccharides. As specific applications of amylose-based materials have been investigated to enable the addition of further functionalities and precise tuning of the materials in biotechnology and therapeutics [22], the non-natural materials described herein can be expected to act as new glycomaterials for cosmetics,

biomedical, and pharmaceutical applications, and for supplements used in animal farming and in food [18].

2. Characteristics of GP-Catalyzed Enzymatic Reactions

GP is the enzyme that catalyzes the reversible phosphorolysis of α(1→4)-glucans at the non-reducing end, such as starch and glycogen, in the presence of Pi, to produce α-D-glucose 1-phosphate (α-Glc-1-P, hereafter simply Glc-1-P) (Figure 2a) [10,11]. Depending on reaction conditions, GP catalyzes the reverse chain-elongation reaction, that is, glycosylation of Glc-1-P and α(1→4)-glucan as a glycosyl donor and a glycosyl acceptor, respectively, to form an α(1→4)-glycosidic linkage, in which a glucose residue enzymatically transfers from Glc-1-P to the non-reducing end of α(1→4)-glucans, such as maltooligosaccharides in regio- and stereocontrolled fashions (Figure 2b). The catalytic mechanism depends on close contact of the phosphate group in the covalently bound cofactor pyridoxal 5′-phosphate to either inorganic phosphate (in the direction of phosphrolysis) or the phosphate group of Glc-1-P (in the direction of glycosylation) [23–26]. Specifically, GP has the smallest DP value in α(1→4)-glucan chains to recognize, and accordingly, maltooligosaccharides with DPs higher than the smallest one should be used as the glycosyl acceptor. The smallest substrates for the phosphorolysis and glycosylation recognized by the most widely studied GP isolated from potato are maltopentaose (Glc_5) and maltotetraose (Glc_4), respectively. On the other hand, it has been found that the smallest DPs of substrates accepted by GP isolated from thermophilic bacteria sources (thermostable GP) for the former and latter reactions are one smaller than those by potato GP, i.e., Glc_4 and maltotriose (Glc_3), respectively [18,27–30]. As enzymes often show weak specificity for the recognition of the substrate structure, GPs have been found to recognize some analog substrates of Glc-1-P (1-phosphates of different monosaccharide residues) depending on their sources [19–21]. Therefore, the extension of the GP-catalyzed enzymatic reactions has been investigated using several monosaccharide 1-phosphates to obtain non-natural oligosaccharides having the different monosaccharide residues at the non-reducing end (Figure 3, vide infra).

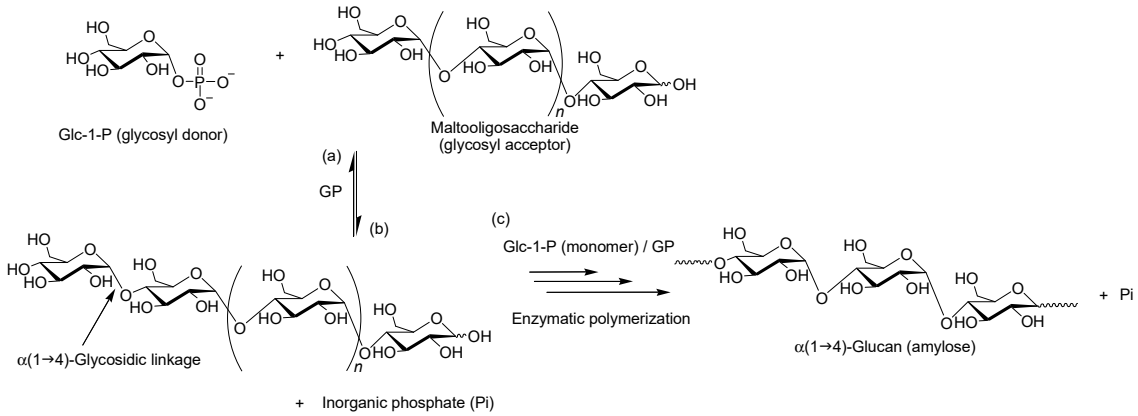

Figure 2. α-Glucan phosphorylase (GP)-catalyzed (**a**) phosphorolysis, (**b**) glycosylation, and (**c**) polymerization.

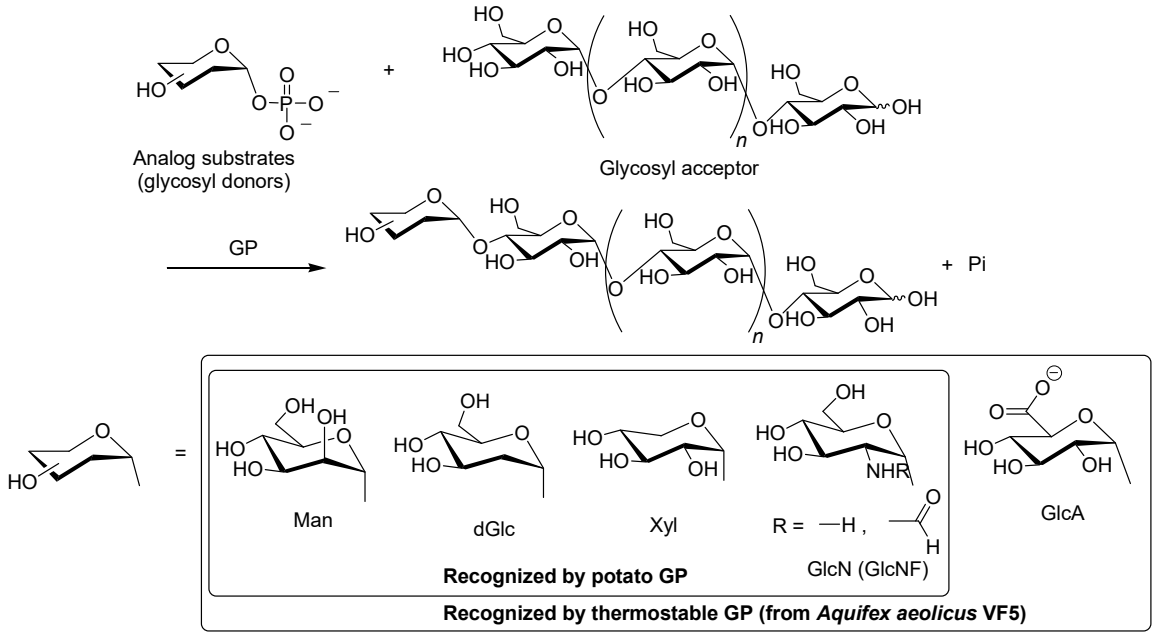

Figure 3. GP-catalyzed enzymatic glycosylations using analog substrates (monosaccharide 1-phosphates) as glycosyl donors to produce non-natural oligosaccharides.

When an excess donor/acceptor (Glc-1-P/maltooligosaccharide) molar ratio is employed in the reaction system, GP catalyzes consecutive glycosylations as the manner of polymerization to produce the α(1→4)-glucan polymer, i.e., amylose (Figure 2c) [30–33], which spontaneously forms a double helical assembly as the precipitate in the reaction media [34,35]. It has been reported that within the double helix, interstrand stabilization is achieved without any steric conflict and through the occurrence of O(2)···O(6) type of hydrogen bonds [35]. The GP-catalyzed enzymatic polymerization of Glc-1-P as a monomer belongs to chain-growth polymerization because the reaction is initiated at the non-reducing end of the acceptor and the propagation consecutively progresses from the non-reducing end of the producing α(1→4)-glucan chain. Accordingly, the glycosyl acceptor is often referred to the "primer" of the polymerization. As the GP-catalyzed polymerization is conceived analogously to a living polymerization, the molecular weight of the produced amylose can be controlled by the monomer/primer feed ratio, and the distribution of the polymeric product is typically narrow ($M_w/M_n < 1.2$) [36].

Polymers having plural non-reducing α(1→4)-glucan chain ends have been used as polymeric primers for GP-catalyzed enzymatic polymerization of Glc-1-P to obtain amylose-grafted and amylose-branched polymeric materials [22,37–43]. For example, glycogen, which is a water-soluble highly branched natural polysaccharide composed of α(1→4)-glucan chains further interlinked by α(1→6)-branching points, has been used as the polymeric primer for GP-catalyzed enzymatic polymerization owing to the presence of a number of non-reducing α(1→4)-glucan chain ends (Figure 4a) [44]. When the GP-catalyzed enzymatic polymerization of Glc-1-P from non-reducing ends of glycogen was conducted in acetate buffer, and the reaction mixture was then left standing at room temperature for 24 h, it turned completely into a hydrogel form. Hydrogelation is reasonably explained by the double-helix formation by the elongated amylose chains among glycogens [34,35], which act as cross-linking points for construction of network structure.

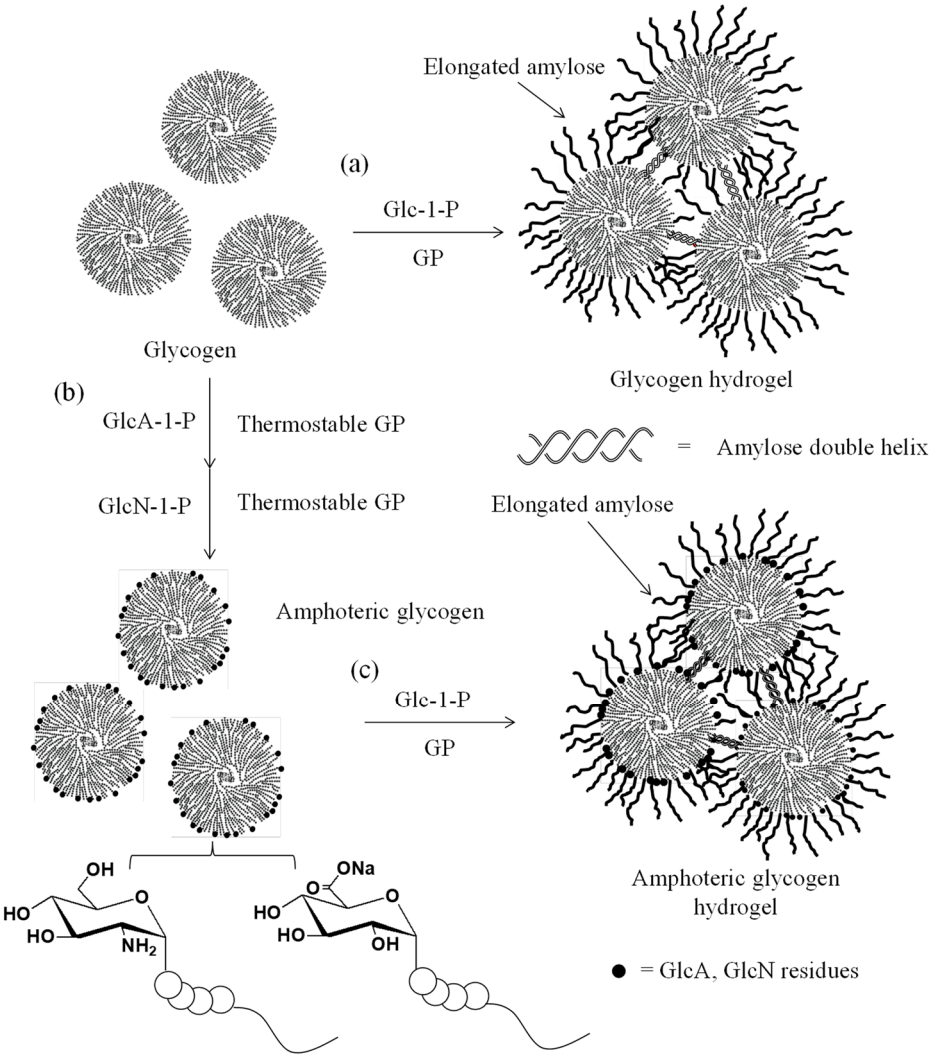

Figure 4. GP-catalyzed (**a**) polymerization to produce glycogen hydrogel, (**b**) glucuronylation and subsequent glucosaminylation to produce amphoteric glycogen, and (**c**) following polymerization to produce amphoteric glycogen hydrogel.

3. Synthesis of Non-Natural Oligosaccharides by Phosphorylase-Catalyzed Enzymatic Glycosylations Using Analog Substrates

Potato GP has been found to recognize several 1-phosphates of monosaccharide residues as the analog substrates of Glc-1-P, such as α-D-mannose (Man), α-D-xylose (Xyl), α-D-glucosamine (GlcN), and *N*-formyl-α-D-glucosamine (GlcNF) 1-phosphates (Figure 3) [45–49]. Potato GP-catalyzed glycosylations using such glycosyl donors have been reported to take place with Glc_4 to produce non-natural pentasaccharides having the respective monosaccharide residues at the non-reducing end. α-Glucosaminylated pentasaccharide obtained from glycosylation using GlcN-1-P (glucosaminylation) is a basic oligosaccharide having an amino group at the C-2 position of a GlcN unit at the non-reducing end.

2-Deoxy-α-D-glucose 1-phosphate (dGlc-1-P) was also found to be recognized by potato GP (Figure 3) [50]. Interestingly, this substrate could be generated by two-step processes in situ using D-glucal as a substrate. In a first step, D-glucal is transferred as a 2-deoxy-D-glucose unit to the non-reducing end of α(1→4)-glucan in the presence of Pi. In a second step, a 2-deoxy-D-glucose residue is released from the non-reducing end of the product by the potato GP-catalyzed phosphorolysis to produce dGlc-1-P, and consequently, α(1→4)-glucan remained intact. Accordingly, D-glucal has been employed as a glycosyl donor in the GP-catalyzed enzymatic glycosylation. In the enzymatic reaction

in the presence of D-glucal, Glc_4, and a small amount of Pi, consecutive glycosylations progressed to produce 2-deoxy-α-D-glucosylated penta-heptasaccharides and higher molecular weight products with an average DP of 12.

Thermostable GP has been found to show more tolerance in the recognition specificities for monosaccharide 1-phosphates than those of potato GP. For example, potato GP does not recognize α-D-glucuronic acid 1-phosphate (GlcA-1-P), while thermostable GP isolated from *Aquifex aeolicus* VF5 recognizes GlcA-1-P in addition to the abovementioned analog glycosyl donors (Figure 3) [51]. Accordingly, the thermostable GP catalyzes glycosylation using GlcA-1-P with Glc_3 (glucuronylation) to produce a tetrasaccharide having a GlcA residue at the non-reducing end, which is an acidic oligosaccharide owing to the presence of a carboxylate group at the C-6 position of a GlcA unit.

By means of the above-mentioned thermostable GP-catalyzed glucosaminylation and glucuronylation, amphoteric polysaccharides with highly branched structure, such as an amphoteric glycogen, having both the basic GlcN and acidic GlcA residues at the non-reducing ends, have been synthesized (Figure 4b) [52,53]. The thermostable GP-catalyzed glucuronylation using GlcA-1-P with highly branched polysaccharides has been conducted to produce acidic materials [54]. Then, the thermostable GP-catalyzed glucosaminylation using GlcN-1-P with the acidic products was performed to obtain amphoteric highly branched polysaccharides. The produced amphoteric glycogen was converted into its hydrogel form by the thermostable GP-catalyzed enzymatic polymerization of Glc-1-P from the pure non-reducing α(1→4)-glucan chain ends without the functionalization by GlcA or GlcN units (Figure 4c) [53]. The elongated amylose chains formed double helical assemblies among the amphoteric glycogen molecules, which acted as cross-linking points for hydrogelation. The resulting amphoteric glycogen and its hydrogel showed pH-responsive properties.

4. Thermostable GP-Catalyzed Enzymatic Oligomerization and Polymerization of Analog Monomers

As mentioned above, the potato GP-catalyzed enzymatic polymerization produces the α(1→4)-glucan chain when the native glycosyl donor, Glc-1-P, is employed. A series of studies on the potato GP-catalyzed glycosylations using analog substrates, on the other hand, suggested that after a monosaccharide residue is transferred from the analog substrates to the non-reducing end of the maltooligosaccharide acceptor, further glycosylations do not take place owing to the loss of recognition of the produced non-reducing end structure being different from the Glc residue by the enzyme [19–21]. Taking the different recognition behavior of thermostable GP (from *Aquifex aeolicus* VF5) from potato into account, it has been found that the former enzyme catalyzed a different reaction manner from the latter enzyme when Man-1-P or GlcN-1-P was used as a glycosyl donor. When the thermostable GP-catalyzed glycosylation of Glc_3 acceptor with Man-1-P or GlcN-1-P was carried out in acetate buffer, consecutive glycosylations took place to obtain non-natural heterooligosaccharides composed of α(1→4)-linked Man/GlcN chains [55]. The MALDI-TOF mass spectra of the products with 10:1 donor/acceptor feed ratio showed several peaks corresponding to the molecular masses of tetra~hepta-octa-saccharides having one–four or five Man or GlcN residues with Glc_3 (Figure 5). The production of longer chains in higher donor/acceptor feed ratios, however, has been inhibited by Pi produced from the glycosyl donors, due to the fact that it is a native substrate for phosphorolysis by the GP catalysis.

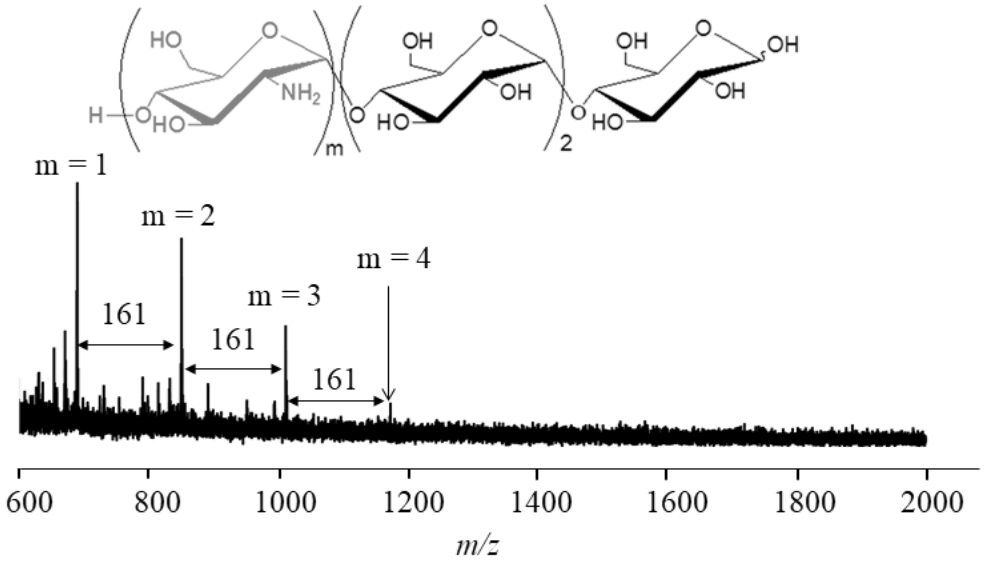

Figure 5. Matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrum of crude product from thermostable GP-catalyzed glucosaminylations using GlcN-1-P in acetate buffer (10:1 donor/acceptor feed ratio).

An attempt has been made to remove Pi as a precipitate in the thermostable GP-catalyzed consecutive glucosaminylations using ammonium buffer (0.5 M, pH 8.6) containing $MgCl_2$ as a reaction solvent system, as the fact that Pi is reported to form an insoluble salt with ammonium and magnesium ions [56]. As a result, the thermostable GP-catalyzed enzymatic polymerization of GlcN-1-P with the Glc_3 primer (30:1) in such buffer system successfully occurred to produce $\alpha(1\rightarrow4)$-linked non-natural (amylose analog) aminopolysaccharide with a DP of the GlcN units of ~20, which was named 'amylosamine' (Figure 6) [57]. The produced amylosamine shows water-solubility because it has not formed a regular higher-order assembly in water as observed for the amylose double helix. However, the cationic amylosamine formed a double helix with anionic amylouronic acid ($\alpha(1\rightarrow4)$-linked GlcA polymer), another amylose analog polysaccharide, by electrostatic interaction between amino and carboxylate groups [58]. Furthermore, reductive amination of amylosamine formed hierarchically controlled assemblies to obtain nanoparticles, microaggregates, and macrohydrogels depending on reaction conditions [59]. The reductive amination of amylosamine was carried out in the presence of $NaBH_3CN$ as a reductant in 0.1 mol/L acetic acid aq. at 60 °C for 1 h. When the lower feed ratios of reductant/reducing end of the substrate were employed for the reaction, nanoparticles were formed. As the feed ratios of the reductant/reducing end increased, a larger number of $\alpha(1\rightarrow4)$-linked GlcN chains assembled to further form the water-insoluble microaggregates and macrohydrogels.

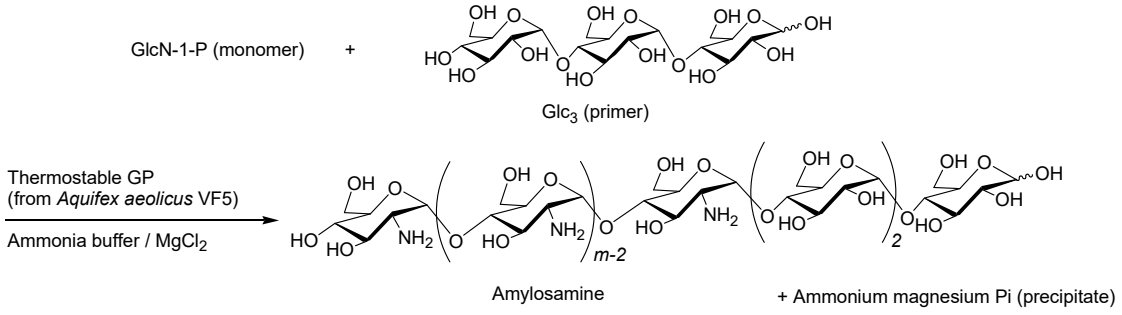

Figure 6. Thermostable GP-catalyzed enzymatic polymerization of GlcN-1-P with removal of Pi to produce amylosamine.

The thermostable GP-catalyzed enzymatic copolymerizations of Glc-1-P with GlcN-1-P, and with Man-1-P, have successfully progressed under the conditions of removal of Pi in ammonia/$MgCl_2$ buffer to obtain non-natural glucosaminoglucan comprising Glc/GlcN units and mannoglucan composed of Glc/Man units [60,61]. The thermostable GP-catalyzed enzymatic polymerization of GlcN-1-P using maltooligosaccharide-functionalized amylouronic acid (amylouronic acid with a short α(1→4)-linked Glc chain at the non-reducing end) as the primer under the same operation yielded an amylose analog amphoteric block polysaccharide composed of GlcN and GlcA chains (Figure 7) [62]. The product showed pH-responsive properties.

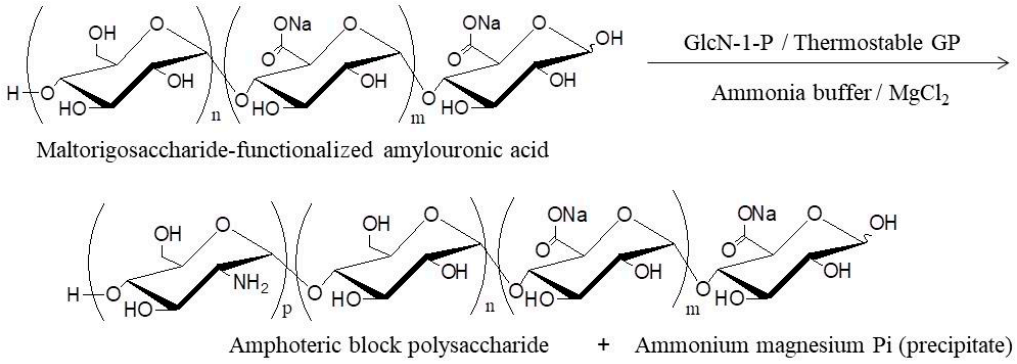

Figure 7. Thermostable GP-catalyzed synthesis of amphoteric block polysaccharide.

5. Conclusions

This review article demonstrated that the GP-catalyzed enzymatic glycosylations and polymerizations using analog substrates are powerful tools to precisely synthesize α(1→4)-linked non-natural oligo- and polysaccharides. As GP shows weak specificity for the recognition of substrates, several monosaccharide 1-phosphates could be employed to enzymatically introduce the corresponding monosaccharide units into the saccharide chains. Such non-natural oligo- and polysaccharides have a high potential to be employed as practical functional materials in the biological, medicinal, and pharmaceutical research fields. Because the analog substrates have been provided efficiently by organic synthetic techniques, the approach from the viewpoint of the synthetic organic chemistry, is in demand for conducting the studies presented in this review. The author, therefore, claims the efficient collaboration between the research fields of synthetic organic chemistry and enzymatic technology will exhibit synergistic effects to contribute to further development in the enzymatic synthesis of functional non-natural oligo- and polysaccharides in the future.

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