Thermostable \( \beta \)-Glucan Phosphorylase-Catalyzed Enzymatic Copolymerization to Produce Partially 2-Deoxygenated Amyloses

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**Keywords:** heteropolysaccharide, d-glucal, \( \beta \)-glucan phosphorylase, enzymatic copolymerization, 2-deoxyamylose

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Thermostable $\alpha$-Glucan Phosphorylase-Catalyzed Enzymatic Copolymerization to Produce Partially 2-Deoxygenated Amyloses

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Keywords: 2-deoxyamylose; enzymatic copolymerization; d-glucal; $\alpha$-glucan phosphorylase; heteropolysaccharide

1. Introduction

Polysaccharides are widely distributed and play important roles in nature as structural materials, suppliers of energy, and key materials for specific biological and vital functions [1]. Natural polysaccharides provide a great variety of chemical structures owing to the many types of monosaccharide units and different stereo- and regio-arrangements of the glycosidic linkages in the polymeric chains, which contributes to their many different in vivo functions [2]. In addition to homopolysaccharides composed of monosaccharide repeating units, such as cellulose, starch, and chitin, there are a variety of natural heteropolysaccharides which are composed of multiple types of monosaccharide units, such as glucomannans and glycosaminoglycans [3–5]. Accordingly, the synthesis of non-natural heteropolysaccharides with well-defined structures has attracted much attention, since they can be expected to exhibit new functions for possible applications as biofunctional materials in the fields of biomedical and tissue engineering.

Since it is well recognized that the enzymatic method is a useful approach to generate well-defined oligo- and polysaccharides [6–10], we investigated $\alpha$-glucan phosphorylase-catalyzed enzymatic reactions using several monosaccharide 1-phosphates as substrates to obtain non-natural oligo- and polysaccharides [11–15]. $\alpha$-Glucan phosphorylase catalyzes the enzymatic polymerization of $\alpha$-d-glucose 1-phosphate (Glc-1-P) monomers from a maltooligosaccharide primer to produce $\alpha$(1→4)-glucan—i.e., amylose—upon the liberation of inorganic phosphate (Pi) [16–23]. This enzymatic
polymerization corresponds to the following reversible chain-elongation (glycosylation) and phosphorolysis: \( [\alpha(1\rightarrow 4)-\text{Glc}]_n + \text{Glc-1-P} \Leftrightarrow [\alpha(1\rightarrow 4)-\text{Glc}]_{n+1} + \text{Pi} \). Owing to the weak specificity for the substrate recognition of this enzyme, some substrate analogs of Glc-1-P (1-phosphates of different monosaccharides) can be used in enzymatic reactions to yield amylose analogs [11–15]. For example, we previously reported the synthesis of an aminopolysaccharide amylose analog composed of \( \alpha(1\rightarrow 4) \)-linked glucosamine (GlcN) (named “amylosamine”) as a repeating unit upon the enzymatic polymerization of \( \alpha-\text{d-glucosamine} \) 1-phosphate (GlcN-1-P), as catalyzed by a thermostable \( \alpha \)-glucan phosphorylase (isolated from *Aquifex aeolicus* VF5 thermophilic bacteria) [24]. The polymerization was achieved by conducting the reaction in an ammonia buffer containing \( \text{Mg}^{2+} \) ions in order to remove the produced Pi from the reaction medium in the form of an ammonium magnesium phosphate precipitate, thus preventing the reverse reaction—namely, phosphorolysis—from taking place.

The copolymerization approach catalyzed by enzymes (enzymatic copolymerization) has been identified as an efficient method for the synthesis of well-defined non-natural heteropolysaccharides [8–10,25]. For example, the hyaluronidase-catalyzed enzymatic copolymerization led to a hyaluronic acid-chondroitin hybrid polysaccharide [26]. In addition, we also revealed that the enzymatic copolymerization catalyzed by a thermostable \( \alpha \)-glucan phosphorylase is a useful tool to synthesize non-natural heteropolysaccharides with a well-defined structure. The thermostable \( \alpha \)-glucan phosphorylase-catalyzed enzymatic copolymerization of analog substrates, such as GlcN-1-P and \( \alpha-\text{d-mannose} \) (Man) 1-phosphate, with the native substrate Glc-1-P, efficiently progressed under the above-mentioned conditions used for the removal of Pi to generate a non-natural glucosaminoglucan and mannoglucon composed of GlcN/Glc and Man/Glc units, respectively [27,28].

It was also reported that \( \alpha \)-glucan phosphorylase isolated from several sources, such as potato, rabbit muscle, and *Escherichia coli*, could catalyze the enzymatic polymerization of 2-deoxy-\( \alpha-\text{d-glucose} \) 1-phosphate (dGlc-1-P), which was produced in situ from 1,2-dideoxy-\( \text{d-glucal} \) (d-glucal) in the presence of Pi, using a maltooligosaccharide primer to afford \( \alpha(1\rightarrow 4) \)-linked 2-deoxyglucose chains—i.e., 2-deoxyamyllose [29–31]. The following mechanism was reported to allow for the generation of 2-deoxyamyllose upon \( \alpha \)-glucan phosphorylase-catalyzed enzymatic polymerization using \( \text{d-glucal} \) to the primer via an addition reaction assisted by Pi. In the second step, a dGlc residue is released upon \( \alpha \)-glucan phosphorylase-catalyzed enzymatic phosphorolysis to yield dGlc-1-P in situ, which acts as substrate of the enzymatic polymerization. It was found that when a thermostable \( \alpha \)-glucan phosphorylase (from *Aquifex aeolicus* VF5) was used in the enzymatic polymerization using \( \text{d-glucal} \), the mechanism of the in situ production of dGlc-1-P most likely differed from that observed using \( \alpha \)-glucan phosphorylases from different sources. In particular, it can be assumed that a thermostable \( \alpha \)-glucan phosphorylase catalyzes the enzymatic addition of a C-4 hydroxy group at the non-reducing end of the primer to \( \text{d-glucal} \) with the consequent formation of an \( \alpha(1\rightarrow 4) \)-glycosidic linkage in the absence of Pi as the first step in the in situ production of dGlc-1-P, as it recognizes \( \text{d-glucal} \) as the only substrate. The in situ production of dGlc-1-P was confirmed by the direct measurement of the \( ^1\text{H} \) NMR spectra of the reaction mixtures in our experiment. Furthermore, we reported that the enzymatically synthesized 2-deoxyamyllose spontaneously formed an antiparallel double-helical crystalline structure, which was completely different from the native parallel double helix built from amylose [33].

Based on the above background, in this study we investigated the thermostable \( \alpha \)-glucan phosphorylase-catalyzed enzymatic copolymerization of dGlc-1-P, which was produced in situ from \( \text{d-glucal} \), with Glc-1-P using maltotriose (Glc3) as a primer to generate new heteropolysaccharides, and particularly partially 2-deoxyxgenated amylloses composed of dGlc/Glc units (Figure 1). Furthermore, the crystalline structures of the products in accordance with the unit ratios of dGlc/Glc residues were evaluated. It was also found that the partially 2-deoxyxgenated amylose, which was composed of an adapted unit ratio, formed a film using a facile casting method. This study highlights the usefulness of the enzymatic copolymerization approach to obtain well-defined heteropolysaccharides.
2. Materials and Methods

2.1. Materials

D-Glucal was prepared by the deacetylation of commercially available tri-O-acetyl-D-glucal (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) in a solution of sodium methoxide with methanol. Thermostable α-glucan phosphorylase from *Aquifex aeolicus* VF5 was supplied from Ezaki Glico Co., Ltd. (Osaka, Japan) [20,34,35]. An amylose sample was prepared by the thermostable α-glucan phosphorylase-catalyzed enzymatic polymerization of Glc-1-P. All other commercial available reagents and solvents—i.e., Glc-1-P (disodium salt, Sigma-Aldrich, St. Louis, MO, USA), Glc3 (Hayashibara CO., LTD., Okayama, Japan), Tris-acetate buffer (Nacalai Tesque, Inc., Kyoto, Japan), KH₂PO₄ (FUJIFILM Wako Pure Chemical Co., Osaka, Japan), and dimethyl sulfoxide (DMSO, FUJIFILM Wako Pure Chemical Co., Osaka, Japan)—were used as received.

2.2. Thermostable α-Glucan Phosphorylase-Catalyzed Enzymatic Copolymerization of D-Glucal with Glc-1-P

A typical experimental procedure was as follows (entry 4 in Table 1). A mixture of d-glucal (43.8 mg, 0.300 mmol), Glc-1-P (disodium salt, 91.2 mg, 0.300 mmol), and Glc3 (2.5 mg, 5.00 μmol) in 20 mM of Tris-acetate buffer (pH 6.9, 1.0 mL) containing KH₂PO₄ (0.70 mg, 5.00 μmol) was incubated in the presence of thermostable α-glucan phosphorylase (122 U) at 40 °C for 24 h. The precipitated product was isolated by centrifugation, followed by lyophilization to give a partially 2-deoxygenated amylose (50.6 mg) in a 53.3% yield based on the amounts of the total dGlc and Glc residues present in the reaction system. ¹H NMR: (Figure 2, DMSO-d₆ + D₂O) δ 1.42–1.56 (m, dGlc-H-2ax), 1.99–2.01 (m, 2dGlc-H-2eq), 2.98–3.79 (m, dGlc-H-3, 4, 5, 6 and Glc-H-2, 3, 4, 5, 6), 4.33 (d, H-1β of reducing end), 4.95 (d, H-1α of reducing end), 5.03 (m, Glc-H-1), 5.30 (m, dGlc-H-1).
Table 1. Thermostable α-glucan phosphorylase-catalyzed enzymatic copolymerization of α-glucal with Glc-1-P. (a)

<table>
<thead>
<tr>
<th>Entry</th>
<th>Feed Ratio (b) Glc3:p-Glucal:Glc-1-P</th>
<th>Feed Ratio (b) α-Glucal:Glc-1-P</th>
<th>Unit Ratio (c) dGlc:Glc</th>
<th>$M_n$ (d)</th>
<th>Yield (%) (e)</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>1:120:0</td>
<td>100:0</td>
<td>100:0</td>
<td>3810</td>
<td>19.6</td>
</tr>
<tr>
<td>2</td>
<td>1:100:20</td>
<td>83:17</td>
<td>62:38</td>
<td>7620</td>
<td>41.6</td>
</tr>
<tr>
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<td>1:80:40</td>
<td>67:33</td>
<td>53:47</td>
<td>12820</td>
<td>55.7</td>
</tr>
<tr>
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<td>1:60:60</td>
<td>50:50</td>
<td>40:60</td>
<td>16790</td>
<td>53.3</td>
</tr>
<tr>
<td>5</td>
<td>1:40:80</td>
<td>33:67</td>
<td>23:76</td>
<td>13560</td>
<td>49.2</td>
</tr>
<tr>
<td>6</td>
<td>1:20:100</td>
<td>17:83</td>
<td>6:94</td>
<td>11080</td>
<td>63.0</td>
</tr>
</tbody>
</table>

(a) Reaction was carried out in Tris-acetate buffer containing an equimolar amount of KH$_2$PO$_4$ with Glc$_3$ at 40 °C for 24 h. (b) Feed ratio of Glc$_3$ to comonomers was 1:120. (c) Determined by $^1$H NMR spectra. (d) The number-average molecular weight values, which were determined by the $^1$H NMR spectra. Values include primer chains. (e) Based on the weights of precipitated products and the amounts of total dGlc and Glc residues present in the reaction systems.

2.3. Film Formation from Partially 2-Deoxygenated Amylose (Entry 4)

A solution of the partially 2-deoxygenated amylose (0.10 g) in DMSO (2.0 mL) was casted on a glass plate and dried under reduced pressure at 60 °C overnight to give a film.

2.4. Measurements

The $^1$H NMR spectra were recorded on JEOL ECA 600 and ECX 400 spectrometers (JEOL, Akishima, Tokyo, Japan). Powder X-ray diffraction (XRD) measurements were conducted using a Rigaku Geigerflex RADIB diffractometer (PANalytical B.V., EA Almelo, The Netherlands) with Ni-filtered CuKα radiation ($\lambda = 0.15418$ nm).

3. Results and Discussion

The thermostable α-glucan phosphorylase-catalyzed enzymatic copolymerization of α-glucal with Glc-1-P was conducted at varying monomer feed ratios with Glc$_3$ as the primer, as shown in Table 1 (entries 2–6), in Tris-acetate buffer at 40 °C for 24 h (Figure 1). The feed ratio of the total comonomers to primer was adjusted to 120:1 to obtain the highest molecular weight products possible in the present reaction system, as it was the highest ratio in the enzymatic homopolymerization of α-glucal catalyzed by the same enzyme in our previous investigation [33]. An equimolar amount of KH$_2$PO$_4$ with the primer as a Pi source was present in the buffer solvent because it was required for the in situ production...
of dGlc-1-P as the actual monomer. For comparison, the enzymatic homopolymerization of α-glucal catalyzed by thermostable α-glucan phosphorylase was performed by the same operation to produce 2-deoxyamyllose (entry 1). As the products were precipitated from the reaction mixtures, they were isolated by centrifugation and dried by lyophilization. The $^1$H NMR spectrum of the product in DMSO-$d_6$ + D$_2$O (entry 4, Figure 2) observed both the signals assignable to α(1→4)-linked dGlc units at δ 1.42–1.56 (H-2ax), 1.99–2.01 (H-2eq), and 5.30 (H-1) and α(1→4)-linked Glc units at δ 5.03 (H-1), supporting the structure of the partially 2-deoxygenated amyllose; the signals’ assignments accorded to the $^1$H NMR analysis for the individual homopolysaccharides, amyllose and 2-deoamyllose, reported in our previous papers [33,36]. From the integrated ratios of the H-1 signals of these two units and the H-1 signals ascribable to the terminal reducing end at δ 4.33 (H-1β) and 4.95 (H-1α), the dGlc/Glc unit ratios and the number-average molecular weight ($M_n$) values of the produced polysaccharides including the primer chain were calculated as listed in Table 1. The unit ratios of dGlc to Glc were always lower than the feed ratios of α-glucal to Glc-1-P, owing to the lower reactivity of dGlc-1-P toward the native substrate, Glc-1-P. The presence of comparable amounts of α-glucal and Glc-1-P at feed gave a higher $M_n$ product (entry 4) than that produced by different feed ratios of comonomers. The copolymerization gave moderate yields in all cases, due to the precipitation of the products during the reaction. The yield and $M_n$ values by the enzymatic homopolymerization of α-glucal (entry 1) were lower than those by the present enzymatic copolymerization (entries 2–6). This is probably due to the low conversion of α-glucal to dGlc-1-P in situ at the non-reducing end of the 2-deoxyamyllose homo-chain present in the progressed reaction system. The above results suggested that the native monomer, Glc-1-P, efficiently copolymerized with the analog monomer, dGlc-1-P, which was produced in situ, to obtain partially 2-deoxygenated amylloses.

The crystalline structures of the partially 2-deoxygenated amylloses were then investigated by powder XRD measurement. The XRD patterns of the products composed of the certainly higher unit ratios of dGlc to Glc (entries 2 and 3, Figure 3b,c) were identical with those of the homopolysaccharide, 2-deoxyamyllose (entry 1, Figure 3a), as mainly detected at 10.5° and 19°, assigned to the arrangement of double helices; 13° and 19°, assigned to the distances between pyranose rings; 16.5°, assigned to the diameter of the double helix; and 21°, assigned to the helical radius. The crystalline structure evaluated by the XRD pattern was precisely reported in our previous study [33]. On the other hand, the XRD results of the products composed of the certainly higher unit ratios of Glc to dGlc (entries 5 and 6, Figure 3d,e,f) exhibited the same patterns as those of amyllose (Figure 3g), as mainly observed at 17° and 22.5°. Such a majority rule in the crystalline structures of the partially 2-deoxygenated amylloses depending on the dGlc/Glc unit ratios is owing to the completely different double helical assembling fashions of the two homopolysaccharides—that is, 2-deoxyamyllose and amyllose—as discussed in our previous publication [33].

On the other hand, the XRD result of the partially 2-deoxygenated amyllose composed of the dGlc/Glc unit ratio = 40/60 (entry 4, Figure 3d) showed a relatively broad pattern, indicating the amorphous nature of this product—specifically, which was probably owing to the random sequence of the two units. Additionally, the observation of such broad pattern, which did not clearly show the diffraction patterns from the individual crystalline structures of amyllose and 2-deoxyamyllose, supported the incorporation of both the units in the same polysaccharide chain by the copolymerization. Owing to the amorphous nature, the film was formed by casting a solution of this product in DMSO on a glass plate, followed by drying under reduced pressure. On the other hand, the other samples, which observed the diffraction patterns corresponding to either amyllose or 2-deoxyamyllose in their XRD results, did not exhibit a film formation property. The produced film showed a flexible nature and was bent as shown in Figure 4. Because only an enzymatically synthesized pure amyllose specifically with a very high molecular weight (e.g., higher than ca. 5 × 10⁵) exhibits an ability in film formation [37], the present copolymerization is a useful method to facilely obtain amyllosic soft materials from α(1→4)-glucan substrates.
Conceived the project, designed the experiments, directed the research, and wrote the manuscript, J.-i.K. and K.Y.; performed the experiments, S.N.; All the authors discussed the results and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

References

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