processes

MDPI

Article

Thermostable α-Glucan Phosphorylase-Catalyzed Enzymatic Copolymerization to Produce Partially 2-Deoxygenated Amyloses

Jun-ichi Kadokawa *, Shota Nakamura and Kazuya Yamamoto

Graduate School of Science and Engineering, Kagoshima University, 1-21-40 Korimoto, Kagoshima 890-0065, Japan; k3539903@kadai.jp (S.N.); yamamoto@eng.kagoshima-u.ac.jp (K.Y.)
* Correspondence: kadokawa@eng.kagoshima-u.ac.jp; Tel.: +81-99-285-7743

Received: 12 August 2020; Accepted: 26 August 2020; Published: 1 September 2020

Abstract: α-Glucan phosphorylase catalyzes the enzymatic polymerization of α-D-glucose 1-phosphate (Glc-1-P) monomers from a maltooligosaccharide primer to produce $\alpha(1\rightarrow4)$-glucan—i.e., amylose. In this study, by exploiting the weak specificity for the substrate recognition of a thermostable α-glucan phosphorylase (from *Aquifex aeolicus* VF5), we investigated the enzymatic copolymerization of 2-deoxy-α-D-glucose 1-phosphate (dGlc-1-P), which was produced in situ from D-glucal, with Glc-1-P to obtain non-natural heteropolysaccharides composed of $\alpha(1\rightarrow4)$-linked dGlc/Glc units—i.e., partially 2-deoxygenated amylose. The reactions were carried out at different monomer feed ratios using a maltotriose primer at 40 °C for 24 h. The products were precipitated from the reaction medium, isolated by centrifugation, and subjected to ^{1}H NMR spectroscopic and powder X-ray diffraction measurements to evaluate their chemical and crystalline structures, respectively. Owing to its amorphous nature, the partially 2-deoxygenated amylose with adapted unit ratios formed a film when subjected to a casting method.

Keywords: 2-deoxyamylose; enzymatic copolymerization; D-glucal; α-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 α-glucan phosphorylase-catalyzed enzymatic reactions using several monosaccharide 1-phosphates as substrates to obtain non-natural oligo- and polysaccharides [11–15]. α-Glucan phosphorylase catalyzes the enzymatic polymerization of α-D-glucose 1-phosphate (Glc-1-P) monomers from a maltooligosaccharide primer to produce $\alpha(1\rightarrow4)$-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: [α(1→4)-Glc]$_n$ + Glc-1-P ⇄ [α(1→4)-Glc]$_{n+1}$ + 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 α(1→4)-linked glucosamine (GlcN) (named "amylosamine") as a repeating unit upon the enzymatic polymerization of α-D-glucosamine 1-phosphate (GlcN-1-P), as catalyzed by a thermostable α-glucan phosphorylase (isolated from *Aquifex aeolicus* VF5 thermophilic bacteria) [24]. The polymerization was achieved by conducting the reaction in an ammonia buffer containing 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 α-glucan phosphorylase is a useful tool to synthesize non-natural heteropolysaccharides with a well-defined structure. The thermostable α-glucan phosphorylase-catalyzed enzymatic copolymerization of analog substrates, such as GlcN-1-P and α-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 mannoglucan composed of GlcN/Glc and Man/Glc units, respectively [27,28].

It was also reported that α-glucan phosphorylase isolated from several sources, such as potato, rabbit muscle, and *Escherichia coli*, could catalyze the enzymatic polymerization of 2-deoxy-α-D-glucose 1-phosphate (dGlc-1-P), which was produced in situ from 1,2-dideoxy-D-glucose (D-glucal) in the presence of Pi, using a maltooligosaccharide primer to afford α(1→4)-linked 2-deoxyglucose chains—i.e., 2-deoxyamylose [29–31]. The following mechanism was reported to allow for the generation of 2-deoxyamylose upon α-glucan phosphorylase-catalyzed enzymatic polymerization using D-glucal [29,32]. First, a dGlc unit is either chemically or enzymatically transferred from D-glucal to the primer via an addition reaction assisted by Pi. In the second step, a dGlc residue is released upon α-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 α-glucan phosphorylase (from *Aquifex aeolicus* VF5) was used in the enzymatic polymerization using D-glucal, the mechanism of the in situ production of dGlc-1-P most likely differed from that observed using α-glucan phosphorylases from different sources. In particular, it can be assumed that a thermostable α-glucan phosphorylase catalyzes the enzymatic addition of a C-4 hydroxy group at the non-reducing end of the primer to D-glucal with the consequent formation of an α(1→4)-glycosidic linkage in the absence of Pi as the first step in the in situ production of dGlc-1-P, as it recognizes D-glucal as the only substrate. The in situ production of dGlc-1-P was confirmed by the direct measurement of the ^{1}H NMR spectra of the reaction mixtures in our experiment. Furthermore, we reported that the enzymatically synthesized 2-deoxyamylose 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 α-glucan phosphorylase-catalyzed enzymatic copolymerization of dGlc-1-P, which was produced in situ from D-glucal, with Glc-1-P using maltotriose (Glc_3) as a primer to generate new heteropolysaccharides, and particularly partially 2-deoxygenated amyloses 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-deoxygenated 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.

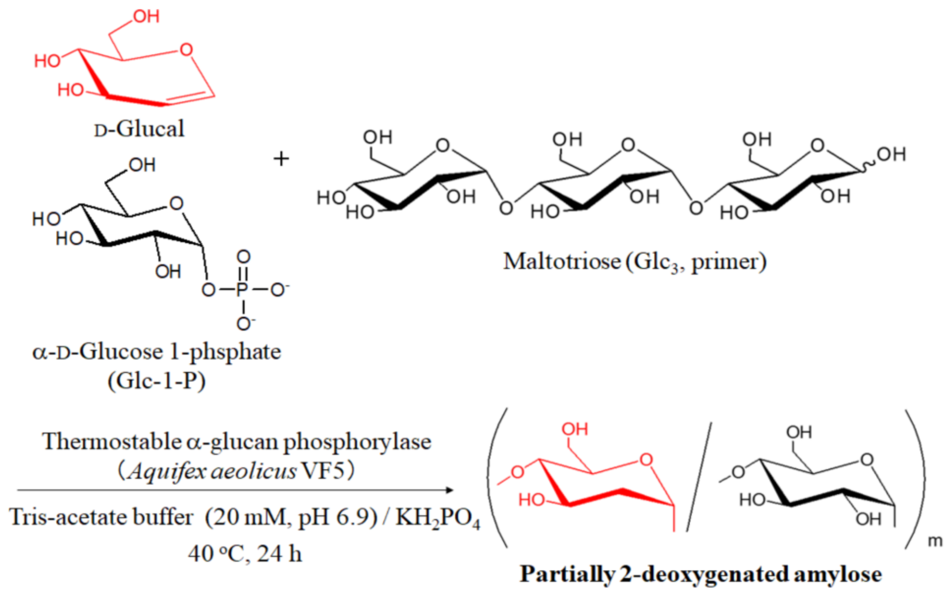

Figure 1. Thermostable α-glucan phosphorylase-catalyzed enzymatic copolymerization of D-glucal with Glc-1-P from Glc_3 to produce partially 2-deoxygenated amyloses; m is the degree of polymerization of the produced polysaccharide, including both dGlc and Glc units.

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), Glc_3 (Hayashibara CO., LTD., Okayama, Japan), Tris-acetate buffer (Nacalai Tesque, Inc., Kyoto, Japan), KH_2PO_4 (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 Glc_3 (2.5 mg, 5.00 µmol) in 20 mM of Tris-acetate buffer (pH 6.9, 1.0 mL) containing KH_2PO_4 (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. ^{1}H NMR: (Figure 2, DMSO-d_6 + D_2O) 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 D-glucal with Glc-1-P [(a)].

Entry	Feed Ratio [(b)] Glc_3:D-Glucal:Glc-1-P	Feed Ratio D-Glucal:Glc-1-P	Unit Ratio [(c)] dGlc:Glc	M_n [(d)]	Yield (%) [(e)]
1	1:120:0	100:0	100:0	3810	19.6
2	1:100:20	83:17	62:38	7620	41.6
3	1:80:40	67:33	53:47	12820	55.7
4	1:60:60	50:50	40:60	16790	53.3
5	1:40:80	33:67	23:76	13560	49.2
6	1:20:100	17:83	6:94	11080	63.0

(a) Reaction was carried out in Tris-acetate buffer containing an equimolar amount of KH_2PO_4 with Glc_3 at 40 °C for 24 h. (b) Feed ratio of Glc_3 to comonomers was 1:120. (c) Determined by ^1H NMR spectra. (d) The number-average molecular weight values, which were determined by the ^1H 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.

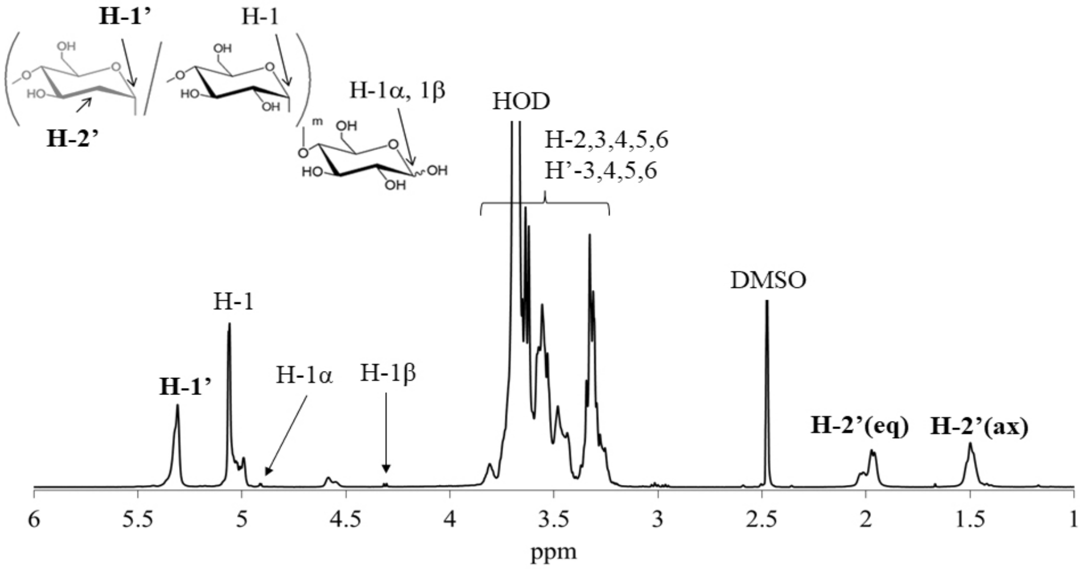

Figure 2. ^1H NMR spectrum of partially 2-deoxygenated amylose (entry 4) in DMSO-d_6/D_2O.

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 ^1H 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 RADIIB diffractometer (PANalytical B.V., EA Almelo, The Netherlands) with Ni-filtered CuKα radiation (λ = 0.15418 nm).

3. Results and Discussion

The thermostable α-glucan phosphorylase-catalyzed enzymatic copolymerization of D-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 D-glucal catalyzed by the same enzyme in our previous investigation [33]. An equimolar amount of KH_2PO_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 D-glucal catalyzed by thermostable α-glucan phosphorylase was performed by the same operation to produce 2-deoxyamylose (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_2O (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 amylose; the signals' assignments accorded to the ^{1}H NMR analysis for the individual homopolysaccharides, amylose and 2-deoxyamylose, 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 D-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 D-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 D-glucal (entry 1) were lower than those by the present enzymatic copolymerization (entries 2–6). This is probably due to the low conversion of D-glucal to dGlc-1-P in situ at the non-reducing end of the 2-deoxyamylose 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 amyloses.

The crystalline structures of the partially 2-deoxygenated amyloses 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-deoxyamylose (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 3e,f) exhibited the same patterns as those of amylose (Figure 3g), as mainly observed at 17° and 22.5°. Such a majority rule in the crystalline structures of the partially 2-deoxygenated amyloses depending on the dGlc/Glc unit ratios is owing to the completely different double helical assembling fashions of the two homopolysaccharides—that is, 2-deoxyamylose and amylose—as discussed in our previous publication [33].

On the other hand, the XRD result of the partially 2-deoxygenated amylose 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 amylose and 2-deoxyamylose, 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 amylose or 2-deoxyamylose 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 amylose specifically with a very high molecular weight (e.g., higher than ca. 5×10^5) exhibits an ability in film formation [37], the present copolymerization is a useful method to facilely obtain amylosic soft materials from α(1→4)-glucan substrates.

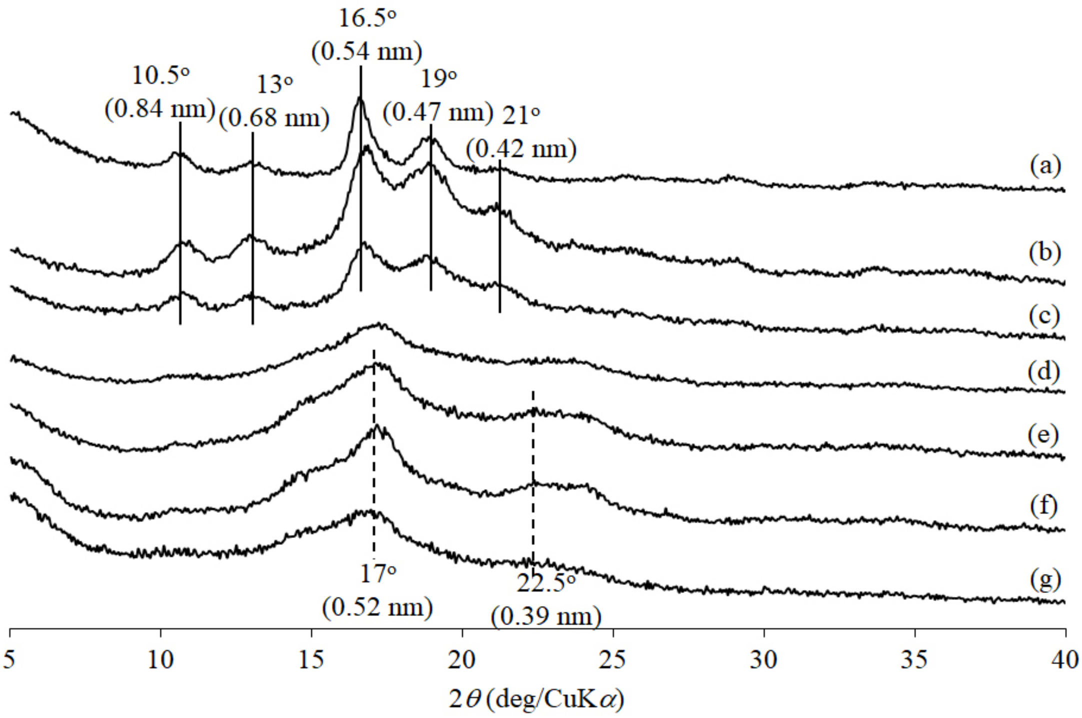

Figure 3. XRD profiles of (**a**) 2-deoxyamylose (entry 1), (**b**–**f**) partially 2-deoxygenated amyloses (entries 2–6), and (**g**) amylose.

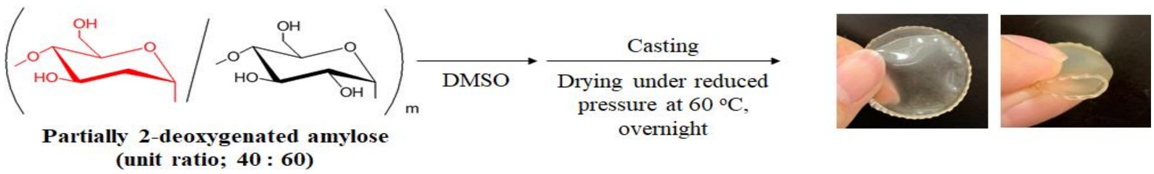

Figure 4. Formation of film from partially 2-deoxygenated amylose (entry 4) by casting DMSO solution and subsequent drying.

4. Conclusions

Because non-natural heteropolysaccharides can be considered as new functional materials, in this study we investigated the thermostable α-glucan phosphorylase-catalyzed enzymatic copolymerization of D-glucal with Glc-1-P from the Glc_3 primer via the in situ production of dGlc-1-P to obtain the partially 2-deoxygenated amyloses. The structures of the products were confirmed by the ^{1}H NMR spectra. The XRD results of the products indicated that the crystalline structures accorded to majority rule, in which the products with certainly higher ratios of dGlc units and Glc units to others formed the same crystalline structures as those of the corresponding homopolysaccharides—i.e., 2-deoxyamylose and amylose, respectively. The amorphous product, which was fabricated by a random sequence of the dGlc/Glc units, formed a flexible film. As the present heteropolysaccharides, the partially 2-deoxygenated amyloses, have a potential to exhibit new functions owing to different crystalline structures depending on the dGlc/Glc unit ratios, we are now working on further functions related to the structures of the present polysaccharides and their possible applications, such as in new practical biocompatible and environmentally benign materials.

Author Contributions: 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.

Funding: This research received no external funding.

Acknowledgments: We acknowledge the supplement of thermostable α-glucan phosphorylase from Ezaki Glico Co. Ltd., Osaka, Japan.

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

References

1. Berg, J.M.; Tymoczko, J.L.; Stryer, L. *Biochemistry*, 7th ed.; W. H. Freeman: New York, NY, USA, 2012.
2. Schuerch, C. Polysaccharides. In *Encyclopedia of Polymer Science and Engineering*, 2nd ed.; Mark, H.F., Bilkales, N., Overberger, C.G., Eds.; John Wiley & Sons: New York, NY, USA, 1986; Volume 13, pp. 87–162.
3. Stephen, A.M.; Phillips, G.O.; Williams, P.A. *Food Polysaccharides and Their Applications*, 2nd ed.; CRC/Taylor & Francis: Boca Raton, FL, USA, 2006; p. 733.
4. DeAngelis, P.L. Glycosaminoglycan polysaccharide biosynthesis and production: Today and tomorrow. *Appl. Microbiol. Biotechnol.* **2012**, *94*, 295–305. [CrossRef]
5. Gómez, B.; Míguez, B.; Yáñez, R.; Alonso, J.L. Manufacture and Properties of Glucomannans and Glucomannooligosaccharides Derived from Konjac and Other Sources. *J. Agric. Food Chem.* **2017**, *65*, 2019–2031. [CrossRef]
6. Kobayashi, S.; Uyama, H.; Kimura, S. Enzymatic polymerization. *Chem. Rev.* **2001**, *101*, 3793–3818. [CrossRef]
7. Shoda, S.; Izumi, R.; Fujita, M. Green process in glycotechnology. *Bull. Chem. Soc. JPN* **2003**, *76*, 1–13. [CrossRef]
8. Kobayashi, S.; Makino, A. Enzymatic polymer synthesis: An opportunity for green polymer chemistry. *Chem. Rev.* **2009**, *109*, 5288–5353. [CrossRef] [PubMed]
9. Kadokawa, J. Precision polysaccharide synthesis catalyzed by enzymes. *Chem. Rev.* **2011**, *111*, 4308–4345. [CrossRef] [PubMed]
10. Shoda, S.; Uyama, H.; Kadokawa, J.; Kimura, S.; Kobayashi, S. Enzymes as green catalysts for precision macromolecular synthesis. *Chem. Rev.* **2016**, *116*, 2307–2413. [CrossRef]
11. Kadokawa, J. Synthesis of non-natural oligosaccharides by α-glucan phosphorylase-catalyzed enzymatic glycosylations using analogue substrates of α-D-glucose 1-phosphate. *Trends Glycosci. Glycotechnol.* **2013**, *25*, 57–69. [CrossRef]
12. Kadoakwa, J. Enzymatic synthesis of non-natural oligo- and polysaccharides by phosphorylase-catalyzed glycosylations using analogue substrates. In *Green Polymer Chemistry: Biobased Materials and Biocatalysis*; ACS Symposium Series 1192; Cheng, H.N., Gross, R.A., Smith, P.B., Eds.; American Chemical Society: Washington, DC, USA, 2015; pp. 87–99.
13. Kadokawa, J. Precision synthesis of functional polysaccharide materials by phosphorylase-catalyzed enzymatic reactions. *Polymers* **2016**, *8*, 138. [CrossRef]
14. Kadokawa, J. α-Glucan phosphorylase: A useful catalyst for precision enzymatic synthesis of oligo- and polysaccharides. *Curr. Org. Chem.* **2017**, *21*, 1192–1204. [CrossRef]
15. Kadokawa, J.I. α-Glucan phosphorylase-catalyzed enzymatic reactions using analog substrates to synthesize non-natural oligo-and polysaccharides. *Catalysts* **2018**, *8*, 473. [CrossRef]
16. Ziegast, G.; Pfannemüller, B. Linear and star-shaped hybrid polymers. Phosphorolytic syntheses with di-functional, oligo-functional and multifunctional primers. *Carbohydr. Res.* **1987**, *160*, 185–204.
17. Kitaoka, M.; Hayashi, K. Carbohydrate-processing phosphorolytic enzymes. *Trends Glycosci. Glycotechnol.* **2002**, *14*, 35–50. [CrossRef]
18. Fujii, K.; Takata, H.; Yanase, M.; Terada, Y.; Ohdan, K.; Takaha, T.; Okada, S.; Kuriki, T. Bioengineering and application of novel glucose polymers. *Biocatal. Biotransform* **2003**, *21*, 167–172. [CrossRef]
19. Seibel, J.; Jordening, H.J.; Buchholz, K. Glycosylation with activated sugars using glycosyltransferases and transglycosidases. *Biocatal. Biotransform* **2006**, *24*, 311–342. [CrossRef]
20. Yanase, M.; Takaha, T.; Kuriki, T. α-Glucan phosphorylase and its use in carbohydrate engineering. *J. Sci. Food Agric.* **2006**, *86*, 1631–1635. [CrossRef]
21. Nakai, H.; Kitaoka, M.; Svensson, B.; Ohtsubo, K. Recent development of phosphorylases possessing large potential for oligosaccharide synthesis. *Curr. Opin. Chem. Biol.* **2013**, *17*, 301–309. [CrossRef]
22. O'Neill, E.C.; Field, R.A. Enzymatic synthesis using glycoside phosphorylases. *Carbohydr. Res.* **2015**, *403*, 23–37. [CrossRef]

23. Puchart, V. Glycoside phosphorylases: Structure, catalytic properties and biotechnological potential. *Biotechnol. Adv.* **2015**, *33*, 261–276. [CrossRef]
24. Kadokawa, J.; Shimohigoshi, R.; Yamashita, K.; Yamamoto, K. Synthesis of chitin and chitosan stereoisomers by thermostable α-glucan phosphorylase-catalyzed enzymatic polymerization of α-D-glucosamine 1-phosphate. *Org. Bimol. Chem.* **2015**, *13*, 4336–4343. [CrossRef]
25. Makino, A.; Kobayashi, S. Chemistry of 2-oxazolines: A crossing of cationic ring-opening polymerization and enzymatic ring-opening polyaddition. *J. Polym. Sci. Polym. Chem.* **2010**, *48*, 1251–1270. [CrossRef]
26. Ochiai, H.; Fujikawa, S.; Ohmae, M.; Kobayashi, S. Enzymatic copolymerization to hybrid glycosaminoglycans: A novel strategy for intramolecular hybridization of polysaccharides. *Biomacromolecules* **2007**, *8*, 1802–1806. [CrossRef] [PubMed]
27. Yamashita, K.; Yamamoto, K.; Kadoakwa, J. Synthesis of non-natural heteroaminopolysaccharides by α-glucan phosphorylase-catalyzed enzymatic copolymerization: α(1->4)-linked glucosaminoglucans. *Biomacromolecules* **2015**, *16*, 3989–3994. [CrossRef] [PubMed]
28. Baba, R.; Yamamoto, K.; Kadokawa, J. Synthesis of α(1–>4)-linked non-natural mannoglucans by alpha-glucan phosphorylase-catalyzed enzymatic copolymerization. *Carbohydr. Polym.* **2016**, *151*, 1034–1039. [CrossRef] [PubMed]
29. Klein, H.W.; Palm, D.; Helmreich, E.J.M. General acid-base catalysis of α-glucan phosphorylases: Stereospecific glucosyl transfer from D-glucal is a pyridoxal 5′-phosphate and orthophosphate (arsenate) dependent reaction. *Biochemistry* **1982**, *21*, 6675–6684. [CrossRef] [PubMed]
30. Evers, B.; Mischnick, P.; Thiem, J. Synthesis of 2-deoxy-α-D-arabino-hexopyranosyl phosphate and 2-deoxy-maltooligosaccharides with phosphorylase. *Carbohydr. Res.* **1994**, *262*, 335–341. [CrossRef]
31. Evers, B.; Thiem, J. Synthesis of 2-Deoxy-maltooligosaccharides with Phosphorylase and Their Degradation with Amylases. *Starch Stärke* **1995**, *47*, 434–439. [CrossRef]
32. Evers, B.; Thiem, J. Further syntheses employing phosphorylase. *Bioorg. Med. Chem.* **1997**, *5*, 857–863. [CrossRef]
33. Uto, T.; Nakamura, S.; Yamamoto, K.; Kadokawa, J. Evaluation of artificial crystalline structure from amylose analog polysaccharide without hydroxy groups at C-2 position. *Carbohydr. Polym.* **2020**, *240*, 116347. [CrossRef]
34. Bhuiyan, S.H.; Rus'd, A.A.; Kitaoka, M.; Hayashi, K. Characterization of a Hyperthermostable Glycogen Phosphorylase from Aquifex aeolicus Expressed in Escherichia coli. *J. Mol. Catal. B Enzym.* **2003**, *22*, 173–180. [CrossRef]
35. Yanase, M.; Takata, H.; Fujii, K.; Takaha, T.; Kuriki, T. Cumulative effect of amino acid replacements results in enhanced thermostability of potato type L α-glucan phosphorylase. *Appl. Environ. Microbiol.* **2005**, *71*, 5433–5439. [CrossRef] [PubMed]
36. Kadokawa, J. Enzymatic synthesis of functional amylosic materials and amylose analog polysaccharides. In *Methods in Enzymology*; Elsevier: Amsterdam, The Netherlands, 2019; Volume 627, pp. 189–213.
37. Suzuki, S.; Shimahashi, K.; Takahara, J.; Sunako, M.; Takaha, T.; Ogawa, K.; Kitamura, S. Effect of addition of water-soluble chitin on amylose film. *Biomacromolecules* **2005**, *6*, 3238–3242. [CrossRef] [PubMed]