J. Appl. Glycosci., **64**, 91–97 (2017)
doi: 10.5458/jag.jag.JAG-2017_010

Regular Paper

Enzymatic Synthesis of 1,5-Anhydro-4-*O*-β-D-glucopyranosyl-D-fructose Using Cellobiose Phosphorylase and Its Spontaneous Decomposition via β-Elimination

(Received July 18, 2017; Accepted August 31, 2017)
(J-STAGE Advance Published Date: September 7, 2017)

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Abstract: Cellobiose phosphorylase from *Cellvibrio gilvus* was used to prepare 1,5-anhydro-4-*O*-β-D-glucopyranosyl-D-fructose [βGlc(1→4)AF] from 1,5-anhydro-D-fructose and α-D-glucose 1-phosphate. βGlc(1→4)AF decomposed into D-glucose and ascopyrone T via β-elimination. Higher pH and temperature caused faster decomposition. However, decomposition proceeded significantly even under mild conditions. For instance, the half-life of βGlc(1→4)AF was 17 h at 30 °C and pH 7.0. Because βGlc(1→4)AF is a mimic of cellulose, in which the C2 hydroxyl group is oxidized, such decomposition may occur in oxidized cellulose in nature. Here we propose a possible oxidizing pathway by which this occurs.

Key words: 1,5-anhydro-4-*O*-β-D-glucopyranosyl-D-fructose, 1,5-anhydro-D-fructose, cellobiose phosphorylase, β-elimination, ascopyrone

INTRODUCTION

The structure of 1,5-anhydro-D-fructose (1,5-anhydro-D-*arabino*-hex-2-ulose; AF, **1**; Fig. 1) is 1-deoxy-2-dehydro-D-glucopyranose. It is now commercially produced from starch or dextrin by reaction with α-1,4-glucan lyase.[1)2)] In general, AF exists as a hydrated form in aqueous solution.[3)4)] It is a functional material, based on its chemical and biochemical properties,[4)5)6)] which include antioxidatant,[4)] and antibacterial[5)] activities.

Oligosaccharides containing AF are also expected to generate new functional materials: 1,5-Anhydro-4-*O*-β-D-glucopyranosyl-D-fructose [1,5-anhydro-4-*O*-β-D-glucopyranosyl-D-*arabino*-hex-2-ulose; βGlc(1→4)AF, **2;** Fig. 1],[7)8)] 1,5-anhydro-4-*O*-β-D-galactopyranosyl-D-fructose,[7)8)] and 1,5-anhydro-4-*O*-α-D-glucopyranosyl-D-fructose[8)] were chemically synthesized as analogs of cellobiose, lactose, and maltose, respectively. However, such chemical synthesis requires a multi-step reaction and is not suitable for the practical production of such oligosaccharides. On the other hand, Richard *et al.*[9)] synthesized mixtures of 1,5-anhydro-6-*O*-α-D-isomaltooligopyranosyl-D-fructose, with various degrees of polymerization, from AF and sucrose using dextransucrase and evaluated their antioxidative activity. Yoshinaga *et al.*[10)] synthesized 1,5-anhydro-3-*O*-α-D-glucopyranosyl-D-fructose from AF and β-cyclodextrin using cyclodextrin glucanotransferase and evaluated the reactivity of the aminocarbonyl reaction of the oligosaccharide. In both cases, the yields of oligosaccharides based on AF were rather low.

Phosphorylases are enzymes that catalyze the reversible phosphorolysis of oligosaccharides.[11)12)13)] These enzymes generally exhibit strict regiospecificity for their substrates and using their reverse reaction, oligosaccharides are effectively prepared from an acceptor sugar and a donor sugar 1-phosphate at good yields. During the present research, we found that cellobiose phosphorylase recognized AF as an acceptor substrate and now describe the synthesis of βGlc(1→4)AF using cellobiose phosphorylase. We also found that the compound was significantly labile under mild conditions and discuss this phenomenon.

RESULTS

Kinetic analysis.

Cellobiose phosphorylase from *Cellvibrio gilvus* used AF as a weak acceptor, as shown in Table 1. The k_{cat}/K_m of AF was much lower than D-glucose (**3**; Fig. 1), but slightly higher than that of 1,5-anhydro-D-glucitol (AG, **4;** Fig. 1) (Table 1). These results are consistent with acceptor recognition by cellobiose phosphorylase, which strongly recognizes the β-hydroxyl group at C1 and weakly recognizes the hydroxyl group at the 2-position.[11)14)15)] Comparison of these parameters on D-mannose (**5;** Fig. 1) and 2-deoxy-D-glucose (**6;** Fig. 1) suggests that the presence of an axial hydroxyl group at C2 slightly increases the k_{cat}/K_m. There-

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Abbreviations: AF, 1,5-anhydro-D-fructose; AG, 1,5-anhydro-D-glucitol; APM, ascopyrone M; APP, ascopyrone P; APT, ascopyrone T; Glc1*P*, α-D-glucose 1-phosphate; βGlc(1→4)AF, 1,5-anhydro-4-*O*-β-D-glucopyranosyl-D-fructose; HPLC, high-performance liquid chromatography; LNB, lacto-*N*-biose I; LPMO, lytic polysaccharide monooxygenase; TLC, thin layer chromatography.

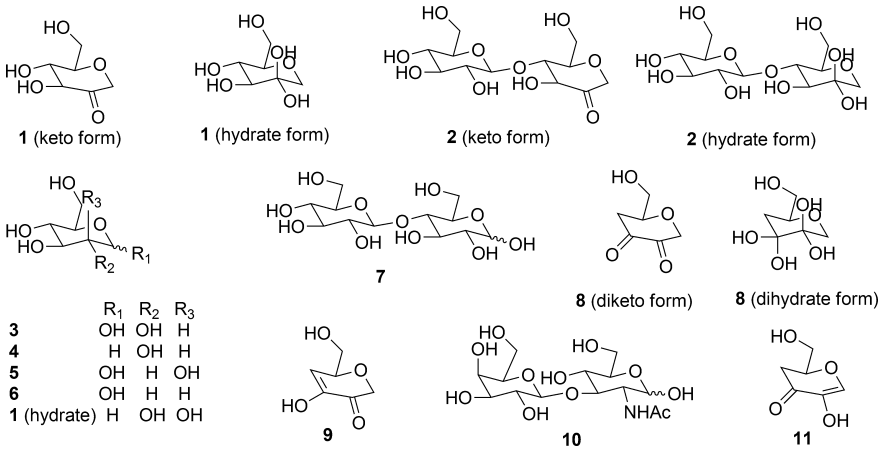

Fig. 1. Formulas of the compounds mentioned in this study.
1, AF; **2**, βGlc(1→4)AF; **3**, D-glucose; **4**, AG; **5**, D-mannose; **6**, 2-deoxy-D-glucose; **7**, cellobiose; **8**, APT; **9**, APM; **10**, LNB; **11**, APP.

Table 1. Kinetic parameters of CBP on various glucosyl acceptors.

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\cdot M^{-1}$)
1,5-Anhydro-D-fructose (**1**)	154 ± 37	16 ± 3	106 ± 8
D-Glucose (**3**)[14]	2.1	98	46,800
1,5-Anhydro-D-glucitol (**4**)[15]	89	5.3	59
D-Mannose (**5**)[14]	115	39	342
2-Deoxy-D-glucose (**6**)[14]	168	37	220

fore, AF (**1;** Fig. 1), which possesses both axial and equatorial hydroxyl groups at C2, was predicted to have a slightly larger k_{cat}/K_m than AG, and the results agree with the prediction.

The disaccharide products of AF.

In the reaction where cellobiose phosphorylase used AF as the acceptor and α-D-glucose 1-phosphate (Glc1*P*) as the donor at pH 7.0 and 30 °C, a single product (Fig. 2, Product I) was observed at the beginning of the reaction. Then another product (Fig. 2, Product II) appeared and Product I levels gradually decreased as the reaction progressed. After a long incubation, e.g. longer than 2 days, Product II became predominant (data not shown). Product II was purified with gel-filtration chromatography and identified to be cellobiose (**7;** Fig. 1) by ^{1}H-NMR analysis.

To obtain pure Product I, reaction conditions were investigated to generate Product I as the single product, without generation of cellobiose. As shown in Fig. 3, the generation of cellobiose was suppressed under lower pH and lower temperature. Preparation of Product I was carried out at pH 5.5 and 10 °C to obtain it as the single product. After the reaction for 12 h, approximately 80 % of Glc1*P* was converted into Product I based on TLC analysis. The separation was carried out at 4 °C by gel-filtration. The fraction containing Product I was treated with cation exchange resin before lyophilization. Without this treatment, partial decomposition of the product was observed during lyophilization, probably due to trace amounts of cations in the fraction. Finally, pure Product I was obtained as a white powder (48 mg, 0.147 mmol, 74 % yield) and identified to be βGlc(1→4)AF (hydrate form in aqueous solution, **2;** Fig. 1).

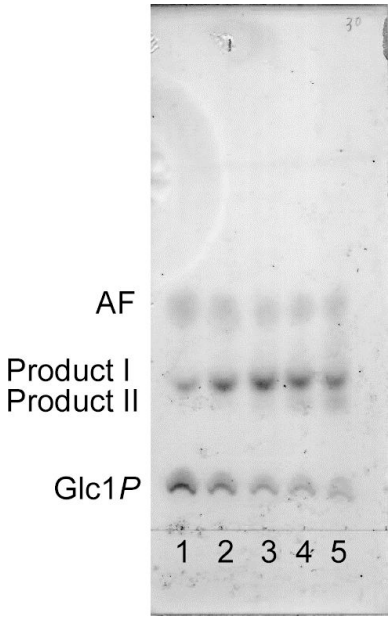

Fig. 2. TLC analysis of the cellobiose phosphorylase reaction with AF and Glc1*P*.
The reaction was carried out in 50 mM 3-morpholinopropanesulfonic acid (MOPS) buffer (pH 7.0) at 30 °C with 0.1 mg/mL cellobiose phosphorylase, 10 mM AF, and 10 mM Glc1*P*. Reaction time: Lane 1,1 h; Lane 2, 3 h; Lane 3, 6 h; Lane 4, 12 h; Lane 5, 24 h.

Spontaneous decomposition of βGlc(1→4)AF.

The treatment of 100 mM βGlc(1→4)AF with 10 mM sodium phosphate buffer (pH 8.0) at 60 °C for 90 min resulted in two products (Fig. 4). One was determined to be D-glucose and the other was the dihydrate form of 1,5-anhydro-4-deoxy-D-*glycero*-2,3-diulose (ascopyrone T,[16,17] APT, **8;** Fig. 1).

The rates of decomposition of βGlc(1→4)AF were measured under various conditions. As shown in Table 2, both higher pH and higher temperature caused a faster reaction rate. The pH dependency suggests the β-elimination, which is catalyzed by OH^- ions.[18] It should be noted that considerable reaction rates were observed under mild conditions, which included normal temperature and neutral pH. For instance, the half-life of βGlc(1→4)AF was only 17 h at pH

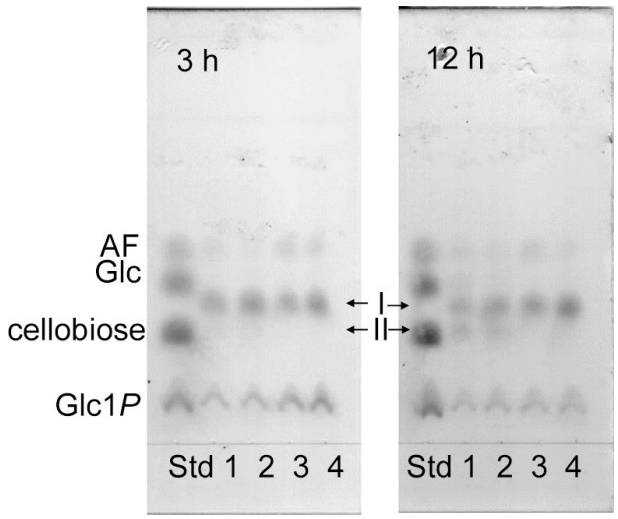

Fig. 3. TLC analysis of the cellobiose phosphorylase reactions under various conditions.

The reaction was carried out in reaction mixtures containing 0.5 mg/mL cellobiose phosphorylase, 100 mM AF, and 100 mM Glc1*P*・2Na (pH adjusted to 7.0 or 5.5 with HCl) under the following conditions: Lane 1, pH 7.0 and 30 C; Lane 2, pH 5.5 and 30 °C; Lane 3, pH 7.0 and 10 °C; Lane 4, pH 5.5 and 10 °C; Standards (Std), AF, D-glucose, cellobiose, and Glc1*P*. Left panel, reaction for 3 h; right panel, reaction for 12 h. I and II indicate the position of Products I and II, respectively.

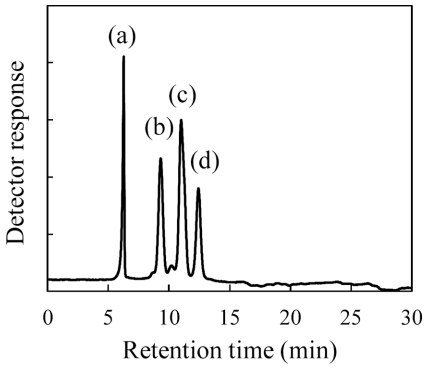

Fig. 4. Chromatogram of the decomposition products of βGlc(1→4)AF.

The HPLC was performed using an MCI GEL CK08S column (eluent, water; flow rate, 1 mL/min) and a reflective index detector (for details, see Experimental section). Peaks were assigned as follows: (a) salts, (b) βGlc(1→4)AF, (c) D-glucose, and (d) ascopyrone T.

7.0 and 30 °C (Table 2). The activation energies of βGlc(1→4)AF decomposition at various pHs were around 104 kJ/mol and they were not dependent on pH.

DISCUSSION

We described here a cellobiose phosphorylase reaction using AF as the acceptor and producing two products, βGlc(1→4)AF and cellobiose. We also found that βGlc(1→4)AF spontaneously decomposed into D-glucose and APT, even under mild conditions. Such decomposition was not described in the previous papers reporting the chemical synthesis of βGlc(1→4)AF.[7)8)]

The formation of D-glucose and APT can be explained by β-elimination of βGlc(1→4)AF, as illustrated in Fig. 5. This β-elimination reaction occurs due to the presence of the carbonyl group at C2 in the keto form of βGlc(1→4)AF. The cleavage occurs at the C–O bond at C4 on the AF residue, with the removal of the H atom at C3, causing β-elimination on βGlc(1→4)AF to generate D-glucose and the 4-deoxy-3-unsaturated compound (1,5-anhydro-4-deoxy-D-*glycero*-hex-3-en-2-ulose; ascopyrone M, APM, **9;** Fig. 1), followed by spontaneous transformation into the 2,3-diketo compound (APT, **8;** Fig. 1) and finally to its dihydrate form, which is stable in the aqueous solution.[17)] Because the amount of APT generated was less than that of D-glucose (Fig. 4), some part of the compound derived from the AF residue might be highly decomposed to be acidic compounds.[6)]

β-Elimination often occurs at a C–O bond two carbons away from a carbonyl residue, as in the case of βGlc(1→4)AF. We have previously reported the β-elimination of free aldoses, with the substitution at the C3 hydroxyl group, such as for lacto-*N*-biose I [β-D-Gal*p*-(1→3)-D-GlcNAc, LNB, **10;** Fig. 1].[18)] Degradation of βGlc(1→4)AF was much faster than that of LNB. For instance, decomposition of LNB was not observed below 55 °C. The activation energy of LNB is 125 kJ/mol,[18)] which is considerably higher than that of βGlc(1→4)AF and can be explained by a difference in the carbonyl group. The carbonyl group of βGlc(1→4)AF is hydrated but does not form hemiacetal (hemiketal), whereas that of LNB mostly exists as hemiacetal to form a pyranose ring (Fig. 6).

It was reported that AF itself caused β-elimination at C4 to form a 3-keto-1-unsaturated compound (1,5-anhydro-4-deoxy-D-*glycero*-hex-1-en-3-ulose; ascopyrone P, APP, **11;**

Table 2. Rate constant and half-life for β-elimination of βGlc(1→4)AF.

	Rate constant (k) (h^{-1}) [Half-life ($t_{1/2}$) (h)]				Activation energy (E_a) ($kJ\ mol^{-1}$)
	50 °C	40 °C	30 °C	20 °C	
pH 8.0	1.0 [0.66]	0.31 [2.2]	0.081 [8.6]	0.018 [39]	107
pH 7.0	0.46 [1.5]	0.14 [4.9]	0.041 [17]	0.0093 [74]	102
pH 6.0	0.11 [6.6]	0.033 [21]	0.0087 [80]	-*	101
pH 5.0	0.027 [25]	0.0081 [85]	0.0022 [315]	-*	103
pH 4.0	0.010 [67]	0.0030 [230]	0.00081 [858]	-*	104

The rates were determined based on the increase in D-glucose. *Not determined due to microbial contamination during the long incubation.

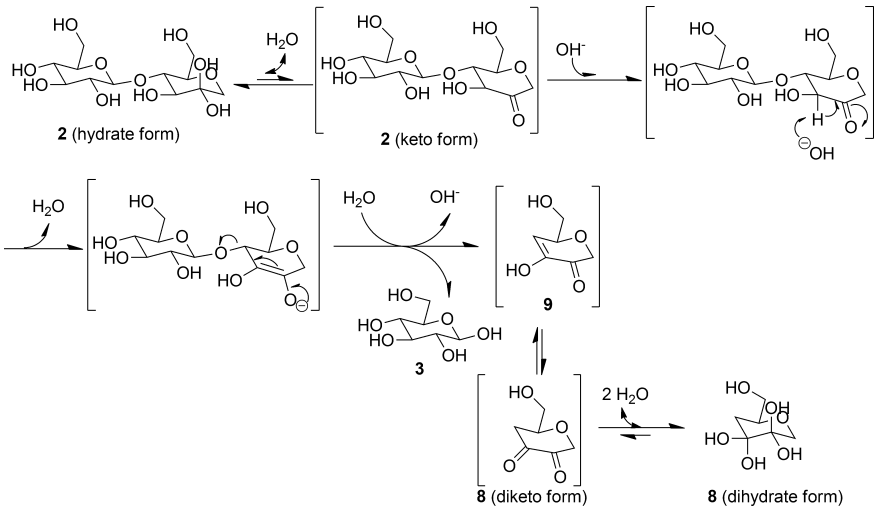

Fig. 5. Reaction mechanism of decomposition of βGlc(1→4)AF (**2**) via β-elimination. Compounds shown in the brackets are intermediates and were not detected.

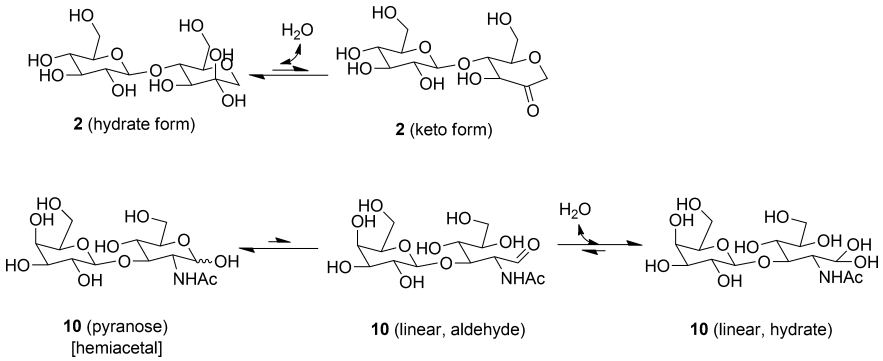

Fig. 6. Difference in the carbonyl groups in βGlc(1→4)AF (**2**) and LNB (**10**).

Fig. 1) under much severe conditions such as at 155 °C.[19] It means that glycosylation at the C4 hydroxyl group greatly enhances β-elimination. Similar enhancement of β-elimination by glycosylation was observed between GlcNAc and LNB.[18)20] The difference in the product (APT and APP) is probably due to the difference in the reaction temperature. High temperature[19] or the enzyme ascopyrone tautomerase[17] is required to pass through the barrier between APT (or APM) and APP.

The formation of cellobiose during the AF-acceptor reaction of cellobiose phosphorylase did not proceed via the hydration of AF or βGlc(1→4)AF, evidenced by the fact that no deuterium was introduced into cellobiose when performing the enzymatic reaction in D_2O (data not shown). We also confirmed the absence of Glc1*P*-hydrolyzing activity in the purified CBP. The formation of cellobiose is, thus, due to the decomposition of βGlc(1→4)AF to generate D-glucose and APT, which also occurs during the cellobiose phosphorylase reaction as well as in the purified compound. The resultant D-glucose reacts with Glc1*P* to generate cellobiose because D-glucose is a much better acceptor substrate for CBP than AF.

We showed here that βGlc(1→4)AF is decomposed by β-elimination at a detectable rate, even under normal biochemical conditions. βGlc(1→4)AF is a structural mimic of cellulose oxidized at the C2 hydroxyl group of a glucosyl residue because of its fixed pyranose ring of the AF residue. Our results suggest that the oxidation of cellulose at C2 may cause the β-elimination to cleave the β-1,4-linkage at a significant rate, judging from the fact that the enzymatic degradation of crystalline cellulose is a very slow process.

In this decade, participation of oxidizing enzymes, the lytic polysaccharide monooxygenases (LPMOs), in degradation of insoluble polysaccharides, including cellulose, has been attracting attention with respect to understanding their natural degradation system and for utilization of biomass resources.[21)22)23)24] LPMOs directly cleave a glycosyl linkage to form glyconolactone and a new non-reducing end, or a new reducing end and a keto sugar by adding an oxygen atom (Fig. 7A). The reaction of LPMOs plays an important role in boosting the degradation of insoluble polysaccharides by increasing the number of entry points for the hydrolases.

If an enzyme that oxidizes the C2 hydroxyl group of cellulose exists, such an enzyme does not directly cleave the glycosyl bond but the resultant 2-ketoglucosyl residue causes β-elimination, degrading the cellulose chain and forming a new reducing end and an APT-like structure (Fig. 7B). We predict the presence of such an oxidizing enzyme, other than known LMPOs, that is involved in the oxidizing pathway for the natural cleavage of cellulose. It should also be noted that the β-elimination would not be significantly affected by the glucosyl linkage, whether it is β or α.[18] A

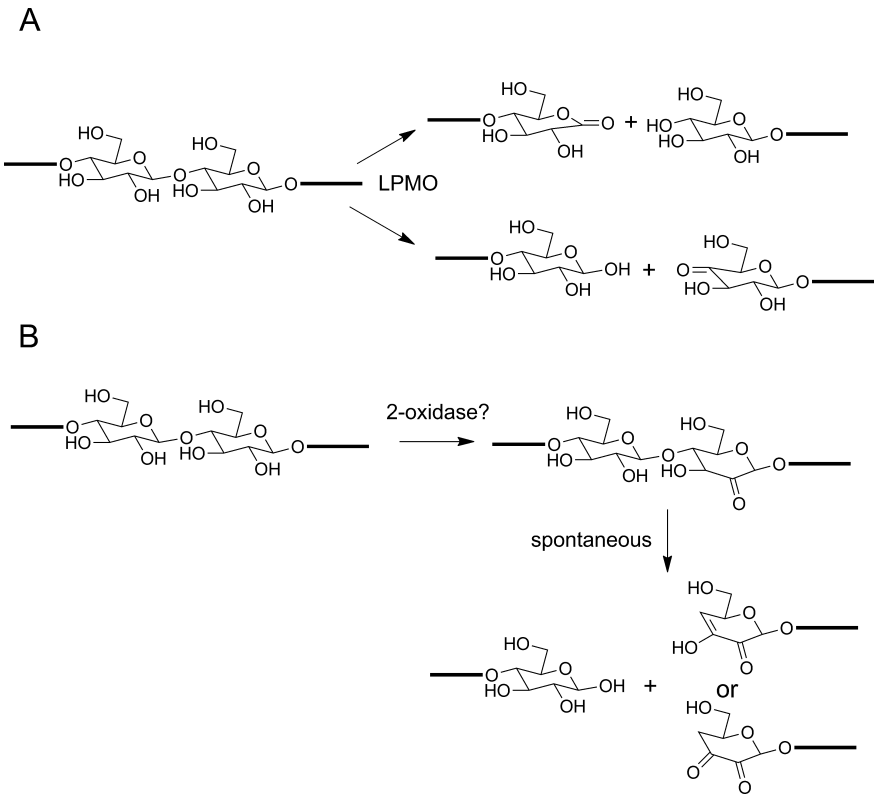

Fig. 7. Reaction scheme of the cleavage of polysaccharide by oxidizing enzyme.
A, Reaction by LPMO; B, Predicted reaction initiated by the oxidization of the C2 hydroxyl group, followed by β-elimination.

similar phenomenon is possibly involved in the degradation of raw starch granules in nature, which is also a slow process.

EXPERIMENTAL

Materials. Recombinant cellobiose phosphorylase from *Cellvibrio gilvus* was prepared as described previously.[15] AF was produced from starch using a glucan lyase from *Gracilaria verrucosa* and purified by gel-filtration as described previously.[4] Other chemicals used were of reagent grade.

General Methods. ^{1}H NMR and ^{13}C NMR spectra were obtained in D_2O on an Avance 400 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) at 400.13 and 100.61 MHz, respectively. Chemical shifts for ^{1}H NMR and ^{13}C NMR signals are given in ppm at 300 K, with 2-methyl-2-propanol as an internal standard (1.23 and 31.3 ppm, respectively). The following abbreviations are used for the characterization of NMR signals: s = singlet, d = doublet, t = triplet, and m = multiplet. ESI-Orbitrap-MS spectra were recorded on a Velos Pro Dual-Pressure Linear Ion Trap Mass Spectrometer (Thermo Fisher Scientific, Inc., Waltham, USA).

Thin layer chromatography (TLC). An aliquot of each reaction mixture containing 10 nmole acceptor-equivalent was spotted on a TLC plate (Kieselgel 60 F254; Merck KGaA, Darmstadt, Germany) and the plate was developed with acetonitrile–water (4:1 by volume). The plate was dipped in a 5 % sulfuric acid–methanol solution and heated in an oven until the bands were visible to detect the products generated.

Kinetic analysis. Measurement of the synthetic reaction was carried out as described previously.[14,15] The initial rate was assayed at 37 °C in 50 mM Tris/HCl buffer (pH 7.0) by measuring the amount of phosphate released from 10 mM Glc1*P*, with various concentrations of a glucosyl acceptor. The kinetic parameters were calculated by regressing the experimental data on the Michaelis–Menten equation using Grafit Ver. 7 (Erithacus Software Ltd., London, UK).

Isolation of cellobiose. A 2.0 mL reaction mixture that consisted of 200 mM AF, 200 mM Glc1*P*·2Na (pH adjusted to 6.5 with HCl), and 0.01 mg/mL cellobiose phosphorylase was incubated for 4 days at 30 °C. The enzyme and salts were removed from the reaction mixture by ultrafiltration using Amicon Ultra-4 Centrifugal Filter Devices (10,000 MW cut off; Merck Millipore, Billerica, USA) and by electrodialysis using Microacylizer S1 with an AC-220-10 membrane cartridge (Astom Corp., Tokyo Japan), respectively. The solution was applied to a Toyopearl HW40S gel-filtration column (2.5 cm φ × 80 cm, Tosoh Corp., Tokyo, Japan) equilibrated with H_2O and fractionated with H_2O at a rate of 2 mL/min at 25 °C. The fractions containing the product were collected and lyophilized to obtain 24 mg of the product, which was identified to be cellobiose.

Preparation of cellobiose in D_2O. A 0.5 mL mixture that consisted of 200 mM AF, 200 mM Glc1*P*·2Na (pH adjusted to 6.5 with HCl) was lyophilized and dissolved in 0.5 mL D_2O containing 0.01 mg/mL cellobiose phosphorylase. The reaction mixture was incubated for 3 days at 30 °C. The enzyme was removed from the reaction mixture using

a Ni-NTA Spin Column (Qiagen, Hilden, Germany). Cellobiose was purified by gel filtration as describes above to yield 0.2 mg of lyophilizate.

Isolation of βGlc(1→4)AF. A 2.0 mL reaction mixture that consisted of 100 mM AF, 100 mM Glc1*P*·2Na (pH adjusted to 5.5 with HCl), and 0.5 mg/mL cellobiose phosphorylase was incubated for 12 h at 10 °C. The enzyme was removed from the reaction mixture using VIVASPIN 20 (10,000 MW cut off; GE Healthcare Life Sciences, Buckinghamshire, UK). Then, approximately 370 mg of cation exchange resin (Dowex 50 W × 8, H^+ form, 200–400 mesh; Wako Pure Chemicals Industries, Ltd., Osaka, Japan) was added to the mixture and the resin was removed by filtration. The filtrate was applied to a Toyopearl HW40S gel-filtration column (2.5 cm φ × 80 cm) equilibrated with H_2O and fractionated with H_2O at a rate of 1 mL/min at 4 °C. Separation of the products was confirmed by TLC analysis. The fractions containing the product were collected and treated with the cation exchange resin to ensure that the solution was not basic. Then, the solution was lyophilized to obtain 48 mg of the product, which was identified to be βGlc(1→4)AF (hydrate form in aqueous solution, **2**; Fig. 1, 0.148 mmol, 74 % yield).

^{1}H NMR (D_2O): 4.48 (d, 1H, $J_{1',2'}$ 8.0 Hz, H-1′), 3.95 (dd, 1H, $J_{5,6a}$ 2.2 Hz, $J_{6a,6b}$ 9.1 Hz, H-6a), 3.92 (dd, 1H, $J_{5',6'a}$ 2.2 Hz, $J_{6'a,6'b}$ 9.3 Hz, H-6′a), 3.75 (dd, 1H, $J_{5,6b}$ 5.8 Hz, $J_{6a,6b}$ 9.7 Hz, H-6b), 3.73 (d, 1H, $J_{1a,1b}$ 12.0 Hz, H-1a), 3.72 (dd, 1H, $J_{5',6'b}$ 6.1 Hz, $J_{6'a,6'b}$ 10.0 Hz, H-6′b), 3.67 (d, 1H, $J_{3,4}$ 8.9 Hz, H-3), 3.63 (t, 1H, $J_{3,4} = J_{4,5}$ 8.9 Hz, H-4), 3.52 (m, 1H, H-5), 3.50 (m, 1H, H-5′), 3.49 (t, 1H, $J_{2',3'} = J_{3',4'}$ 9.2 Hz, H-3′), 3.45 (d, 1H, $J_{1a,1b}$ 12.0 Hz, H-1b), 3.40 (t, 1H, $J_{3',4'} = J_{4',5'}$ 9.4 Hz, H-4′), 3.30 (dd, 1H, $J_{1',2'}$ 8.0 Hz, $J_{2',3'}$ 9.4 Hz, H-2′); ^{13}C NMR (D_2O): 104.3 (C-1′), 94.3 (C-2), 81.0 (C-5), 80.4 (C-4), 77.7 (C-5′), 77.2 (C-3 and C-3′), 74.8 (C-2′), 73.2 (C-1), 71.2 (C-4′), 62.3 (C-6′), 62.2 (C-6); ESIMS: *m/z* 365.11 $[M+Na]^+$ (calculated for $C_{12}H_{22}O_{11}Na^+$ [hydrate form], 365.11).

Isolation of products of decomposed βGlc(1→4)AF. A 1.0 mL reaction mixture that consisted of 100 mM βGlc(1→4)AF in 10 mM sodium phosphate buffer (pH 8.0) was incubated at 60 °C for 90 min. After the reaction, the products were purified twice by high-performance liquid chromatography (HPLC) equipped with a reflective index detector (Model 504, GL Sciences Inc., Tokyo, Japan) using an MCI GEL CK08S column (eluent, water; flow rate, 1 mL/min; Mitsubishi Chemical Corporation, Tokyo, Japan). The fractions containing a product other than glucose were lyophilized. Approximately 1 mg of the product was obtained and identified to be APT (dihydrate form in aqueous solution, **8**; Fig. 1).

^{1}H NMR (D_2O): 3.77 (ddt, 1H, $J_{4a,5}$ 10.6 Hz, $J_{4b,5} = J_{5,6a}$ 3.5 Hz, $J_{5,6b}$ 7.0 Hz, H-5), 3.64 (d, 1H, $J_{1a,1b}$ 11.7 Hz, H-1a), 3.62 (dd, 1H, $J_{5,6a}$ 3.3 Hz, $J_{6a,6b}$ 12.0 Hz, H-6a), 3.59 (d, 1H, $J_{1a,1b}$ 11.8 Hz, H-1b), 3.55 (dd, 1H, $J_{5,6b}$ 7.0 Hz, $J_{6a,6b}$ 12.1 Hz, H-6b), 1.82 (dd, 1H, $J_{4a,4b}$ 13.5 Hz, $J_{4a,5}$ 10.5 Hz, H-4a), 1.77 (dd, 1H, $J_{4a,4b}$ 13.5 Hz, $J_{4b,5}$ 3.5 Hz, H-4b); ^{13}C NMR (D_2O): 95.0 (C-3), 94.0 (C-2), 77.5 (C-5), 71.9 (C-1), 65.3 (C-6), 38.5 (C-4); ESIMS: *m/z* 203.05 $[M+Na]^+$ (calculated for $C_6H_{12}O_6Na^+$ [dihydrate form], 203.05).

Rate constant and half-life measurement of β-elimination of βGlc(1→4)AF. βGlc(1→4)AF was dissolved and made to 1 mM with the following buffers: 50 mM sodium phosphate buffer (pH 8.0, 7.0, and 6.0) and 50 mM sodium acetate buffer (pH 5.0 and 4.0). The solutions (1.0 mL) were incubated at temperatures of 50, 40, 30, and 20 °C. For each measurement time, aliquots (100 μL) of the reaction mixtures were diluted with 20 mM sodium acetate buffer (900 μL, pH 4.0). Glucose produced by the decomposition was quantified using a glucose oxidase-peroxidase method with a Glucose CII-test Wako (Wako Pure Chemicals), with the following modification. The phosphate buffer solution containing 500 mg/L phenol (pH 7.1) in the kit was substituted with 50 mM 2-morpholinoethanesulfonic acid (MES) buffer (pH 6.0) containing 1 mM *N*-ethyl-*N*-(3-sulfopropyl)-3-methylaniline sodium salt (TOPS, Dojindo Laboratories, Mashiki, Japan). The working reagent was mixed with an equal volume of the sample and incubated at 30 °C for 10 min, followed by measurement of the absorbance at 550 nm. The concentration of βGlc(1→4)AF was calculated by subtracting the concentration of Glc measured from the initial concentration of βGlc(1→4)AF. The rate constant (k) was determined through liner regression of the following equation (1).

$$\ln([\beta\mathrm{Glc}(1\rightarrow4)\mathrm{AF}]/[\beta\mathrm{Glc}(1\rightarrow4)\mathrm{AF}]_0) = -k\mathrm{t} \qquad (1)$$

The half-life ($t_{1/2}$) was calculated as $\ln 2/k$.

The activation energy (E_a) at each pH was determined through linear regression of the Arrhenius equation (2).

$$\ln k = \ln \mathrm{A} - E_a/\mathrm{RT}(\mathrm{A}: \text{frequency factor}) \qquad (2)$$

ACKNOWLEDGMENTS

The NMR and MS spectra were recorded by the staff of the Advanced Analysis Center, NARO. We thank Professor Shinya Fushinobu for the discussion on LPMO. The authors would like to thank Enago (www.enago.jp) for the English language review.

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