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Stability of (1 → 3)-β-polyglucuronic acid under various pH and temperature conditions

Erika Watanabe[a], Naoyuki Tamura[a], Shuji Fujisawa[a], Tsuguyuki Saito[a], Naoto Habu[b], Akira Isogai[a,*]

[a] Department of Biomaterial Sciences, The University of Tokyo, Tokyo 113-8657, Japan
[b] Department of Applied Biological Chemistry, Utsunomiya University, Tochigi 321-8505, Japan

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ABSTRACT

Regioselective oxidation of C6 primary hydroxyls to carboxyls was applied to curdlan, to prepare a water-soluble (1 → 3)-β-polyglucuronic acid Na salt [(1,3)-β-PGluA], using a 4-acetamido-TEMPO/NaClO/$NaClO_2$ oxidation system at pH 4.7. Changes in the chemical structure and degree of polymerization of (1,3)-β-PGluA treated in water at various temperatures and pHs were studied to evaluate the stability of (1,3)-β-PGluA to these treatments. This polyuronic acid was found to be stable, without any depolymerization, to treatment in water at pHs 1–9 and room temperature for up to 128 h; slight depolymerization was observed at pHs 11 and 13. When heated in water at pH 1 and high temperatures (1,3)-β-PGluA molecules were randomly depolymerized by hydrolysis, primarily forming glucuronic acid. In contrast, dicarboxylic-acid-type monomers containing ethylene carbons were formed from the C1-carboxyl or C1 reducing ends of (1,3)-β-PGluA molecules by treatment under alkaline conditions; this was initiated by β-alkoxy elimination, similar to the peeling-off reaction of cellulose.

1. Introduction

Curdlan, a linear (1 → 3)-β-glucan, is an extracellular bacterial polysaccharide, and is insoluble in water at room temperature (Harada, Misaki, & Saito, 1968; McIntosh, Stone, & Stanisich, 2005; Stasinopoulos, Fisher, Stone, & Stanisich, 1999). Because of its triple-helical structure, curdlan has peculiar functionalities. Much research has been carried out on curdlan based on its unique structure and functionalities (Chuah, Sarko, Deslandes, & Marchessault, 1983; Deslandes, Marchessault, & Sarko, 1980; Shibakami, Sohma, & Hayashi, 2012). Aqueous suspensions of curdlan form two types of gel, namely low-setting and high-setting gels, on heating, depending on the temperature. Curdlan has therefore been used as an ingredient in various types of processed foods for many years (Fan et al., 2006; Funami, Funami, Yada, & Nakao, 1999; Konno & Harada, 1991; Maeda, Saito, Masada, Misaki, & Harada, 1967). In addition, curdlan has some bioactivities such as immune modulation and antitumor effects, so its derivatives have been used as anticoagulant, antithrombotic, and anti-HIV agents (Bohn & BeMiller, 1995; Jagodzinski et al., 1994; Kataoka, Muta, Yamazaki, & Takeshige, 2002; Ooi & Liu, 2000; Toida, Chaidedgumjorn, & Linhardt, 2003).

Oxidation of the C6 primary hydroxyl groups of polysaccharides using the 2,2,6,6-tetramethylpiperidin-1-oxyl radical (TEMPO), or an analog, as a catalyst under aqueous conditions has opened up a new field of polysaccharide chemistry. The TEMPO-mediated oxidation of polysaccharides has some advantages such as high reaction rates, high regioselectivity, and environmental compatibility (Bragd, Besemer, & van Bekkum, 2001; de Nooy, Besemer, & van Bekkum, 1995). The TEMPO/NaBr/NaClO oxidation system has been applied to various polysaccharides such as cellulose, starch, chitin, and regenerated cellulose (Isogai & Kato, 1998; Kato, Kaminaga, Matsuo, & Isogai, 2004; Muzzarelli, Muzzarelli, Cosani, & Terbojevich, 1999).

When curdlan was subjected to TEMPO/NaBr/NaClO oxidation in water at pH 10, water-soluble (1 → 3)-β-polyglucuronic acids [(1,3)-β-PGluA] were obtained quantitatively within 100 min. The C6 primary hydroxyl groups of curdlan were mostly converted to carboxylate groups by the oxidation. However, significant depolymerization was inevitable during the oxidation (Tamura, Wada, & Isogai, 2009). In contrast, when curdlan was subjected to oxidation using a 4-acetamido-TEMPO/NaClO/$NaClO_2$ system in water at pH 4.7, completely C6-carboxylated (1,3)-β-PGluA samples with high degrees of polymerization (DPs) were obtained quantitatively (Watanabe, Tamura, Saito, Habu, & Isogai, 2013). Because water-soluble polysaccharides, which originally have triple-helical structures, can be used as effective dispersants of carbon nanotubes and metal nanoparticles in water (1,3)-β-PGluA is also expected to

* Corresponding author. Tel.: +81 3 5841 5538; fax: +81 3 5841 5269.
E-mail address: aisogai@mail.ecc.u-tokyo.ac.jp (A. Isogai).

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have applications as a dispersant (Le et al., 2011; Li, Zhang, Xu, & Zhang, 2011). Investigation of the stability of (1,3)-β-PGluA in water under various temperature and pH conditions is therefore of significance for understanding the fundamental properties and applications of (1,3)-β-PGluA.

In this study (1,3)-β-PGluA samples prepared using the above method were treated in water under various temperature and pH conditions for up to 128 h. The molecular weight parameters and chemical structures of the treated products were analyzed using size-exclusion chromatography with multi-angle laser light-scattering (SEC-MALLS) and photodiode-array detectors and ^{13}C nuclear magnetic resonance (NMR) spectroscopy of the treated products was used to evaluate the stability of (1,3)-β-PGluA. The depolymerization rate constants of this polyuronic acid in water under various conditions were obtained and compared with those of cellouronic acid (Fujisawa, Isogai, & Isogai, 2010).

2. Materials and methods

2.1. Materials

Curdlan was a commercial product (Wako Pure Chemicals Industries Ltd., Japan). 4-Acetamide-TEMPO, sodium chloride, 80% sodium chlorite, 12% sodium hypochlorite solution, and other reagents and solvents were of laboratory grade and used without further purification. Distilled water of HPLC grade was purchased from Wako Pure Chemicals.

2.2. TEMPO-mediated oxidation of curdlan

Curdlan (1 g, 6 mmol C6-OH) was placed in an Erlenmeyer flask with a magnetic stirring bar. Acetate buffer (0.2 M) at pH 4.7 (100 mL) containing $NaClO_2$ (80%, 0.68 g, 6 mmol) and 4-acetamido-TEMPO (0.096 g, 0.45 mmol) was added to the flask. NaClO solution (0.62 mL, 1 mmol) was added at once to the curdlan suspension, and the flask was immediately capped with a universal stopper. The mixture was stirred at 35 °C for 24 h. Oxidation was quenched by adding an excess of ethanol (100 mL) to the mixture, and the precipitate formed was collected by centrifugation. The oxidized product was dialyzed with deionized water and freeze-dried. The oxidized curdlan obtained was re-oxidized to completely oxidize the small amount of remaining primary hydroxyls, using the method described above, and a (1,3)-β-PGluA Na salt consisting of only glucuronosyl units was obtained in 85% yield (Watanabe et al., 2013).

2.3. Stability tests under various pH and temperature conditions

(1,3)-β-PGluA Na salt solutions (1%, w/v) in 0.1 M acetate buffer at pH 4, 0.1 M phosphate buffer at pH 7, and 0.1 M bicarbonate buffer at pHs 9 and 11 were prepared. A 1% (w/v) (1,3)-β-PGluA solution at pH 13 was prepared by adding appropriate amounts of 0.2 M NaOH and water to a 2% (w/v) (1,3)-β-PGluA/water solution. To the 2% (w/v) (1,3)-β-PGluA Na salt/water solution, 0.2 M HCl was added to prepare a 1% (w/v) (1,3)-β-PGluA suspension at pH 1. When the pH of the (1,3)-β-PGluA Na salt solution was adjusted to 1, a white precipitate was formed in the solution as a result of conversion of the (1,3)-β-PGluA Na salt to (1,3)-β-PGluA with protonated carboxyl groups. Each (1,3)-β-PGluA solution or suspension was put in a vial and shaken at room temperature (25 °C) for up to 128 h. In the case of heating above room temperature, each solution or suspension was put in a test-tube with a Teflon-sealed screw cap, and heated in a block-type heating apparatus for the test-tubes (DTU-1C TAITEC, Japan) at 40–105 °C. After treatment, the sample solution or suspension was neutralized with dilute HCl or NaOH solution. These samples were immediately frozen with liquid nitrogen to stop further structural changes, and kept at −20 °C until use. After defrosting at room temperature, the sample solutions were diluted with 0.1 M NaCl. The molecular weight parameters of the products were determined using SEC-MALLS.

2.4. SEC-MALLS analysis

The sample solution was diluted with 0.1 M NaCl to a concentration of 0.1%. The solutions were subjected to SEC-MALLS analysis (DAWN EOS, λ 690 nm; Wyatt Technologies, USA) using one of the following SEC columns for aqueous systems: SB-806MHQ and SB-802.5HQ, 8 mm $\varphi \times$ 30 cm, Shodex, Japan. A 0.1 M NaCl solution was used as the eluent for measuring the molecular weight parameters of the products. The eluent and sample solutions were filtered using 0.2-μm poly(tetrafluoroethylene) membranes (Millipore, USA) before the SEC-MALLS analysis. The weight- and number-average molecular-weights of the samples were calculated from the SEC-MALLS data using ASTRA software (Wyatt Technologies, USA), with a specific refractive index increment (dn/dc) value of 0.158 mL g^{-1} for (1,3)-β-PGluA (Watanabe et al., 2013). A pullulan standard (weight-average molecular-weight 22,800; Shodex, Japan) was exclusively used to normalize the MALLS photodetectors (ASTRA for Windows user's guide, version 4.90). Details of the SEC-MALLS system used and operating conditions have been described elsewhere (Isogai, Yanagisawa, & Isogai, 2009; Tamura, Hirota, Saito, & Isogai, 2010). The DPs of the (1,3)-β-PGluA-related products were obtained by SEC-MALLS analysis; it was assumed that the products had the same structures as that of the original (1,3)-β-PGluA but with different molecular weights.

2.5. Other analysis

After freeze-drying the sample solutions, D_2O containing 3-trimethylsilyl-2,2,3,3-d_4-propionic acid sodium salt (Aldrich, USA) was added to the samples. ^{13}C NMR spectra of the solutions were recorded on an ALPHA-500 (JEOL, Japan). The data accumulation times of the ^{13}C NMR spectra were approximately 25,000. The carboxyl contents of the samples were determined by electric conductivity titration using 0.05 M NaOH (Saito & Isogai, 2004).

3. Results and discussion

3.1. Stability of (1,3)-β-PGluA at room temperature under various pH conditions

The (1,3)-β-PGluA Na salt was treated in water at pHs 1–13 and room temperature for 0–128 h to evaluate its stability under various pH conditions. Fig. 1 shows the relationships between the treatment time and weight-average DP (DP_w) of the products. When (1,3)-β-PGluA was treated in water at pHs 1–9 for 0–128 h, the DP_w values were mostly unchanged and almost the same as the original value. Hence (1,3)-β-PGluA was stable without any depolymerization in water at room temperature under these pH conditions. Slight decreases in the DP_w values with increasing treatment time were observed when (1,3)-β-PGluA was treated under alkaline conditions at pH 11 or 13; the DP_w values decreased from 300 to 220 and 140 at pHs 11 and 13, respectively, after treatment for 128 h. Thus, the higher the treatment pH, the lower the DP_w value of the products obtained by treatment in these pH regions, even at room temperature.

Fig. 2 shows the changes in the SEC elution pattern of the products obtained from (1,3)-β-PGluA by treatment in water at pHs 1, 9, 11, and 13 and room temperature for 0–128 h. When (1,3)-β-PGluA was treated at pHs 1–7, no shifts in the peak-top positions of the SEC elution patterns were observed. (1,3)-β-PGluA was therefore

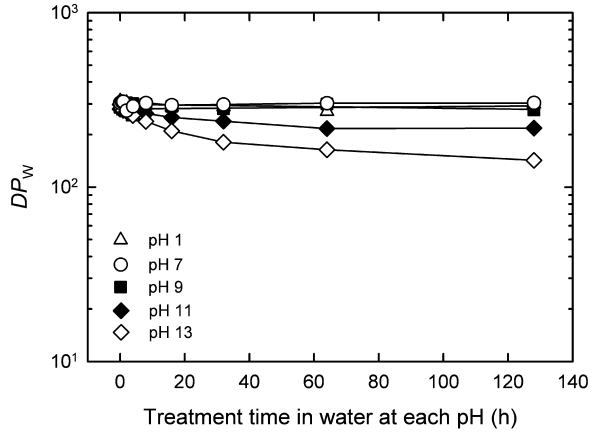

Fig. 1. Weight-average degree of polymerization (DP_w) of the products obtained from (1 → 3)-β-polyglucuronic acid by treatment in water at room temperature for 0–128 h under various pH conditions.

stable, and almost no depolymerization took place at room temperature under these pH conditions. When (1,3)-β-PGluA was treated at pHs 9–13, the peak-top positions of the SEC elution patterns clearly shifted in the higher elution volume direction or lower DP direction as the treatment time increased; slight random depolymerization of (1,3)-β-PGluA molecules along the chains occurred under these conditions.

The SEC patterns of the products obtained by the treatment of (1,3)-β-PGluA at pH 13 (Fig. 2, pH 13) were somewhat different from the others. The peak at an elution volume of 10.9 mL, roughly corresponding to monomers, increased with increasing treatment time. This elution volume was slightly lower than that of glucuronic acid. These results indicate that (1,3)-β-PGluA is depolymerized from the C1-carboxyl ends, forming monomers, by reactions similar to the peeling-off reaction of cellulose under alkaline conditions, together with slight random depolymerization in water at pH 13. The details are discussed in the following section.

3.2. Stability of (1,3)-β-PGluA at high temperatures under various pH conditions

(1,3)-β-PGluA was treated in water at pHs 1–13 and high temperatures (50 and 105 °C) for 0–128 h to investigate the stability of (1,3)-β-PGluA under various pH and high temperature conditions. When (1,3)-β-PGluA was treated in water at pHs 1, 7, 9, and 13 and 50 °C for 128 h, the DP_w values decreased from 250 to 120, 190, 130, and 140, respectively (data not shown). Depolymerization of the (1,3)-β-PGluA molecules therefore clearly took place under conditions at 50 °C for 128 h; the temperature effect on depolymerization of (1,3)-β-PGluA was decisive.

Fig. 3 shows the DP_w values of the products obtained from (1,3)-β-PGluA by treatment in water at a constant temperature of 105 °C and various pH values of 1–13. When (1,3)-β-PGluA was treated in water at pH 1, the DP_w sharply decreased to less than 20 within 20 h and to 12 after treatment for 128 h. The DP_w of (1,3)-β-PGluA decreased sharply from 250 to 120–190 at pHs 7, 9, and 13 within 0.5 h, in a similar manner to that at pH 1. However, the changing patterns of DP_w after treatment for 0.5 h were explicitly different from that at pH 1. The DP_w values of the products treated at pHs 9 and 13 were mostly unchanged, at 110–120, even after extended treatment times from 0.5 to 128 h; the treated products had quite stable DP_w values under these conditions. When (1,3)-β-PGluA was treated in water at 105 °C and pH 7 for 128 h, the DP_w decreased to 75. However, the decreased in the DP_w value was not as drastic as

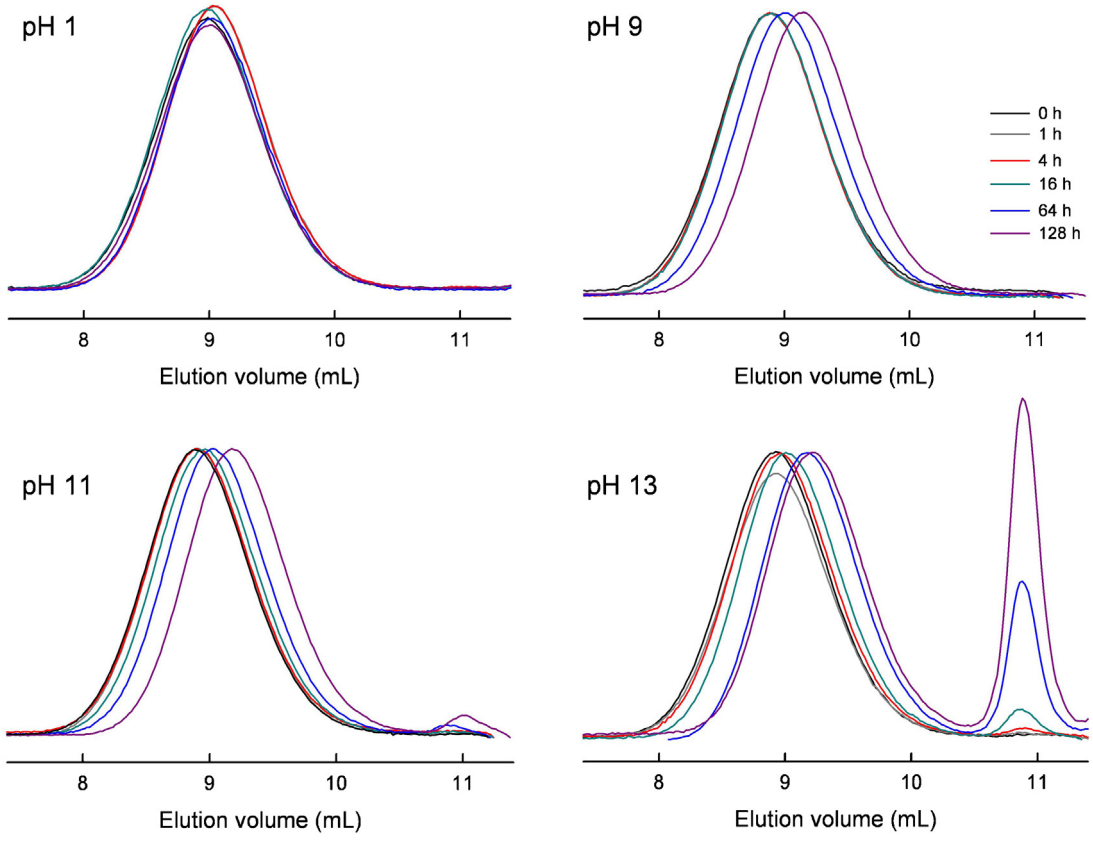

Fig. 2. SEC elution patterns of products obtained from (1 → 3)-β-polyglucuronic acid by treatment in water at room temperature for 0–128 h under various pH conditions. SB-806MHQ was used as the SEC column.

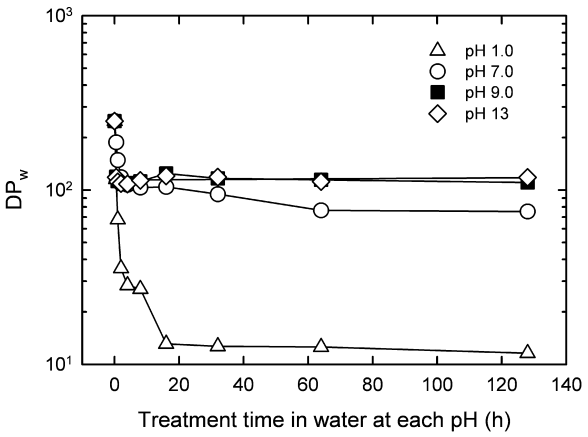

Fig. 3. Weight-average degree of polymerization (DP_w) of the products obtained from (1 → 3)-β-polyglucuronic acid by treatment in water at 105 °C for 0–128 h under various pH conditions.

that at pH 1. These results indicate that the depolymerization mechanisms of (1,3)-β-PGluA at pH 1 and other pH values are clearly different.

Fig. 4 shows the changes in the SEC elution patterns of the products obtained from (1,3)-β-PGluA by treatment in water at pHs 1, 7, 9, and 13 and 105 °C for 0–128 h. An SB-806MHQ column was used in these studies to obtain SEC elution patterns with sufficiently separated high-molecular-weight fractions. When (1,3)-β-PGluA was treated at pH 1, the peak-top position of the SEC elution pattern gradually shifted to lower DP with increasing treatment time, mostly maintaining the normal distribution pattern. The peak position at an elution volume of 10.9 mL was consistent with that of an authentic glucuronic acid sample. These results revealed that depolymerization of (1,3)-β-PGluA occurred randomly along each molecular chain via acid hydrolysis to form glucuronic acid, the main monomer component (Fig. 4, pH 1).

The treatment at pH 7 resulted in shifts of the peak-top position of the high-molecular-weight fraction to lower DP with increasing treatment time, and correspondingly two overlapping peaks appeared at elution volumes of 10.7 and 10.9 mL, probably from dicarboxylic and monocarboxylic acid monomers, respectively (Fig. 4, pH 9). However, because the SB-806MHQ column for separation of high-molecular-weight fractions was used in these experiments, these peaks located at a high elution volume (or low-molecular-weight fractions) may have overlapped with those of the phosphate salts used as buffers during treatment in water at 105 °C and pH 7. The details are discussed later. When (1,3)-β-PGluA was treated in water at pHs 9 and 13, the shifting of the peak-top position of the high-molecular-weight fractions to lower DPs stopped in the early stages of the treatment (<0.5 h), and the peak at an elution volume of 10.7 mL (probably from dicarboxylic acid monomers) increased with increasing treatment time (Fig. 4, pHs 11 and 13).

In order to investigate the detailed monomer fractions formed from (1,3)-β-PGluA by treatment in water at 105 °C and pHs 1–13, the SB-802.5HQ SEC column for separation of low-molecular-weight fractions was used in the SEC-MALLS analysis of the treated products (Fig. 5). When (1,3)-β-PGluA was treated in water at pH 1, the peak at an elution volume of 9.2 mL was consistent with that of an authentic glucuronic acid sample. In the SEC elution patterns for the products treated for 4, 16, and 64 h, not only monomers but also dimers and trimers were probably present, at elution volumes of 8.5 and 8 mL, respectively. The depolymerization mechanism of (1,3)-β-PGluA in water at 105 °C and pH 1 was therefore again the result of acid hydrolysis.

In contrast, only a single peak appeared at an elution volume of at 8.6 mL, i.e., the monomer fraction of the products obtained from (1,3)-β-PGluA by treatment in water at pHs 9 and 13; this peak corresponded to dicarboxylic-acid-type monomers. This peak increased with increasing treatment time (Fig. 5, pHs 11 and 13). These results showed that only dicarboxylic-acid-type monomers were formed from (1,3)-β-PGluA by treatment in water at 105 °C under alkaline conditions such as pHs 9 and 13. Because the hydrophilic poly(hydroxymethacrylate) gel particles in the SEC column have anionic surface charges, dicarboxylic acid monomers have elution volumes slightly smaller than that those of monocarboxylic acid monomers as a result of electrostatic repulsion between the surface anionic polymer gels and anionic monomers in the aqueous eluent (Homma, Isogai, Saito, & Isogai, 2013; Kagawa & Tokunaga, 2009).

When (1,3)-β-PGluA was treated in water at pH 7, two peaks appeared at elution volumes of 8.6 and 9.2 mL, from dicarboxylic acid and glucuronic acid monomers, respectively, as low-molecular-weight fractions (Fig. 5, pH 7). This result indicates that two mechanisms occurring at pHs 1, 9–13, participated in the depolymerization of (1,3)-β-PGluA at pH 7.

3.3. Depolymerization mechanism of (1,3)-β-PGluA at 105 °C under various pH conditions

Each glucuronosyl unit in (1,3)-β-PGluA has no glycoside bond to the adjacent glucuronosyl unit at the β-position from the C6-carboxyl. It is therefore not plausible that (1,3)-β-PGluA molecules are randomly depolymerized via β-alkoxy elimination under alkaline conditions, which is one of the primary depolymerization mechanisms of cellouronic acid or (1 → 4)-β-polyglucuronate under alkaline conditions. However, it may be possible that some of the C4-hydroxyl groups of (1,3)-β-PGluA molecules are removed via β-elimination under alkaline conditions to form C4—C5 double-bonds in the products treated in water at pHs 9–13. The ultraviolet absorbance at 235 nm is an indicator of the presence of double bonds in the water-soluble products obtained from cellouronic acid treated with exo- and endo-type degradation enzymes (Konno, Habu, Iihashi, & Isogai, 2008; Konno, Habu, Maeda, Azuma, & Isogai, 2006). The absorbances at 235 nm of the high-molecular-weight products obtained from (1,3)-β-PGluA by treatment in water at pH 13 and 105 °C for 1–128 h were therefore measured using a photodiode array detector attached to the SEC-MALLS system. The results showed that no absorbance at 235 nm was observed for the high-molecular-weight fractions at the peak-top of the 9.5-mL elution volume (Fig. 4, pH 13). No β-elimination to form C4—C5 double-bonds therefore took place in the high-molecular-weight fractions of the products during the alkaline treatment.

^{13}C NMR spectra of the original (1,3)-β-PGluA and the products obtained by treatment in water at room temperature and 105 °C and pHs 1, 7, and 13 are shown in Fig. 6. When (1,3)-β-PGluA was treated in water at room temperature and pHs 1–7, the NMR spectrum of the original (1,3)-β-PGluA was mostly unchanged even after the treatment for 128 h; this polyuronic acid is stable to these treatments. However, the products obtained at room temperature and pH 13 had some new signals due to a methylene carbon at 38.4 ppm and C=O carbons at 180–190 ppm, which might arise from depolymerization products similar to α-keto-glucuronic acid (Konno et al., 2006, 2008).

When (1,3)-β-PGluA was treated in water at 105 °C and pH 1, the C1, C3 and C4 signals mostly disappeared, and two anomeric carbon signals were clearly detected in the ^{13}C NMR spectrum, indicating the formation of glucuronic acid monomer by acid hydrolysis. However, because a methylene carbon at 38 ppm was present, and no clear C6-carboxyl signal was detected, some side reactions may also have taken place during the treatment.

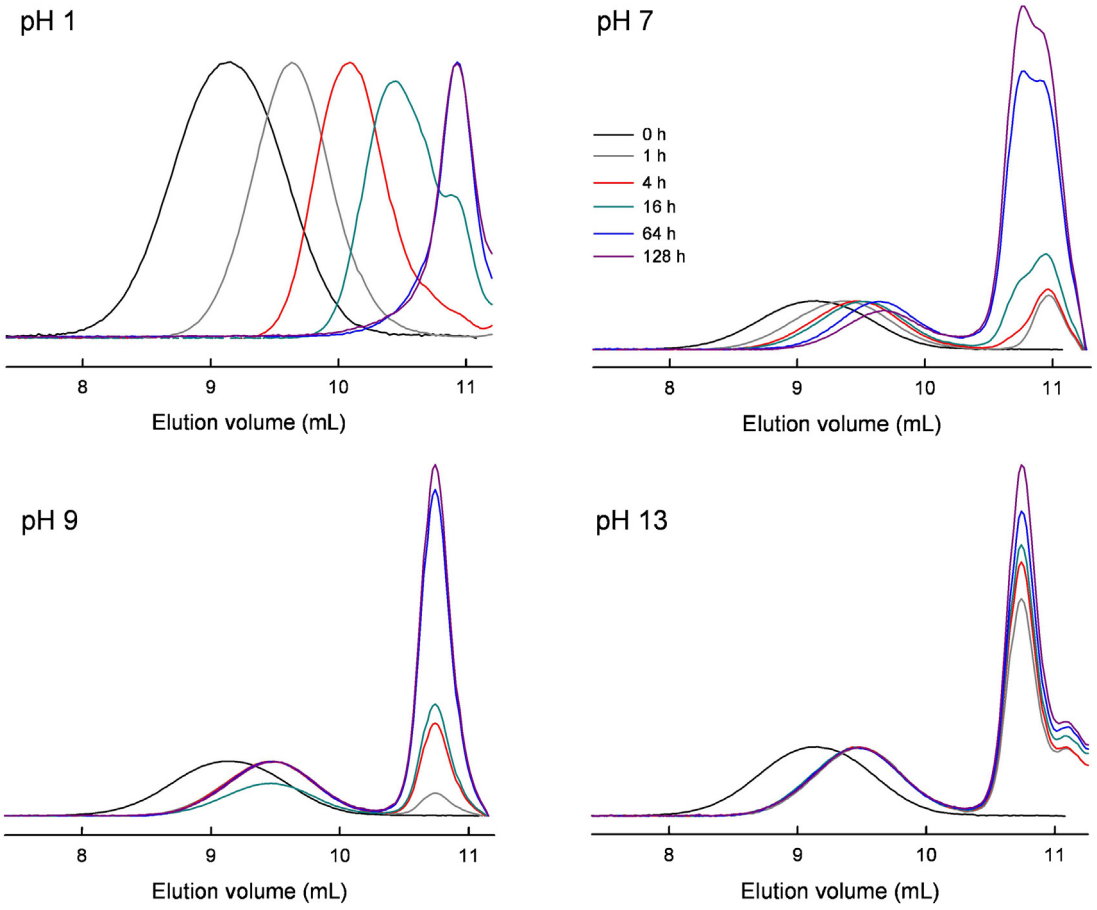

Fig. 4. SEC elution patterns of products obtained from (1 → 3)-β-polyglucuronic acid by treatment in water at 105 °C for 0–128 h under various pH conditions. SB-806MHQ was used as the SEC column.

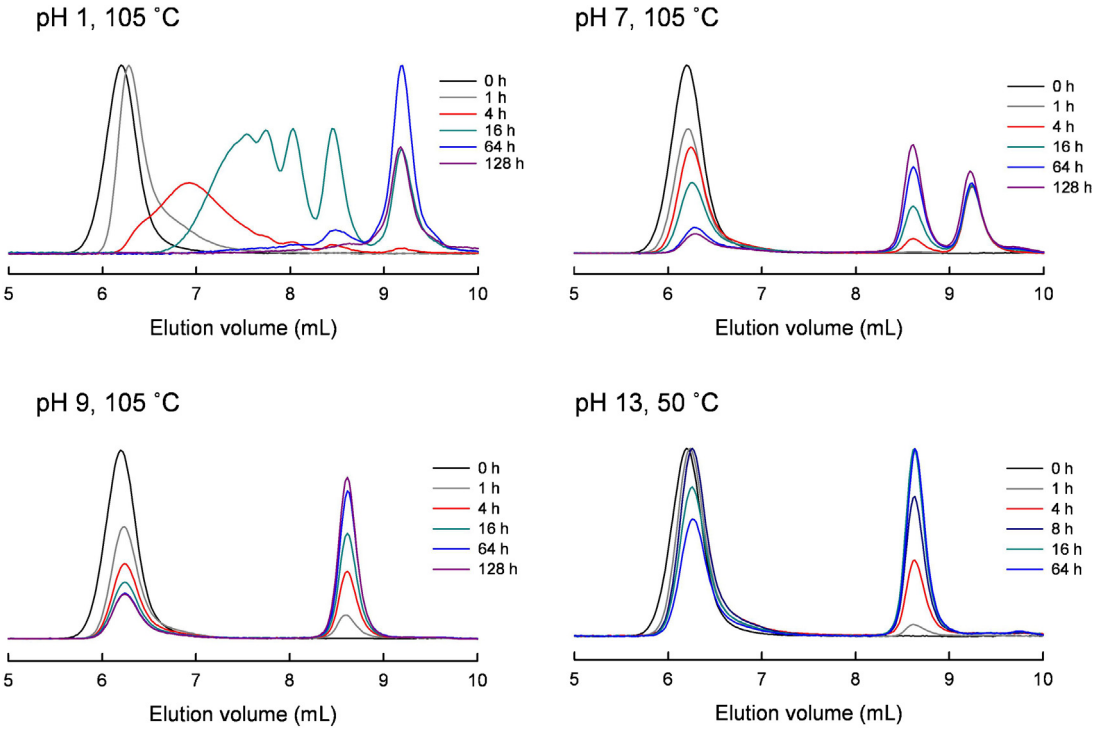

Fig. 5. SEC elution patterns of products obtained from (1 → 3)-β-polyglucuronic acid by treatment in water at 50 or 105 °C for 0–128 h under various pH conditions. SB-802.5HQ was used as the SEC column.

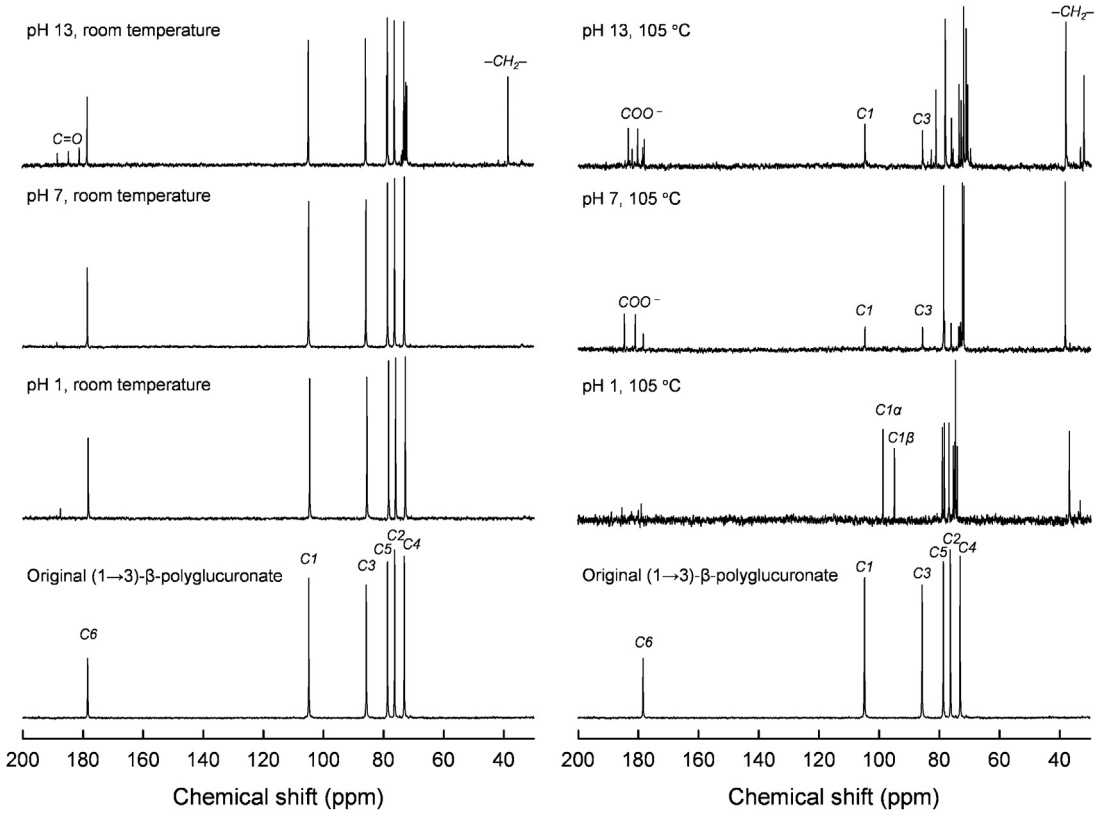

Fig. 6. ^{13}C NMR spectra of the original (1 → 3)-β-polyglucuronic acid and products obtained from (1 → 3)-β-polyglucuronic acid by treatment in water at room temperature (left) or 105 °C (right) under various pH conditions for 128 h. D_2O was used as the solvent.

The product obtained by the treatment of (1,3)-β-PGluA in water at 105 °C and pH 13 had two methylene carbon signals at 32 and 38 ppm and multiple carboxyl carbons at 178–184 ppm. This result and the SEC-MALLS data in Figs. 4 and 5 (pH 13) indicate that some dicarboxylic-acid-type monomers with two methylene carbons in the molecules were formed from the C1-carboxyl ends of (1,3)-β-PGluA molecules at 105 °C under alkaline conditions. All the carbon signals ascribed to the original (1,3)-β-PGluA were also present in this spectrum, showing that the (1,3)-β-PGluA-type structure remained in the product to some extent. The product obtained at pH 7 had a ^{13}C NMR spectrum rather similar to that obtained at pH 13; the depolymerization mechanism of (1,3)-β-PGluA at pH 7 is similar to that at pH 13.

Because the second adjacent glucuronosyl unit has a glycoside bond at the C3 or β-position from the C1-carboxyl ends of (1,3)-β-PGluA molecules prepared from curdlan by TEMPO-mediated oxidation, it is possible that β-alkoxy elimination takes place from the C1-carboxyl ends under alkaline conditions, forming dicarboxylic-acid-type monomer(s) ***A*** containing ethylene carbon(s) (Fig. 7). Once new reducing ends with C1-aldehyde groups are formed from (1,3)-β-PGluA molecules via the above reaction under alkaline conditions, removal of the newly formed reducing end monomer probably proceeds more easily under alkaline conditions to form dicarboxylic-acid-type monomer(s) ***B*** containing ethylene carbon(s) shown in Fig. 7. These removal reactions of monomers from the C1-carboxyl ends and C1-aldehyde reducing ends of (1,3)-β-PGluA molecules are similar to the peeling-off reaction of cellulose, which occurs from its reducing ends under alkaline conditions. Because significant depolymerization of (1,3)-β-PGluA at 105 °C and pHs 9–13 took place within 0.5 h, and thereafter the DP_w values were almost unchanged (Fig. 3), some depolymerization-terminating reactions may take place at the reducing ends. However, the detailed mechanism of termination of the removal reactions (Fig. 3) is unknown at present. The termination reaction of the peeling-off reaction of cellulose under alkaline conditions, forming *meta*-saccharic acid ends (Knill & Kennedy, 2003; Machell & Richards, 1957, 1960), is not applicable in the case of (1,3)-β-PGluA, because there is a structural difference between the end groups of these two polymers.

The depolymerization mechanisms of (1,3)-β-PGluA are therefore clearly different at pH 1 and pH 13; the mechanisms are random acid hydrolysis to form glucuronic acid, and removal of monomers from the C1-carboxyl and C1-aldehyde reducing ends of the (1,3)-β-PGluA molecules to form dicarboxylic-acid-type monomers containing ethylene carbons, with some termination reactions, respectively. However, detailed structures of the dicarboxylic-acid-type monomers containing ethylene carbons are unknown at present.

3.4. Depolymerization kinetics of (1,3)-β-PGluA

The depolymerization rate constants *k* of (1,3)-β-PGluA in water under various pH and temperature conditions are listed in Table 1;

Table 1
Depolymerization rate constant ($\times 10^5$ min^{-1}) of (1 → 3)-β-polyglucuronic under various pH and temperature conditions.

	Temperature (°C)							
	25	40	50	60	70	80	90	105
pH 1	0.09	0.92	1.14	1.36	1.76	3.16	6.64	20.0
pH 4	0.44	0.62	0.07	0.71	0.86	1.43	2.75	9.54
pH 7	0.43	0.44	0.80	0.80	1.04	1.90	4.68	12.1
pH 9	0.00	1.61	2.94	2.81	6.22	10.6	17.6	18.8
pH 11	0.33	1.27	3.30	10.4	13.5	15.0	15.4	16.2
pH 13	0.84	3.15	8.80	16.0	16.3	17.0	17.9	18.1

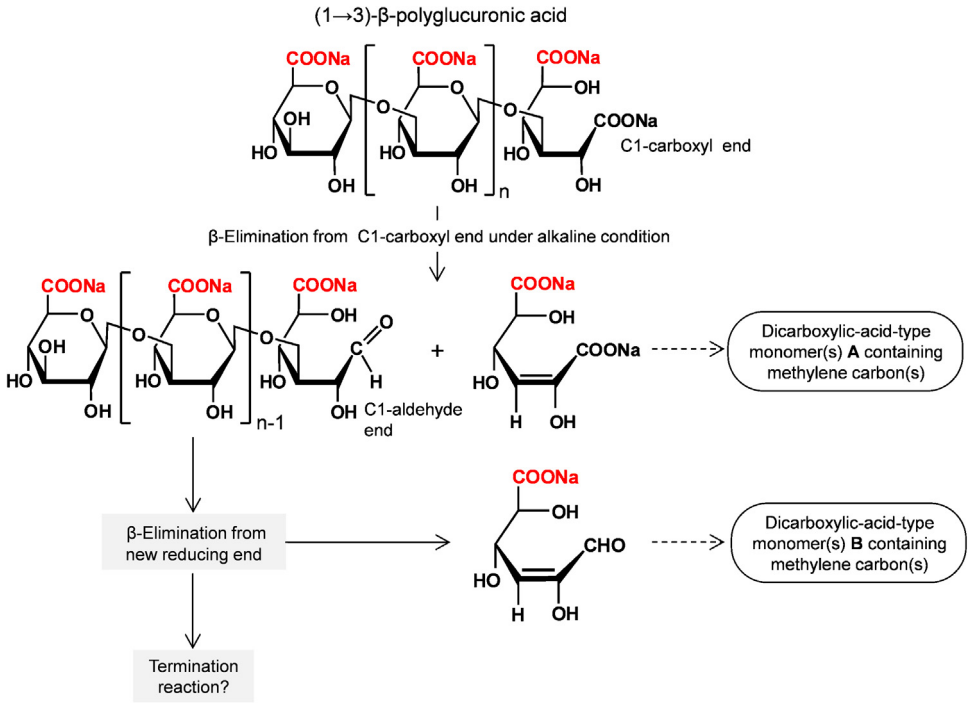

Fig. 7. Possible mechanisms to form dicarboxylic-acid-type monomers containing methylene carbons from C1-carboxyl end of (1 → 3)-β-polyglucuronic chain via β-elimination under alkaline conditions.

these were obtained from the initial plots for the treatments at 0–0.5 h, according to the following equation (Sharples, 1971),

$$\ln\left(1-\frac{1}{\mathrm{DP}_{n0}}\right)-\ln\left(1-\frac{1}{\mathrm{DP}n_t}\right)=kt \quad (1)$$

where k is the rate constant for bond cleavage, and DP_{n0} and DP_{nt} are the original DP_n (DP_n is the number-average DP) and the DP_n after treatment time t, respectively. The data in Table 1 show that the higher the temperature, the greater the depolymerization rate constant. However, because the depolymerization mechanisms under acid and alkaline conditions are different, the k values cannot be compared directly in terms of the treatment pH values. Across the whole temperature range, the k values were low at pH 4, probably because the effects of both acid hydrolysis and alkaline depolymerization were lowest at this pH. The data in Table 1 were obtained in the initial stage, and prolonged treatment times brought about different results, especially at 105 °C, as shown in Fig. 3.

The k values obtained for (1,3)-β-PGluA were compared with those for (1 → 4)-β-polyglucuronic acid or cellouronic acid (Fujisawa et al., 2010). For example, the k value for (1,3)-β-PGluA at 40 °C and pH 13 ($3.15 \times 10^{-5}\ \mathrm{min}^{-1}$) was two orders of magnitude lower than that of cellouronic acid ($295 \times 10^{-5}\ \mathrm{min}^{-1}$). This is because cellouronic acid is randomly depolymerized at the (1 → 4)-β-glycoside bonds along the chains by β-alkoxy elimination under alkaline conditions, whereas this random β-alkoxy elimination cannot take place on (1,3)-β-PGluA molecules; the glycoside bond of (1,3)-β-PGluA is not located at the β-position from the C6-carboxyl group. As described in the previous section, depolymerization of (1,3)-β-PGluA primarily occurs from the C1-carboxyl ends under alkaline conditions, the same as the peeling-off reaction of cellulose. Moreover, the k value of (1,3)-β-PGluA at 105 °C and pH 1 ($20 \times 10^{-5}\ \mathrm{min}^{-1}$) was one order of magnitude lower than that of cellouronic acid ($481 \times 10^{-5}\ \mathrm{min}^{-1}$). Thus (1,3)-β-PGluA is more stable to depolymerization than cellouronic acid under both acidic and alkaline conditions.

The activation energies of depolymerization of (1,3)-β-PGluA, which were calculated from the linear slopes of $\ln k$ versus $1/T$, primarily by the hydrolysis mechanism, were 76, 75, and 77 kJ mol^{-1} at 70–105 °C and pHs 1, 4, and 7, respectively. These values were quite similar to those for curdlan (70 kJ mol^{-1}; Prieto, Vázquez, & Murado, 2011) in acid hydrolysis at high temperatures; probably the same (1 → 3)-β-glycoside bond in curdlan and in (1,3)-β-PGluA primarily affects the activation energies of depolymerization as a result of acid hydrolysis. In contrast, the activation energies of the peeling-off-type depolymerization of (1,3)-β-PGluA at 70–105 °C and pHs 11 and 13 were quite low, 5.3 and 3.2 kJ mol^{-1}, respectively, indicating that depolymerization from the C1-carboxyl ends proceeds more easily in the initial stage.

4. Conclusions

When (1,3)-β-PGluA was treated in water at pHs 1–9 and room temperature for up to 128 h, almost no depolymerization occurred, indicating that this polyuronic acid is stable under these conditions. In contrast (1,3)-β-PGluA was depolymerized from a DP_w of 250 to 220 and 140 after treatment in water at room temperature at pHs 11 and 13, respectively, for 128 h. When (1,3)-β-PGluA was treated in water at 105 °C and pHs 9–13, the original DP_w (250) sharply decreased to ≈120 by removal of monomers from the C1-carboxyl ends of (1,3)-β-PGluA molecules as a result of β-elimination. Keto-dicarboxylic-acid-type monomers with methylene carbons were eventually formed by the above reactions. In contrast (1,3)-β-PGluA was hydrolyzed randomly along the chains in water at 105 °C and pH 1, and the original DP_w value (250) was drastically reduced to 13 and 12 after treatment for 16 and 128 h, respectively, primarily forming the glucuronic acid monomer. The depolymerization rate constant of (1,3)-β-PGluA at each temperature was clearly lower than that of cellouronic acid, showing that (1,3)-β-PGluA is more stable to these treatments than cellouronic acid is.

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