Carbohydrate Polymers 100 (2014) 74–79

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Preparation of completely C6-carboxylated curdlan by catalytic oxidation with 4-acetamido-TEMPO

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ARTICLE INFO

Article history:
Received 17 July 2012
Received in revised form 20 October 2012
Accepted 21 November 2012
Available online 7 December 2012

Keywords:
4-Acetamido-TEMPO
Curdlan
(1→3)-β-Polyglucuronic acid
Oxidation
Molecular weight

ABSTRACT

Pure (1→3)-β-polyglucuronic acid sodium salt was prepared from curdlan by oxidation with 4-acetamido-TEMPO/NaClO/$NaClO_2$ in water at pH 4.7 and 35 °C. The oxidation conditions, including the reaction time and amounts of reagents added, were optimized for the preparation of (1→3)-β-polyglucuronic acids with high molecular weights. The primary C6 hydroxyl groups of curdlan were completely oxidized to the corresponding C6-carboxylates using a one- or two-step reaction process by controlling the oxidation conditions, thus providing pure (1→3)-β-polyglucuronic acids consisting only of D-glucuronosyl units. Unfortunately, however, the increased amounts of reagents and long reaction time led to significant depolymerization of the curdlan during the oxidation process, and the resulting (1→3)-β-polyglucuronic acids had weight-average degrees of polymerization of 340–360. The ^{13}C and ^{1}H NMR chemical shifts of the products were successfully assigned using pure (1→3)-β-polyglucuronic acid.

1. Introduction

The regioselective oxidation of the C6 primary hydroxyl groups of polysaccharides using 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO) or its analogue as a catalyst under aqueous conditions has received considerable interest because of the high reaction rates and levels of regioselectivity achieved by the reaction, together with the aqueous reaction conditions (Bragd, Besemer, & van Bekkum, 2001; de Nooy, Besemer, & van Bekkum, 1995). This TEMPO-mediated oxidation process has created novel opportunities in polymer science for research focused on functional polysaccharides. Various water-insoluble polysaccharides, including starch, chitin and regenerated cellulose become water-soluble following the application of the TEMPO-mediated oxidation process under the appropriate reaction conditions (Isogai & Kato, 1998; Kato, Matsuo, & Isogai, 2003; Kato, Kaminaga, Matsuo, & Isogai, 2004; Muzzarelli, Muzzarelli, Cosani, & Terbojevich, 1999).

Curdlan, a linear (1→3)-β-glucan, is a water-insoluble extracellular bacterial polysaccharide. Curdlan is composed of repeating glucose units in a similar way to the biomass resources cellulose and amylose, which are (1→4)-β- and (1→4)-α-glucans, respectively (Harada, Misaki, & Saito, 1968; McIntosh, Stone, & Stanisich, 2005). Curdlan has a triple-helical structure that gives rise to specific functionalities because of its unique glycoside bonds. Curdlan has consequently been the subject of multiple research projects that have focused specifically on its unique structural and functional properties (Chuah, Sarko, Deslandes, & Marchessault, 1983; Deslandes, Marchessault, & Sarko, 1980). Furthermore, curdlan has been reported to exhibit a number of different biological activities, including immunomodulation and antitumor efficacy, and its derivatives have been applied as anticoagulants, antithrombotics and anti-HIV polymers (Bohn & BeMiller, 1995; Ooi & Liu, 2000; Kataoka, Muta, Yamazaki, & Takeshige, 2002; Toida, Chaidedgumjorn, & Linhardt, 2003).

When the TEMPO/NaBr/NaClO oxidation conditions were applied to curdlan at pH 10 (Tamura, Wada, & Isogai, 2009), water-soluble oxidized products were obtained quantitatively within 100 min containing (1→3)-β-glucuronosyl units. The C6 primary hydroxyl groups of curdlan were predominantly converted to the corresponding C6-carboxylate groups by the oxidation process. Unfortunately, however, significant levels of depolymerization occurred on the curdlan molecules during the oxidation process. Tamura, Hirota, Saito, and Isogai (2010) reported that the regioselective oxidation of C6-OH groups of curdlan to carboxylate groups could be achieved with lower levels of depolymerization when a 4-acetamido-TEMPO/NaClO/$NaClO_2$ system was used in water at pH 4.7 (Fig. 1). The oxidation ratios of C6-OH groups in the resulting products, however, were less than 95%.

Water-soluble curdlan derivatives with triple-helical structures have been applied to the dispersion of carbon nanotubes and metal nanoparticles in water (Le et al., 2011; Li, Zhang, Xu, & Zhang, 2011).

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http://dx.doi.org/10.1016/j.carbpol.2012.11.094

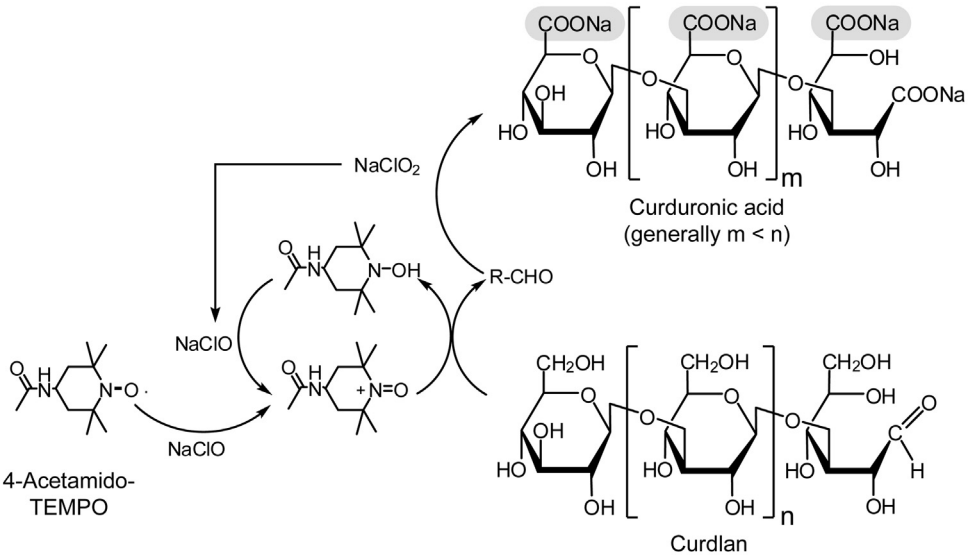

Fig. 1. Oxidation of C6 primary hydroxyls of curdlan by 4-acetamido-TEMPO/NaClO/$NaClO_2$ system at pH 4.7.

Although oxidized curdlans with almost entirely homogeneous structures have been obtained to date, to the best of our knowledge, oxidized curdlans containing carboxylate groups exclusively at the C6-positions have never been obtained. The development of techniques for the fabrication of chemically homogenous structures in water-soluble polysaccharides is important for their accurate characterization and future applications.

Herein, we describe the application of a 4-acetamido-TEMPO/NaClO/$NaClO_2$ oxidation system to curdlan under a variety of different conditions to obtain oxidized curdlans containing carboxylate groups exclusively at the C6-positions by controlling reaction time and amounts of reagents added. The degrees of polymerization (DP) of the resulting oxidized curdlans were studied in terms of the C6-oxidation ratios and oxidation conditions.

2. Materials and methods

2.1. Materials

Commercially sourced curdlan (DP_w 6790; Wako Pure Chemicals Co., Japan) was used as the starting material. 4-Acetamido-TEMPO, sodium chloride, sodium chlorite (>80%), 12% sodium hypochlorite solution, and other reagents and solvents were of laboratory grade and used as received. HPLC grade distilled water was purchased from Wako Pure Chemicals.

2.2. TEMPO-mediated oxidation of curdlan

Curdlan (1.0 g) was placed in an Erlenmeyer flask (200 mL) equipped with a magnetic stirrer bar and a 0.2 M acetate buffer at pH 4.7 (100 mL) containing $NaClO_2$ (0.34–1.36 g) and 4-acetamido-TEMPO (0.048–0.192 g) was added to the flask. A 12% NaClO solution (0.31–1.24 mL) was then added to the curdlan suspension in a single portion, and the flask was immediately capped with a universal stopper. The mixture was then stirred at 35 °C for 1–3 days. The oxidation was quenched by the addition of an excess of ethanol (100 mL), and the resulting precipitate was collected by centrifugation. Alternatively, the amount of curdlan was reduced from 1.0 to 0.5 g, and the oxidation was carried out under the same conditions as described above to study the effect of curdlan concentration in the aqueous reaction medium on the oxidation efficiency. The oxidized product was then dialyzed against de-ionized water for 3 days and subsequently isolated by freeze-drying.

2.3. SEC-MALLS analysis

The water-soluble TEMPO-oxidized curdlans were dissolved in a 0.1 M NaCl solution at concentration of 0.1%. The resulting solutions were subjected to size-exclusion chromatography equipped with a multi-angle laser-light scattering detector (SEC-MALLS, DAWN EOS, λ 690 nm; Wyatt Technologies, USA) using a SEC column for aqueous systems (DB-806MHQ, 8 mm $\varphi \times 30$ cm, Shodex, Japan) and 0.1 M NaCl solution as the eluent. The solvents were filtered using 0.2-μm polytetrafluoroethylene membranes (Millipore, USA) prior to injection. The weight- and number-average molecular mass values (M_w and M_n, respectively) of the oxidized curdlans were calculated from the SEC-MALLS data using the ASTRA software (Wyatt Technologies) with a specific refractive index increment (d_n/d_c) value of 0.158 mL g^{-1} (Tamura et al., 2010). A pullulan standard (M_w 22,800; Shodex, Japan) was used to normalize the MALLS photo-detectors (ASTRA for Windows user's guide version 4.90). Details of the SEC-MALLS system used and operation conditions have been described elsewhere in the literature (Isogai, Yanagisawa, & Isogai, 2009; Tamura et al., 2010). The weight- and number-average degrees of polymerization (DP_w and DP_n, respectively) of the oxidized curdlans were calculated from their M_w and M_n values, respectively, using the corresponding C6-oxidation ratios.

2.4. Other analyses

The carboxyl contents of the oxidized curdlans were determined by electric conductivity titration using a 0.05 M NaOH solution (Tamura et al., 2010). The ^{13}C and ^{1}H NMR spectra of the water-soluble oxidized curdlans dissolved were recorded in D_2O on an ALPHA-500 (JEOL, Japan) using 3-trimethylsilyl-2,2,3,3-d_4-propionic acid sodium salt (Aldrich, USA) as an internal standard. The data accumulation times for the ^{13}C NMR spectra were about 25,000. The C—H and H—H correlation spectra were recorded on the same NMR apparatus using the hetero-nuclear single quantum coherence (HSQC) method and H-H correlation spectroscopy (COSY).

Table 1
Reaction conditions for the TEMPO-mediated oxidation of curdlan together with the corresponding *DP* values[a], carboxylate contents and C6-oxidation ratios of the oxidized products. The bold font indicates conditions providing high C6-oxidation ratios.

Sample no.	Curdlan (g)	4-Acetamido-TEMPO (g)	NaClO (mL)	$NaClO_2$ (g)	Reaction time (day)	DP_w content (mmol g^{-1})	DP_n ratio (%)	DP_w/DP_n	Carboxylate	C6-oxidation
1	1	0.096	0.62	0.68	1	612	388	1.58	3.85	72
2	1	0.096	0.62	0.68	2	521	344	1.51	3.98	75
3	1	0.096	0.62	0.68	3	535	351	1.53	3.98	75
4	1	0.192	0.62	0.68	1	515	316	1.63	4.14	79
5	1	0.096	1.24	0.68	1	333	215	1.55	4.45	86
6	1	0.096	0.62	1.36	1	529	340	1.56	4.44	81
7	1	0.192	1.24	0.68	1	386	248	1.56	4.13	79
8	**1**	**0.192**	**0.62**	**1.36**	**1**	**628**	**405**	**1.55**	**4.95**	**98**
9	1	0.096	1.24	1.36	1	647	424	1.53	4.64	90
10	1	0.192	1.24	1.36	1	525	327	1.61	4.78	94
11	**1**	**0.192**	**1.24**	**1.36**	**2**	**337**	**202**	**1.67**	**5.05**	**100**
12	0.5	0.048	0.31	0.34	2	667	418	1.62	4.68	91
13[b]	**1**	**0.096**	**0.62**	**0.68**	**1**					
		0.096	**0.62**	**0.68**	**1**	**361**	**218**	**1.60**	**5.05**	**100**

[a] DP_w and DP_n values were calculated from the corresponding M_w and M_n values, respectively, using the corresponding C6-oxidation ratios.
[b] Sample 13: The isolated, purified and dried Sample 1 was re-oxidized using the same oxidation treatment as that of Sample 1.

3. Results and discussion

3.1. TEMPO-mediated oxidation of curdlan

In all of the cases investigated in the current study, the water-insoluble curdlan particles became water-soluble following the TEMPO-mediated oxidation of their C6-OH groups to the corresponding C6-carboxylates (Fig. 1). The reaction conditions for the TEMPO-mediated oxidation of curdlan together with the characteristics of the resulting water-soluble oxidized products are shown in Table 1. The yields of the oxidized curdlans were in the range of 84–87%. Yield losses occurred primarily as a consequence of the handling processes during isolation, purification and dialysis states. Sample 1 was prepared as a reference material via the TEMPO-mediated oxidation according to the previously reported method (Tamura et al., 2010).

When the total amounts of NaClO and $NaClO_2$ added were 7 mmol g^{-1} of curdlan (containing 6.1 mmol of C6-OH), the C6-oxidation ratios were found to be only 72–75% following an oxidation period of 1–3 days (Samples 1–3, Table 1). An extension to the oxidation time therefore represented an ineffective strategy for increasing the C6-oxidation ratio under these conditions. In the preparation conditions of Samples 4–6, the amount of one of the three reagents added (4-acetamido-TEMPO, NaClO or $NaClO_2$) was increased to be double that used in Sample 1 and an increase in the amount of NaClO was found to provide an effective increase in the C6-oxidation ratio (Sample 5, Table 1). In the preparation of Samples 7–9, the amounts of two of the three reagents added were doubled simultaneously to identify any additive effects. When the amounts of 4-acetamido-TEMPO and $NaClO_2$ were doubled (Sample 8, Table 1), the C6-oxidation ratio increased to 98%. Furthermore, when the amounts of all the three reagents were doubled, the C6-oxidation ratios clearly increased further (Samples 10 and 11, Table 1), reaching 100% when the oxidation was conducted over a 2-day period, which indicates the complete conversion of the C6-OH groups to the corresponding C6-carboxylates and the formation of pure (1→3)-β-polyglucuronic acid.

When the concentration of curdlan in the reaction mixture was reduced by 50% relative to that of Sample 2, the viscosity of the mixture decreased and the magnetic stirring of the reaction mixture became smoother. Consequently, the C6-oxidation ratio increased from 75% (Sample 2, Table 1) to 91% (Sample 12,

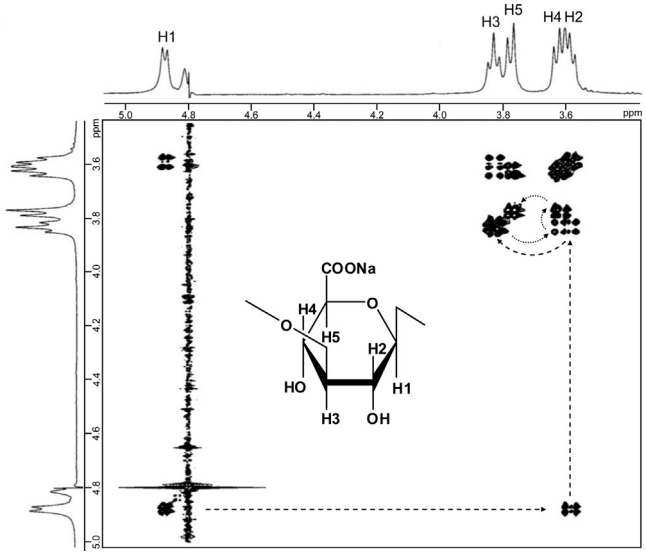

Fig. 2. COSY spectrum of (1→3)-β-polyglucuronic acid in in D_2O.

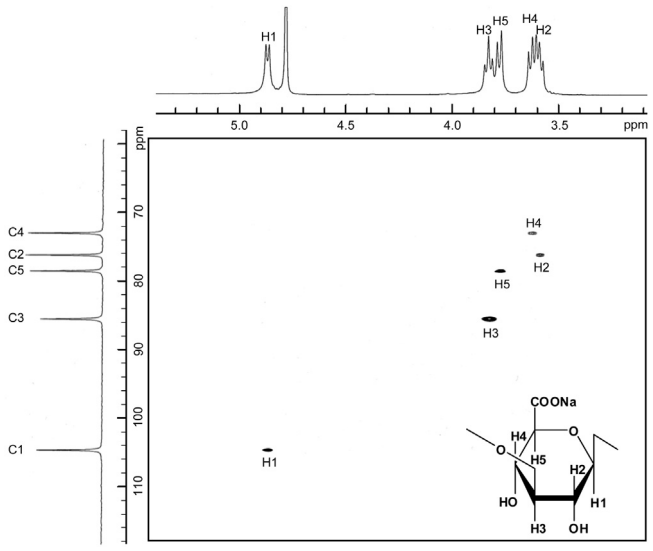

Fig. 3. HSQC spectrum of (1→3)-β-polyglucuronic acid in D_2O.

Table 1). The material oxidized during the preparation of Sample 1 (C6-oxidation ratio of 72%) was isolated, purified and dried and subsequently subjected to the same set of oxidation conditions to generate (1→3)-β-polyglucuronic acids with a C6-oxidation ratio of 100% (Sample 13, Table 1). The synthesis of (1→3)-β-polyglucuronic acids with C6-oxidation ratios of 100% could therefore be achieved in a one- or a two-step process. Repeating of the oxidation process and an extension in the reaction time were considered to be intrinsically the same in terms of the oxidation ratio. Based on the results in Table 1, it was concluded that some of the reagents were being consumed in side reactions such as depolymerization, because the DP_w of the original curdlan was as high as 6790 (Tamura et al., 2010). The relationships between the C6-oxidation ratios and the *DP* values of the oxidized curdlans will be discussed in greater detail in Section 3.3.

3.2. NMR analysis of (1→3)-β-polyglucuronic acid

The COSY spectrum of the pure (1→3)-β-polyglucuronic acid is shown in Fig. 2. The H1 and H5 protons appeared as doublets. The H2, H3 and H4 protons were sequentially and completely assigned from the COSY spectrum. Based on the analysis of spectrum in Fig. 2, the chemical shifts of all of the carbons of (1→3)-β-polyglucuronic acid were successfully assigned using the HSQC spectrum in Fig. 3. The ^{13}C and ^{1}H NMR chemicals shifts obtained in the current study for (1→3)-β-polyglucuronic acid in D_2O obtained and the ^{13}C NMR chemical shifts reported elsewhere for curdlan in DMSO-d_6 (Tamura et al., 2010) are listed in Table 2.

Based on the results from Figs. 2 and 3, the ^{13}C and ^{1}H NMR spectra of some of the oxidized curdlans with a variety of different C6-oxidation ratios are shown in Fig. 4. In the ^{13}C NMR

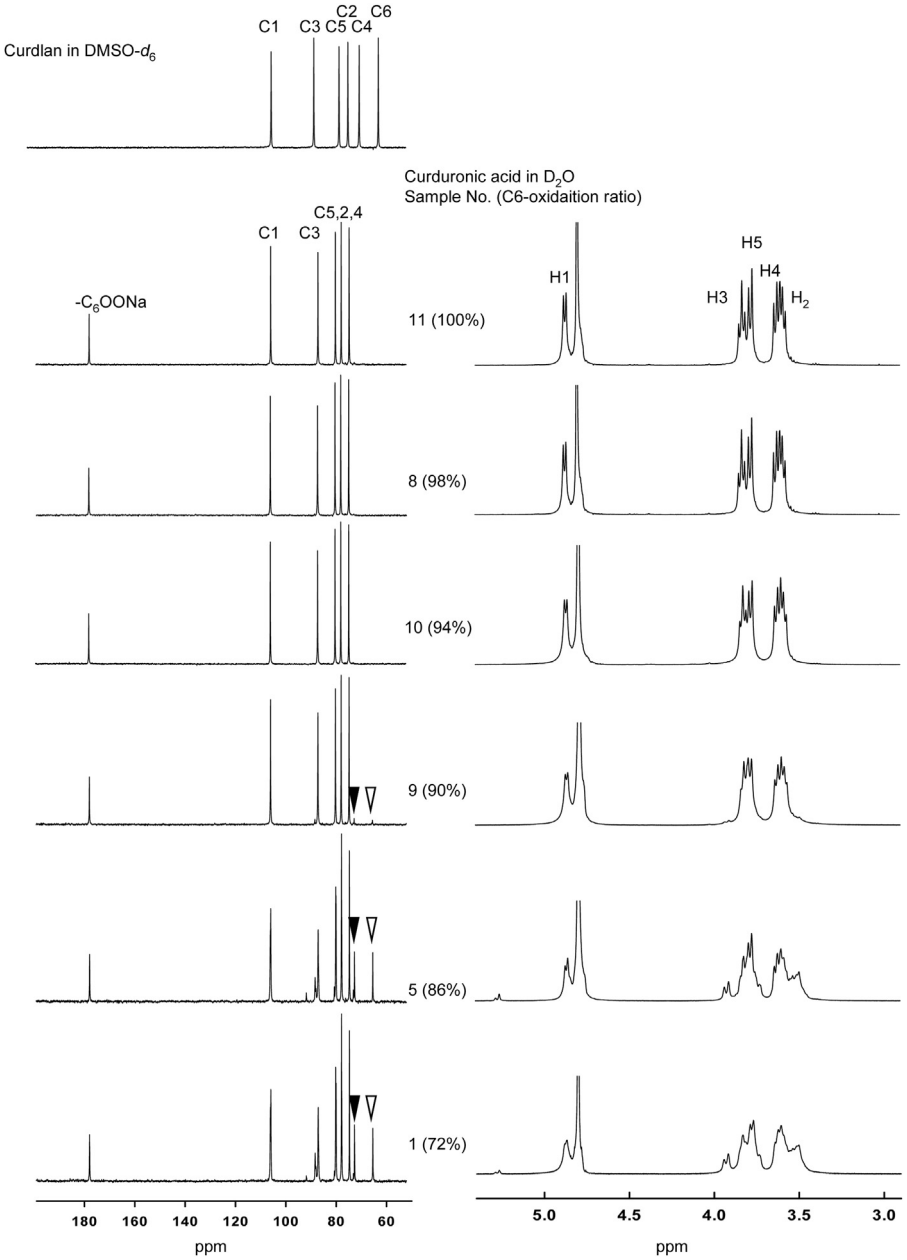

Fig. 4. ^{1}H and ^{13}C NMR spectra of oxidized curdlans prepared at various conditions. The C6-oxidation ratios (%) were determined by electric conductivity titration. The original and oxidized curdlans were dissolved in DMSO and D_2O, respectively. Filled and open arrows indicate the C4 and C6 carbons, respectively, of the unoxidized glucosyl units.

Table 2
^{13}C and ^{1}H NMR chemical shifts and ^{1}H vicinal coupling constants of (1→3)-β-polyglucuronic acid in D_2O and the ^{13}C NMR chemical shifts of curdlan in DMSO-d_6.

(1→3)-β-Polyglucuronic acid in D_2O						
^{13}C chemical shift (ppm)	C1	C2	C3	C4	C5	C6
	104.9	76.3	85.7	73.1	78.7	178.4
^{1}H chemical shift (ppm)	H1	H2	H3	H4	H5	
	4.88	3.59	3.83	3.62	3.78	
^{1}H coupling constant (Hz)	7.95	8.50	8.85	9.20	9.75	
Curdlan in DMSO-d_6[a]						
^{13}C chemical shift (ppm)	102.9	72.7	86.2	68.3	76.2	66.8

[a] Tamura et al. (2010).

spectra of Samples 1, 5 and 9, which had C6-oxidation ratios of 72, 86 and 90%, respectively, the C4 and C6 resonances of the unoxidized glucosyl units were detected at 73 and 64 ppm, respectively. Multiple and complicated C3 resonance signals were observed for the oxidized curdlans with lower C6-oxidation ratios. Although the C6-oxidation ratios of Samples 8 and 10 were in the range of 94–98%, no C4 or C6-OH resonance signals were seen in their ^{13}C NMR spectra. Furthermore, the ^{13}C NMR spectra of Samples 8 and 10 were almost identical to that of Sample 11, which had C6-oxidation ratio of 100%, indicating that ^{13}C NMR was not a sensitive enough technique to effectively detect the C4 or C6-OH resonance signals of the residual unoxidized glucosyl units. The electric conductivity titration method was therefore used for determining the carboxylate contents of the TEMPO-oxidized curdlans because it is a much more sensitive technique with a lower limit of detection.

The ^{1}H NMR spectra of the oxidized curdlans also showed similar results to those of the corresponding ^{13}C NMR spectra, although more quantitative information can be discerned from the ^{1}H NMR spectra. Only the well-split H1–H5 protons of (1→3)-β-polyglucuronic acid were detected for Samples 8 and 10, which had C6-oxidation ratios greater than 94%. In contrast, the ^{1}H NMR spectra of Samples 1, 5 and 9, which had C6-oxidation ratios of less than 90%, contained resonance signals from the unoxidized glucosyl units at approximately 3.5 and 3.9 ppm.

3.3. *Effect of the reaction conditions on the DP values of the oxidized curdlans*

The DP_w and DP_n values of the oxidized curdlans prepared under a variety of different conditions were measured by SEC-MALLS using the same d_n/d_c value, and are shown in Table 1. As described in the previous sections, pure (1→3)-β-polyglucuronic acid sodium salt was successfully prepared by controlling the oxidation conditions. Unfortunately, however, remarkable depolymerization was inevitable during the oxidation of curdlan even under the weakly acidic conditions at pH 4.7. Fig. 5 shows the SEC elution patterns and the corresponding molecular mass plots of Samples 6, 8 and 11, which had different C6-oxidation ratios and DP_w values (Table 1). It was envisaged that the depolymerization would occur randomly along the curdlan or oxidized curdlan chains during the oxidation, irrespective of the *DP* value, because all of the SEC-elution patterns had similar normal distribution patterns. It has been reported that HClO, $NaClO_2$ and several other oxidative species formed in situ from $NaClO_2$ can attack the glycosidic bonds of cellulose suspended in water and decrease its molecular weight (Duan & Kasper, 2010; Kantouch, Hebeish, & El-Rafie, 1970; Lewin, 1997; Shenai & Date, 1976). The low *DP* values presented in Table 1 for the oxidized curdlans were attributed to the oxidative cleavage of the glycosidic bonds.

The C6-carboxylate contents of Samples 6, 8 and 11 varied considerably and were 4.44, 4.95 and 5.05 mmol g^{-1}, respectively. Although the three oxidized curdlans had different anionic charge densities, the molecular mass plots were effectively the same and the difference in charge density within the above range exerted little influence on the degree of molecular expansion or molecular conformation in the oxidized curdlans in a 0.1 M NaCl solution.

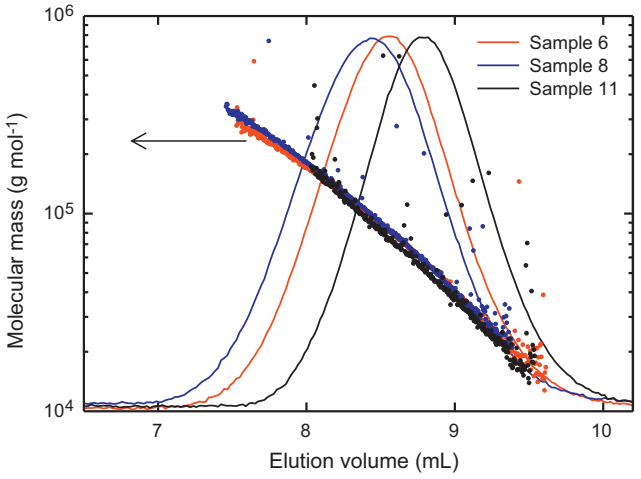

Fig. 5. SEC-MALLS elution patterns and the corresponding molecular mass plots of oxidized curdlans, Samples 6, 8 and 11.

The C6-oxidation ratios and the corresponding DP_w values of the oxidized curdlans from Table 1 were plotted against each another (Fig. 6). When the amount of NaClO added was doubled (Sample 5, Table 1), the C6-oxidation ratio increased from 72 to 86%, whereas the DP_w value clearly decreased from 612 to 333. For Samples 7, 8 and 9, the amounts of two of the three reagents

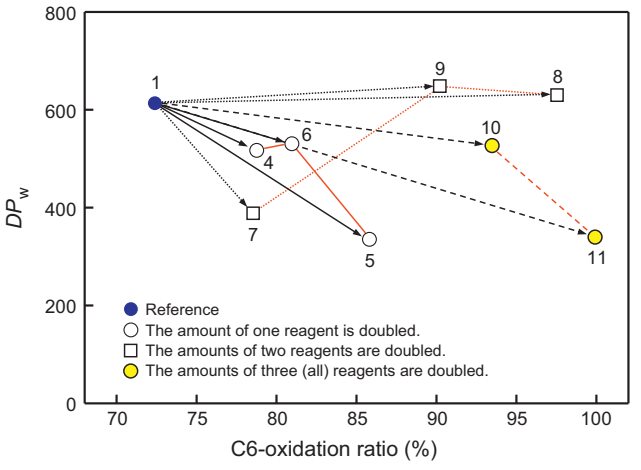

Fig. 6. Relationships between the C6-oxidation ratio and DP_w of oxidized curdlans. Each number corresponds to that of Sample in Table 1.

added were simultaneously doubled. In these cases, remarkable depolymerization only occurred when the C6-oxidation ratio was low (Sample 7, Table 1) or when the amount of $NaClO_2$ added was as low as 0.68 g. Furthermore, Sample 8 had the highest C6-oxidation ratio and DP_w value of the three samples at 98% and 628, respectively. When the amounts of all three reagents added were doubled, a C6-oxidation ratio of 100% was achieved following a reaction period of 2 days (Sample 11, Table 1). The DP_w value, however, decreased significantly to 340. Sample 13, which was prepared by repeating the reaction conditions for Sample 1, also gave a C6-oxidation ratio of 100%, but the DP_w value was found to be similar to that of Sample 11 at 360. Taken together, the results suggested that the increased amounts of reagents added were being consumed not only for increases in the C6-oxidation ratio but also for depolymerization during the oxidation. The results in Table 1 and Fig. 6 indicated that the best conditions for the preparation of 100% C6-oxidized (1→3)-β-polyglucuronic acids with a high DP_w value would be represented by the amounts of the reagents added for Sample 8 with an oxidation time of 1.5 days.

4. Conclusions

Pure polyglucuronic acids were successfully prepared from curdlan using a 4-acetamido-TEMPO/NaClO/$NaClO_2$ oxidation system in water at pH 4.7 and 35 °C by controlling the amounts of reagents added and the reaction time. Significant levels of depolymerization, however, occurred during the oxidation process and the polyglucuronic acids obtained had DP_w values of 340–360, whereas the value of the original curdlan was as high as 6790. The increased amounts of reagents were therefore not only being consumed to provide the increased C6-oxidation ratio but also in the observed depolymerization processes. The SEC-MALLS analysis showed that the depolymerization was taking place randomly along the curdlan or oxidized curdlan chains during the oxidation process. Based on the COSY and HSQC spectra, a complete assignment of ^{1}H and ^{13}C NMR chemical shifts of pure polyglucuronic acid was achieved. Electric conductivity titration was used for determining the carboxyl contents of the polyglucuronic acids because the NMR signals were not sensitive enough to detect small amounts of unoxidized glycosyl units in the oxidized curdlans.

Acknowledgment

This study was supported by the Japan Society for the Promotion of Science (JSPS) with a Grant-in-Aid for Scientific Research S (grant number 21228007).

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