Carbohydrate Polymers 81 (2010) 592–598

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Oxidation of curdlan and other polysaccharides by 4-acetamide-TEMPO/NaClO/NaClO$_2$ under acid conditions

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ARTICLE INFO

Article history:
Received 20 January 2010
Received in revised form 25 February 2010
Accepted 8 March 2010
Available online 15 March 2010

Keywords:
(1 → 3)-β-D-Glucan
Curdlan
TEMPO
4-Acetamide-TEMPO
Oxidation
SEC-MALLS

ABSTRACT

Regioselective oxidation of C6 primary hydroxyls to carboxylate groups was applied to curdlan to prepare water-soluble oxidized products with less depolymerization by 4-acetamide-TEMPO/NaClO/NaClO$_2$ treatment in water at pH 4.7 and 35 °C. Approximately 40–95% of the C6-OH groups of curdlan were converted to C6-carboxylate groups by the oxidation under the above conditions for 4–24 h, and these C6-oxidized curdlans became water-soluble. Although partial depolymerization was inevitable during the oxidation, the oxidized curdlan with 95% C6-carboxylation had a weight-average degree of polymerization of more than 1000. This value was much higher than that prepared by the conventional TEMPO/NaBr/NaClO oxidation at pH 10. When the 4-acetamide-TEMPO/NaClO/NaClO$_2$ oxidation at pH 4.7 and 35 °C for 24 h was applied to amylose, starches and pullulan, all the oxidized products except amylose became water-soluble. Carboxylate contents of the oxidized products were, however, lower than that for curdlan. Thus, the C6-OH groups of (1 → 3)-β-glucan are more susceptible to the oxidation than those of glucans with (1 → 4)-α- and (1 → 6)-α-glycoside bonds.

1. Introduction

Curdlan is a linear (1 → 3)-β-D-glucan, and produced as water-insoluble and extracellular bacterial polysaccharide with low crystallinity of about 30% (Harada, Masada, Fujimori, & Maeda, 1966; Harada, Misaki, & Saito, 1968; Marchessault & Deslandes, 1979). Some (1 → 3)-β-D-glucans produced by fungi have branch structures, which sometimes lead to increases in water-solubility (Barbosa, Steluti, Dekker, Cardoso, & da Silva, 2003; Hirokawa et al., 2008; Johansson et al., 2000; Saito, Yoshioka, & Uehara, 1991; Tada et al., 2007) and have some bioactivities such as immunomodulation and anti-tumoral efficacy (Bohn & BeMiller, 1995; Wood, 1994). Thus, chemical modifications of water-insoluble curdlan have been studied to increase water-solubility and the corresponding bioactivities (Gao et al., 2008; Ohya, Nishimoto, Murata, & Ouchi, 1994; Suzuki et al., 1991; Usui, Matsunaga, Ukai, & Kiho, 1997).

Regioselective oxidations of C6 primary hydroxyls of polysaccharides to carboxylate groups have been developed, in which 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO) and NaBr are used as catalysts under aqueous conditions at pH 8.5–11 (Bragd, Besemer, & van Bekkum, 2001; Bragd, van Bekkum, & Besemer, 2004; de Nooy, Besemer, & van Bekkum, 1995). This TEMPO-mediated oxidation has opened a new field of carbohydrate chemistry. Water-insoluble β-cyclodextrin, chitins and regenerated celluloses become water-soluble by partial or complete conversion to C6 carboxylate groups by the TEMPO/NaBr/NaClO oxidation system at pH 10 (Fraschini & Vignon, 2000; Isogai & Kato, 1998; Kato, Kaminaga, Matsuo, & Isogai, 2004). However, remarkable depolymerization of the polysaccharides was inevitable during the oxidation especially under the conditions for preparation of completely C6-oxidized products, *i.e.* polyuronates (de Nooy, Besemer, van Bekkum, van Dijk, & Smit, 1996; Kato et al., 2004; Shibata & Isogai, 2003; Shibata, Yanagisawa, Saito, & Isogai, 2006; Isogai, Yanagisawa, & Isogai, 2009). Cellouronic acids, *i.e.* (1 → 4)-β-D-polyglucuronic acid Na salts, prepared from regenerated, mercerized and ball-milled native celluloses by TEMPO/NaBr/NaClO oxidation at pH 10 had weight-average degrees of polymerization (DP$_w$) of 40–80, which were far lower than those of the original celluloses. Such depolymerization may take place by β-elimination under alkaline conditions (de Nooy et al., 1996) and/or by some active species such as hydroxyl radicals formed *in situ* as side reactions (Shibata & Isogai, 2003).

When the TEMPO/NaBr/NaClO oxidation at pH 10 was applied to paramylon and curdlan, low and high crystalline (1 → 3)-β-D-glucans, respectively, with a sufficient amount of NaClO, water-soluble TEMPO-oxidized products were obtained quantitatively within 100 min. ^{13}C NMR analysis revealed that the C6 primary hydroxyl groups of both paramylon and curdlan were completely converted to carboxylate groups by the oxidation.

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doi:10.1016/j.carbpol.2010.03.016

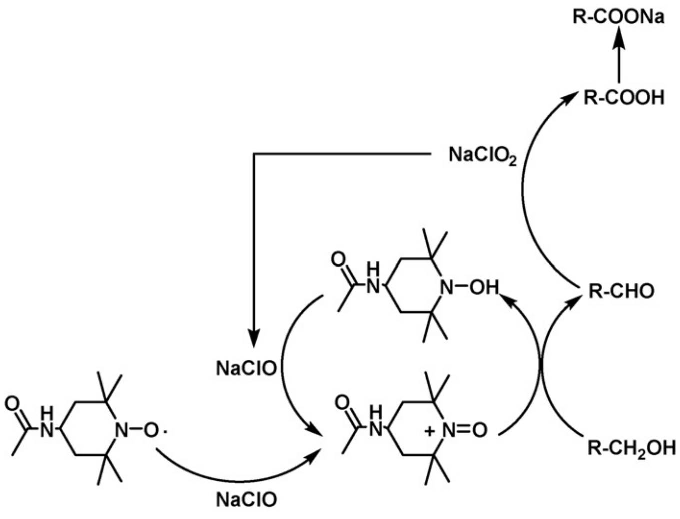

Fig. 1. Oxidation of primary hydroxyls by 4-acetamide-TEMPO/NaClO/$NaClO_2$ system under acid-neutral conditions.

Thus, new (1 → 3)- β-D-polyglucuronic acid sodium salts having almost homogeneous chemical structures were obtained quantitatively. However, remarkable depolymerization occurred also on the (1 → 3)- β-D-glucan chains during the oxidation, and the DP_w values were decreased from 6790 and 1680 for the original curdlan and paramylon to only 86 and 68, respectively by the oxidation (Tamura, Wada, & Isogai, 2009).

Recently, new TEMPO-mediated oxidation of regenerated, mercerized and native celluloses in water under acidic-neutral conditions has been reported to prepare the corresponding oxidized products with higher DP values (Hirota, Tamura, Saito, & Isogai, 2009; Hirota, Tamura, Saito, & Isogai, 2010; Saito et al., 2009). In this oxidation, catalytic amounts of TEMPO or 4-acetamide-TEMPO and NaClO are used with $NaClO_2$ as the primary oxidant (Fig. 1) (Zhao et al., 1999; Zhao, Li, Mano, Song, & Tschaen, 2005). Even though longer reaction times were required, cellouronic acid Na salts with higher DP values were obtained (Hirota et al., 2009). When TEMPO/NaClO/$NaClO_2$ oxidation was applied to native wood celluloses, transparent and highly viscous dispersions were obtained by moderate mechanical disintegration of the oxidized native cellulose fibers in water. Microscopic observation revealed that mostly individualized cellulose nanofibrils approximately 4 nm in width were present in the dispersions. These TEMPO-oxidized cellulose nanofibrils prepared under neutral conditions had higher aspect ratios and higher DP values than those prepared by the TEMPO/NaBr/NaClO oxidation at pH 10 (Saito et al., 2009). Hirota et al. (2009) reported that 4-acetamide-TEMPO was effective in oxidation of C6-OH groups of regenerated celluloses more than TEMPO.

In this study, therefore, 4-acetamide-TEMPO/NaClO/$NaClO_2$ oxidation under acid-neutral conditions was applied to curdlan to prepare C6-oxidized curdlans with higher DP values. Reaction time, pH, temperature, and the addition levels of reagents varied to obtain optimum conditions. This oxidation was applied also to amylose, pullulan and starches from various origins under the same conditions, and carboxylate contents of the oxidized products were determined to study difference in reactivity of C6-OH groups between the polysaccharides.

2. Materials and methods

2.1. Materials

Curdlan, pullulan, amylose and starches originating from potato, corn and wheat are commercial products (Wako Pure Chemicals, Co., Japan). 4-acetamide-TEMPO, sodium chloride, 80% sodium chlorite, 12% sodium hypochlorite solution, and other reagents and solvents were of laboratory grade, and used as received. Distilled water of HPLC grade (Wako Pure Chemicals) was used for dissolution of water-soluble compounds and the following SEC-MALLS analysis.

2.2. Oxidation of polysaccharides

A standard procedure for oxidation of curdlan, amylose and starches is as follows. Curdlan, amylose (1 g, 6.0 mmol C6-OH) or starch (1 g, <6 mmol C6-OH because of the presence of (1 → 6)-α-glycoside branch structures due to amylopectin) was placed in an Erlenmeyer flask with a magnetic stirrer bar. 100 mL of 0.2 M acetate buffer at pH 4.7 containing $NaClO_2$ (80%, 0.68 g, 6.0 mmol) and 4-acetamide-TEMPO (0.096 g, 0.45 mmol) were added to the flask. The NaClO solution (0.62 mL, 1.0 mmol) was added at once to the polysaccharide suspension, and immediately the flask was capped with an universal stopper. The mixture was stirred at 35 °C for 24 h. All the products except amylose became soluble in the aqueous reaction medium during the oxidation process, and transparent solutions were obtained. Amylose was insoluble in the aqueous reaction medium even after extended reaction time, and maintained the suspension state. Oxidation was quenched by adding an excess amount of ethanol. The precipitate thus formed or oxidized amylose powder was collected by centrifugation. The oxidized products were dialyzed with de-ionized water, and freeze-dried. The oxidation factors such as reaction time, pH of the acetate buffer solution, temperature and others varied to obtain the optimum conditions. When pullulan (1 g) was used as the starting material, the amount of $NaClO_2$ added was reduced to 4 mmol/g, because 1 g pullulan has 4 mmol C6 primary hydroxyls.

2.3. SEC-MALLS analysis

Curdlan and starches were dissolved in 1% LiCl/1,3-dimethyl-2-imidazolidinone (LiCl/DMI) at 0.1% concentration by heating the mixtures at about 100 °C for 10 min. The curdlan and starch solutions were subjected to size-exclusion chromatography with multi-angle laser-light scattering method (SEC-MALLS, DAWN EOS, $\lambda = 690$ nm: Wyatt Technologies, USA) using 1% LiCl/DMI as an eluent. A polystyrene-divinylbenzene copolymer gel (KD-806M, 8 mm Ø × 30 cm, Shodex, Japan) was used as the SEC column. The solvent and polysaccharide solutions were filtered using 0.2 μm polytetrafluoroethylene (PTFE) membranes (Millipore, USA) before injection. Weight and number-average molecular mass values of curdlan and starches were calculated from the SEC-MALLS data by ASTRA software (Wyatt Technologies, USA) with a specific refractive index increment (*dn*/*dc*) value of 0.087 mL/g (Yanagisawa & Isogai, 2005). A pullulan standard (M_w 22,800; Shodex, Japan) was exclusively used to normalize the MALLS photo-detectors (ASTRA for Windows user's guide version 4.90). On the other hand, the water-soluble oxidized products and the original pullulan were dissolved in 0.1 M NaCl at 0.1% concentration, and the solutions were subjected to the SEC-MALLS analysis using a SEC column for aqueous systems (DB-806MHQ, 8 mm Ø × 30 cm, Shodex, Japan) and 0.1 M NaCl as the eluent. The *dn*/*dc* value of 0.125 mL/g for cellouronic acid (Shibata et al., 2006; Isogai et al., 2009) was adapted to the water-soluble oxidized products. Details of the SEC-MALLS system used and operation conditions were described elsewhere (Yanagisawa & Isogai, 2005; Shibata et al., 2006).

2.4. Other analyses

Carboxyl contents of the oxidized products were determined by electric conductivity titration using 0.05 M NaOH (Saito &

Table 1
Carboxylate contents and yields of oxidized products prepared from curdlan, starches, amylose and pullulan by 4-acetamide-TEMPO/NaClO/$NaClO_2$ system at pH 4.7 and 35 °C for 24 h.

Original sample	Carboxylate content (mmol/g)	Oxidation ratio of C6-OH (%)	Yield (%)
Curdlan	4.85	95.0	90
Starch from			
Potato	2.23	>39.3[a]	83
Corn	1.64	>28.2[a]	85
Wheat	1.34	>22.8[a]	84
Amylose	2.74	49.2	96
Pullulan	0.32	8.0	90

[a] Because starches have some (1 → 6)-α-glycoside bonds due to amylopectin, accurate oxidation ratios of C6-OH groups cannot be calculated from the carboxylate contents.

Isogai, 2004). Curdlan, amylose and potato starch were dissolved in deuterated dimethylsulfoxide (DMSO-d_6) at about 100 °C for several min followed by cooling to room temperature. ^{13}C NMR spectra of these glucan/DMSO solutions were recorded on an ALPHA-500 (JEOL, Japan) using tetramethylsilane (Aldrich, USA) as an internal standard for 0 ppm. Water-soluble products and the original pullulan were dissolved in D_2O containing 3-trimethylsilyl-2,2,3,3-d_4-propionic acid sodium salt (Aldrich, USA) used as an internal standard for 0 ppm, and the solutions were subjected to the ^{13}C NMR measurement. Data accumulation times were about 15,000.

3. Results and discussion

3.1. 4-acetamide-TEMPO/NaClO/$NaClO_2$ oxidation of polysaccharides

Table 1 shows carboxylate contents of the oxidized curdlan, starches, amylose and pullulan. In all cases examined, carboxylate groups were formed in the products by the 4-acetamide-TEMPO/NaClO/$NaClO_2$ oxidation at pH 4.7. Because the theoretically maximum carboxylate content is 5.05 mmol/g for curdlan, 95.0% of the C6-OH groups were converted to carboxylate groups by the oxidation for 24 h. On the other hand, carboxylate contents of the oxidized starches were as low as 1.34–2.23 mmol/g. From these values, only 23–39% of the C6-OH groups were converted to carboxylate groups by the oxidation, when all starches were assumed to be linear glucans by ignoring the branch structures due to (1 → 6)-α-glycoside bonds. Even though the branched structures of amylopectin in the starches were taken into account, the oxidation ratios of C6-OH for the starches were likely to be lower than 50%. The oxidized amylose was insoluble in water even after extended reaction time. Carboxylate content was 2.74 mmol/g, which corresponds to the C6-OH oxidation ratio of 49.2%. Even though significant amounts of carboxylate groups were present in the oxidized amylose, its (probably high) molecular mass value and mostly linear structures might have brought about the water-insolubility.

The results for curdlan, starches and amylose show that the C6-OH groups of (1 → 3)-β-glucan are more susceptible to the 4-acetamide-TEMPO/NaClO/$NaClO_2$ oxidation at pH 4.7, compared with (1 → 4)-α-glucan and those containing (1→6)-α-glycoside bonds. Especially, because the C6-OH oxidation ratios of starches were lower than that of amylose, the (1 → 6)-α-glycoside branch structures are likely to prevent the oxidation of C6-OH groups located adjacent to the branch structures. This hypothesis was justified by the following results of pullulan oxidation. Pullulan is a linear glucan consisting of maltotriose units connected to each other by (1 → 6)-α-glycoside bond, and kept the dissolution state in the aqueous reaction medium from beginning to end of the oxidation. When pullulan was used as the starting material, the carboxylate content was only 0.32 mmol/g, which corresponds to 8.0% of C6-OH groups of the original pullulan. Thus, again the presence of (1 → 6)-α-glycoside bonds in polysaccharides might cause clear restriction of the oxidation of C6-OH groups, especially when the 4-acetamde-TEMPO/NaClO/$NaClO_2$ oxidation at pH 4.7 was adopted even under homogeneous conditions like pullulan. Steric hindrance due to the (1 → 6)-α-glycoside bonds for the covalent bond formation as the intermediate structure between N-oxonium ions and C6-OH groups is presumably the reason for the restriction of the oxidation.

Because almost all C6-OH groups of pullulan and starches were convertible to C6-carboxylate groups by the TEMPO/NaBr/NaClO oxidation at pH 10, the above restriction or incomplete oxidation of C6-OH groups is characteristic for the 4-acetamide-TEMPO/NaClO/$NaClO_2$ oxidation at pH 4.7. Similar results were obtained also in 4-acetamide-TEMPO/NaClO/$NaClO_2$ oxidation of regenerated and mercerized celluloses under weakly acid and neutral conditions (Hirota et al., 2009, 2010). When the TEMPO/NaBr/NaClO oxidation at pH 10 was applied to amylose, starches and pullulan, almost complete conversion of the C6-OH groups to carboxylate groups can be achieved but always in association with remarkable depolymerization (Shibata & Isogai, 2003; Shibata et al., 2006; Isogai et al., 2009). As described later, the 4-acetamide-TEMPO/NaClO/$NaClO_2$ oxidation at pH 4.7 resulted in less depolymerization of polysaccharide chains, and thus remarkable depolymerization occurring during the TEMPO/NaBr/NaClO oxidation at pH 10 might be one of the necessary conditions for complete and efficient oxidation of C6-OH groups of polysaccharides to carboxylate groups.

3.2. NMR analysis of the oxidized polysaccharides

^{13}C NMR spectra of the original and oxidized polysaccharides in Table 1 are shown in Fig. 2. Because the oxidized amylose was insoluble in water, it was treated with a dilute HCl solution to convert the sodium carboxylate groups to free carboxyl groups, and dissolved in DMSO-d_6. Curdlan was mostly converted to the corresponding polyuronate by the 4-acetamide-TEMPO/NaClO/$NaClO_2$ oxidation at pH 4.7 for 24 h, as shown in Table 1.

Although the signal due to C6 carbons linked to C1 by the (1 → 6)-α-glycoside bonds was not detected in the NMR spectrum of potato starch dissolved in DMSO by overwrapping with other C2, C3 and C5 carbon signals, it was detected at 72.8 ppm in the spectrum of the oxidized potato starch. Thus, the (1 → 6)-α-glycoside bonds of starch are more stable under the conditions in the 4-acetamide-TEMPO/NaClO/$NaClO_2$ oxidation at pH 4.7 than those in the TEMPO/NaBr/NaClO oxidation at pH 10. In the latter case, almost all the (1 → 6)-α-glycoside bonds of water-soluble starch were cleaved, and almost linear (1 → 4)-α-polyglucuronate (*i.e.* amylouronic acid Na salt) was obtained from a water-soluble starch (Kato, Matsuo, & Isogai, 2003).

The oxidized pullulan had a carboxylate content of 0.32 mmol/g (Table 1). However, because the ^{13}C NMR spectra were recorded with the non-quantitative mode, only a small signal due to C6 carboxylate groups at 178 ppm was detected for the oxidized pullulan.

3.3. Effect of oxidation time on structures of oxidized curdlans

As the oxidation time increased from 4 to 24 h, carboxylate content of the oxidized curdlan was increased from 2.27 to 4.85 mmol/g (Fig. 3). The oxidized curdlan with the carboxylate content of 2.27 mmol/g became water-soluble. Thus, water-insoluble curdlan can be converted to water-soluble oxidized products with various carboxylate contents by controlling the oxidation time. Although the oxidation of the C6-OH groups of curdlan to carboxylate

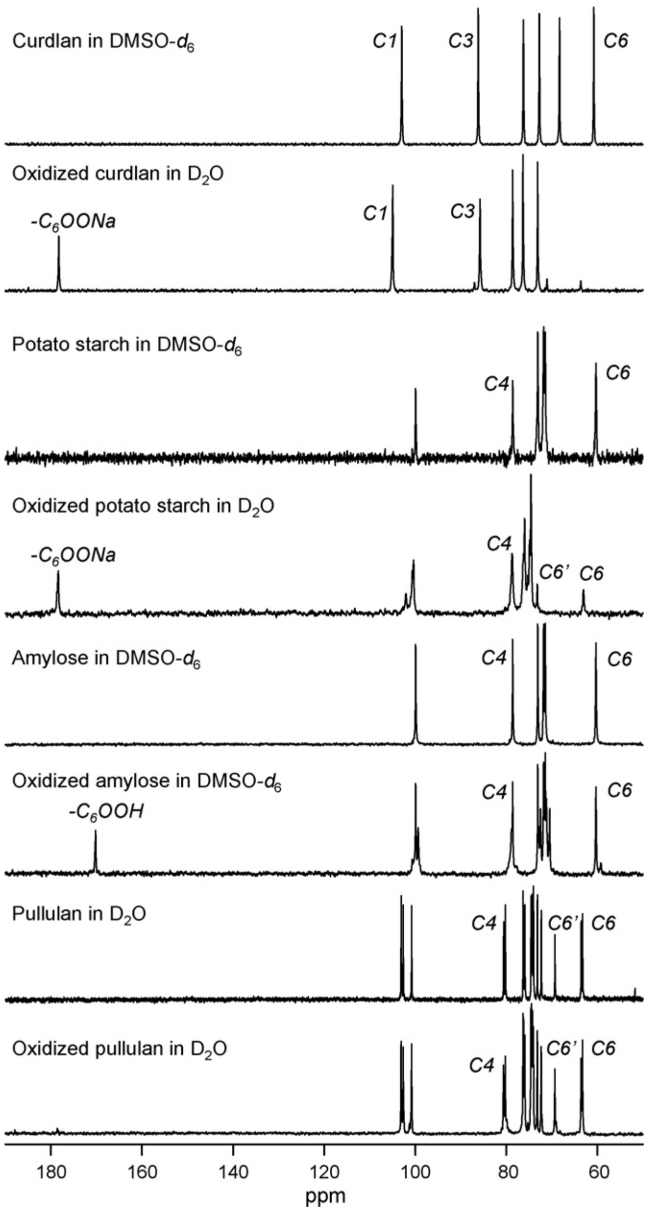

Fig. 2. ^{13}C NMR spectra of the original curdlan, potato starch, amylose and pullulan, and their oxidized products prepared by 4-acetamide-TEMPO/NaClO/NaClO$_2$ at pH 4.7 and 35 °C for 24 h.

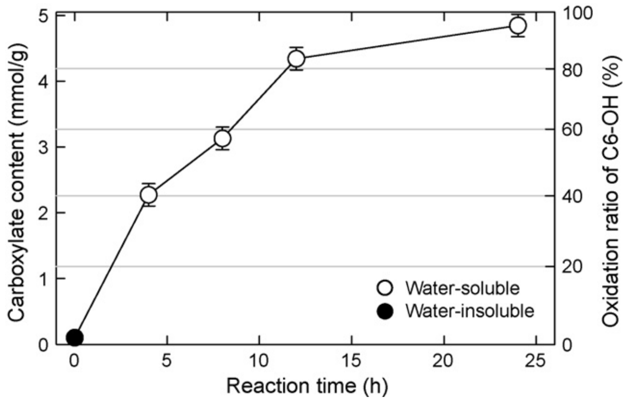

Fig. 3. Carboxylate content and the corresponding C6-carboxylation ratio of the oxidized products prepared from curdlan by 4-acetamide-TEMPO/NaClO/NaClO$_2$ at pH 4.7 and 35 °C for 0–24 h.

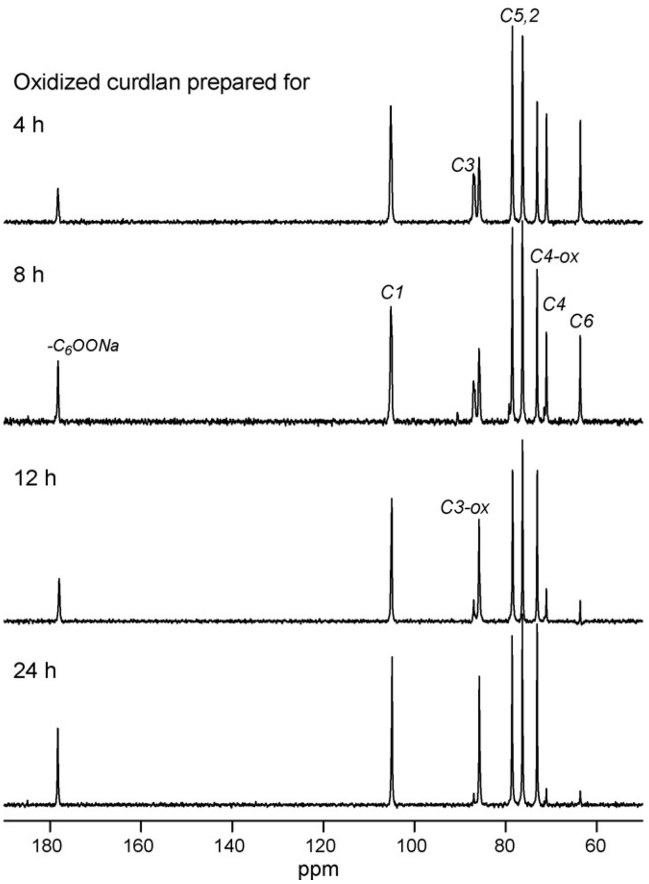

Fig. 4. ^{13}C NMR spectra of the oxidized products prepared from curdlan by 4-acetamide-TEMPO/NaClO/NaClO$_2$ at pH 4.7 and 35 °C for 4-24 h. C3-ox and C4-ox: C3 and C4, respectively, of (1 → 3)-β-anhydroglucuronate unit. D_2O was used as the solvent.

groups can be completed within 50 min by the TEMPO/NaBr/NaClO oxidation at pH 10 (Tamura et al., 2009), the 4-acetamde-TEMPO/NaClO/NaClO$_2$ oxidation at pH 4.7 and 35 °C took longer time of approximately 24 h for 95% conversion of the C6-OH groups to carboxylate groups.

Fig. 4 displays ^{13}C NMR spectra of the oxidized curdlans prepared by the oxidation for 4–24 h. As the oxidation proceeded, the signal intensity due to C6-OH at 64 ppm was decreased, and inversely that due to C6 carboxylate groups at 178 ppm was increased. Because the ^{13}C NMR spectra were recorded with non-quantitative mode, the peak intensities due to C6 carboxylate groups were not quantitative. On the other hand, the signal intensity ratios between the C3 carbons at 86 and 87 ppm due to anhydroglucose and anhydroglucuronate units, respectively, corresponded well to the carboxylate contents of the oxidized curdlans in Fig. 3, probably because these two C3 carbons of different units were in similar circumstances. Likewise, the signal intensity ratios between the C4 carbons at 71 and 73 ppm due to anhydroglucose and anhydroglucuronate units, respectively, were well related to the carboxylate contents in Fig. 3. Thus, approximate carboxylate contents of the oxidized curdlans can be calculated from these C3 or C4 carbon intensity ratios in the NMR spectra.

DP values of the oxidized curdlans were measured by SEC-MALLS. As the oxidation time increased, the peak top position of the DP distribution pattern was shifted to lower DP direction (Fig. 5), showing that depolymerization of curdlan was inevitable even during the 4-acetamide-TEMPO/NaClO/NaClO$_2$ oxidation at pH 4.7. Molecular mass values and the corresponding DP values of the original curdlan and the oxidized curdlans prepared by the

Table 2
Weight- and number-average molecular weights (M_w and M_n, respectively) and the corresponding degrees of polymerization (DP_w and DP_n, respectively) of the original curdlan and its oxidized products prepared by TEMPO/NaBr/NaClO at pH 10 and 25 °C, and by 4-acetamide-TEMPO/NaClO/$NaClO_2$ at pH 4.7 and 35 °C.

Sample	Reaction time (h)	Oxidation ratio of C6-OH (%)	M_w (DP_w)	M_n (DP_n)	M_w/M_n
Original curdlan	–	–	1,100,000 (6790)	890,000 (5490)	1.24
Oxidized curdlan prepare by					
TEMPO/NaBr/NaClO at 25 °C and pH 10[a]	0.8	100	17,000 (86)	13,500 (68)	1.26
4-AcNH-TEMPO/NaClO/$NaClO_2$ at 35 °C and pH 4.7	24	95	197,000 (1020)	130,000 (670)	1.52

[a] Tamura et al. (2009).

two oxidation procedures at pH 10 and 4.7 are summarized in Table 2. Although the original curdlan had a high DP_w of 6790, the oxidized curdlan prepared by the new oxidation at pH 4.7 for 24 h had a DP_w of 1020: depolymerization still took place on curdlan chains even by the new oxidation. However, this DP_w was much higher than that (DP_w 86) of the oxidized curdlan prepared by the TEMPO/NaBr/NaClO oxidation at pH 10. Hence, the 4-acetamide-TEMPO/NaClO/$NaClO_2$ oxidation at pH 4.7 can bestow mostly C6-oxidized curdlans with sufficiently higher DP_w values than those prepared by the TEMPO/NaBr/NaClO oxidation at pH 10. Curdlan, one of (1 → 3)-β-glucans, has no chances to allow depolymerization by β-elimination even if C6, C2 or C4 hydroxyls are oxidized to aldehydes or ketones by TEMPO-mediated oxidation. Thus, the remarkable depolymerization of curdlan during the TEMPO/NaBr/$NaClO_2$ oxidation at pH 10 might not be engendered by β-elimination but probably by some active radical species formed as by-products during the oxidation (Shibata & Isogai, 2003).

3.4. *Effect of other oxidation conditions on structures of oxidized curdlans*

The 4-acetamide-TEMPO/NaClO/$NaClO_2$ oxidation was applied to curdlan in water at 35 °C and pH 3.7–6.7 for 8 h. As shown in Fig. 6, water-soluble oxidized products were obtained by the oxidation at pH 4.7, 5.7 and 6.7. The carboxylate contents were 3.13 and 3.00 mmol/g at pH 4.7 and 5.7, respectively, whereas those at 3.7 and 6.7 were as low as 0.69 and 1.87 mmol/g, respectively: the oxidation conditions at pH 4.7 and 5.7 were preferable in terms of efficient oxidation of the C6-OH groups of curdlan.

The DP distribution patterns of the oxidized curdlans are depicted in Fig. 7. Even though the carboxylate contents were similar, the oxidized curdlan prepared at pH 4.7 had a DP_w higher than that prepared at pH 5.7. If the aforementioned hypothesis concerning depolymerization of curdlan is correct, some active radical species might be formed much more at pH 5.7. Because the oxidized curdlan prepared at pH 6.7 had the carboxylate content of only 1.87 mmol/g, the incomplete oxidation might have brought about such a broad DP distribution pattern. This result indicates that the conversion of the C6-OH groups to carboxylate groups by the oxidation is always accompanied by depolymerization of curdlan, or some depolymerization is inevitable as long as high C6-oxidation ratios are achieved by the 4-acetamide-TEMPO/NaClO/$NaClO_2$ oxidation.

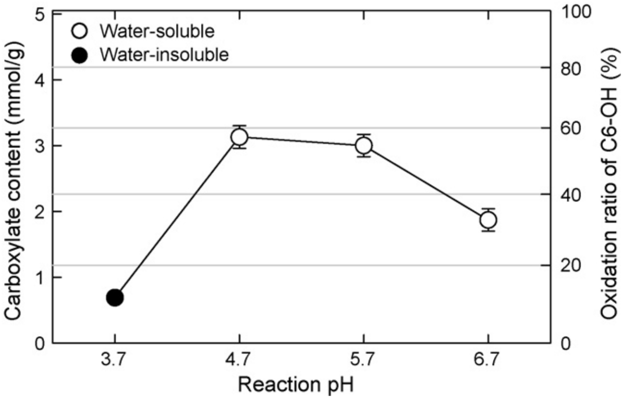

Fig. 6. Carboxylate content and the corresponding C6-carboxylation ratio of the oxidized products prepared from curdlan by 4-acetamide-TEMPO/NaClO/$NaClO_2$ at pH 3.7–6.7 and 35 °C for 8 h.

As the oxidation temperature increased from room temperature (ca. 20 °C) to 35, 50 and 65 °C, the carboxylate content of the oxidized curdlan was increased from 2.11 mmol/g to 3.13, 3.53 and 3.72 mmol/g, respectively. Thus, the temperature increase is effective in formation of C6 carboxylate groups of curdlan by the oxidation. The DP distribution patterns of the oxidized curdlans

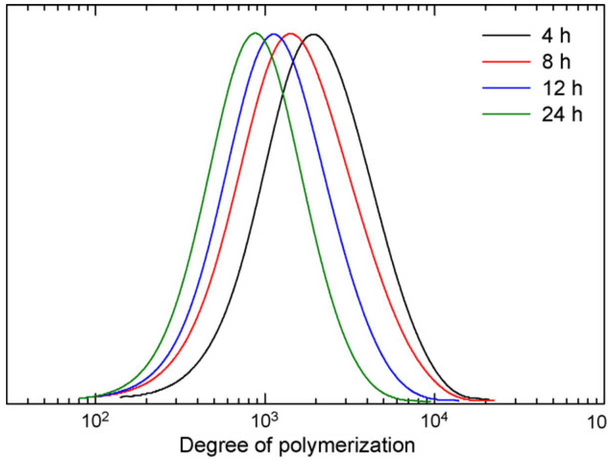

Fig. 5. DP distribution patterns of the oxidized products prepared from curdlan by 4-acetamide-TEMPO/NaClO/$NaClO_2$ at pH 4.7 and 35 °C for 4-24 h.

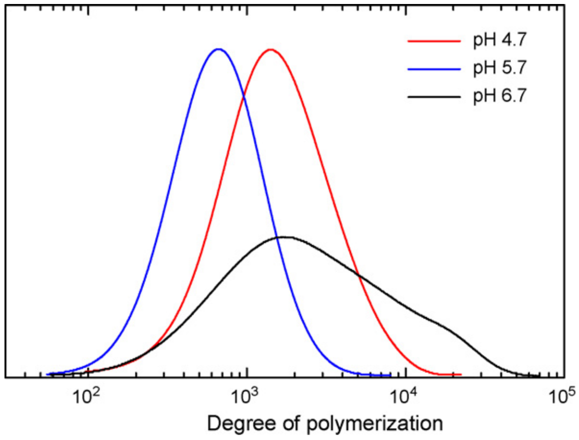

Fig. 7. DP distribution patterns of the oxidized products prepared from curdlan by 4-acetamide-TEMPO/NaClO/$NaClO_2$ at pH 4.7–6.7 and 35 °C for 8 h.

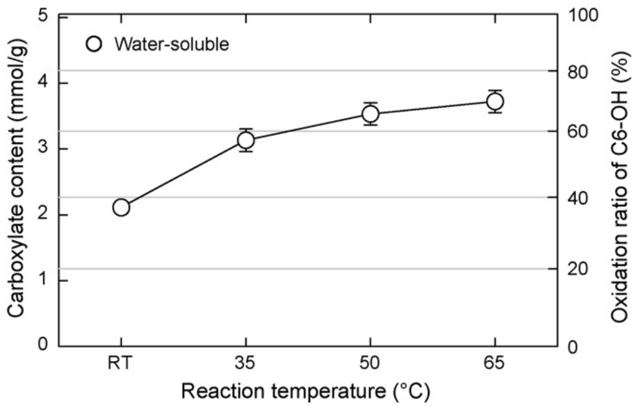

Fig. 8. Carboxylate content and the corresponding C6-carboxylation ratio of the oxidized products prepared from curdlan by 4-acetamide-TEMPO/NaClO/$NaClO_2$ at pH 4.7 and 20–65 °C for 8 h.

are shown in Fig. 9. Even though the difference in the carboxylate content between the oxidized curdlans prepared at 35, 50 and 65 °C was not so remarkable, the oxidized curdlans prepared at 50 and 65 °C had DP values clearly lower than that prepared at 35 °C.

Because the oxidized curdlan prepared at room temperature had the carboxylate content as low as 2.11 mmol/g, such insufficient oxidation might have led to such a broad DP distribution pattern with higher DP. Even though the oxidized curdlan prepared at pH 4.7 and 35 °C for 4 h (Fig. 3) and that prepared at pH 4.7 and room temperature for 8 h (Fig. 8) had similar carboxylate contents of 2.27 and 2.11 mmol/g, respectively, the DP distribution patterns were so different to each other (Figs. 5 and 9); the decrease in the oxidation temperature may be effective in preparation of the oxidized curdlans with higher DP values. However, because oxidized curdlans with carboxylate contents of more than 4 mmol/g could not be prepared by the oxidation at room temperature for more than 3 days, we concluded from the obtained results that the oxidation conditions at pH 4.7 and 35 °C were preferable for preparation of the oxidized products with higher DP values and higher carboxylate contents.

The addition levels of NaClO and 4-acetamide-TEMPO varied in the range of 0.25–1.0 and 0.1–0.9 mmol/g-curdlan, respectively. The oxidation efficiency was clearly improved with the addition level of 4-acetamide-TEMPO (Fig. 10). When the addition levels of 4-acetamide-TEMPO were 0.1 and 0.2 mmol/g-curdlan, the oxidized products were insoluble in water because of low carboxylate contents, and the addition level of NaClO had nearly no influence on the degree of C6-OH oxidation under these conditions. When the addition level of 4-acetamide-TEMPO was 0.45 mmol/g-curdlan, the degree of C6-OH oxidation was increased with the increase of the NaClO addition level. The efficiency of the C6-OH oxidation was remarkably improved by the increase of the 4-acetamide-TEMPO addition level to 0.9 mmol/g-curdlan. The NaClO addition level had nearly no influence on the degree of C6-OH oxidation in these cases examined so far. However, because the addition level of TEMPO for the oxidation of curdlan by the TEMPO/NaBr/NaClO system at pH 10 was only 0.1 mmol/g-curdlan, the addition level of 0.9 mmol/g-curdlan for 4-acetamide-TEMPO seemed to exceed the catalytic level.

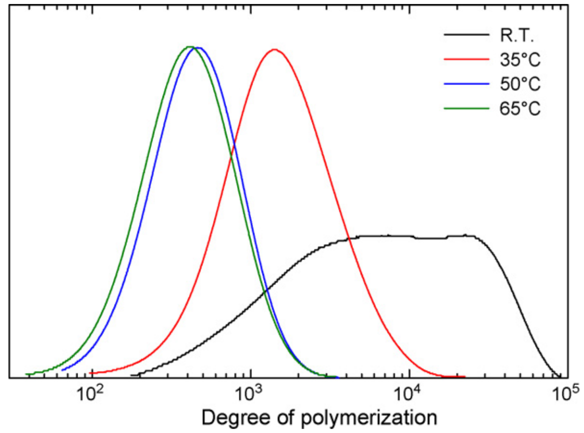

Fig. 9. DP distribution patterns of the oxidized products prepared from curdlan by 4-acetamide-TEMPO/NaClO/$NaClO_2$ at pH 4.7 and 20–65 °C for 8 h.

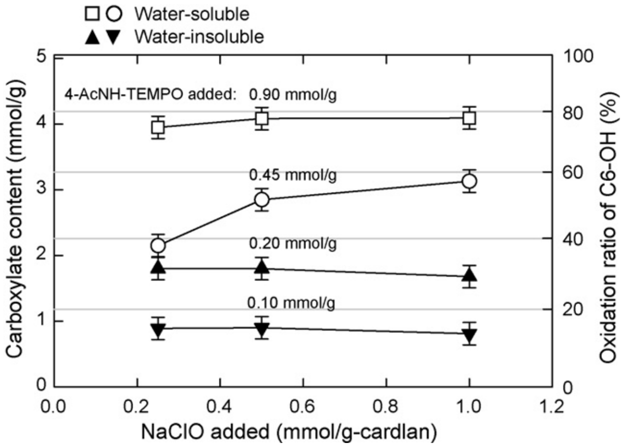

Fig. 10. Carboxylate content and the corresponding C6-carboxylation ratio of the oxidized products prepared from curdlan by 4-acetamide-TEMPO/NaClO/$NaClO_2$ at pH 4.7 and 35 °C for 8 h, with different addition levels of 4-acetamide-TEMPO or NaClO.

The DP distribution patterns of the oxidized curdlans prepared by the new oxidation system with 4-acetamide-TEMPO and NaClO of 0.9 and 0.25–1.0 mmol/g-curdlan, respectively, are depicted in Fig. 11. Even though the oxidation efficiency was improved by the increase in the addition level of 4-acetamide-TEMPO from 0.45 to 0.9 mmol/g-curdlan, the DP distribution patterns were shifted to lower DP direction from that prepared with 4-acetamide-TEMPO and NaClO of 0.45 and 1.0 mmol/g-curdlan in Figs. 7 and 9.

Because the increased addition level of 4-acetamide-TEMPO from 0.45 to 0.90 mmol/g-curdlan was effective in the C6-

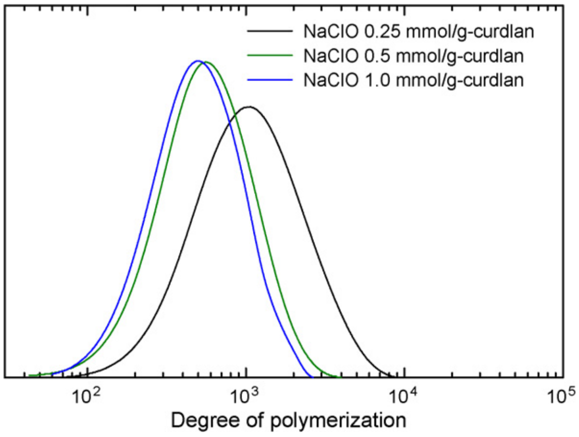

Fig. 11. DP distribution patterns of the oxidized products prepared from curdlan by 4-acetamide-TEMPO/NaClO/$NaClO_2$ at pH 4.7 and 35 °C for 8 h. The NaClO addition level varied from 0.25 to 1.0 mmol/g-curdlan, while the addition level of 4-acetamide-TEMPO was fixed to 0.9 mmol/g-curdlan.

carboxylation but negative for preparation of the oxidized curdlans with higher DP values, the addition level of 4-acetamide-TEMPO was fixed to 0.45 mmol/g-curdlan in this study as preferable conditions. However, oxidized curdlans with higher DP values and higher carboxylate contents may be obtained with shorter reaction time by the addition of 4-acetamide-TEMPO with relatively higher addition levels. Moreover, the addition levels of NaClO and/or $NaClO_2$ may be reduced to some extent and/or the oxidation time required for complete oxidation of C6-OH may be somewhat decreased by the oxidation with higher addition level of 4-acetamide-TEMPO. Thus, further investigations of the curdlan oxidation by the 4-acetamide-TEMPO/NaClO/$NaClO_2$ system at pH 4.7 should be carried out to obtain more optimum oxidation conditions to prepare oxidized curdlans with higher carboxylate contents and higher DP values under more moderate conditions. Viscosities and viscoelastic properties of the oxidized curdlan/water solutions, their stabilities by temperature, pH, co-existing ions and other factors, and bioactivities of the water-soluble oxidized curdlans will be studied also in the nest stage.

4. Conclusions

The regioselective oxidation of C6-OH groups of curdlan to carboxylate groups with less depolymerization was achieved by 4-acetamide-TEMPO/NaClO/$NaClO_2$ system in water at pH 4.7 and 35 °C for 4–24 h. Carboxylate contents of the oxidized curdlan ranged from 2.27 to 4.85 mmol/g by the above oxidation from 4 to 24 h, which corresponded to 40 and 95% conversions, respectively, of the C6-OH groups to carboxylate groups. The C6-oxidized curdlans with carboxylate contents of more than approximately 2 mmol/g became water-soluble. Although partial depolymerization was inevitable during the oxidation, the oxidized curdlan with the 95% C6-carboxylation had the DP_w value of more than 1000. This value was much higher than that prepared by the TEMPO/NaBr/NaClO oxidation at pH 10. When the 4-acetamide-TEMPO/NaClO/$NaClO_2$ oxidation was applied to starches, amylose and pullulan under the same conditions, the degrees of C6-oxidation were lower than that for curdlan. The C6-OH groups of $(1 \rightarrow 4)$-α-glucan were, therefore, less susceptible to the oxidation than those of $(1 \rightarrow 3)$-β-glucan, and those located adjacent to $(1 \rightarrow 6)$-α-glycoside bonds might be also highly resistant to the oxidation.

Acknowledgement

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

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