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Antioxidant activities of a polyglucuronic acid sodium salt obtained from TEMPO-mediated oxidation of xanthan

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ABSTRACT

A xanthouronic acid sodium salt called xanthouronan was produced from xanthan by regioselective oxidation with NaOCl/NaBr using 2,2,6,6-tetramethylpiperidine-1-oxy radical (TEMPO) as catalyst. The efficiency of the one pot TEMPO-mediated oxidation was confirmed by HPAEC-PAD, ^{13}C NMR, and FT-IR. The oxidation degree was close to 98% and the mass yield of this new polyglucuronic acid was higher than 90% (w/w). The macromolecular characterization of xanthouronan using SEC-MALLS showed a molecular size reduced by a third due to the oxidation treatment and the degree of polymerization (DP) of the xanthouronan form was about 665. The evaluation of the enzymatic degradation of this C-6 carboxylated xanthan by various polysaccharide hydrolases and one polysaccharide lyase showed its high resistant to biodegradation. The antioxidant activity of xanthouronan was also tested by using the 2,2′-diphenyl-1-picrylhydrazyle (DPPH) and hydroxyl radical procedures. At 1 g/L, xanthouronan presented 75% of the ascorbic acid antioxidant activity.

1. Introduction

Xanthan is an anionic gum produced by *Xanthomanas campestris pv campestris* (Sutherland, 1997). This high molecular weight ramified heteropolysaccharide is made up of a cellulose backbone ([,4)-β-D-Glc-(1,4)-β-D-Glc-(1,]$_n$), substituted on every second unit with a trisaccharide side chain (β-D-Man-(1,4)-β-D-GlcA-(1,2)-α-D-Man-(1,]), linked on C-3 position of the glucosyl units (Jansson, Kenne, & Lindberg, 1975). Generally speaking, mannose residues were *O*-acetylated on C-6 whereas around 15–30% of the terminal mannose residues were pyruvated between C-6 and C-4 position. It is appropriate to mention that the substitution level (acetates and/or pyruvates groups) could fluctuate according to the strain fermentation conditions (Cadmus, Knutson, Lagoda, Pittsley, & Burton, 1978; Delattre, Laroche, & Michaud, 2008). This microbial polysaccharide is extensively used in food applications either alone or in combination with others polysaccharides such as glucomannans (Sutherland, 1998). One of the most important characteristic of xanthan is high viscosity at low concentrations in water and its strong shear-thinning behavior partly due to its polyelectrolyte nature. The high viscosity pseudoplastic solutions are very resistant to high temperature and stable over a large pH range conditions (Delattre et al., 2008). Moreover, as related by Katzbauer (1998), xanthan has the ability to be stable in food applications under high salt amount or high enzymatic activity. These unique rheological properties have resulted in the successful marketing of xanthan as a gelling, emulsifying, thickening, and stabilizing agent (Morris, 1995; Sutherland, 2001). The last two decades, some studies in functional foods have described the putative antioxidant capacity of xanthan insofar as mentioned by Shimaida, Fujikawa, Yahara, and Nakamura (1992), it could first reduce the Fe^{2+} inducing the peroxidation of oil from soybean and secondly, inhibit the autoxidation of soybean oil in emulsion system. Furthermore, Paquet, Turgeon, and Lemieux (2010) have investigated the study of the protective effect of xanthan against hydroxyl radical. Others studies have recently described the hydroxyl and superoxide anion radical scavenging properties of xanthan derivatives (He, Zhang, Bai, Du, & Li, 2005; Xiong et al., 2013).

It was recently reported that C6-carboxylation of polysaccharides such as gellan (Elboutachfaiti, Delattre, Petit, & Michaud,

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2011; Elboutachfaiti, Petit, et al., 2011) or curdlan (Yan et al., 2014) considerably increase the scavenging effects of free radicals. This effect was explained by the chelates formation between metal cations (such as Fe^{2+} and Fe^{3+}) but also by the decreasing of intra- and intermolecular hydrogen bonds between polysaccharidic chains promoting their antioxidant activities.

These polyglucuronic acid sodium salts families have attracted increasing interest in the biotechnological field (Elboutachfaiti, Delattre, et al., 2011; Elboutachfaiti, Petit, et al., 2011). The most attractive method to produce polyglucuronic acid sodium salts was the catalytic use of the radical 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO®) in the presence of sodium bromide/sodium hypochlorite (Delattre, Michaud, Elboutachfaiti, Courtois, & Courtois, 2006; Delattre et al., 2009; Delattre, Chaisemartin, Favre, Berthon, & Rios, 2012; Elboutachfaiti, Delattre, et al., 2011; Elboutachfaiti, Petit, et al., 2011; Isogai & Kato, 1998; Muzzarelli, Muzzarelli, Cosani, & Terbojevich, 1999; Pierre et al., 2013).

In this study, a new kind of C-6 carboxylated xanthan called xanthouronan was produced by TEMPO oxidation of xanthan in NaOCl/NaBr system. After structural characterization using ^{13}C nuclear magnetic resonance (NMR), Fourier transform infrared (FT-IR) spectroscopy and high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), antioxidant capacity of xanthouronan has been evaluated in order to investigate the relationship between carboxylation and antioxidant activity. Finally, various polysaccharide hydrolases and one polysaccharide lyase were used to degrade the oxidized derivative by enzymes.

2. Materials and methods

2.1. Chemical and enzymatic materials

Commercial xanthan (200 meshes) was from Jungbunzlauer (Switzerland). Glucose (assay > 99%), L-serine (assay > 99%), deuterated water (isotopic purity > 99.99%), potassium acetate (assay ≥ 99%), sodium chloride (assay > 98%), Tris buffer (assay > 98%), 2,2′-diphenyl-1-picrylhydrazyle (DPPH), ascorbic acid (assay > 99%), $FeSO_4$ (assay > 99%), H_2O_2 (30%, m/v) and ethanol 96% were from Sigma–Aldrich (France). Disodium phosphate (assay > 98%), sodium phosphate (assay > 99%), copper sulfate (assay > 99%), sodium hydroxide (granules, assay > 98%), citric acid (assay ≥ 98%) and sodium bromide (ultra pure) were provided by Merck (France). Sodium hypochlorite as bleach (9.6° chl) was from ETS Richet (France) and chlorhydric acid 37% was supplied by Riedel de Haën (Germany). Sodium carbonate (assay > 99%), sodium bicarbonate (assay > 99%) and TEMPO (assay > 98%) were provided by Acros Organics (Belgium). Celluclast 1.5 L (C2730), endocellulase (C9422), Macerozyme R-10 (P2401) and hyaluronidase (H3506) were from Sigma–Aldrich (France). Glucanex® was from Novo Nordish Ferment Ltd (Switzerland) and alginate lyase (E-ALGS) was from Megazyme (France). Glucuronan lyase was extracted by the method of Delattre, Michaud, Keller, et al. (2006).

2.2. Oxidation procedure

C6-carboxylated xanthan (xanthouronan) was prepared according to the oxidation process described by Delattre et al. (2009). Briefly, xanthan (10 g) was dissolved at room temperature in distilled water (500 mL) for 60 min at pH 10 by adding NaOH (4 M). TEMPO (100 mg) and NaBr (2 g) were added to the xanthan solution. The solution was kept below 4 °C in an ice bath during oxidation step. NaOCl (100 mL at 9.6%) were added at 1.7 mL/min. The pH was kept at 10 by addition of NaOH (1 M) during the oxidation step. The reaction was quenched by adding absolute ethanol (50 mL) after 1 h and neutralized with HCl (5 M). Xanthouronan was precipitated with cold ethanol (3 volumes). The precipitate was dissolved in distilled water (200 mL) and precipitated with cold ethanol (3 volumes). After centrifugation (10,000 × *g*, 30 min, 4 °C) the pellet of xanthouronan was dispersed in acetone (100 mL), triturated, filtered under-vacuum and dried at 40 °C overnight.

2.3. Determination of oxidation degree by HPAEC-PAD

Xanthan (10 mg) and xanthouronan (10 mg) dissolved in 4 M TFA (1 mL) were heated at 100 °C during 8 h. The hydrolysates were neutralized with ammonia solution (4 M). Monosaccharide composition of xanthan and xanthouronan was evaluated by High Pressure Anion Exchange Chromatography (HPAEC) on an ICS 3000 (Dionex, USA) equipped with pulsed amperometric detection and AS 50 autosampler. It was assembled with a guard CarboPac™ PA1-column (4 × 50 mm) and analytical CarboPac™ PA1-column (4 × 250 mm). Samples (10 mg/mL) were filtered using 0.2 μm membrane filter and injection volume was fixed at 25 μL. Before each injection, columns were equilibrated by running during 15 min with 18 mM NaOH. Samples were eluted with 18 mM NaOH for 30 min, followed by a linear gradient between 0 and 1 M sodium acetate in 200 mM NaOH for 20 min to elute acidic monosaccharides. The column was then washed for 15 min with 200 mM NaOH. The eluent flow rate was kept constant at 1 mL/min. Columns were thermostated at 25 °C. Data were collected and analyzed with Dionex Chromeleon 6.80 software (Sunnyvale, USA).

2.4. FT-IR and NMR analyses

FT-IR analysis was performed using a Nicolet 380 FT-IR instrument (Thermoelectron Corporation, France). Three milligrams of dried xanthouronan were dispersed into 100 mg of anhydrous KBr and pressed to form a pellet. Fifty IR spectra were recorded and averaged at room temperature in the wavenumber range of 500–4000 cm^{-1} and referenced against air.

^{13}C measurements were carried out at 60 °C using a Bruker advance 400 MHz apparatus equipped with a QNP probe (5 mm) with Z-gradient. ^{13}C spectra were recorded with a spectral width of 30 KHz, an acquisition time of 1.3 s, a pulse width of 7 μs, a relaxation time of 2 s and a number of 30,000 scans. Xanthan and xanthouronan were previously dissolved in D_2O at 50 g/L.

2.5. SEC-MALLS analyses

Average molecular weights and molecular weight distributions of xanthan and xanthouronan were determined by high pressure size exclusion chromatography (HPSEC) with on line multi-angle laser light scattering (MALLS) filled with a K5 cell (50 μL) and two detectors: a He–Ne laser (λ = 690 nm) and a differential refractive index (DRI). Columns [OHPAK SB-G guard column, OHPAK SB806, 804 and 803 HQ columns (Shodex)] were eluted with $NaNO_3$ 0.1 M at 0.7 mL/min. Solvent was filtered through 0.1 μm filter unit (Millipore), degassed and filtered through a 0.45 μm filter upstream column. The sample was injected at 5 g/L through a 100 μl full loop with a flow rate of 0.4 mL/min. The collected data were analyzed using the Astra 4.90.08 software package and a d*n*/dc of 0.15 as referenced in the literature (Delattre et al., 2009; Tavernier et al., 2008).

Degrees of polymerization (DPs) were defined considering the pentasaccharidic repeat units of xanthan and xanthouronan (Fig. 1) and determined using (Eq. (1)) for xanthan and (Eq. (2)) for xanthouronan.

$$\mathrm{DP(xanthan)} = \frac{M_w}{894} \quad (1)$$

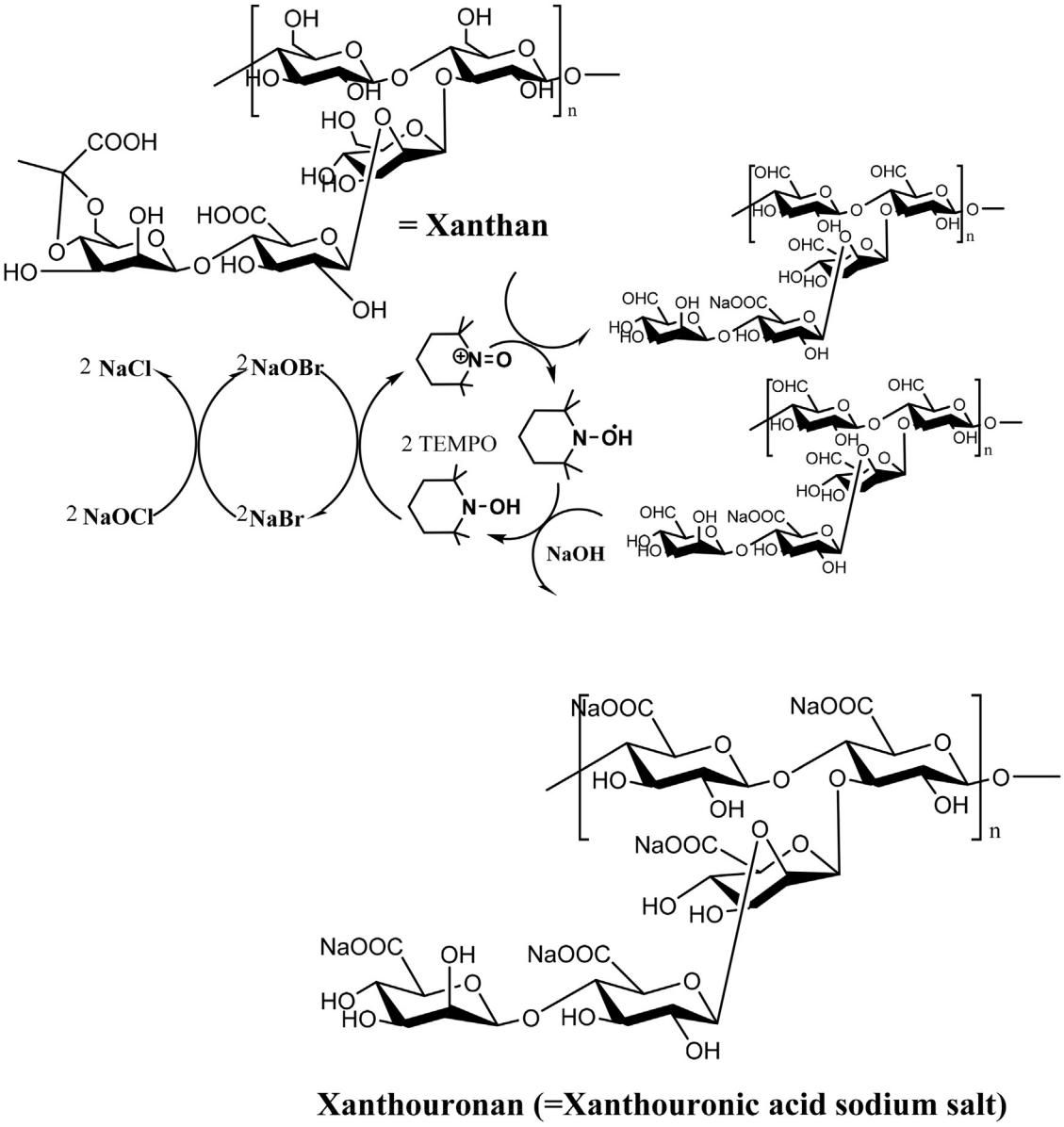

Fig. 1. Proposed process to produce xanthouronan by regioselective oxidation of xanthan catalyzed by TEMPO/NaOCl/NaBr chemical system.

$$\mathrm{DP(xanthouronan)} = \frac{M_w}{880} \quad (2)$$

2.6. Enzymatic degradation of xanthouronan

Celluclast 1.5 L (8.4 U/mL), endocellulase C9422 (0.84 U/mL), Macerozyme R-10 (0.1 U/mL), hyaluronidase H3506 (4–10 U/mL), alginate lyase E-ALGS (0.25 U/mL) and Glucanex® (0.15 BGXU/mL) were added to 0.2% (w/v) of xanthan or xanthouronan previously suspended in 10 mL of appropriate buffer, *i.e.* 50 mM citrate-phosphate buffer pH 4.6 for Celluclast 1.5 L, endocellulase C9422 and Glucanex® and pH 4 for Macerozyme R-10 and 300 mM phosphate buffer pH 5.35 for hyaluronidase H3506 and 100 mM Tris HCl pH 7.2 for alginate lyase E-ALGS.

One unit (1 U) of Celluclast 1.5 L is defined as the amount of enzyme releasing 1 μmol of glucose per min; one unit (1 U) of endocellulase C9422 is defined as the amount of enzyme releasing 1 μmol of glucose from cellulose in 1 h; one unit (1 U) of Macerozyme R-10 liberates 1 μmol of galacturonic acid from polygalacturonic acid per min and; one unit BGXU of Glucanex® is the quantity of enzyme required to produce 1 mmol of reducing sugars per min.

The experiments were performed in 10 mL total reaction volume using a Carousel 12 Plus Reaction Station Radleys (Radleys, France). The tubes were sealed and incubated at 37 °C with Macerozyme R-10 (0.10 U/mL), hyaluronidase H3506 (4–10 U/mL) and alginate lyase E-ALGS (0.25 U/mL), and 50 °C for Celluclast 1.5 L (8.4 U/mL), endocellulase C9422 (0.84 U/mL) and Glucanex® (0.15 BGXU/mL) under agitation (700 rpm) during 1140 min For each sampling, 500 μL were taken and heated 10 min at 95 °C to denature enzymes then stored at −20 °C until colorimetric assays were performed. Denatured enzymes (2 h, 95 °C) were used as controls. Enzymatic hydrolysis was followed through the determination of reducing sugars content by using the bicinchoninic acid (BCA) method (Waffenshchmidt & Jaenicke, 1987) and a Shimadzu UV-1700 spectrophotometer (PharmaSpec). Glucose was used as representative monosaccharide for the expression of reducing sugars equivalents. Initial reducing sugars amount measured at $t = 0$ min was reset. All reactions were carried out at least in duplicate.

Table 1
Monosaccharides analysis (molar ratio) of xanthan and xanthouronan by HPAEC-PAD.

	Monosaccharides (mol%)[a]				Oxidation rate (%)
	Glc (mol%)	Man (mol%)	GlcA (mol%)	ManA (mol%)	
Xanthan	40	40	20	0	20
Xanthouronan	~1	~1	58.8	39.2	98

Glc: glucose; Man: mannose; GlcA: glucuronic acid, ManA: mannuronic acid.
[a] Monosaccharides composition was estimated by HPAEC-PAD.

Glucuronan lyase activity was also followed by measuring the increase of absorbance at 235 nm, corresponding to the absorbance of the C-4/C-5 unsaturated non-terminal reducing unit generated by the lyase activity, using a Shimadzu UV-1700 spectrophotometer (PharmaSpec). The reaction mixture was composed of 1 mL of 0.2% (w/v) xanthouronan in 50 mM potassium acetate buffer (pH 5.5) and 3.2×10^{-3}–3.2×10^{-2} U/mL of glucuronan lyase. A deacetylated bacterial glucuronan was used as standard (Delattre, Michaud, Keller, et al., 2006). All reactions were performed in triplicate.

2.7. Antioxidant activity assay

The antioxidant activities of xanthouronan, xanthan and ascorbic acid were evaluated by using 2,2′-diphenyl-1-picrylhydrazyle (DPPH) procedure adapted from Yamaguchi, Takamura, Matoba, and Terao (1998) and hydroxyl radical procedure adapted from Luo et al. (2010). Briefly, for the adapted DPPH method, the fractions were previously dissolved at different concentrations (0–10 g/L) in ultra pure water. One milliliter of the solution (sample or control) was added into 1 mL of a DPPH solution at 0.1 mM in ethanol. The solution was strongly stirred and incubated 30 min at room temperature (25 °C) in obscurity. The absorbance was measured at 517 nm using a Shimadzu UV-1700 spectrophotometer (PharmaSpec). The DPPH inhibition (%) was calculated by using Eq. (3).

$$\text{DPPH inhibition}\,(\%) = \left(1 - \left(\frac{A_{\text{sample}}}{A_{\text{conrol}}}\right)\right) \times 100 \quad (3)$$

where A_{sample} and A_{control} are respectively the absorbances at 517 nm of 1 mL of the sample (0–10 g/L) and 1 mL of ultra pure water with 1 mL of DPPH at 0.1 mM in ethanol.

Concerning the adapted hydroxyl radical method, the fractions were previously dissolved at different concentrations (0–10 g/L) in ultra pure water. A volume of 0.2 mL of the solution (sample or control) was added into 0.2 mL of an aqueous solution of $FeSO_4$ at 5 mM. After stirring, 0.2 mL of an aqueous solution of H_2O_2 at 1% (v/v) was added to the mixture. The solution was stirred again then incubated at room temperature. After 60 min, 1 mL of ultra pure water was added and the absorbance was measured at 510 nm using a Shimadzu UV-1700 spectrophotometer (PharmaSpec). The hydroxyl radical inhibition (%) was calculated by using Eq. (4).

$$\text{Hydroxyl radical inhibition}\,(\%) = \left(\frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}}\right) \times 100 \quad (4)$$

where A_{sample} and A_{control} are the absorbances at 510 nm of 0.2 mL of the sample (0–10 g/L) or 0.2 mL of ultra pure water with 0.4 mL of a solution (v/v) of $FeSO_4$ (5 mM)/H_2O_2 (1%).

3. Results and discussion

3.1. One pot TEMPO-mediated oxidation of xanthan

β-(1,4)-Polyglucuronic acid sodium salt (xanthouronan) was easily produced from xanthan with high yield (~90%) after 1 h of oxidation time at pH 10 and 4 °C using TEMPO/NaOCl/NaBr system as described previously and outlined in Fig. 1. As summarized in Table 1, the HPAEC analyses have shown that the native xanthan was composed of glucose (40%), mannose (40%) and glucuronic acid (20%) as described in literature (Jansson et al., 1975). The oxidized xanthan was almost made up of mannuronic (39.2%) and glucuronic (58.8%) acids validating a principal polyglucuronic backbone ([,4)-β-D-GlcA-(1,4)-β-D-GlcA-(1,]$_n$), substituted on every second unit with a trisaccharide side chain (β-D-ManA-(1,4)-β-D-GlcA-(1,2)-α-D-ManA-(1,]), linked on C-3 position of the glucuronyl units.

To confirm the full oxidation of xanthan, xanthouronan was analyzed by ^{13}C NMR and FT-IR. First of all, as shown in Fig. 2, the ^{13}C NMR analysis of xanthouronan (Fig. 2B) was less complex than xanthan (Fig. 2A) indicating a homogenous modification by oxidation of xanthan. ^{13}C NMR spectra shown characteristic resonance peaks for xanthan (Horton, Mols, & Walaszek, 1985) and C-6 carboxylated xanthan (xanthouronan) corresponding to ([,4)-β-D-Glc-(1,4)-β-D-Glc-(1,] and (β-D-Man-(1,4)-β-D-GlcA-(1,2)-α-D-ManA-(1,]) residues and, ([,4)-β-D-GlcA-(1,4)-β-D-GlcA-(1,] and (β-D-ManA-(1,4)-β-D-GlcA-(1,2)-α-D-ManA-(1,]) residues respectively. The C-6 signals from **C**H_2OH groups of xanthan were observed at 63.5–64.6 ppm and 67.3–68.0 ppm for C-6 glucoses residues and C-6 mannoses residues, respectively according to Horton et al. (1985). The full oxidation of the primary hydroxyl groups C-6 ($\underline{C}H_2$-OH) for ([,4)-β-D-Glc-(1,4)-β-D-Glc-(1,] and ([β-D-Man-(1,4)] residues of xanthan (Fig. 2A) were clearly confirmed by the absence of the specific C-6 resonance peaks at around 63.5–68.0 ppm after 60 min of oxidation step (Fig. 2B). Moreover, the spectrum of xanthouronan showed new signals due to the carboxyl group around 178–179 ppm as chemical signature of C-6 oxidation for [→4)-β-D-Glc-(1→] and ([β-D-Man-(1,4)] residues of xanthan.

On the other hand, FT-IR analysis (Fig. 3) was carried out to confirm the production of xanthouronan. Xanthan FT-IR analysis (Fig. 3A) was correlated with literature (Ahuja, Kumar, & Singh, 2012) where the absorption band observed at 3423.92 cm^{-1} was assigned to the stretching frequency of hydroxyl group as well as water adsorption. The signal at 2920.41 cm^{-1} was due to the CH stretching of alkyl group and the signal at 1409.29 cm^{-1} was attributed to the CH bending of methyl group. The signals at 1614.56 cm^{-1} and 1730.04 cm^{-1} were attributed to C=O stretching of alkyl ester and asymmetric stretching of carboxylate. As for xanthouronan, the FT-IR spectrum (Fig. 3B) showed a specific increase in the intensity of the bands at 1707.49 cm^{-1}, which are assigned to the carboxyl groups of ([,4)-β-D-GlcA-(1,4)-β-D-GlcA-(1,] and (β-D-ManA-(1,4)-β-D-GlcA-(1,2)-α-D-ManA-(1,]) residues.

Consequently, the presence of specific signals from oxidized [→4)-β-D-Glc-(1→] and ([β-D-Man-(1,4)] residues as related in ^{13}C NMR and FT-IR analyses have validated the fully oxidation of native xanthan mediated by TEMPO/NaOCl/NaBr system.

As generally described in literature focusing on TEMPO oxidation of natural polysaccharides (Delattre, Chaisemartin, et al., 2012; Delattre, Michaud, Chaisemartin, Berthon, & Rios, 2012; Elboutachfaiti, Delattre, et al., 2011; Isogai & Kato, 1998), this chemistry leads to an increasing of solubility of modified polysaccharides compared to natives ones due to modification of macromolecular conformation and also to alkaline depolymerisation of polymers during the treatment (Delattre et al., 2009; Elboutachfaiti et al.,

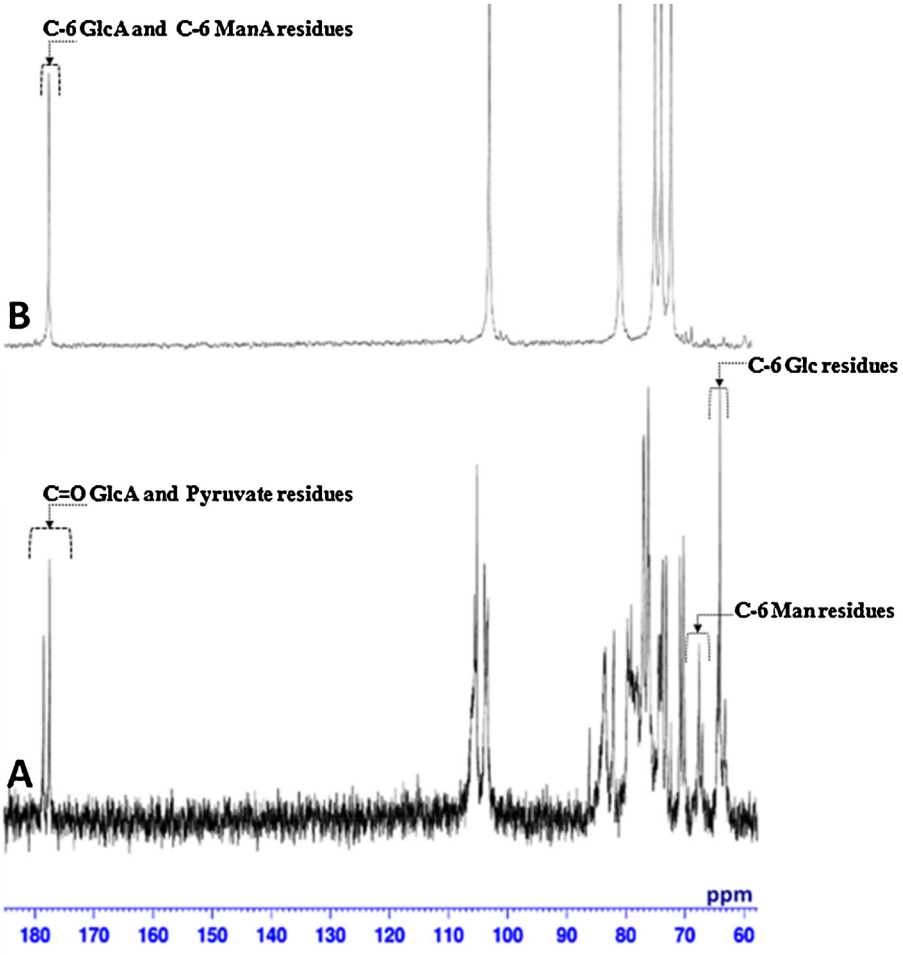

Fig. 2. ^{13}C NMR spectra at 60 °C of (A) xanthan (50 g/L in D_2O) and (B) xanthouronan (50 g/L in D_2O). Glc, Man, GlcA and ManA referred to glucose, mannose, glucuronic acid and mannuronic acid residues respectively.

2010; Elboutachfaiti, Delattre, et al., 2011; Pierre et al., 2013). SEC-MALLS analysis was then carried out in order to estimate the molecular weight of xanthouronan. As summarized in Table 2, the average molecular weight of xanthouronan was evaluated as 5.85×10^5 g/mol which is about one third of the parent xanthan from *Xanthomonas campestris pv campestris* (1.91×10^6 g/mol). This size exclusion chromatography analysis has clearly confirmed that the TEMPO oxidation process of xanthan reduced the molecular

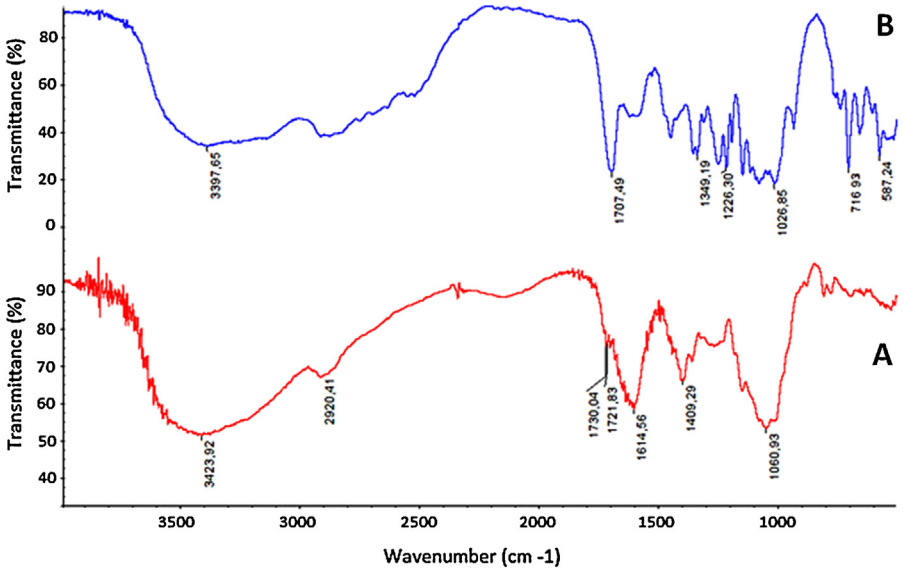

Fig. 3. FT-IR spectra of (A) xanthan and (B) xanthouronan.

Table 2
Macromolecular characterization of xanthan and xanthouronan by SEC-MALLS analyses.

	Water solubility[a]	Mn[b] (g/mol)	Mw[c] (g/mol)	Ip[d]	DP[e]
Xanthan	Soluble	1.86×10^6	1.91×10^6	1.0	2136
Xanthouronan	Soluble	4.51×10^5	5.85×10^5	1.3	665

[a] Evaluated at room temperature after dissolution in water under agitation at 5 g/L.
[b] Number average molecular weight.
[c] Average molecular weight.
[d] Polydispersity index.
[e] Degree of polymerization.

weight and consequently reduced the degree of polymerization (DP). Indeed, according to Eqs. (1) and (2) previously mentioned (Section 2.5), we could then evaluate an average DP of 2136 and 665 for xanthan and xanthouronan respectively.

3.2. Enzymatic depolymerization of xanthouronan

Xanthan depolymerization by various commercial enzymes, enzymatic mixes or new original enzymes from natural or modified microorganisms has been extensively reported (Kool et al., 2013; Li et al., 2009; Nankai, Hashimoto, Miki, Kawai, & Murata, 1999). But to date, enzymatic degradation of the oxidized form of xanthan has not been investigated. Thus, various polysaccharide hydrolases and one polysaccharide lyase were used to depolymerize the xanthouronan. The hydrolysis yields of xanthan and xanthouronan using Celluclast 1.5 L, endocellulase C9422, Glucanex®, Macerozyme R-10, hyaluronidase H3506, alginate lyase E-ALGS and glucuronan lyase are described in Table 3. On the one hand, the primary structure of xanthan and especially its molecular composition, in terms of acetyl and/or pyruvate groups, is of importance for enzyme-based studies (Kool et al., 2013). It is well known that xanthan could be more or less degraded by cellulases depending on this primary structure (Cheetham & Mashimba, 1991). In this way, acetyl and/or pyruvate groups-rich xanthan can be resistant to enzymatic degradation as it was observed with our xanthan (Table 3). On the other hand, previous works showed that oxidation procedure in alkaline media remove acetyl and/or pyruvate groups (Horton et al., 1985), thus we suspected that the xanthouronan form could be potentially depolymerized by enzymes. Previous studies on partly and/or fully oxidized oligo- and polysaccharides, *e.g.* oxidized cellulose and chitosan, were consistent with this hypothesis (Delattre, Michaud, Elboutachfaiti, et al., 2006; Pierre et al., 2013; Westereng et al., 2013). However, we observed that the fully oxidized form of xanthan was highly resistant to polysaccharide hydrolases. Even the glucuronan lyase (EC 4.2.2.14), which catalyses the chemical reaction of eliminative cleavage of (1→4)-β-D-GlcA, did not depolymerize the main backbone of xanthouronan composed of [,4)-β-D-GlcA-(1,4)-β-D-GlcA-(1,]$_n$, probably because of the high degree of substitutions by trisaccharide side chains of (β-D-ManA-(1,4)-β-D-GlcA-(1,2)-α-D-ManA-(1,]) on every second unit of β-D-GlcA. Indeed, glucuronan lyases are very sensitive to surrounding groups, such as acetyl groups, and also need a repeat at least of three recognizing units of (1→4)-β-D-GlcA to start the eliminative cleavage (Da Costa et al., 2001; Delattre, Michaud, Lion, Courtois, & Courtois, 2005; Michaud et al., 1997). Anyway, this result highlights the potential of xanthouronan as a new line of enzymes resistant biopolymers for the generation of antifouling bio-based material. Further experiments on the mechanical properties and physico-chemical applications of our new xanthouronan could be of immediate interest.

Table 3
Final hydrolysis yields of xanthan and xanthouronan using various enzymes.

Enzymes	Enzyme concentrations	Final hydrolysis yields (%) xanthan/xanthouronan
Macerozyme R-10	0.10 U/mL	0/0.18
Glucuronan lyase	3.2×10^{-3}–3.2×10^{-2} U/mL	0/1.25
Celluclast 1.5 L	8.4 U/mL	0.10/0.05
Endocellulase C9422	0.84 U/mL	0.1/0.1
Glucanex®	0.15 BGXU/mL	0.1/0.16
Hyaluronidase H3506	4–10 U/mL	0/0
Alginate lyase E-ALGS	0.25 U/mL	0/0

3.3. Antioxidant activity of xanthouronan

In recent years, extraction or synthesis of polysaccharides with antioxidant capacity has attracted increasing attention for food and pharmaceutical applications (Elboutachfaiti, Petit, et al., 2011; Jin et al., 2012; Paquet et al., 2010; Yan et al., 2014). It was largely described that specific groups such as sulfate, amino, hydroxyl and carboxyl were responsible of the antioxidant effects of polysaccharides (Elboutachfaiti, Petit, et al., 2011; Petit, Delattre, Papy-Garcia, & Michaud, 2005; Sun, Zhu, Xie, & Yin, 2011). Nevertheless, according to previous reports, the antioxidant activity could be related to not only the functional groups of polysaccharides, but also the molecular weight (Chen, Tsai, Huang, & Chen, 2009). In this present study, xanthouronan was then evaluated for its antioxidant activity in order to establish the carboxylate/antioxidant relationships.

3.3.1. Scavenging effect on DPPH radical

The most common method for evaluating the radical scavenging power of polysaccharides is unequivocally the DPPH assay (Elboutachfaiti, Petit, et al., 2011; Yan et al., 2014). It was well-established that the DPPH radical scavenging by antioxidants polysaccharides was related to their hydrogen-donating capacity. In this context, the scavenging capability of xanthouronan on DPPH radical was compared with xanthan and ascorbic acid (Fig. 4.A). Xanthouronan possessed a stronger antioxidant activity than the native xanthan for each concentration tested. It was important to mention that the anti-DPPH radical activities of xanthouronan were lower than ascorbic acid (positive standard) at low concentration (0.1–2.5 g/L) which represented 40–90% of the ascorbic acid antioxidant activity. Nevertheless, for concentration higher than 2.5 g/L of xanthouronan, the DPPH radical scavenging effect of xanthouronan was similar to that of ascorbic acid up to a value close to 98%. This evaluation of anti-DPPH radical properties of xanthouronan, confirmed that the modification of polysaccharides by regioselective oxidation could considerably enhance the antioxidant activities. In fact, as already shown by Elboutachfaiti, Petit, et al. (2011) and confirmed by Yan et al. (2014), it was reported that the presence of carboxylate groups of oxidized polysaccharides could activate the hydrogen atom of the anomeric carbon and then increase their hydrogen donating capability. One of the most commonly hypotheses is the decrease of the intramolecular/intermolecular hydrogen bond of polysaccharide through the addition of carboxylate groups catalyzed by TEMPO/NaOCl/NaBr system, thus promoting its antioxidant properties.

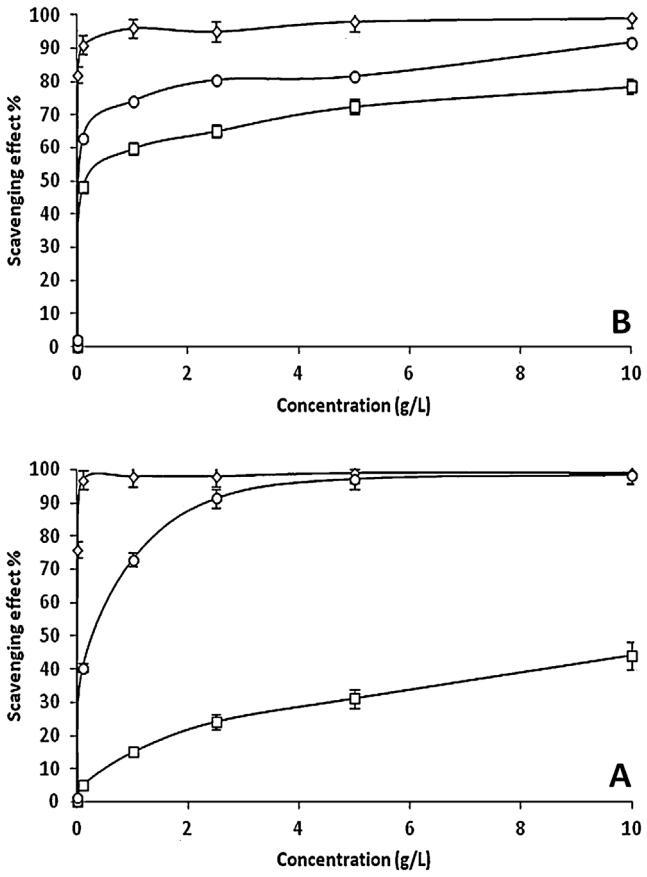

Fig. 4. Scavenging effects on (A) DPPH radical and (B) hydroxyl radical for (□): xanthan; (○): xanthouronan and, (◊): ascorbic acid.

3.3.2. *Scavenging effect on hydroxyl radical*

In our study, the hydroxyl radical scavenging effect of xanthouronan was evaluated by comparison with xanthan and ascorbic acid. As shown in Fig. 4B, all scavenging hydroxyl radical effects were concentration-dependent. The scavenging ability of xanthouronan appeared stronger than xanthan whatever the tested concentration in the range 0–10 g/L. Nevertheless, the difference was not significant since we observed only 10% less for xanthan and the anti-hydroxyl radical properties of xanthouronan were lower than ascorbic acid. For example, at low concentration from 0.1 to 2.5 g/L, the activities reached around 60–80% of the ascorbic acid anti-hydroxyl radical activity. So, the study of anti-hydroxyl radical capability of xanthouronan *versus* xanthan tends to prove that the structural modification of polysaccharide by carboxylation had an effect on the scavenging hydroxyl radical ability, which was in consistent with studies already described for gellan and curdlan oxidation (Elboutachfaiti, Petit, et al., 2011; Yan et al., 2014).

4. Conclusion

In this present study, we have described for the first time, the synthesis of a new family of polyglucuronic acid sodium salt from the oxidation of xanthan catalyzed by TEMPO/NaOCl/NaBr system with a yield of 90%. This oxidation process has shown beneficial effect to improve antioxidant activities of native xanthan allowing to explore xanthouronan as a novel antioxidant agent. Moreover, due to the high molecular weight of xanthouronan, this new polyelectrolyte polysaccharide will be characterized in the near future for its rheological properties.

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