Carbohydrate Research 344 (2009) 1670–1675

Contents lists available at ScienceDirect

Carbohydrate Research

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

Improved isolation of glucuronan from algae and the production of glucuronic acid oligosaccharides using a glucuronan lyase

Elboutachfaiti Redouan [a,*], Delattre Cedric [b,c], Petit Emmanuel [a], El Gadda Mohamed [d], Courtois Bernard [a], Michaud Philippe [b], El Modafar Cherkaoui [d], Courtois Josiane [a]

[a] LPMV, Université de Picardie Jules Verne, IUT-GB Amiens, Avenue des facultés, Le Bailly, F-80025 Amiens Cedex 1, France
[b] LGCB, Université Blaise Pascal-Polytech Clermont Ferrand, 24 rue des Landais, F-63174 Aubière, France
[c] GREENTECH, Biopôle Clermont Limagne 63360 Saint Beauzire, France
[d] LBVPA, Université Cadi Ayyad, Faculté des Sciences et Techniques Guéliz B.P. 549, Avenue Abdelkarim El Khattabi 40 000 Marrakech, Maroc

ARTICLE INFO

Article history:
Received 9 March 2009
Received in revised form 26 April 2009
Accepted 8 May 2009
Available online 7 June 2009

Keywords:
Glucuronan
Green seaweed
Oligosaccharides
Polysaccharide lyase
Glucuronic acid oligosaccharides
Ulva lactuca

ABSTRACT

A new source for the production of bioactive glucuronic acid oligosaccharides (GlcUAOs) from the depolymerization of green seaweed *Ulva lactuca* glucuronan (Algal glucuronan) has been investigated. Algal glucuronan purification was optimized by the acidic precipitation method which allowed us to separate the polysaccharide mixture extracted from the cell wall of *Ulva lactuca* using hot water containing sodium oxalate. A series of the GlcUAOs were obtained by enzyme degradation of algal glucuronan with a glucuronan lyase (GL) isolated from *Trichoderma* strain. The putative bioactive GlcUAOs generated were then purified by size-exclusion chromatography in gram quantity and characterized by $^{1}H/^{13}C$ NMR spectroscopy and ESI-Q/TOF-mass spectrometry.

1. Introduction

Homopolyuronides are anionic polysaccharides presenting many biological interests and industrial applications. It is described in the literature that apart from polygalacturonate and alginate, the structural variability of this class of acidic polysaccharides is poor and low. Consequently, new generation of the homopolyuronides has been developed. Firstly, the emergence of 2,2,6,6-tetramethylpiperidine-1-oxy (TEMPO) chemistry has led to regioselective oxidation of polysaccharides.[1] When applied to cellulose, this specific oxidation of primary alcohol function generated β-(1,4)-D-polyglucuronic acids (synthetic glucuronans).[2] Secondly, the bacterial strain *Sinorhizobium meliloti* M5N1CS was isolated for its ability to excrete variably acetylated β-(1,4)-D-polyglucuronic acids called glucuronans (bacterial glucuronans).[3,4] The interest of the scientific community for this new family of acidic polysaccharides was mostly motivated by its rheological and biological properties. Effectively, this glucuronan form may be employed as gelifying, thickening, hydrating, stabilizing, chelating, or flocculating agent,[5] and also as a biologically active polysaccharide in therapy and agronomy.[5–9] In the two latter cases, low-molecular weight glucuronan and glucuronic acid oligosaccharides (GlcUAOs) seem to be more active than the native polymer. This phenomenon is often described for oligosaccharides showing biological activities.[10]

It is true that bacterial glucuronans appear to have some advantages over the synthetic glucuronans, generally because they are non-toxic, less expensive, and freely available. Moreover, appropriate strains can be genetically modified to acquire a product with desired properties.[11] In addition, several bacterial oligo- and polysaccharides have been reported, while only a few of them have been developed on a commercial scale because of the pathogenic nature of certain producer organisms.[12] For this reason, new sources of natural glucuronan have been explored.

Previous studies have revealed the presence of β-(1,4)-D-poly glucuronic acids (algal glucuronans) in the cell walls of a number of green seaweeds.[13] It was reported that the algal glucuronan was co-extracted together with ulvans, major water-soluble polysaccharides usually extracted from the cell wall of *Ulva* sp. using hot water often containing a calcium chelating agent such as sodium oxalate.[14] This algal glucuronan poses several problems for the fine chemical structure analysis of the ulvans. Thus, in previous studies some authors have successfully used the ion-exchange chromatography to eliminate the algal glucuronan.[14,15] Nevertheless, these chromatographic techniques, which are labor-intensive and time-consuming, pose real obstacles and limit the industrial scale valorization of algal glucuronan.

* Corresponding author. Tel.: +33 3 2253494; fax: +33 3 22956254.
E-mail address: redouan_elboutachfaiti@hotmail.com (E. Redouan).

0008-6215/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2009.05.031

The purpose of this study was to develop a rapid, technically simple, and cost-effective method to separate the ulvans/glucuronans mixture from the most widely distributed green algae (*Ulva lactuca*) extracts using acid precipitation. Moreover, the large-scale production of bioactive GlcUAOs is proposed by enzymatic depolymerization of algal glucuronan using glucuronan lyase (GL).

2. Results and discussion

2.1. Extraction of algal glucuronan

A new procedure (Fig. 1) was developed to efficiently and quantitatively extract the glucuronan from *Ulva lactuca*. By combining oxalate buffer extraction (50 mM, pH 6) at 90 °C for 3 h with acid precipitation, we were able to produce a high yield of pure glucuronan in few reaction steps. Table 1 gives the details of the main fractions extracted according to the procedure developed in our laboratory. The yield of the different polysaccharide-enriched fractions was determined based on the dry weight of the algae. As described in the literature,[14] fraction A corresponds to an ulvan/glucuronan mixture. To separate this mixture simply and rapidly into two distinct polysaccharides, the acid precipitation was employed due to the non-solubility of glucuronan at a lower pH. As we can observe, after sugar and sulfate analysis of fractions A, B, and C that were obtained, the separation of glucuronan and ulvan was confirmed. In fact, the high degrees of purity, estimated at 94% by the uronic acid contents associated with the absence of sulfate groups give evidence for the presence of pure glucuronan in fraction C (Table 1). On the other hand, the uronic acids and neutral sugar ratio in fraction B conform to ulvan structure.[14,15] Extraction yields are in agreement with previous reports concerning the study of water-soluble cell wall polysaccharides from *Ulva* sp.[14] Consequently, the algal glucuronan was obtained in 2.5% yield. It has to be mentioned that contrary to other *Ulva* sp. glucuronan extraction processes, the method presented here does not need any chromatographic purification steps.

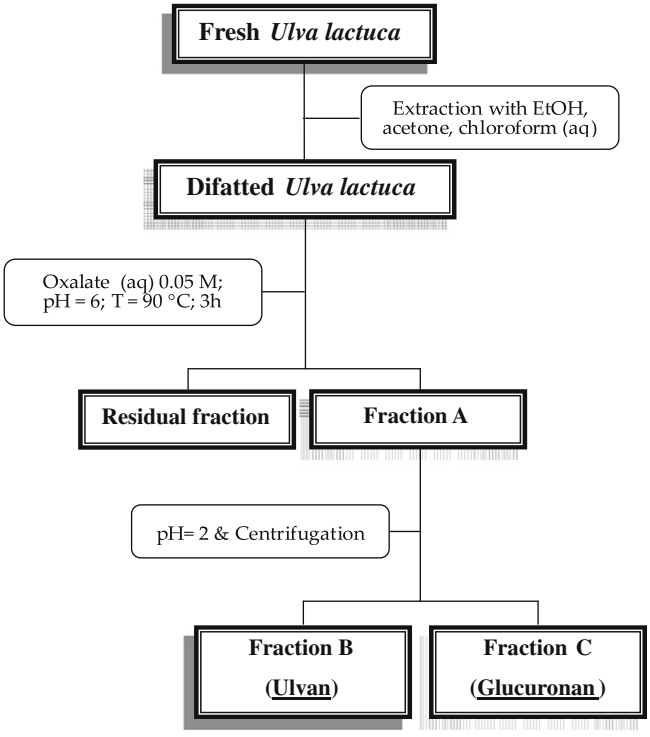

Figure 1. Extraction procedure of ulvans and glucuronans from *Ulva lactuca*.

Table 1
Composition of polysaccharide fraction preparations from *Ulva lactuca*

Fraction	A	B	C
Yields[a]	20	17.5	2.5
Proteins[b]	8	1	4
Neutral sugars[b]	51	60	2
Uronics acids[b]	23	19	94
Sulfates[b,c]	18	20	Nd

Nd = not detected.
[a] Yield of crude polysaccharides, based on milled seaweed.
[b] Based on dry weight of the polysaccharide fraction preparation.
[c] Expressed as SO_3Na.

2.2. ^{1}H NMR analyses of extracted fractions

To confirm the efficiency of the separation process, fractions B and C were analyzed by ^{1}H NMR as shown in Figure 2. Concerning fraction C, five resonance peaks, characteristics of glucuronan, were observed according to the previous work.[16] These signals were attributed to: H-1 (5.25 ppm), H-2 (4.08 ppm), H-3 (4.33 ppm), H-4 (4.42 ppm), and H-5 (4.58 ppm). The NMR analysis

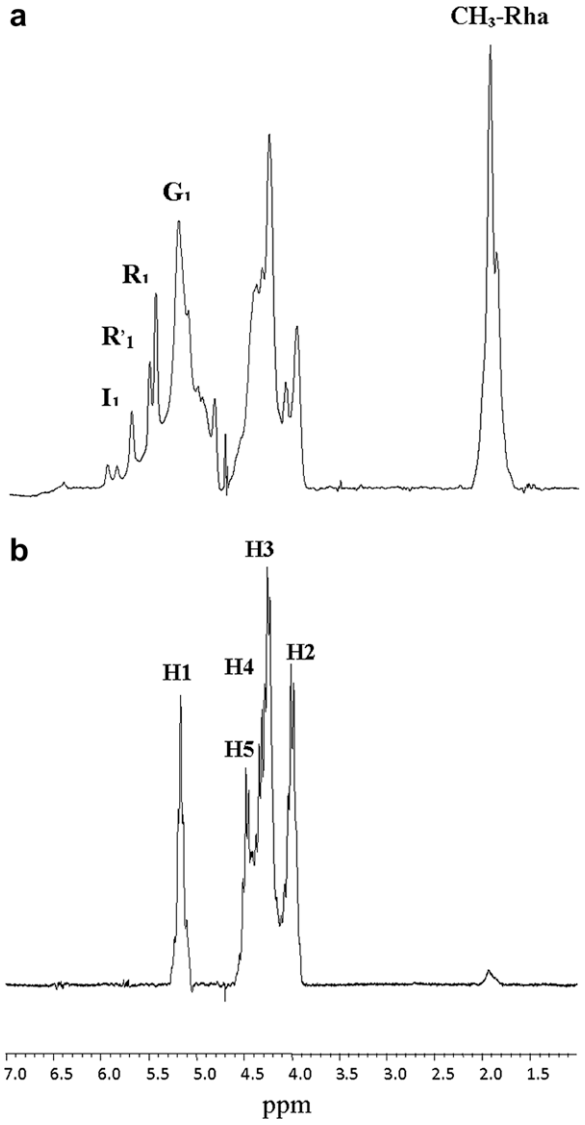

Figure 2. ^{1}H NMR spectra of (a) fraction B and (b) fraction C. HOD refers to the signal of the residual water in the sample.

provides Supplementary data on the presence of glucuronan structure in fraction C. This interpretation is based on the comparison of ^{1}H NMR spectrum presented here with those obtained from bacterial and synthetic glucuronans.[16,17] On the other hand, the ^{1}H NMR spectrum of fraction B shows the presence of a signal at about 1.76 ppm which is a characteristic of the methyl group of rhamnose (CH_3-Rha). The result of this analysis is the first indication of the presence of ulvans, which are mainly composed, in addition to rhamnose, of glucuronic acid, xylose, iduronic acid, and sulfate.[14,18–23] Then, ^{1}H NMR resonance peaks were assigned to ulvans with specific signals at 5.60, 5.61, 5.30, and 5.5 ppm, respectively. These resonance peaks were attributed to H-1 of rhamnose 3-sulfate linked to iduronic acid (R1′), H-1 of iduronic acid (I_1), H-1 of glucuronic acid (G_1), and H-1 of rhamnose linked to glucuronic acid (R_1).[24] This NMR analysis confirms and validates the fact that we have obtained ulvans and glucuronans form after this extraction process, supporting a successful separation of polysaccharides by using acidic precipitation.

2.3. Biodegradation study of algal glucuronan

Having at our disposal an important new source of glucuronan from *Ulva lactuca*, we investigated the biodegradation of this algal glucuronan . As previously described in the literature,[25–28] numerous enzymatic activities have been discovered and employed for the depolymerization of the glucuronan. In this study, we used a glucuronan lyase (GL) from *Trichoderma* isolated from compost using bacterial glucuronan as the sole carbon source.[29]

This GL (4.2.2.14) allows the production of large amounts of GlcUAOs that are acetylated or not acetylated by depolymerization of the bacterial[27,30,31] and synthetic glucuronans.[26,32] They have been induced by an enzymatic β-elimination leading to the formation of 4-deoxy-L-*erythro*-hex-4-enopyranosyluronic acid. Then, biodegradation of algal glucuronan was followed by the measurement of the absorbance at 235 nm and comparison with the bacterial glucuronans and ulvans. Logically, ulvans (fraction B) depolymerization is very low with regard to the evolution of the absorbance at 235 nm. On the contrary, similar activities were observed after 15 min for bacterial and algal glucuronans (data not shown). Therefore, this high level of GL activity on algal glucuronan authorized us to envisage the large-scale production and purification of GlcUAOs as described previously for bacterial and synthetic glucuronans.

Table 2
Time course of relative yields (mass %) of various GlcUAOs

Oligosaccharides	Samples (mass %)			
	12[a]	**24**[a]	**48**[a]	**72**[a]
GlcUA	13.1	15.3	16.5	17.2
GlcUA2	20.8	29.1	33.1	30.5
GlcUA3	28.6	43.2	49.2	52.3
GlcUA4	8.5	3.5	1.2	–
GlcUA5	10.6	6.3	–	–
GlcUA6	5.8	2.6	–	–
GlcUA7	9.2	–	–	–
GlcUA8	3.4	–	–	–

[a] Reaction time (h).

2.4. Large- scale production of GlcUAOs

Algal glucuronan was depolymerized for 72 h by GL in order to generate bioactive GlcUAOs. Fractions were collected each at 12 h and GlcUAOs mixtures were analyzed and quantified. In Table 2 the yield values for each individual oligosaccharide present at different time intervals are resumed. As observed, after 12 h, we produced GlcUAOs with a degree of polymerization (dp) of up to 8 whereas after an incubation period of 72 h with GL activity, the oligosaccharides mixtures were essentially made up of monosaccharides (GlcUA), disaccharides (GlcUA2), and trisaccharides (GlcUA3) with molecular weights of 175, 351, and 527, respectively (Fig. 3). This result conforms with those obtained with bacterial and synthetic glucuronans where the complete biodegradation gave unsaturated disaccharide and trisaccharide as the most abundant final products.[30,32]

The GlcUAOs mixture obtained after 72 h was loaded on Biogel P-2 column to purify the oligosaccharide families (Fig. 4). The chromatograms obtained confirm the results of the mass

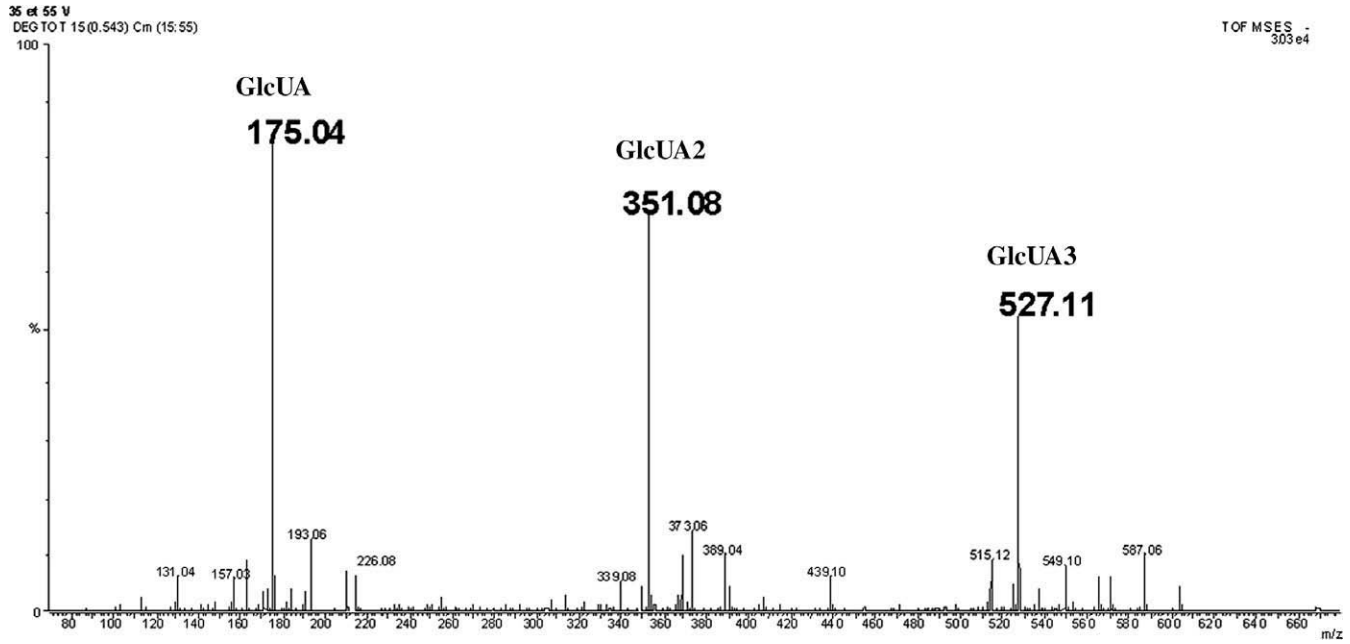

Figure 3. ESI-Q/TOF-mass spectra of GlcUAOs mixture (72 h of incubation).

spectrometry analysis with the detection of two major products (GlcUA2 and GlcUA3). These oligosaccharides were collected and dried under vacuum after five injections on Biogel P-2. Purity and dp of each oligosaccharide were assessed and confirmed by ESI-Q/TOF-mass spectrometry.

Finally, GlcUA2 and GlcUA3 were characterized by NMR spectroscopy. Similar resonances were observed for each oligosaccharide ^{1}H NMR spectrum such as the characteristic doublet at 6.4 ppm (Fig. 5) attributed to H-4 of an unsaturated unit (H-4Δ), and signals at 5.6 ppm, 5.0 ppm, 5.8 ppm, and 5.2 ppm assigned to H-1 of an unsaturated residue (H-1Δ), H-1 of the repeating unit (H-1), and anomeric H-1 protons (H-1α and H-1β).[30] The dp was then confirmed at 2 and 3 for GlcUA2 and GlcUA3, respectively, by comparison of H-1 signal integration of β-Δ-(4,5)-glucuronic acid (H-1Δ) and all H-1 signal integrations (H-1Δ, H-1β, H-1α, and H-1).

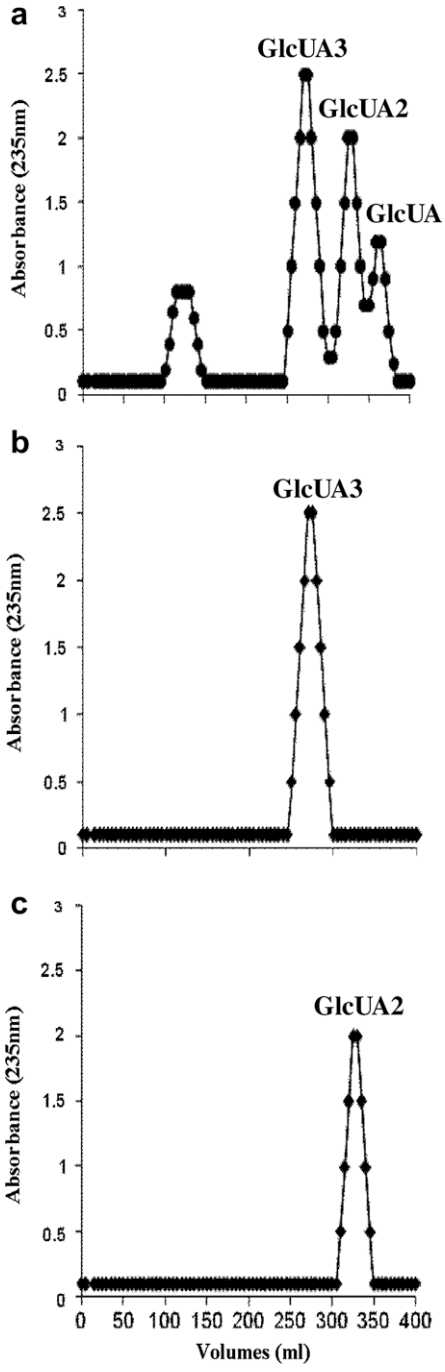

Figure 4. Gel chromatograms of GlcUAOs mixture (72 h of incubation) (a) and each pooled fraction (GlcUA3, GlcUA2) was separately applied to the same Biogel column (b,c).

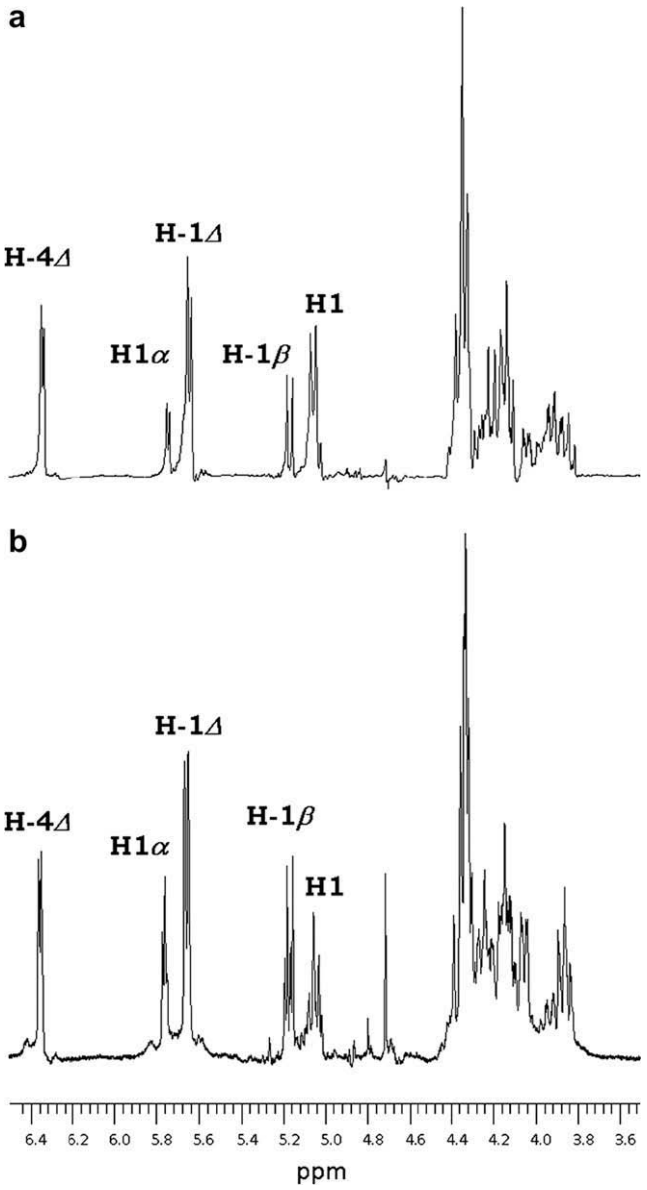

Figure 5. ^{1}H NMR spectra of GlcUA2 (a) and GlcUA3 (b) (20 g/L in D_2O).

The structure information of the products was further confirmed by ^{13}C NMR. A typical pattern for GlcUA2 and GlcUA3 was observed according to the NMR spectrum (Fig. 6). The resonances of the unsaturated oligosaccharides at 178 and 170 ppm were assigned to the carbon of sodium carboxylate at the reducing end, influenced by the α- and β-anomeric structures, and C-6Δ at the non-reducing end, respectively. Obviously the C-4Δ resonance was observed at around 108 ppm which is the characteristic signal of the unsaturated glucuronic acid unit at the non-reducing end. These results show that GlcUA2 and GlcUA3 produced by GL are dimer and trimer, respectively.[28]

Consequently, on the basis of the production yield, we can envisage the large-scale production of pure GlcUAOs by biodegradation of algal glucuronan.

In conclusion, we explored seaweeds such as *Ulva lactuca* as putative natural abundant sources of glucuronan to substitute

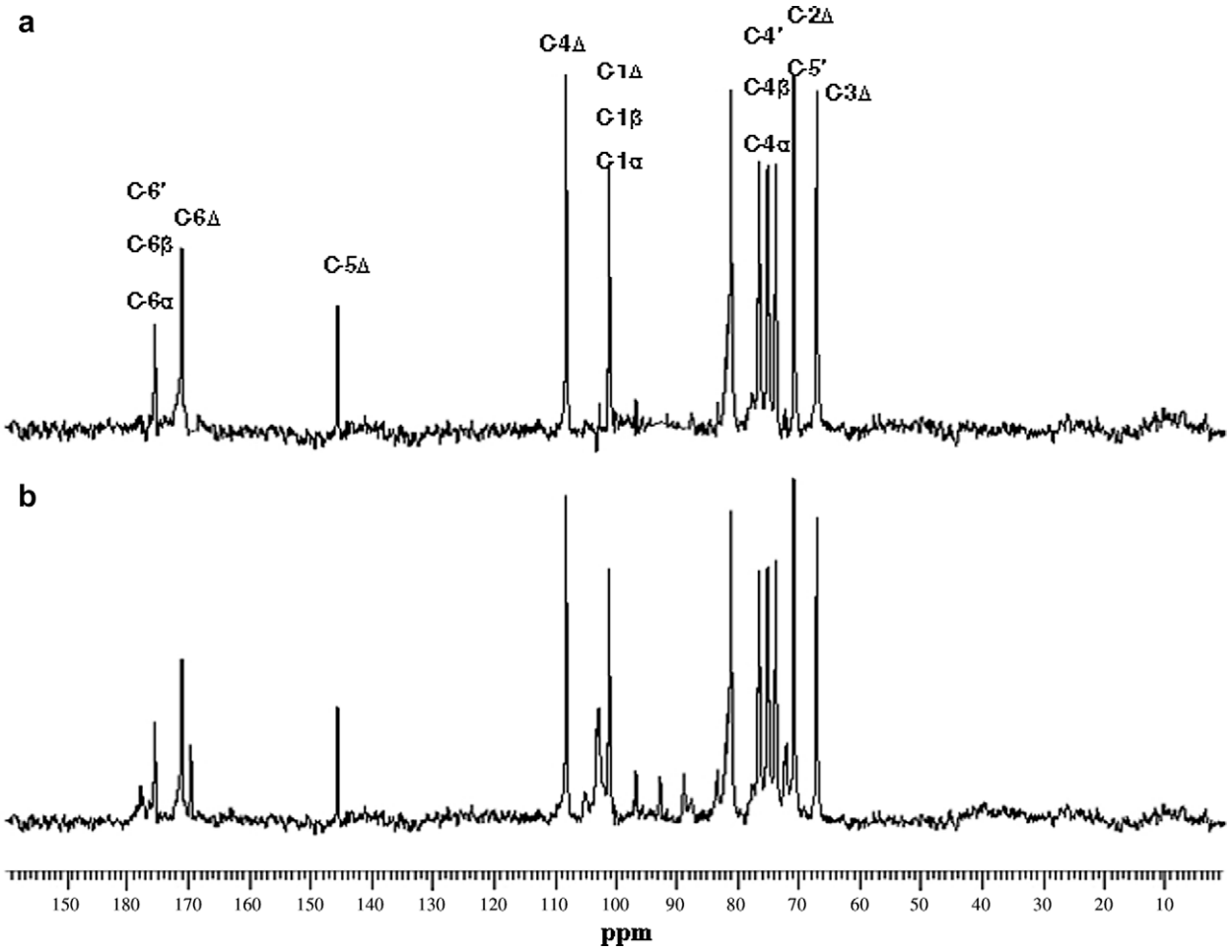

Figure 6. ^{13}C NMR (80 °C) spectra of GlcUA2 (a) and GlcUA3 (b) (100 g/L in D_2O).

bacterial or synthetic production way. We developed a new and fast process to extract the algal glucuronan selectively in large amounts. The major novelty of this method, with respect to the previously reported one, is that it avoids the difficulty of the chromatography step exploiting the precipitating power of glucuronan at a low pH. Disposing of a new and inexhaustive way to produce pure algal glucuronan associated with the high yield of this enzymatic degradation is a good means for the large-scale bioactive GlcUAOs production. In the relatively near future, GL activity from *Trichoderma* sp. could be a good tool for the biodegradability and industrial valorization of *Ulva lactuca*.

3. Experimental

3.1. Isolation and separation of *Ulva lactuca* water-soluble polysaccharides

The method was carried out according to the scheme shown in Figure 1. Fresh seaweeds were pretreated with EtOH, acetone, and chloroform. Samples of defatted algae (100 g) were extracted with hot oxalate solution (3 L, 0,05 M, pH 6, 90 °C, 3 h). The resultant extracts (fraction A) were subjected to acid precipitation. HCl (4 M) was slowly added dropwise to give a final pH of 2 and the precipitate glucuronan was collected (fraction C). The ulvans (fraction B) were precipitated from the supernatant with aqueous EtOH (80%).

The yields of fractions A, B, and C were determined from the dry weight of the algae. These results are given in Table 1.

3.2. Colorimetric assays

The following colorimetric assays were used: The uronic acid (UA) and neutral sugar (NS) contents of fractions A, B, and C were measured in duplicate with both the *meta*-hydroxydiphenyl (*m*-HDP)[33] and the resorcinol micromethods.[34] Rhamnose and glucuronic acid were used as the standards. The content of UA was directly determined with the *m*-HDP test. The content of NS was calculated after correcting the UA interference in the resorcinol assay. Protein concentrations were measured using the Bradford assay with BSA as the standard.[35]

3.3. Sulfate content

For sulfate analysis, the appropriate fractions were quantitatively assayed by turbidity measurement after hydrolysis with HCl (4 M) and addition of gelatin–barium chloride ($BaCl_2$).[36]

3.4. Preparation of crude enzyme fraction

To produce GL activity, *Trichoderma* strain GL2, isolated from compost, was cultured sequentially on *Trichoderma* complete medium and on *Trichoderma* minimal medium supplemented with glucuronan as previously described.[27] After 72 h, the culture was filtered successively through 160 and 0.2 μm filters to remove the mycelia. Extracellular proteins were recovered and concentrated (40-fold) on a 1×10^4 NMWCO (Normal Molecular Weight Cut Off) polyethersulfone membrane (Amicon, Beverly, Mass.) in

a stirred Amicon cell (Amicon). This protein preparation was stored at 80 °C for up to 6 months.

3.5. Enzyme assay

The GL activity was measured by monitoring the increase in absorbance at 235 nm using an Uvikon 930 spectrophotometer (Kontron, Montigny Lebretonneux, France). The reaction mixture was composed of 1 mL of 0.2% (w/v) glucuronan solution in 50 mM potassium acetate buffer (pH 5.5) and an appropriate volume (5–10 μL) of enzyme preparation equivalent to 2 U per gram of glucuronan. One unit (U) of the enzyme activity was defined as the GL amount required to cause an increase in the absorbance by 1 at 235 nm in a minute under the above conditions.

3.6. Production of GlcUAOs

Solutions of algal glucuronan (100 mL, 20 g/L) in 50 mM potassium acetate buffer (pH 5.5) were incubated at 20 °C with the enzymatic preparation (6 U per gram of glucuronan) for various reaction times (12, 24, 48, and 72 h). After incubation, the GL activity was stopped by dipping the reaction medium into a 100 °C water bath. The hydrolysis mixtures were then centrifuged (15000*g*, 15 min, 20 °C) and the supernatants (containing GlcUAOs) were recovered.

3.7. Purification of GlcUAOs

All the purifications were performed at room temperature using low-pressure liquid chromatography system (Proteam LC system 210, Lincoln, NE). The GlcUAOs were size-fractionated by low-pressure gel-permeation chromatography on a Biogel P-2 and P-6 fine (Biorad) column (2.6 × 100 cm, Amersham Bioscience). Each GlcUAOs mixture was loaded (100–500 mg in 10 mL) and eluted with a 50 mM ammonium formate solution at a flow rate of 0.8 mL/min. Detection was achieved with a UV detector (UA-6 from ISCO) at 254 nm and with a RI detector (Melz). Fractions (5 mL) were collected with a Foxy 200 (ISCO) collector. Fractions belonging to the same peak were pooled and freeze-dried.

3.8. Nuclear magnetic resonance (NMR) spectroscopy

NMR analyses were performed at 80 °C with a Bruker Avance 300 spectrometer of 300 MHz equipped with a $^{13}C/^{1}H$ dual probe according to the conditions described previously.[14] The NMR experiments were recorded with a spectral width of 3000 Hz, an acquisition time of 1.36 s, a pulse width of 7 s, a relaxation time of 1 s, and a number of 256 scans. The HOD signal was presaturated by a presaturation sequence. All samples were previously dissolved in D_2O (99.9% D) and lyophilized to replace exchangeable protons with deuterium. The lyophilized samples were then dissolved in D_2O at a 10–100 g/L concentration.

3.9. Mass spectrometry

The GlcUAOs mixture (72 h incubation) was analyzed by Electrospray Ionization Quadrupole Time-of-flight Mass Spectrometry (ESI-Q/TOF-MS). Experiments were carried out on a Q-TOF Ultima global (Micromos-waters) equipped by electrospray source (Z-spray) using negative and positive modes for ionization. The source temperature and desolvation gas (N_2) temperature were 80 and 150 °C, respectively. Gas flows used for cone and desolvatation were 50 and 350 L/h, respectively.

Samples were diluted in ultra high quality water (UHQ-water) and injected into electrospray source at a flow rate of 5 μml/min with a capillary tension of 2.5 kV and a cone tension of 35–75 V. Spectra were accumulated at 2 s/scan. The mass range was scanned from 50 to 2050 atomic mass units (amus). All acquisitions were recorded in the negative mode and treated by MassLynx V 4.0 software.

Acknowledgment

Financial support for this work was provided by the French-Moroccan cooperation (PRAD 05-13 and PAI Volubilis MA/05/135).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2009.05.031.

References

1. De Nooy, A. E. J.; Besemer, A. C.; van Bekkum, H. *Carbohydr. Res.* **1995**, *269*, 89–98.
2. Isogai, A.; Kato, Y. *Cellulose* **1998**, *5*, 153–164.
3. Heyraud, A.; Courtois, J.; Dantas, L.; Colin-Morel, P.; Courtois, B. *Carbohydr. Res.* **1993**, *240*, 71–78.
4. Courtois, J.; Séguin, J. P.; Declomesnil, S.; Heyraud, A.; Colin-Morel, P.; Dantas, L.; Barbotin, J. N.; Courtois, B. *J. Carbohydr. Chem.* **1993**, *12*, 441–448.
5. Tavernier, M. L.; Delattre, C.; Petit, E.; Michaud, P. *Open Biotechnol. J.* **2008**, *2*, 73–86.
6. Courtois-Sambourg, J.; Courtois, B. FR. Patent 2781673, 2000.
7. Lintner, K. FR. Patent 2768335, 1999.
8. Petit, E.; Papy-Garcia, D.; Muller, G.; Courtois, B.; Caruelle, J. P.; Courtois, J. *Biomacromolecules* **2004**, *5*, 445–452.
9. Lienart, Y.; Heyraud, A.; Sevenou, O. U.S. Patent 2006270844, 2006.
10. Delattre, C.; Michaud, P.; Courtois, B.; Courtois, J. *Minerva Biotechnol.* **2005**, *17*, 107–117.
11. Khan, T.; Hyun, S. H.; Park, J. K. *Enzyme Microb. Technol.* **2007**, *42*, 89–92.
12. Sutherland, I. W. *Microbiology* **2001**, *147*, 3–9.
13. Ray, B. *Carbohydr. Polym.* **2006**, *66*, 408–416.
14. Ray, B.; Lahaye, M. *Carbohydr. Res.* **1995**, *274*, 251–261.
15. Ray, B.; Lahaye, M. *Carbohydr. Res.* **1995**, *274*, 313–318.
16. Dantas, L.; Courtois, J.; Courtois, B.; Séguin, J. P.; Gey, C.; Heyraud, A. *Carbohydr. Res.* **1994**, *265*, 303–310.
17. Fraschini, C.; Vignon, M. R. *Carbohydr. Res.* **2000**, *328*, 585–589.
18. Lahaye, M.; Jegou, D. *J. Appl. Phycol.* **1993**, *5*, 195–200.
19. Lahaye, M.; Ray, B.; Baumberger, S.; Quemener, B.; Axelos, M. A. V. *Hydrobiologia* **1996**, *326*, 473–480.
20. Lahaye, M.; Brunel, M.; Bonnin, E. *Carbohydr. Res.* **1997**, *304*, 325–333.
21. Lahaye, M.; Inizan, F.; Vigouroux, J. *Carbohydr. Polym.* **1998**, *36*, 239–249.
22. Lahaye, M.; Cimadevilla, E. A. C.; Kuhlenkamp, R.; Quemener, B.; Lognoné, V.; Patrick-Dion, P. *J. Appl. Phycol.* **1999**, *11*, 1–7.
23. Quemener, B.; Lahaye, M.; Bobin-Dubigeon, C. *J. Appl. Phycol.* **1997**, *9*, 179–188.
24. Chang, P. S.; Robyt, J. F. *J. Carbohydr. Chem.* **1996**, *15*, 819–830.
25. Da costa, A.; Michaud, P.; Petit, E.; Heyraud, A.; Colin-Morel, P.; Courtois, B.; Courtois, J. *Appl. Environ. Microbiol.* **2001**, *67*, 5197–5203.
26. Konno, N.; Habu, N.; Maeda, I.; Azuma, N.; Isogai, A. *Carbohydr. Polym.* **2006**, *64*, 589–596.
27. Delattre, C.; Michaud, P.; Keller, C.; Courtois, B.; Courtois, J. *Biotechnol. Prog.* **2005**, *21*, 1775–1781.
28. Konno, N.; Habu, N.; Maeda, I.; Azuma, N.; Isogai, A. *Cellulose* **2008**, *15*, 453–463.
29. Michaud, P.; Delattre, C.; Courtois, B.; Sambourg-courtois, J. FR. Patent 2885911, 2006.
30. Delattre, C.; Michaud, P.; Lion, J. M.; Courtois, B.; Courtois, J. *J. Biotechnol.* **2005**, *118*, 448–457.
31. Delattre, C.; Michaud, P.; Courtois, J.; Courtois, B. *Enzyme Microb. Technol.* **2007**, *41*, 250–257.
32. Delattre, C.; Michaud, P.; Elboutachfaiti, R.; Courtois, B.; Courtois, J. *Cellulose* **2006**, *13*, 63–71.
33. Van den Hoogen, B. M.; van Weeren, P. M.; Lopes-Cardozo, M.; van Golde, L. M. G.; Barneveld, A.; van de Lest, C. H. A. *Anal. Biochem.* **1998**, *257*, 107–111.
34. Monsigny, M.; Petit, C.; Roche, A. C. *Anal. Biochem.* **1998**, *175*, 525–530.
35. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248–254.
36. Dodgson, K. S.; Price, R. G. *Biochem. J.* **1962**, *84*, 106–110.