Carbohydrate Polymers 83 (2011) 277–283

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Structural analysis of the degradation products of porphyran digested by *Zobellia galactanivorans* β-porphyranase A

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ARTICLE INFO

Article history:
Received 14 June 2010
Received in revised form 28 June 2010
Accepted 28 July 2010
Available online 6 August 2010

Keywords:
Porphyran
Porphyranase
β-Agarase
Algal cell wall
Glycoside hydrolase

ABSTRACT

Porphyran from the red seaweed *Porphyra umbilicalis* was degraded with β-porphyranase A derived from the marine flavobacterium *Zobellia galactanivorans*. β-Porphyranase A produces a new series of sulfated oligosaccharides that we characterized by NMR, HPAEC and size-exclusion chromatography. In contrast to previously used β-agarases, which produce predominantly non-sulfated oligosaccharides of the neoagarobiose series and hybrid oligosaccharides, β-porphyranase A produced oligosaccharides of the α-L-Gal*p*-6-sulfate (1 → 3) ß-D-Gal*p* (L6S-G) series and the L6S-G disaccharide was the major final product. The newly identified β-porphyranase A was further used in combination with β-agarase B to revise porphyran polysaccharide structure.

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1. Introduction

The cell wall of numerous red algae (Rhodophyta) is composed of sulfated galactans (Craigie, 1990). These linear polysaccharides consist of alternating 3-linked beta-D-galactose (G) and 4-linked alpha-galactose (D or L). The disaccharide repetition moiety of carrageenans is composed of two D-galactose residues (G-D), whereas in agarans the alpha-galactose belongs to the L-series (G-L). According to the nomenclature introduced by Knutsen, Myslabodski, Larsen, and Usov (1994), carrageenan (G-D) and agaran (G-L) are distinct from carrageenose (G-DA) and agarose (G-LA), respectively, which differ by the occurrence of a 3,6-anhydro-α-D/L-galactose. The galactan backbones are often masked by ester sulfate groups (S), methyl groups (M) or pyruvic acid acetal groups (P), thereby increasing the number of possible carrabiose and agarobiose structures (Lahaye, Yaphe, Viet, & Rochas, 1989; Usov, 1998; van de Velde, Knutsen, Usov, Rollema, & Cerezo, 2002).

Porphyran refers to a water-soluble agaran extracted from the cell wall of red seaweeds belonging to the genus *Porphyra* (Anderson & Rees, 1965). Unlike agarose and carrageenan, porphyran is not utilized as ingredient for its gelling or texturizing properties. However, it is one of the most consumed algal polysaccharides because it is the main component of edible *P. yezoensis* (susabinori) and *P. tenera* (asakusanori), which are extensively used in the preparation of sushi (Nisizawa, Noda, Kikuchi, & Watanabe, 1987; Fukuda et al., 2007). As other sulfated galactans, porphyran may also have some potential pharmacological applications (Pomin & Mourao, 2008), based on, for example, its hypolipidemic (Inoue et al., 2009) and anti-allergic properties (Ishihara, Oyamada, Matsushima, Murata, & Muraoka, 2005).

Extensive structural analysis of the oligosaccharides produced after incubation of porphyran with β-agarases has demonstrated its hybrid copolymer structure (Morrice, McLean, Long, & Williamson, 1983, 1984). The backbone is composed of about 30% of agarose repetition moieties (LA-G) with the remaining moieties being 4-linked α-L-galactopyranose-6-sulfate (L6S) and 3-linked ß-D-galactopyranose (G) residues (Fig. 1). In addition, the galactan backbone is masked by methyl ether groups (M) at the C6 position of the G residues whether it is linked to LA or L6S units. The L6S residue of porphyran is cyclisized into a 3,6-anhydro-ring by hot alkaline treatment, producing a methylated agarose (Noseda, Viana, Duarte, & Cerezo, 2000). *In vivo*, the formation of the 3,6-anhydro-ring is catalyzed by the galactose-6-sulfurylase enzyme (EC 2.5.1.5, Rees, 1961a, 1961b), giving insight into the biosynthetic relationship between the L6S-G and the LA-G moieties.

Structural analysis of carrageenans has greatly benefited from the use of κ-, ι- and λ-carrageenases, which have made it possible to dissect the hybrid nature of these complex polysaccharides (Guibet et al., 2007, 2008; Jouanneau et al., 2010; Michel, Helbert, Kahn, Dideberg, & Kloareg, 2003). In contrast, the currently

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doi:10.1016/j.carbpol.2010.07.060

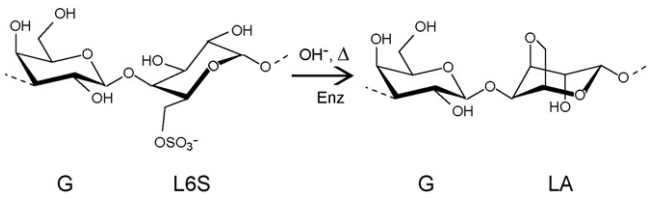

Fig. 1. Structure of the G-L6S and G-LA (agarobiose) repetition moieties encountered in porphyran. The L6S residues are converted into 3,6-anhydro-ring in hot alkaline solution or by the action of galactose-6-sulfurylase.

available enzymes for the structural analysis of agarans are limited in specificity, since only agarose-degrading enzymes, α- or β-agarases depending on the configuration of the glycosidic bond they cleave, have been reported so far (Michel, Nyvall-Collen, Barbeyron, Czjzek, & Helbert, 2006). The use of β-agarases has been helpful in revealing the structural diversity of agarose-containing polysaccharides, such as porphyrans; however these investigations are based on the characterization of degradation products of low abundance that are not representative of the overall polysaccharide structure (Lahaye, 2001; Lahaye et al., 1989). In this context, an in-depth analysis of the composition, as well as the distribution of the various substituted moieties along the hybrid polysaccharide chain would greatly benefit from new enzymes with other specificities.

The marine bacterium *Zobellia galactanivorans* has been isolated from the surface of the red algae *Delesseria sanguinea* for its ability to grow in minimal medium-containing agar, with κ- or ι-carrageenans as its sole carbon source (Barbeyron et al., 2001). Genome analysis has revealed numerous genes belonging to family 16 glycoside hydrolases (GH16) that have strong similarities with the known *Z. galactanivorans* β-agarases, and also some closely related enzymes that form new gene clusters with different specificities (Hehemann et al., 2010). The corresponding recombinant proteins have been shown to be inactive on standard agarose (LA-G), but highly specific to porphyran degradation through the cleavage of the L6S-G linkages. Here, we apply this recent discovery to revise the structure of porphyran from *P. umbilicalis* by characterizing porphyran degradation products using the new recombinant β-porphyranase A (Hehemann et al., 2010) together with β-agarase B (Jam et al., 2005) from *Z. galactanivorans*.

2. Experimental

2.1. Purification of Porphyra umbilicalis porphyran

P. umbilicalis was collected in spring 2008 in the intertidal zone of Perharidy Point (Roscoff, France). The specimens were extensively washed in freshwater and cleaned of sand and epiphytes. They were dried in an oven at 60 °C. Dried material (100 g) was ground with a blender to a fine powder. The resulting powder was kept for 15 h in 1 L 7.5% (v/v) formalin/water solution. Then, an equal volume of water was added and the suspension was boiled under reflux for 8 h. This suspension was filtered through a sieve to remove most algal fragments. A clear solution was obtained after a centrifugation at 10,000 × *g* for 30 min at 20 °C and filtration through diatomaceous earth followed by a second filtration through activated carbon. The pH of the solution was adjusted to pH 7.5 with NaOH (1 M). The volume of the sample was reduced to about 1/6 of the starting volume by rotary evaporation (65 °C). Polysaccharides were precipitated by adding 4 volumes of pure methanol, and maintained at 4 °C overnight. The precipitate was recovered by filtration and extensively washed with pure methanol and finally with acetone, prior to air drying. This porphyran preparation will be hereafter referred to as native porphyran.

2.2. Purification of the enzyme

Recombinant β-porphyranase A from *Z. galactanivorans* was prepared as previously described (Hehemann et al., 2010). Briefly, *Escherichia coli* BL21(DE3) cells, which carried the PFO4 plasmid containing the β-porphyranase A gene, were grown to high density (Studier, 2005). The His-tagged porphyranase was purified from about 50 mL of the cell supernatant by immobilized metal-affinity chromatography on 10 mL of a nickel-charged IMAC HyperCell resin column (Pall Corporation). The protein was eluted with a linear gradient of 0–500 mM nickel sulfate and fractions which corresponded to the major eluted peak were analyzed by SDS-PAGE. The protein was concentrated to a volume of ∼5 mL by ultra-filtration on an Amicon membrane (polyethersulfone, 30 kDa cutoff). For final protein polishing, a Sephacryl S-200 column (GE Healthcare) pre-equilibrated with buffer C at a flow rate of 1 mL min^{-1} was used. The purified enzyme was concentrated to ∼3 mg mL^{-1} by ultra-filtration on an Amicon membrane (10 kDa cutoff). All chromatographic steps were carried out on an ÄKTA Explorer Chromatography system (GE-Healthcare) at 20 °C. β-Agarase B was produced as previously described (Jam et al., 2005).

2.3. Enzymatic degradation

Porphyran (1%, w/v, in de-ionized water) was incubated for 12 h at 30 °C with pure β-porphyranase A at an enzyme concentration of 0.15 μg mL^{-1}. Degradation kinetics were followed using the reducing-end sugar assay and size-exclusion chromatography (SEC). The completion of the enzyme reaction was verified by adding of more enzyme to check that no further degradation could be induced. Degradation of porphyran with β-agarase B (0.15 μg mL^{-1}) was conducted following the same experimental conditions.

The degradation of agarose by β-agarase B was adapted from Jam et al. (2005). Agarose (0.5%, w/v, Eurogentec) was melted in de-ionized water at 80 °C for 30 min and then kept at 40 °C to prevent gelation. β-Agarase B was added at 1 μg mL^{-1} for an incubation of 7 h at 40 °C.

2.4. Analytical size-exclusion chromatography

Analytical size-exclusion chromatography was conducted with an Ultimate 3000 (Dionex) chromatography system using a Optilab Tex refractive index detector (Wyatt). After filtration through 0.45 μm filter membrane, 250 μL of sample was injected on two analytical columns, a Superdex 200 (10/300) and a Superdex Peptide HR (10/300) mounted in series (GE Healthcare). Elution was carried out with a 100 mM ammonium carbonate [$(NH_4)_2CO_3$] solution at a flow rate of 0.3 mL min^{-1}. All data were recorded with the Chromeleon software (Dionex).

2.5. Purification of standard and hybrid oligosaccharides

The freeze-dried hydrolysis product was dissolved in de-ionized water at a concentration of 4% (w/v). After filtration through 0.45 μm, 4 mL of sample was injected. The purification of porphyran oligosaccharides was carried out by preparative SEC with three Superdex 30 (26/60) (GE Healthcare) columns in series, integrated on a HPLC system liquid injector/collector (Gilson). Detection was carried out using a refractive index detector (Spectra System RI-50). The Gilson system and the detector data were monitored by Unipoint software (Gilson). Ammonium carbonate [$(NH_4)_2CO_3$] at 50 mM was used as a running buffer at a flow rate of 1.5 mL min^{-1} for 650 min. Fractions of oligosaccharides were collected and frozen until further analysis. The fractions were subsequently freeze-dried

before being analyzed by electrophoresis techniques and nuclear magnetic resonance (NMR).

2.6. High-performance anionic-exchange chromatography-pulsed amperometric detection (HPAEC-PAD)

For high-resolution oligosaccharide analysis, the oligosaccharide fractions (filtered on 0.22 μm) were analyzed with a Dionex system (ICS2500), equipped with a Carbopac PA100 (4/250 mm) column associated with a PA100 guard column. The system was equilibrated in 150 mM NaOH and the elution of samples (20 μL) was performed at a flow rate of 0.5 mL min^{-1} with 150 mM NaOH and a step gradient from 0 to 1 M sodium acetate (0–5 min = 0–60%; 5–10 min = 60–100%; 10–30 min = 100%). Oligosaccharide detection was carried out with an electrochemical detector (gold electrode) and data were recorded using Chromeleon Peak Net software.

2.7. NMR spectroscopy

All samples were solubilized in D_2O and exchanged twice, before being analyzed on the 500 MHz Bruker NMR spectrometer (Service de RMN, University Bretagne Occidentale, Brest, France). For the porphyran polysaccharide, proton NMR spectra were recorded at 70 °C using 64 scans. The oligosaccharide spectra were recorded at 25 °C using 16 scans. Chemical shifts are expressed in ppm in reference to the external standard TSP (trimethylsilylpropionic acid). The NMR signals of the porphyran disaccharide were fully assigned, using a complete set of correlation spectrums: COSY (double quantum-filtered correlation spectroscopy), HMQC (heteronuclear multiple quantum correlation) and HMBC (heteronuclear multiple bond correlation). Other oligosaccharides were characterized using the same techniques and compared to the structure obtained for the porphyran disaccharide.

3. Results

3.1. NMR characterization of porphyran

Porphyran was extracted from the cell wall of *P. umbilicalis* in boiling water, yielding about 12–15% polysaccharide with respect to the dry weight of algae. The ^{1}H NMR spectrum of this polysaccharide (Fig. 2) showed the characteristic downfield signals at 5.19 and 5.30 ppm from the α-anomeric proton of the 4-linked 3,6-anhydro-alpha-L-galactose (LA-H1) and α-L-galactopyranose-6-sulfate (L6S-H1) (Maciel et al., 2008; Murano, 1995; Zhang et al., 2005). Relative amounts of the two repeating moieties, L6S-G or LA-G, were deduced by integrating the signal of the α-anomeric protons. The measured L6S-H1/LA-H1 ratio of 1:0.4 accounts for about one-third of LA-G and two-thirds of L6S-G repeating moieties (Fig. 2A). After incubation with β-agarase B, almost all LA-G moieties were eliminated and only the L6S-H1 signal remained (Fig. 2B). Agars and porphyran have previously been shown to carry methyl ether groups (Morrice et al., 1983; Lahaye et al., 1989), which were detected in the 3.5–3.40 ppm region of ^{1}H NMR spectra. In the case of the porphyran described here, two signals resonating at 3.46 and 3.44 ppm were clearly visible. However, after the β-agarase B treatment, our porphyran was predominantly composed of L6S-G moieties and contained almost exclusively methyl ether groups at 3.44 ppm.

3.2. Chromatographic analysis of enzymatic degradation products

The enzymatic degradation of porphyran by *Z. galactinovorans* β-porphyranase A was monitored both by assaying the production

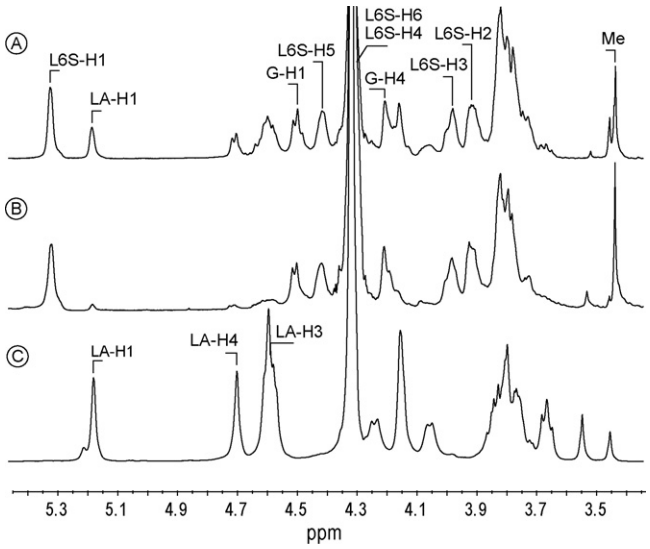

Fig. 2. ^{1}H NMR (500 MHz) spectra of porphyran from *Porphyra umbilicalis*. (A) Native porphyran without enzymatic pre-treatment. (B) High-molecular-weight fraction obtained by digestion of native porphyran with β-agarase B. (C) NMR spectrum of agarose. The characteristic anomeric protons of the G-L6S and G-LA moieties are indicated, as well as the signal characteristic of the L6S-G moiety.

of reducing ends and by chromatography. The amount of reducing sugars produced after incubation with β-prophyranase was about twice the amount released after degradation with β-agarase. In addition, the rate of cleavage was faster with the porphyranase, since completion of polysaccharide degradation was reached in about 1 h, while 6 h were necessary with the β-agarase (not shown).

When porphyran was completely digested with β-porphyranase A, several peaks could be separated by SEC and appeared between 450 and 550 min (Fig. 3A). A low amount of undigested high-molecular-weight fraction eluting at 220–250 min, which was not further characterized here, shows that almost all polysaccharides were fragmented into oligosac-

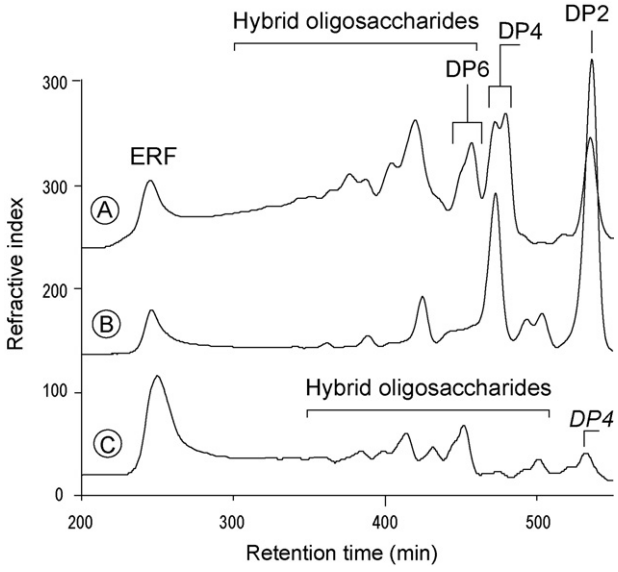

Fig. 3. Size-exclusion chromatography of the reaction products obtained by digestion of native and β-agarase B pre-treated porphyran with β-porphyranase A. (A) Chromatogram of native porphyran incubated with β-porphyranase A. (B) Chromatogram of β-agarase B pre-treated porphyran digested with β-porphyranase A. (C) Chromatogram of the reaction products of native porphyran digested with β-agarase B. DP2, DP4 and DP6 designate fractions of di-, tetra- and hexasaccharides, respectively. *DP4* refers to neo-agarotetraose. ERF: enzyme-resistant fraction.

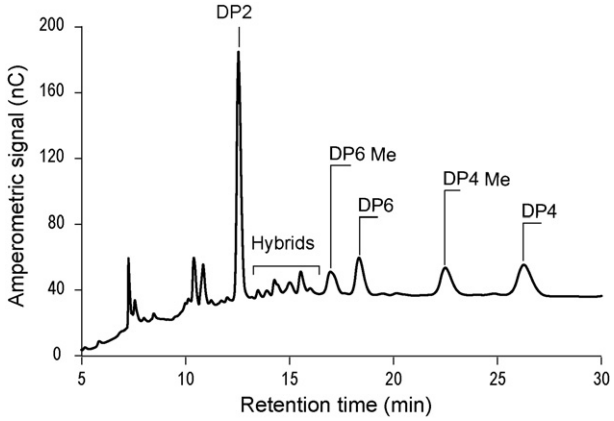

Fig. 4. Anion-exchange chromatography of the reaction products obtained by degradation of native porphyran incubated with β-porphyranase A. DP2, DP4 and DP6 designate (L6S-G), $(L6S\text{-}G)_2$ and (L6S-G–LA-G–L6S-G) oligosaccharides, respectively. Me: methylated oligosaccharides.

charides. To remove LA-G moieties and to reduce the hybrid nature of the porphyran, we used β-agarase B and pre-digested the porphyran. The resistant fraction enriched in L6S-G moieties was then used as substrate for β-porphyranase A. β-Porphyranase A enzymatic digestion of the enriched L6S-G porphyran led to a simplified chromatogram (Fig. 3B), indicating that the complexity of the chromatogram of the unmodified porphyran (Fig. 3A) was correlated with the co-occurrence of LA-G and L6S-G moieties in the polysaccharide. Consequently, the additional peaks in Fig. 3A were assigned to oligosaccharides having a hybrid structure. The chromatogram of the digestion of porphyran with β-agarase B (Fig. 3C) clearly shows a different peak profile, lower peak sizes and

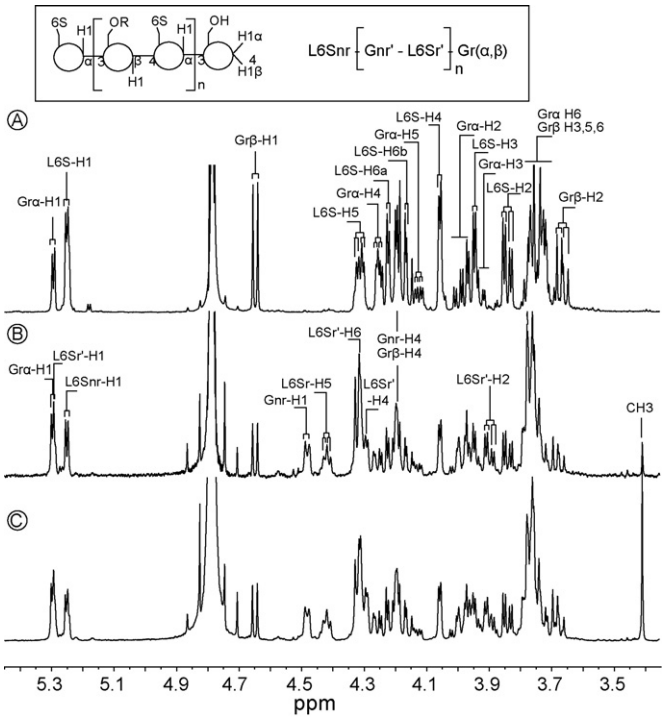

Fig. 5. ^{1}H NMR (500 MHz) spectra of disaccharide and tetrasaccharide of the L6S-G series. (A): L6S-G disaccharide; (B): non-methylated $(L6S\text{-}G)_2$ tetrasaccharide; and (C): methylated $(L6S\text{-}G)_2$ tetrasaccharide. Assignments of the disaccharide are indicated in (A) and the assignments of the internal G-L6S moiety of the tetrasaccharide in (B). Inset: schematic structure of the standard $(L6S\text{-}G)_n$ oligosaccharide series showing H-1 protons and the methyl group position R.

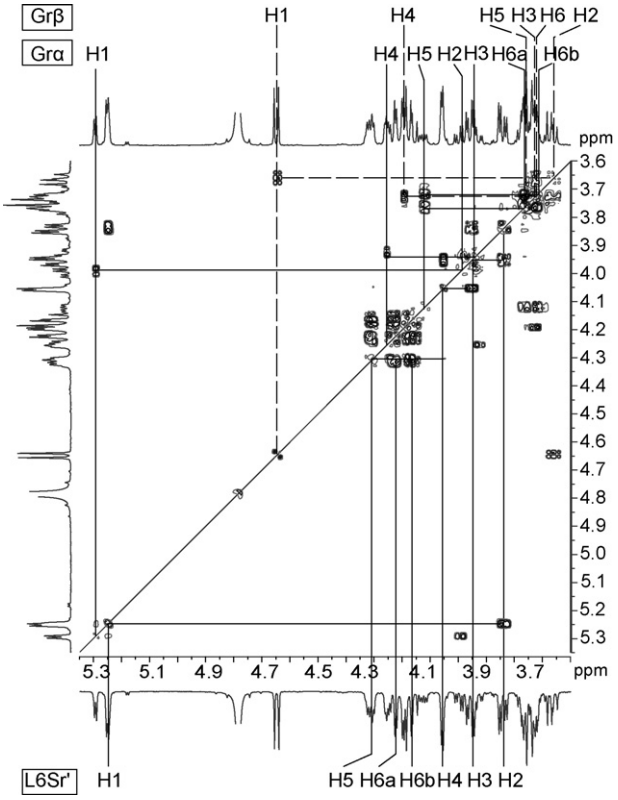

Fig. 6. . ^{1}H COSY (500 MHz) spectrum of the disaccharide (L6S-G) at 25 °C. Correlation systems of the protons belonging to the L6S and G residues are drawn.

a pronounced peak corresponding to the L6S-G-enriched resistant fraction.

The most abundant oligosaccharides were purified by preparative SEC at the mg scale and their purity was estimated to be in the range of 90–95% by HPAEC (data not shown). The oligosaccharides that correspond to the different peaks were analyzed by ^{1}H NMR (Figs. 5 and 7, for analysis see below). As shown in Fig. 3B, we identified the two major peaks as DP2 and DP4, composed solely of

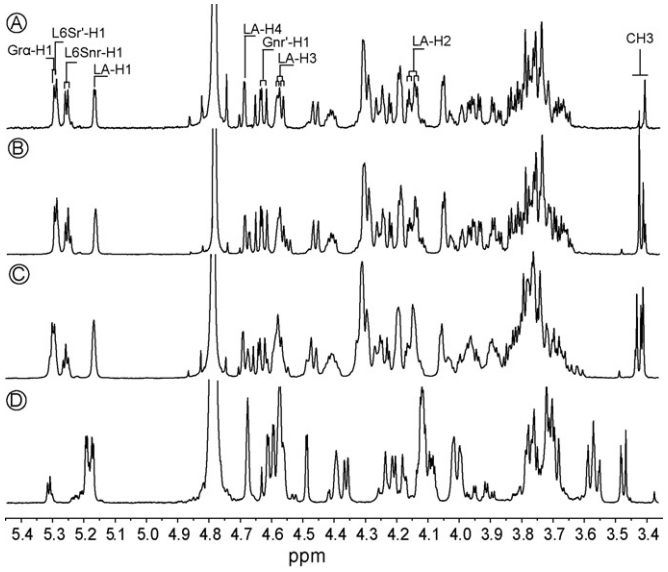

Fig. 7. ^{1}H NMR (500 MHz) spectra of the non-methylated (A) and methylated (B) L6S-G–LA-G–L6S-G oligosaccharides, high molecular fraction of hybrid oligosaccharides (C), and neo-agarotetraose (D).

Table 1
^{1}H NMR data (δ) ppm of the L6S and G residues at 25 °C in standard disaccharide (L6S-G), tetrasaccharide (L6S-G)$_2$ and polymer (L6S-G)$_n$.

		(L6S-G)	(L6S-G)$_2$	(L6S-G)$_n$			(L6S-G)	(L6S-G)$_2$	(L6S-G)$_n$
Grα	H-1	5.29	5.30		L6Sr′α	H-1	5.25	5.30	
Grβ	H-1	4.65	4.65		L6Sr′β	H-1	5.24	5.30	
Gnr′	H-1	–	4.49		L6Snr	H-1		5.25	
G	H-1	–	–	4.50	L6S	H-1			5.33
Grα	H-2	3.99	4.00		L6Sr′α	H-2	3.84	3.90	
Grβ	H-2	3.66	3.68		L6Sr′β	H-2	3.84	3.90	
Gnr′	H-2	–	3.76		L6Snr	H-2		3.84	
G		–	–	3.76	L6S	H-2			3.92
Grα	H-3	3.94	3.95		L6Sr′α	H-3	3.95	3.98	
Grβ	H-3	3.73	3.75		L6Sr′β	H-3	3.95	3.98	
Gnr′	H-3	–	3.75		L6Snr	H-3		3.95	
G		–	–	3.76	L6S	H-3			3.98
Grα	H-4	4.25	4.26		L6Sr′α	H-4	4.05	4.31	
Grβ	H-4	4.19	4.20		L6Sr′β	H-4	4.05	4.31	
Gnr′	H-4	–	4.20		L6Snr	H-4		4.06	
G		–	–	4.21	L6S	H-4			4.31
Grα	H-5	4.12	4.13		L6Sr′α	H-5	4.31	4.42	
Grβ	H-5	3.72	3.76		L6Sr′β	H-5	4.31	4.42	
Gnr′	H-5	–	3.74		L6Snr	H-5		4.30	
G		–	–	3.82	L6S	H-5			4.42
Grα	H-6a	3.75	3.78		L6Sr′α	H-6a	4.16	4.32	
Grβ	H-6a	3.75	3.76		L6Sr′β	H-6a	4.16	4.32	
Gnr′	H-6a	–	3.74		L6Snr	H-6a		4.16	
G		–	–	3.78	L6S	H-6a			4.31
Grα	H-6b	3.77	3.78		L6Sr′α	H-6b	4.23	4.32	
Grβ	H-6b	3.77	3.76		L6Sr′β	H-6b	4.23	4.32	
Gnr′	H-6b	–	3.74		L6Snr	H-6b		4.23	
G		–	–	3.78	L6S	H-6b			4.31

the L6S-G moiety (Fig. 5), and the peak eluting at 450 min (Fig. 3A) corresponded to a hybrid oligosaccharide composed of L6S-G and LA-G moieties (DP6, Fig. 7). As expected, the oligosaccharides were separated according to their molecular mass in permeation gel chromatography. Notably, the peaks corresponding to DP4 and DP6 were not symmetrical and shoulders were clearly visible in both cases, indicating the co-elution of oligosaccharides.

The samples corresponding to Fig. 3A were further analyzed by HPAEC and we could indeed separate more distinct peaks. In comparison to the elution time when injecting the purified and characterized oligosaccharides, all major peaks could be assigned (Fig. 4). While the oligosaccharides were separated according to their molecular mass in the SEC experiment, the correlation between SEC and HPAEC chromatograms was not clear. We observed that the DP6, containing the neutral LA-G moiety, eluted between DP2 and DP4 in the HPAEC experiment. This effect may be due to differences of charges: molecular mass ratio and distribution of charge density along the oligosaccharides. We further identified two additional peaks in the HPAEC profile, which corresponded to methylated species of the DP6 and DP4 oligosaccharides (see NMR analysis below). The co-elution of methylated and non-methylated from DP4 and DP6 were thus the cause of the asymmetrical shape of the SEC. Interestingly, a methylated form of DP2 was not observed.

3.3. Complete assignment of standard L6S-G oligosaccharides based on ^{13}C and ^{1}H NMR

The smallest oligosaccharide purified was a disaccharide and was the first characterized by NMR (Table 1). In accordance with the ^{1}H/^{13}C correlations and with the integration ratio, the assignment of the anomeric protons was straightforward. The most downfield signals correspond to the Grα-H1 (5.29 ppm) and L6Sr′-H1 (5.25 ppm) peaks. The coupling constant of these doublets, which result from the typical axial-gauche H1/H2 conformation of D/L-galactose, was about 3.9 Hz. The doublet at 4.65 ppm ($^3J_{H1,H2}$ = 7.8 Hz) was assigned to the reducing residue in the β-anomeric configuration (Grβ-H1). The anomeric equilibrium (α/β 0.3/0.7) was determined from the integration of anomeric signals and showed displacement towards the β-anomer.

The protons of the galactose rings were assigned based on the COSY spectra using the anomeric protons as starting point. The H1–H4 correlation systems of the Grα/β and L6S were clear and are indicated in Fig. 6. The corresponding carbons were deduced from the heteronuclear ^{1}H/^{13}C chemical shift correlation (HMQC, not shown). The connection between H4 and H5–H6 systems were obtained based on long-range heteronuclear ^{1}H/^{13}C chemical shift correlations (HMBC). Evidence that the neutral residue was at the reducing end was provided by the difference of chemical shift encountered at the H6 and C6 positions. The most downfield H6 and C6 were observed at the residue's non-reducing end, which therefore most likely carries the sulfate ester group. The L6Sr′-H1 had an α-anomeric configuration suggesting that the two residues were linked by an α(1 → 3) linkage. This was supported by HMBC experiments which showed, for example, the connection of H3 (not H4) from the reducing galactose (Grα-H3 and Grβ-H3) with the anomeric carbon of the L6Sr′ residue. All together, these results were consistent with a L6S-G disaccharide structure, obtained through the cleavage of the β(1–4) glycosidic bond of porphyran.

The ^{1}H NMR spectra of the L6S-G di- and tetrasaccharides share several similarities, such as the chemical shift of the anomeric protons localized at the reducing and non-reducing end, respectively. Based on the DP2 spectra, the three α-anomeric protons were easily assigned to the internal and non-reducing L6S (L6Sr′ = 5.30 ppm; L6Snr = 5.25 ppm) and to the reducing-end Grα (5.30 ppm). The chemical shift of the β-anomeric proton at the reducing end was found at 4.65 ppm for DP4 and DP2. The additional signal, measured at 4.49 ppm, was assigned to the anomeric proton of the internal G residue in DP4. Similar to that in DP2, the COSY spectra demonstrated the connection of the H1–H4 ring protons and using HMQC, the corresponding carbons could be assigned. We further

used HMBC experiments to connect the H5–H6 correlation system with the H1–H4 system and finally determined the chemical shift of the corresponding carbons (not shown). These data allowed to completely assign the spectrum of the polymer and values were similar to those reported previously (Usov et al., 1980; Morrice et al., 1983).

3.4. Structure of hybrids L6S-G/LA-G oligosaccharides

The various ^{1}H NMR spectra illustrated in Fig. 7 all display characteristics similar to those in the L6S-G oligosaccharides (Fig. 5). Consequently, we identified the signals corresponding to the α-anomeric protons of the reducing and non-reducing ends (Grα-H1, L6Sr′-H1 and L6Snr-H1 respectively) in the most downfield region of the spectra. Similarly, the β-anomeric protons (Gnr′-H1 = 4.62 ppm Grβ-H1 = 4.65 ppm) were also identified. The integration of the signal of the anomeric protons revealed a ratio similar to that observed in DP4, suggesting that one L6S-G moiety is located at each end of the oligosaccharide. Based on the recorded data for pure porphyran oligosaccharides, proton signals as well as carbons were assigned using well-resolved COSY, HMBC and HMQC spectra (data not shown). In addition to the L6S-G signals, the signal resonating at 5.16 ppm, which is very close to the LA-H1 signal measured in the agarose polymer, was ascribed to the α-anomeric proton of an internal anhydro-galactose residue (LA-H1). The integration of LA-H1 and L6Snr-H1 signals led to similar values, indicating that this hybrid oligosaccharide is a hexasaccharide composed of one LA-G moiety positioned between two L6S-G moieties (L6S-G–LA-G–L6S-G). The protons and carbons of the internal LA-G moiety were completely assigned and the values were comparable to values determined for agarose polymers and for agaro-oligosaccharides (Kazlowski et al., 2008).

Longer oligosaccharides that eluted between 350 and 420 min in the SEC experiment (Fig. 3A) were not purified to homogeneity. However, fractions were collected and analyzed by NMR as shown in Fig. 7C. Interestingly, the NMR spectra revealed that the oligosaccharides were systematically terminated by L6S-G moieties at the reducing and the non-reducing end, giving a clear indication of the substrate specificity of the enzyme. The integration of the anomeric protons suggested that the internal moieties are composed of both LA-G and L6S-G moieties, in a ratio of about two LA-G units for one L6S-G moiety.

3.5. Methylation of L6S-G/LA-G oligosaccharides

The extracted porphyran was decorated by methyl groups (Fig. 2) that were estimated by integration of the ^{1}H NMR signals, indicating that about 18% of D-galactose residues were substituted. Similarly, some of the produced oligosaccharides, obtained after enzymatic digestion, were methylated. Notably, a methylated form of the L6S-G–L6S-G tetrasaccharide and a hybrid hexasaccharide eluted as shoulder of their corresponding non-methylated form in SEC (Fig. 3). Therefore, these two oligosaccharides were purified to determine the precise position of the methyl group on the oligosaccharides.

The protons of the methyl group present in the tetrasaccharide resonated as a single peak at 3.4 ppm in the ^{1}H NMR spectrum (Fig. 5C), and the 61.25 ppm chemical shift of the corresponding carbon can be deduced from the HMQC spectrum. The carbon carrying the methyl group was identified as a C6 carbon of a G unit using long-range ^{1}H/^{13}C correlation of the HMBC spectra. The chemical shift of 74.5 ppm of this carbon indicated that the non-reducing-end Gnr′-C6 unit was methylated, while the reducing-end Grα and Grβ-C6 signals, at 64.28 and 64.11 ppm, respectively, indicated the absence of methylation.

In the case of the purified fraction of methylated hybrid DP6, three methyl signals were observed with different chemical shifts and signal intensities, suggesting a mixture of oligosaccharides. The protons of the abundant methyl group had exactly the same chemical shift (3.41 ppm) of that encountered in the tetrasaccharide, suggesting that the most abundant hybrid hexasaccharide most likely carried a methyl group on the non-reducing Gnr′ unit. This signal was also present in the spectra of longer hybrid oligosaccharides. However, in this case, the intensity was lower than that of the other signals at 3.42–3.43 ppm. The chemical shifts of these less intense methyl signals were also present in the undigested porphyran, but absent after incubation with the β-agarase, suggesting that this methyl was possibly carried by agarobiose moieties.

4. Discussion

In this study, ^{1}H and ^{13}C NMR spectra of standard L6S-G oligosaccharides were assigned for the first time. Based on an internal standard, our data confirmed previous ^{13}C NMR investigations carried out on enriched L6S-G oligosaccharides obtained after incubation with β-agarases (Morrice et al., 1983). We completed the set of ^{13}C NMR data with chemical shifts of L6S-G moieties located at the ends of the oligosaccharides. ^{1}H NMR spectra of L6S-G oligosaccharides were completely elucidated and allowed further assignment of the chemical shifts of the proton signal from the polymer. In this context, we determined that the internal G-L6S moiety of the standard DP4 (L6Snr-G–L6S-Gr) can be seen as a model of the repeated moiety, as encountered in the polymer. Indeed, the chemical shifts of the L6Sr′-H1 (5.33 ppm), L6Sr′-H2 (3.92 ppm) and L6Sr′-H3 (3.98 ppm) proton signals were very similar, compared to those reported by Maciel et al. (2008). In contrast, the undefined L6Sr′-H4, -H5 and -H6 protons in the polymer were straightforwardly measured on the oligosaccharides. Moreover, chemical shifts of the neutral galactose residues could also be unambiguously assigned, such as the G-H1 (4.5 ppm) and G-H3 (3.76 ppm), which resonated similarly to the internal G unit, as encountered in the polymer (Table 1).

Unlike degradation with β-agarase B, β-porphyranase A almost completely degraded the porphyran. The purification of oligosaccharides and the characterization of the most abundant degradation products by NMR revealed that β-porphyranase A predominantly produces products of the L6S-G series and hybrid L6S-G/LA-G oligosaccharides of the DP6 type. The smallest oligosaccharide indentified was the L6S-G disaccharide, subsequently used as standard, which was also obtained by degradation of the pure tetrasaccharide of structure L6S-G–L6S-G. In addition, our NMR data showed that the hybrid oligosaccharides are systematically terminated at both ends by at least one L6S-G moiety. Altogether, these results clearly demonstrate that β-porphyranase A selectively cleaves the β(1 → 4) glycosidic bond between L6S-G moieties – as expected since all members of family GH16, including agarases (Jam et al., 2005), κ-carrageenases (Michel et al., 2001), and lichenases (Malet et al., 1993) are known to cleave the β-linkage – and does not efficiently bind LA-G moieties in binding subsites close to the point of cleavage, i.e. subsites −2, −1 and +1, +2.

Given the absence of methylated disaccharides and the fact that the methylated tetrasaccharides (L6S-GMe–L6S-G) were not further degraded by β-porphyranase A, C6 methylated D-galactose units thus cannot bind in subsite −1. However, the enzyme can efficiently bind oligosaccharides that are methylated further away from the point of cleavage, since hybrid DP6 appeared to be methylated on the C-6 of the D-galactose units, but never on the reducing-end sugar. This suggests that methylated D-galactose can be bound at least in subsite +2 of β-porphyranase A.

Based on the complete digestion of natural porphyran using the combination of a β-agarase and a β-porphyranase, the overall description of the polysaccharide was determined in more detail. The complexity of porphyran can be attributed to the co-occurrence of two repeating moieties, which are LA-G (agarobiose) and L6S-G, as well as by additional decoration with methyl groups on the hybrid copolymer chain. Previous enzymatic digestion of porphyran using β-agarases already highlighted this complexity, but only on a limited fraction of the polysaccharide, as the agarose fraction represents only about one-third of the porphyran. Using β-agarases, Duckworth & Turvey (1969) – and later Morrice et al. (1983) – isolated a low-molecular-weight fraction that accounted for about 20–30% of the starting material. We observed a similarly low rate of porphyran degradation using β-agarase B (Fig. 3), and showed that an abundant enzyme-resistant fraction of high molecular weight remains, containing almost exclusively L6S-G moieties (Fig. 3). This means that most of the LA-G moieties occur in the oligosaccharides and in the form of neo-agarotetraose and hybrid LA-G/L6S-G oligosaccharides, as previously reported (Morrice et al., 1983). Interestingly, the hybrid oligosaccharides produced by β-agarase B can be terminated by L6S-G moieties at the non-reducing end, showing that the specificity of β-agarase is less restrictive. However, we can conclude that blocks of agarose, if present, are very short in porphyran, since the amount of pure agarose oligosaccharides was low compared to that of hybrid LA-G/L6S-G oligosaccharides. Overall, using a combination of two enzymes, a β-agarase and the newly identified β-porphyranase, a more complete view of the algal polysaccharide porphyran has now been elucidated.

Acknowledgements

The work leading to this invention has received funding from the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement no. 222628. J.H. Hehemann was supported by a European Marie Curie Ph.D. grant. We also thank the NMR Service, University Bretagne Occidentale, Brest, France for access to the Bruker NMR spectrometer and Dr. Diane Jouanneau for helpful discussions.

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