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Structural characterisation and bioactivities of hybrid carrageenan-like sulphated galactan from red alga *Furcellaria lumbricalis*

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A B S T R A C T

The red alga, *Furcellaria lumbricalis* from the coast of the Prince Edward Island (PEI) in Atlantic Canada, was extracted with hot water and fractionated with 0.125 M KCl to obtain a carrageenan-like polysaccharide. The polysaccharide was further purified on ion-exchange and gel-permeation chromatography to yield a fraction (FB1) of uniform size and charge, with an average molecular weight of 428 kD. Oligosaccharides generated with acid hydrolysis of FB1 were sequenced using the electrospray ionisation collision induced dissociation tandem mass spectrometry (ES-CID-MS/MS) technique. On the basis of chemical and spectroscopic analysis, FB1 was characterised to be composed of 1,4-linked 3,6-anhydro-galactose (40%), 1,3-linked 4-sulphated-galactose (30%), 1,3-linked galactose (20%), 1,4-linked galactose (8%) and 1,4-linked 3-O-methyl-galactose (2%), which makes it be a novel sulphated galactan hybrid. The β-secretase (BACE) inhibition and immunomodulation activities of FB1-derived oligosaccharides were evaluated *in vitro*.

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1. Introduction

Carrageenans, the sulphated galactans isolated from marine red algae, are widely used as food additives due to their gel-forming ability and other rheological properties. Similar to many sulphated polysaccharides (e.g. glycosaminoglycans) in vertebrate tissues, carrageenans consist of linear, repeating disaccharide blocks of alternating 3-linked β-D-galactopyranose (β-Gal, unit G) and 4-linked α-D-galactopyranose (α-Gal, unit D), with the D unit often occurring as its 3,6-anhydro form (AnGal, unit A). The classification of carrageenans is based on the presence of a D or an A form of the 4-linked galactose and the differing sulphate contents and substitutions. For example, κ-, ι- and λ-carrageenans have different disaccharide building blocks $-[G4S–A]_n-$, $-[G4S–A2S]_n-$, and $-[G2S–D2S6S]_n-$, respectively (De Ruiter & Rudolph, 1997). However, native polysaccharides are rarely in their uniform or "ideal" form. The structural complexity of carrageenans is attributed to a mixed combination of different biose units or copolymeric chains. The heterogeneity of carrageenan largely depends on the algal species, growing stage, and environmental conditions. Structural complexity occurs when the hydroxyls in D-galactose are substituted by methyl and pyruvate groups (Usov, 1998). The presence of hybrid or "non-ideal" biose sequences introduces structural heterogeneity, which confers a wide range of physicochemical properties and biological activities. Carrageenans or their sulphated galactan oligomers are reported to possess a number of bioactivities including antiviral (Buck et al., 2006), antitumour (Hirayasu, Yoshikawa, Tsuzuki, & Fushiki, 2005; Zhou, Sheng, Yao, & Wang, 2006) and immunomodulatory activities (Yuan, Song, Li, Li, & Dai, 2006). Sulphated polysaccharides, such as chondroitin sulphate, heparan sulphate and fucoidan, have also been reported to reduce amyloid beta peptide toxicity and thus may have potential application in Alzheimer's disease (Bravo et al., 2008).

The red alga *Furcellaria lumbricalis* is widely distributed in both the eastern and western North Atlantic, from the Barents Sea to the Bay of Biscay, and particularly in the brackish waters of the Baltic Sea (Laos & Ring, 2005). On the shores of Prince Edward Island (PEI; Canada) *F. lumbricalis* has infiltrated for many areas, and its presence is often viewed as a nuisance or damaging the viability of commercial seaweed species. On the other hand, *F. lumbricalis* is also commercially important, being used for many decades by European food manufacturers as a source for agar, carrageenan

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and sulphated galactan (Bird, Saunders, & McLachlan, 1991). Sulphated galactan (e.g. furcellaran) can make milk gels smoother and less brittle and generates a specific "mouthfeel" sensation of diary products due to its interaction with milk protein. Although the crude polysaccharides from *F. fastigiata* and *F. lumbricalis* can be used as food additives (Laos, Brownsey, & Ring, 2007), their precise structures are still to be elucidated.

In our previous study, four types of polysaccharides were extracted and purified from *F. lumbricalis* (Yu, Yang et al., 2007). The polysaccharide FB, which was obtained by hot water extraction and fractionation with 0.125 M KCl, proved to be a low sulphated κ-carrageenan-like galactan, yet its viscosity in the presence of casein at different pH and temperature was different from that of standard κ-carrageenan (Yu, Ren, Yang, Zhou, & Zhao, 2007). In this paper, the precise structure of FB was determined, six low-molecular-weight fractions were prepared by a chemical degradation method, and their immunomodulation and neuroprotection activities were evaluated *in vitro*.

2. Materials and methods

2.1. Materials and reagents

The red seaweed *Furcellaria lumbricalis* was collected from the coast of PEI or obtained from the PEI Food Technology Center (Charlottetown, PEI). Monosaccharide standards (rhamnose, mannose, arabinose, fucose, xylose, galactose, glucose, 3,6-anhydrogalactose), κ-carrageenan standard, methylmorpholine-borane (MMB), *N*-(1-Naphthyl) ethylenediamine dihydrochloride, and ion-exchange resin Amberlite IR 120 (H+form) were purchased from Sigma Company (Shanghai, China). Dextran standards (788, 404, 212, 112, 47.3, 22.8, 11.8, and 5.9 kD) were purchased from Shodex Company (Tokyo, Japan). Chlorotrimethylsilane (CTMS) and acetal were obtained from J&K Chemical (Beijing, China). Superdex 30 preparation- grade columns (1.6 × 60 cm), Superdex Peptide HR column (1.0 × 30 cm), Sepharose 4B Fast Flow, Q-Sepharose Fast Flow ion-exchange and Sephadex G10 resin were purchased from Pharmacia Bioscience (Uppsala, Sweden). PL aquagel–OH column (8 μm, 300 × 8 mm) was from Perkin Elmer Company (Massachusetts, USA). Fused-silica capillary columns DB-225 (30 m × 0.32 mm × 0.25 μm) and DB-225MS (30 m × 0.32 mm × 0.25 μm) were purchased from J&W Scientific (Folsom, CA, USA). The Raw 264.7 macrophage cell line was obtained from the American Tissue Culture Collection (Rockville, MD); recombinant human BACE-1 and fluorogenic peptide substrate IV were purchased from R&D Systems (Shanghai, China). All other reagents and solvents used were of analytical-grade.

2.2. Extraction, fractionation and purification of polysaccharides

Extraction of polysaccharides was carried out as previously described (Yu et al., 2007b). Briefly, *F. lumbricalis* was pulverised and passed through a 60 mesh sieve, extracted with 95% ethanol for 4 h at 80 °C (3 times) to remove lipids, and the residue dried. The residue (50 g) was extracted with 20 volumes of cold water, filtered, and further extracted with distilled water (1 l) at 85 °C for 1 h (3 times). The pH of the extraction was adjusted to 8.0 with 1 M NaOH, the three volumes combined, neutralised, evaporated, and precipitated with 4 volumes of ethanol. The precipitate was dissolved in distilled water to make a 1% (w/v) solution and an equal volume of 0.25 M KCl was added with stirring. The solution was allowed to stand for 1 h before centrifuging. The precipitated polysaccharide (FB) was dialysed (10 kD MWCO) against water and freeze-dried.

For purification, FB (300 mg) was dissolved in 50 ml of distilled water, applied to a Q-Sepharose Fast Flow column (XK 5.0/10 cm) connected to an AKTA–FPLC system (Amersham Pharmacia Biotech, Sweden), and washed with water, followed by a stepwise addition of increasing concentrations of NaCl (0.5, 1.0, 1.5 and 2 M) at a flow rate of 120 ml/h. Each fraction (8 ml) was tested for polysaccharide by the phenol–sulphuric acid method. Fractions containing polysaccharide were combined, dialysed, concentrated and subjected to gel filtration separation using a Sepharose 4B Fast Flow column (XK 2.6/90 cm). Elution was carried out with 0.2 M NaCl at a flow rate of 12 ml/h. The purified fraction (FB1) was dialysed against distilled water and lyophilised (242 mg, yield 80.6%).

2.3. Purity analysis and molecular weight determination

The purity and relative molecular weight (M_W) of FB1 was determined by high-performance gel-permeation chromatography (Agilent 1100, USA) with a PL aquagel–OH column eluted with 0.2 M NaCl at a flow rate of 0.5 ml/min at 35 °C. The column was calibrated with dextran standards, and the corrected regression equation was $\log M_W = 12.944–0.5183\,Rt$ (Rt, retention time; $r^2 = 0.998$).

2.4. Monosaccharide composition analysis

Monosaccharide composition analysis was carried out by a two-step reductive hydrolysis method (Stevenson & Furneaux, 1991), as previously described (Yu et al., 2007b). The monosaccharide alditols (2 mg) were further acetylated with a mixture of 500 μl EtOAc, 1.5 ml Ac_2O and 50 μl $HClO_4$. The mixture was sonicated at room temperature for 10 min, then 5 ml H_2O was added, and the solution was extracted with 2 ml dichloromethane (DCM) three times. The DCM fraction, which contained the alditol acetates, was analysed by GC (Agilent HP5890 II, USA) using a fused-silica capillary column DB-225 and by GC/MS (Agilent HP6890, USA) using a DB-225MS column. The sample was detected with a flame-ionisation detector (FID) at 240 °C, with the injector and oven temperatures set at 240 °C and 210 °C, respectively. For GC analysis, the composition and content of monosaccharide were determined by retention time and peak area, based on a comparison with monosaccharide standards. For GC/MS analysis, the ion source temperature was set at 280 °C and ionisation energy at 70 eV. The MS spectrum acquired was compared with the NIST library spectrum.

2.5. General analysis

Total sugar content was determined by the phenol–sulphuric acid method using galactose as standard (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956). The content of AnG was determined by the resorcinol method using fructose as standard (Arsenault & Yaphe, 1965). The sulphate content was determined by the $BaCl_2$–Gelatin method (Dodgson & Price, 1962). The content of crude protein was determined by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951). For FTIR analysis, a sample (1–2 mg) was dried in a P_2O_5 desiccator for 48 h, then mixed with 100 mg KBr and pressed under 7 kg/cm^2 to make a transparent film. The film was put into an FTIR instrument (Nicolet Nexus 470, Thermo Electron, USA) and scanned from 400 to 4000 cm^{-1}.

For NMR analysis, mild acid hydrolysis of FB1 was done to reduce its high viscosity. FB1 (40 mg) was dissolved in 8 ml water, then adjusted to pH 1.0 with 2 M HCl, and kept for 10 min at 60 °C, then neutralised, desalted, and freeze-dried. The hydrolysed sample (30 mg) was dissolved in 1 ml D_2O and freeze-dried twice to replace all exchangeable protons with deuterium. The ^1H NMR, ^{13}C NMR, ^1H–1H COSY and HMQC were all acquired at

20 °C using a JEOL 600 MHz equipment. The chemical shift values were calibrated using acetone-d6 as an internal standard.

2.6. Desulphation and methylation analysis

Desulphation was operated as described (Kolender & Matulewicz, 2004), but with some modification. In brief, FB1 (20 mg) was passed through an Amberlite IR 120 column (20 ml). The column was washed with water and the eluate was adjusted to pH 9.0 with pyridine, rotatory evaporated and then freeze-dried. The desulphation was carried out in anhydrous pyridine with CTMS (molar ratio of CTMS:sulphate was 400:1) in a sealed Teflon tube at 100 °C for 8 h. The excess CTMS was washed with water, and the desulphated product was recovered by dialysis and then freeze-dried.

Methylation was performed as previously described (Hakomori, 1964). The methylated polysaccharide was hydrolysed and acetylated as described above. Thereafter, partial methylated alditol acetates were analysed by GC–MS equipped with a DB-225MS fused-silica capillary column. The injector temperature was set at 220 °C. Helium was used as the carrier gas at a constant flow rate of 1.0 ml/ min through the column. The initial oven temperature was 100 °C (for 4 min) and was then increased to 240 °C at the rate of 58 °C/min. The transfer line temperature was 220 °C and the ion source temperature was 280 °C. Ions were generated by a 70 eV electron beam at a current of 2.0 mA. Masses were acquired from 30 to 800 *m/z* at a rate of 30 spectra/s, and the acceleration voltage was turned on after a solvent delay of 180 s. Mass spectra of the compounds were analysed using the Complex Carbohydrate Structural Database (CCSD) of the Complex Carbohydrate Research Centre (http://www.ccrc.uga.edu/).

2.7. Preparation and purification of oligosaccharides

2.7.1. Preparation of oligosaccharides for mass spectrum analysis

FB1 was hydrolysed with 0.1 M H_2SO_4 (10 mg/ml) at 60 °C for 2 h, followed by neutralisation with 2 M NaOH. The same volume of ethanol was added to the above reaction solution and then centrifuged. The supernatant (FB1S) was collected and freeze-dried. FB1S was dissolved in 0.1 M NH_4HCO_3 and separated on a Superdex 30 column eluted with 0.1 M NH_4HCO_3 at a flow rate of 0.2 ml/min, using a refractive index detector. Ten oligosaccharides fractions (FB1S1–10) were acquired. The ethanol precipitate fraction (FB1P) was hydrolysed with 0.2 M H_2SO_4 (10 mg/ml) at 80 °C for 4 h and separated on a Superdex peptide column eluted with 0.1 M NH_4HCO_3 at a flow rate of 0.1 ml/min. Eight oligosaccharide fractions (FB1P1–8) were collected, concentrated and freeze-dried.

2.7.2. Preparation of low molecular weight oligosaccharides for bioassays

FB1S was fractionated on a Superdex 30 column to give six oligosaccharide fractions, M1–M6. Their average molecular weights were determined with a Superdex peptide column eluted with 0.2 M NaCl at a flow rate of 0.5 ml/min at 30 °C, using a refractive index detector. The column was calibrated with standard κ-carrageenan oligosaccharides (dp3–dp17) (Yang et al., 2009). The regression equation was log M_W = 5.0521–0.0677 Rt (r^2 = 0.997).

2.8. ES-TOF-MS analysis

Negative-ion electrospray ionisation mass spectrometry (ES–MS) analysis on a Micromass Q-Tof Ultima instrument (Waters, Manchester, UK) was performed for all oligosaccharide sequence analysis (Yu et al., 2006). Nitrogen was used as the desolvation and nebulizer gas at a flow rate of 500 l/h and 50 l/h, respectively. The source temperature was 80 °C and the desolvation temperature was 150 °C. Samples were dissolved in CH_3CN/2 mM NH_4HCO_3 (1:1, v/v), typically at a concentration of 5–10 pmol/μl, of which 5 μl was loop-injected. The mobile phase (CH_3CN/2 mM NH_4HCO_3, 1:1, v/v) was delivered by a syringe pump at a flow rate of 5 μl/min. The capillary voltage was maintained at 3 kV, while the cone voltage was 150 eV, depending on the size of the oligosaccharides. For CID-MS/MS product-ion scanning, argon was used as the collision gas at a pressure of 1.7 bar and the collision energy was adjusted between 17–100 eV for optimal sequence information.

In order to get the oligosaccharide sequence, deuterium reduction is needed. Typically, $NaBD_4$ reagent (20 μl, 0.05 M $NaBD_4$ in 0.01 M NaOH) was added to the freeze-dried oligosaccharide (20 μg), and the reduction carried out at 4 °C overnight. The reaction solution was then neutralised to pH 7 with a solution of HAc/H_2O (1:1) to destroy borohydride before being passed through a mini-column of cation exchange (AG50 W-X8, H form, Bio-Rad). Boric acid was removed by repeated co-evaporation with methanol under nitrogen gas.

2.9. Bioassays

The β-secretase (BACE) inhibition assay was carried out according to Sinha et al. (1999) and Vassar et al. (1999) with some modifications. Briefly, 0.1 M sodium acetate buffer (pH 4.0) was added (5 μl/well) to a 384-well black plate, followed by 5 μl of samples (1 mg/ml) and 20 μl BACE-1 (10 μg/ml). The reaction was started by the addition of 20 μl of substrate (25 μM). After shaking for 5 min, the fluorescence (Ex at 320 nm and Em at 405 nm) was measured with a POLARstar Galaxy fluorimeter (BMG Lab technologies GmbH, Offenburg, Germany).

Raw 264.7 macrophage cells were used to screen for immune enhancing activities. Cells were grown in 96-well plates to 70–80% confluences and then treated for 24 h with various concentrations of the low-molecular-weight fractions. Activation was determined by assessing the nitrite accumulation in the culture medium using Griess reagent (1.0% sulphanilamide, 0.10% *N*-(1-Naphthyl) ethylenediamine dihydrochloride, and 2.5% phosphoric acid). Sodium nitrite was used as standard.

3. Results and discussion

3.1. Extraction and general analysis of polysaccharide FB1

In our previous study, we reported that the polysaccharide fraction (FB) in *F. lumbricalis* was the main component, representing about 34% (w/w) of the alga dry mass. Physicochemical analysis showed that FB is similar to κ-carrageenan but has a higher molecular mass and a relatively low sulphate content compared with standard κ-carrageenan (Yu et al., 2007b). To further investigate the structure of FB, the polysaccharide was fractionated on Q-Sepharose FF (Fig. 1A) and Sepharose 4B FF columns (Fig. 1B). Based on size and charge, a fraction of FB1was acquired with the yield about 81%. FB1 showed a symmetric peak on a PL aquagel–OH column, with average molecular weight of 428 kD (Fig. 1C). The chemical composition analysis show that FB1 contained 83.6% sugar and 15.2% sulphate, with a small amount of crude protein (0.3%). Monosaccharide composition analysis by GC indicated that FB1 is composed of 58.1% Gal, 39.8% AnG and 2.1% of an unknown constituent. By comparing the GC–MS analysis with the entries in the CCRC database, the identity of this unknown constituent was revealed to be 3-O-methyl-galactose (3MeGal). The FTIR spectrum of FB1 revealed an intense band at 1230–1260 cm^{-1}, indicative of the existence of a sulphate ester ($\nu_{S=O}$). The peaks at 930 and 850 cm^{-1} suggested the presence of AnG and axial sulphate ester at C4 of D-galactopyranose (G4S), respectively. Compared with

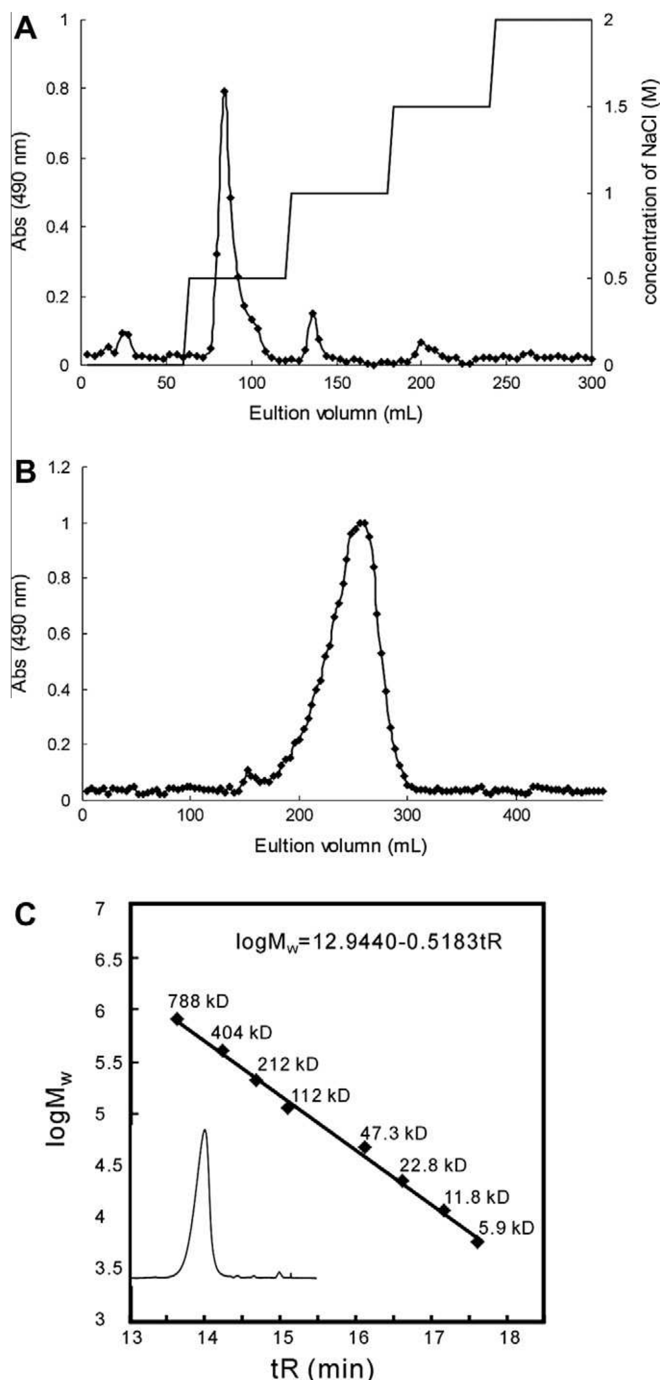

Fig. 1. Separation and purification of polysaccharide (FB) from *Furcellaria lumbricalis*. (A) Elution profiles of polysaccharide FB on Q-Sepharose Fast Flow ion-exchange chromatography column; (B) Gel filtration chromatography of polysaccharide FB1 on Sepharose 4B Fast Flow column. (C) The average molecular weight of FB1 was analysed by the HPGPC method on PL aquagel–OH column.

standard κ-carrageenan, the content of galactose in FB1 was relatively high, but the proportions of AnG and sulphate were lower. Therefore, FB1 appears to be under-sulphated carrageenan.

The predominant structure of polysaccharide from *F. lumbricalis* has been characterised as a mixture of κ-, β- and θ-carrageenan (Craigie, 1990). The monosaccharide composition and structural character of polysaccharide purified from *F. lumbricalis* (Huds.) Lamour, which grows in the northern Baltic Sea, was studied using ^{13}C and ^{1}H NMR spectroscopy (Knutsen, Myslabodski, & Grasdalen, 1990; van de Velde, Knutsen, Usov, Rollema, & Cerezo, 2002). Small amounts of methylated monosaccharides, such as 6-O-methyl-galactose (2.0–2.7%), and 2-O-methyl-3,6-anhydro galactose have been found in *F. lumbricalis*. Whereas 3MeGal has been found in the polysaccharides of a number of algal species, including the red alga *Porphyridium aerugineum* and the green alga *Chlorella vulgaris* (Popper, Sadler, & Fry, 2001), to our knowledge this is the first report of its presence in *F. lumbricalis.*

3.2. Linkage analysis of polysaccharide FB1

For sulphated polysaccharides, it is difficult to get reliable proportions of methylated alditol due to sulphated esters. Harsh conditions are necessary to remove sulphated esters. A comparative analysis between the native polysaccharide FB1 and its desulphated product dsFB1 provided key information for the correct linkage position assignments of each monosaccharide, and also for the position of the sulphates. The identification and the proportions of the partially methylated alditol acetates of FB1 and dsFB1 are listed in Table 1. The native FB1 consisted of 1,4,5-Ac_3-2-Me-3,6-AnG, 1,3,5- Ac_3-2,4,6-Me_3-Gal, 1,4,5-Ac_3-2,3,6-Me_3-Gal and 1,3,4,5-Ac_4-2, 6-Me_2-Gal, while only a few 1,3,4,5-Ac_4-2, 6-Me_2-Gal components were found in dsFB1. This indicates that the sulphate group is mainly located in C4 of 1,3-Gal of FB1, a result that is in agreement with FTIR analysis. NMR data (Supplementary Tables 1 & 2, Figs. 1 & 2) further confirmed the identity of FB1. According to the ^{1}H-^{1}H COSY and HMQC analysis, apart from the major residues of 1,3-β-G4S, and 1,4-α-AnG, FB1 also contained 1,3-β-Gal (G) and 1,4-α-Gal (D). Owing to its low content, the signal of 3MeGal was not found in ^{13}C NMR, though it was found by GC/MS (Table 1) and ES–MS analysis (Table 2 and Fig. 3C). Based on these data, FB1 consisted of 1,4-linked 3,6-AnG (40%), 1,3- linked G4S (30%), 1,3-linked Gal (20%), 1,4-linked Gal (8%), and 1,4- linked-3MeGal (2%), which makes it structurally different from other carrageenans reported.

It is generally believed that furcellaran contains low sulphated κ-carrageenan, and has also been characterised as a hybrid κ/β-carrageenan containing a small amount of ω-carrageenan (Craigie, 1990; Knutsen, Myslabodski, & Grasdalen, 1990). Recently, the structure and functional properties of hybrid carrageenans have attracted the attention of the industry due to their unique rheological and conformational characteristics (van de Velde, 2008).

3.3. Preparation of oligosaccharides and MS analysis

Detailed investigation of oligosaccharide fragments, including their preparation and sequence determination, are important for further structure–function relationship studies. As we previously reported 3,6-anhydro-galactose containing polysaccharides, such as agarose and κ-carrageenan, are easily hydrolysed with mild acid

Table 1
Composition of partially methylated monosaccharides produced by permethylation of a purified polysaccharide fraction (FB1) and its desulfated counterpart (dsFB1) from *Furcellaria lumbricalis.*

Linkages	Partially methylated alditol acetates	Main ion (m/z)	Proportions (%)	
			FB1	dsFB1
→4) A(1→	1,4,5-Ac_3-2-Me-3,6-AnGal	43,103,145	40.2	38.4
→3)G4S(1→	1,3,4,5-Ac_4-2,6- Me_2-Gal	117,129,305	29.7	3.2
→3)G(1→	1,3,5-Ac_3-2,4,6- Me_3-Gal	117,161,233,277	20.1	46.6
→4)D(1→	1,4,5-Ac_3-2,3,6- Me_3-Gal	117,161,189,233	7.9	9.7
→4)3MeGal(1→			2.1	2.1

A, 1,4-linked-3,6-anhydro-galactose; G4S, 1,3-linked-4-sulfated-galactose; G, 1,3-linked-galactose;
D, 1,4-linked-galactose.

Table 2
Negative-ion ES-MS of mild acid hydrolysis products from a purified polysaccharide fraction (FB1) derived from *Furcellaria lumbricalis.*

Fractions	Found ions[a] (charge)	Calculated mol mass (H form)	Assignment			Theoretical mol mass (H form)
			DP[b]	Sequences		
FB1S1[c]	565.1 (−1)	566.1	3	G4S-A-G		566.1
	583.1 (−1)	584.1	3	G4S-D-G		584.1
FB1S2	322.0 (−2)	646.0	3	G4S-A-G4S		646.1
	827.3 (−1)	828.3	5	G-D-G-D-G	D-G-D-G-D	828.3
FB1S3	745.3 (−1)	746.3	4	G-D-G-D (1S)	D-G-D-G (1S)	746.2
	773.4 (−1)	774.4	4	G4S-D3Me-G-D3Me		744.2
	907.4 (−1)	908.4	5	G-D-G-D-G (1S)	D-G-D-G-D (1S)	908.2
FB1S4	475.0 (−2)	952.0	5	G-A-G-A-G (2S)		952.2
FB1S5	343.0 (−3)	1032.0	5	G4S-A-G4S-A-G4S		1032.1
FB1S6	668.1 (-2)	1338,0	7	G-A-G-A-G-A-G (3S)		1338.2
	445.0 (−3)					
FB1S7	463.7 (−3)	1394.1	8	G-D-G-D-G-D-G-D(1S) D-G-D-G-D-G-D-G (1S)		1394.4
FB1S8	410.1 (−4)	1644.4	9	G-A-G-A-G-A-G-A-G (3S)		1644.3
FB1P1	341.1	342.1	2	G-D	D-G	342.1
FB1P2	503.1	504.1	3	G-D-G	D-G-D	504.2
FB1P3	665.2	666.2	4	G-D-G-D	D-G-D-G	666.2
FB1P4	827.4	828.4	5	G-D-G-D-G	D-G-D-G-D	828.3
FB1P5	989.4	990.4	6	G-D-G-D-G-D	D-G-D-G-D-G	990.3
FB1P6	1151.6	1152.6	7	G-D-G-D-G-D-G	D-G-D-G-D-G-D	1152.4

G4S, 1,3-linked-4-sulfated-galactose; A, 1,4-linked-3,6-anhydro-galactose; G, 1,3-linked-galactose; D, 1,4-linked-galactose.
[a] Major ion detected; other ions with different charge states and sodiated ion species had much lower intensities and therefore are not listed.
[b] Degree of polymerisation.
[c] FB1S1–10 and FB1P1–6 denote oligosaccharide fractions derived from supernatant and precipitate, respectively, following ethanol precipitation of FB1.

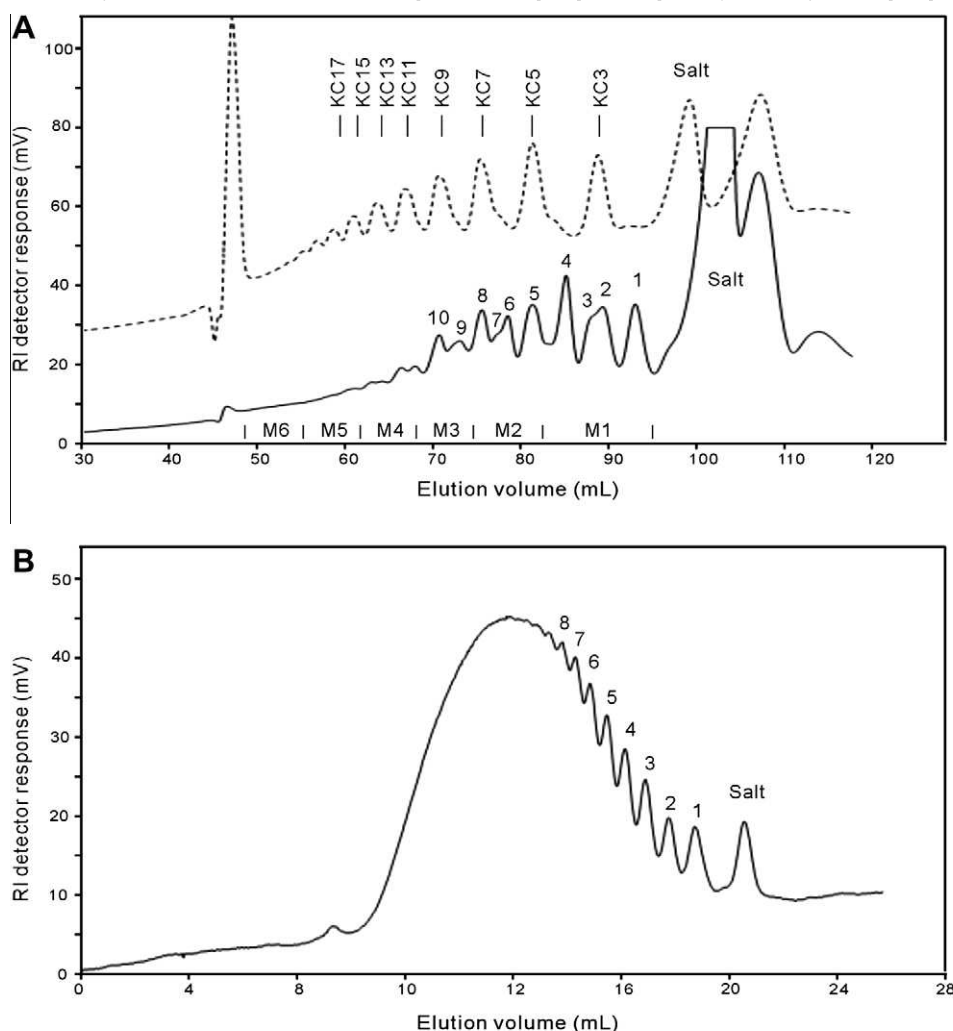

Fig. 2. Low pressure gel-permeation chromatography of acid hydrolysates of polysaccharide (FB1) from *Furcellaria lumbricalis.* (A) FB1 was hydrolysed with 0.1 M H_2SO_4 (10 mg/ml) at 60 °C for 2 h, followed by ethanol precipitation. The supernatant of the ethanol precipitation (FB1S) was applied to a Superdex 30 prep-grade column (solid line); for comparison, acid hydrolysis products from κ-carrageenan are also shown (dotted line). Six oligosaccharide fractions (M1-M6) were prepared for bioassay as denoted. (B) The pellet of the ethanol precipitation (FB1P) was applied to a Superdex peptide column.

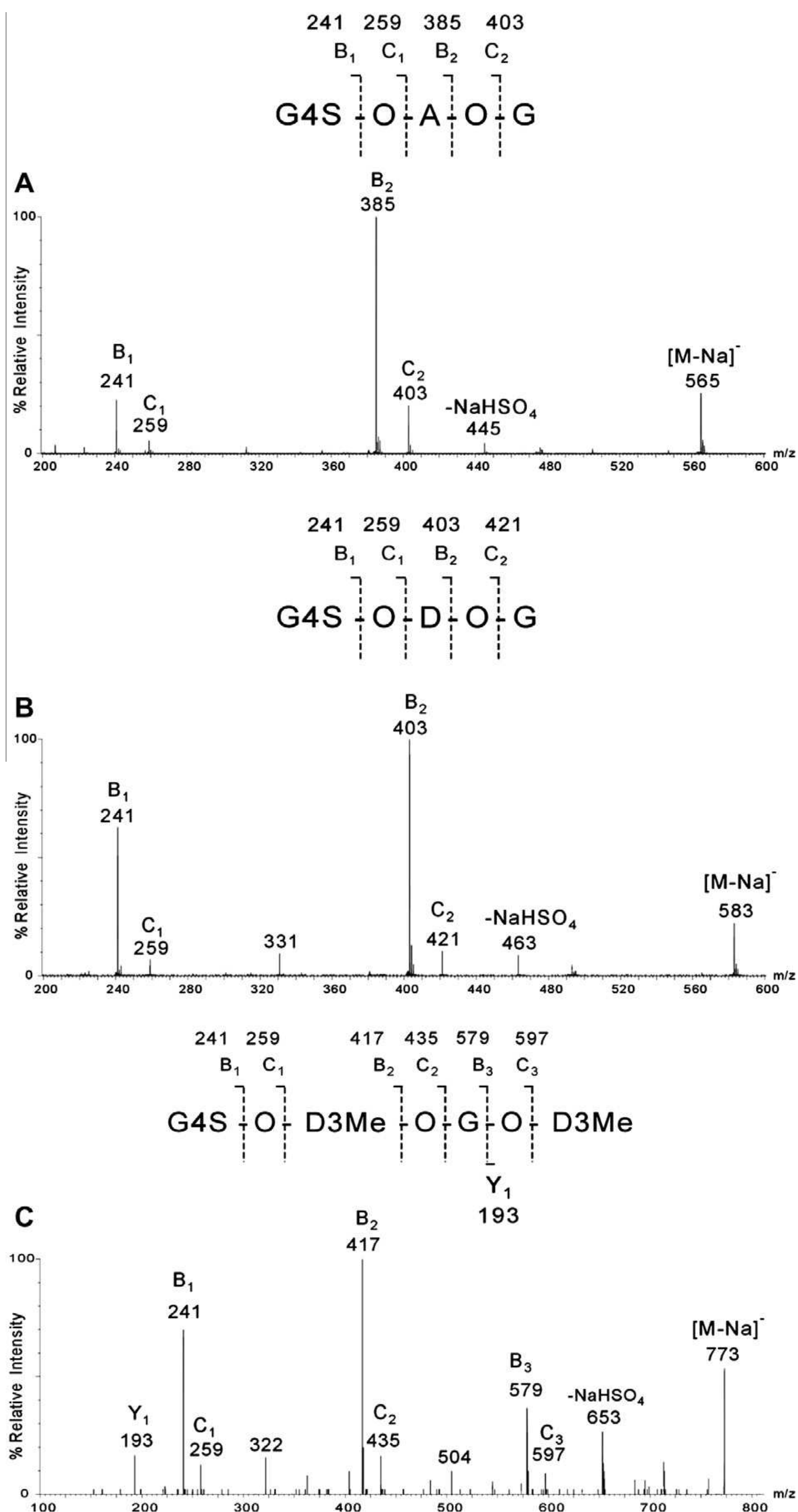

Fig. 3. Negative-ion ES CID MS/MS product-ion spectrum of acid-hydrolysed oligosaccharides of polysaccharide FB1, derived from *Furcellaria lumbricalis*. FB1 was hydrolysed with 0.1 M H_2SO_4 (10 mg/ml) at 60 °C for 2 h, followed by ethanol precipitation. The oligoscharides were separated on Superdex 30 prep-grade column resulting in 10 oligosaccharide peaks (FB1S1–10). (A) Sequence analysis of a trisaccharide from FB1S1; (B) sequence analysis of another trisaccharide from FB1S1; (C) sequence analysis of tetrasaccharide from FB1S3.

to get a series of oligosaccharides (Yang et al., 2009; Yu et al., 2002). In order to study its fine structure, FB1 was subjected to mild acid hydrolysis with subsequent generation of FB1S, and fractions FB1S1 to FB1S10 (Fig. 2A). Compared with the oligosaccharides from mild acid hydrolysis of κ-carrageenan (dotted line), those of FB1S showed a series of irregular patterns (solid line). The oligosaccharide fractions FB1S1–FB1S8 were analysed by negative-ion ES–MS. The presence of multiple sulphates in each fraction gave rise to spectra in which multiple charged ions dominated, and from which the molecular mass and the degree of polymerisation (DP) of components was determined (Table 2). In the mass spectrum of the slowest eluting fraction FB1S1, two main ions at *m/z* 565.1 and 583.1 were detected (not shown). The ion at *m/z* 565.1 indicated a component of two G units, one A unit, and one sulphated ester. Its detailed sequence was corroborated by negative-ion ES-CID-MS/MS. Due to the liability of the free acid forms of the sulphated molecules, singly charged molecular ions $[M–Na]^-$ were selected as the precursors. The product-ion spectrum of κ/β-carrageenan trisaccharide $[M–Na]^-$ at *m/z* 565, is shown in Fig. 3A. A terminal fragment ion, either reducing or non-reducing, was assigned by following the reduction of product-ion spectrum of its alditol, in which the reducing terminal ions would have a three mass unit increment. The intense B2 and C2 ions clearly identified the non-reducing terminal G4S residues, an internal A and reducing terminal G residues, yielding the sequence G4S–A–G. The ion at *m/z* 583.1 indicated a composition of three Gal residues and one sulphated ester with the sequence of G4S–D–G (Fig. 3B). In the mass spectrum of FB1S2 (not shown), the double charged ion at m/z 322.0 identified a κ-carra-trisaccharide with a molecular mass of 646.0, indicating a composition of two G4S and one A, with a sequence of G4S–A–G4S. The ion at *m/z* 827.3 indicated a pentasaccharide with five Gal residues. In the mass spectrum of FB1S3, the ion at *m/z* 773.4 indicated a composition of two G units, two 3MeGal units, and one sulphated ester. Its detailed sequence was also confirmed by negative-ion ES-CID-MS/MS (Fig. 3C).

The polysaccharide FB1P was hydrolysed and fractionated on a Superdex peptide column to yield eight oligosaccharide fractions, FBP1–FBP8 (Fig. 2B). Their molecular mass and structural features were confirmed by ES–MS (Table 2). The results showed that FB1P1–8 were DP2–DP9 neutral oligosaccharides, each consisting of Gal without AnGal, with the sequence of -[G-D]_n- or -[D-G]_n-. The composition of FB1P was also determined by GC–MS analysis of its methylated derivative. The partially methylated alditol acetates consisted of 1,3,5- Ac_3-2,4,6-Me_3-Gal and 1,4,5-Ac_3-2,3,6-Me_3-Gal, with the approximate molar proportion of 1:1, suggesting that FB1P is a linear repeating sequence of alternating 3-linked galactopyranose and 4-linked galactopyranose.

Based on the above analysis, FB1 appears to be a hybrid sulphated galactan with two major blocks, $[\text{G4S–A–G–A}]_n$ (75%) and $[\text{G–D}]_n$ (10%), and small building blocks of [G4S–D], [G–3MeGal], and[G4S–3MeGal]. Thus, FB1 is characterised as a copolymer of κ/β-carrageenan and non-sulphated galactan.

3.4. Bioassays

The sulphated oligosaccharide mixture FB1S was separated into six mixed fractions M1–M6 (shown in Fig. 2 A). Their average molecular weights were determined as M1 (1.0 kD), M2 (1.5 kD), M3 (2.0 kD), M4 (3 kD), M5 (48 kD) and M6 (6 kD). As a point of departure to examine these oligosaccharide fractions for bioactivity we tested M1–M6 for their ability to inhibit β-secretase (BACE) and for their immunomodulatory bioactivties, *in vitro*. BACE is the key rate-limiting enzyme that initiates the formation of β-amyloid peptide Aβ, excess production of which is central to the pathophysiology of Alzheimer's disease. At the test concentration of 100 μg/ml, the lower molecular weight fractions M1, M2 and M3 inhibited BACE by 42.5%, 31.1% and 47.6%, respectively. The higher molecular weight oligosaccharide fractions M4–M6 did not inhibit BACE. The immune enhancing activity was evaluated using RAW264.7 macrophage cells. The fractions M1–M5 showed little or no ability to stimulate nitrite production in macrophage cells at the test concentration of 100 μg/ml. However, M6 produced a strong stimulation that was dose-dependent (Fig. 4). These activities are being confirmed in further studies.

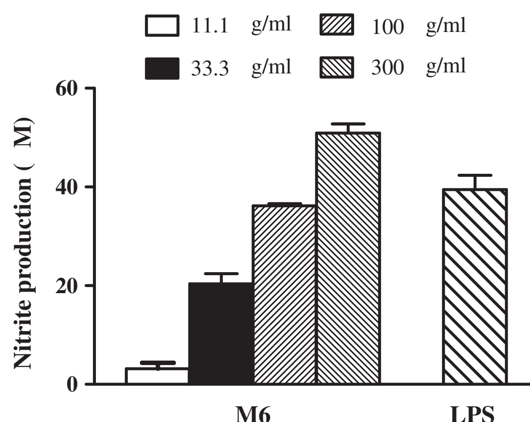

Fig. 4. Dose–response of the immune stimulation activity of *Furcellaria lumbricalis* oligosaccharide fraction (M6), derived from the fractionation of FB1S on Superdex 30. RAW264.6 macrophage cells were treated for 24 h with various concentrations of M6 as specified. Nitric oxide production was monitored by quantifying the nitrite released into the culture medium. Results are the mean ± s.e.m. of three determinations.

4. Conclusion

We conclude that FB1 is composed of 1,4-linked-3,6-An-Gal(40%), 1,3-linked-Gal4S (30%), 1,3-linked-Gal (20%), 1,4-linked-Gal (8%), and 1,4-linked-3MeGal (2%), which makes it a hybrid sulphated galactan distinguishing it from common carrageenans. Based on a detailed sequence analysis of the FB1-derived oligosaccharides, its structure was further characterised as a linear hybrid carrageenan-like sulphated galactan, with two major blocks: κ/β-carrageenan units $[\text{G4S–A–G–A}]_n$ (75%) and non-sulphated galactan units $[\text{G–D}]_n$ (10%), and also small building blocks of [G4S–D], [G–3MeGal] and [G4S–3MeGal], distributed in the main polysaccharide chain. In addition, the oligosaccharide derivatives, prepared from FB1, showed immunomodulatory activity and inhibition against BACE *in vitro*. Fraction M6 demonstrated the most potent immunomodulation, and could be investigated further as a candidate for use in dietary supplements or functional foods.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2010.05.102.

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