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Technical Note

Ion-exchange purification and structural characterization of five sulfated fucoidans from brown algae

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Abstract

Fucoidans are a diverse class of sulfated polysaccharides integral to the cell wall of brown algae, and due to their various bioactivities, they are potential drugs. Standardized work with fucoidans is required for structure–function studies, but remains challenging since available fucoidan preparations are often contaminated with other algal compounds. Additionally, fucoidans are structurally diverse depending on species and season, urging the need for standardized purification protocols. Here, we use ion-exchange chromatography to purify different fucoidans and found a high structural diversity between fucoidans. Ion-exchange chromatography efficiently removes the polysaccharides alginate and laminarin and other contaminants such as proteins and phlorotannins across a broad range of fucoidans from major brown algal orders including *Ectocarpales*, *Laminariales* and *Fucales*. By monomer composition, linkage analysis and NMR characterization, we identified galacturonic acid, glucuronic acid and O-acetylation as new structural features of certain fucoidans and provided a novel structure of fucoidan from *Durvillaea potatorum* with α-1,3-linked fucose backbone and β-1,6 and β-1,3 galactose branches. This study emphasizes the use of standardized ion-exchange chromatography to obtain defined fucoidans for subsequent molecular studies.

Key words: brown algae, ion-exchange chromatography, fucoidan, sulfated polysaccharides

Introduction

Fucoidans are the major cell wall polysaccharides of brown algae accounting for up to 23% of algal dry weight (Deniaud-Bouët et al. 2014). Within the cell wall, fucoidans tightly interact and are consequently co-extracted with proteins, phlorotannins and alginates (Deniaud-Bouët et al. 2014, 2017). Fucoidans are of high pharmaceutical interest as they display anticoagulant, antiviral, antitumor and immune-inflammatory bioactivities (Synytsya et al. 2010; Ale et al. 2011; Vishchuk et al. 2011; Fitton et al. 2015; Kopplin et al. 2018; Dörschmann et al. 2019). However, impurities such as co-extracted

phlorotannins can exhibit bioactivity (Lahrsen et al. 2018), and hence, fucoidan extracts require further purification for structure–function studies of this potential marine drug.

Fucoidans are a diverse class of polysaccharides and are broadly classified into homofucans and heterofucans. Homofucans from the brown algal order *Laminariales* and *Ectocarpales* have a backbone of α-1,3 L-fucose with sulfate groups mainly at C2 and C4, whereas homofucans from the order *Fucales* have an alternating α-1,3/α-1,4 linked L-fucose sulfated at C2 and C3 (Chevolot et al. 2001; Deniaud-Bouët et al. 2017; Kopplin et al. 2018). Homofucans have branches of fucose and/or of galactose, glucuronic acids, xylose, mannose and acetate (Nishino et al. 1991; Nagaoka et al. 1999; Sakai et al. 2003; Bilan et al. 2013; Bilan et al. 2014). Heterofucans have a non-fucose backbone of, e.g., galactose or glucuronic acid with side branches of sulfated fucose (Bilan et al. 2010; Bilan et al. 2017; Deniaud-Bouët et al. 2017). Owing to their structural diversity and complexity, most fucoidans are poorly characterized and their structural analysis requires a combination of analytical methods, such as chemical desulfation, mass spectrometry of native oligosaccharides and NMR spectroscopy (Usov et al. 1971; Yuguchi et al. 2016; Kopplin et al. 2018).

Due to variation in sampling and extraction methods, current fucoidan preparations are often contaminated and molecularly poorly defined requiring further purification. Often, brown algal biomass is harvested without taking into account that its composition varies between seasons (Rioux et al. 2009; Skriptsova et al. 2010; Mak et al. 2013; Fletcher et al. 2017). Different extraction methods such as chemical fractionation, enzyme-assisted extractions or microwave-assisted extractions result in different fucoidan preparations, even when using the same starting material (Hahn et al. 2012; Deniaud-Bouët et al. 2014). A variety of purification methods is reported in the literature, for example calcium chloride precipitates alginates or dye affinity chromatography directly captures fucoidans (Ale and Meyer 2013; Fitton et al. 2015; Hahn et al. 2016; Zayed et al. 2016). Ion-exchange chromatography has been repeatedly used to purify fucoidans and other sulfated polysaccharides (Nardella et al. 1996; Ermakova et al. 2011; Soares et al. 2018), but its applicability across different fucoidan extracts has not yet been evaluated.

Here, we use ion-exchange chromatography (IEX) to purify highly sulfated fucoidans by a stepwise protocol. We obtained eight highly sulfated fucoidans from major brown algal orders including *Ectocarpales*, *Laminariales* and *Fucales*. By characterizing their structure, we confirmed their purity, assessed their diversity and showed that IEX is broadly applicable to purify substantial amounts of fucoidans providing a basis for further molecular studies.

Results and discussion

IEX purification of highly sulfated fucoidans

We exploited the high negative charge of sulfate groups to develop a stepwise IEX purification protocol. To test if IEX can be used to separate fucoidans by their sulfate content, we separated fucoidan from *Fucus vesiculosus* and its desulfated derivative using an anion exchange column at a pH of 7.5 and a salt gradient from 0 to 5 M NaCl (Supplementary Figure S1A). The native fucoidan eluted between 2 and 5 M NaCl, whereas the desulfated fucoidan eluted between 0.5 M and 2 M NaCl. Interestingly, fractions with different sulfate-to-fucose ratio that increases with retention could be separated, suggesting that sulfate groups are key for column binding (Supplementary Results, Supplementary Figure S1B). Notably, we observed the elution of proteins in the UV detector below, a salt concentration of 0.5 M NaCl, suggesting a wash step with 0.5 M NaCl sufficiently removes contaminating proteins. Additionally, brown phenolic compounds such as phlorotannins (Koivikko et al. 2005) were strongly retained by the column and could only be eluted with an NaOH wash step, ion-exchange purification of fucoidans, leading to the characteristic change from brown- to white-colored fucoidan before and after purification (Figure 1A). The 0.5 M NaCl wash step removes less charged contaminations, and pure fucoidans can be eluted with 5 M NaCl.

Next, we demonstrated that IEX is sufficient to remove impurities from eight commercial fucoidan extracts (Supplementary Table SI). Over the course of the purification, we quantified the yield of carbohydrates and their monosaccharide composition (Figure 1B). Overall, the relative fucose content increased and glucose, mannose and mannuronic acid decreased. As monosaccharides generally do not bind to the column, glucose, mannose and mannuronic acid indicate multiple polysaccharides present in the extracts such as laminarin, alginates and/or mannans as these are the major polysaccharides of brown algae (Kloareg and Quatrano 1988; Duarte et al. 2001; Kadam et al. 2015). In fact, the preparation of *F. serratus* contained more than 95% glucose illustrating the high degree of contaminations in commercial fucoidan preparations. Alternatively, fucoidans could be extracted from ground seaweeds by 2 M NaCl (Deniaud-Bouët et al. 2014) followed by dilution with ddH_2O below 100 mM NaCl and IEX to remove contaminations.

Compositional analysis of purified fucoidans

The purified fucoidans are excessively sulfated, are diverse in monosaccharide composition and have a high molecular mass. Fucose and sulfate account between 10% and 45% (w/w), and other monosaccharides, which can be as abundant as fucose, are detected in varying amounts across fucoidans (Figure 1C, Supplementary Table SII). For example, fucoidan from *Undaria pinnatifida* has almost equal ratios of fucose, galactose and sulfate agreeing well with its putative structure as galactofucan with branches of sulfated galactose (Hemmingson et al. 2006; Synytsya et al. 2010). Fucoidans from *Cladosiphon okamuranus, Ecklonia maxima* and *F. serratus* are rich in uronic acids. We newly identified galacturonic acid in *C. okamuranus* fucoidan (Sakai et al. 2003), and we further specified the previously reported uronic acids of *F. serratus* fucoidan to be glucuronic acid (Cumashi et al. 2007). The molar ratio of fucose:sulfate is an average of 1.8 sulfate groups per fucose, similar to fucoidan from *Laminaria hyperborea* with 1.7 sulfate groups per fucose (Kopplin et al. 2018). The sulfate content significantly (two-sided paired *t*-test, P-value<0.01) increased over the course of the purification ranging from 10% to 45% of the dry weight (Figure 1D). The molecular mass of 95 up to 418 kDa (Figure 2A) is higher than fucoidan purified by, e.g., dye-affinity chromatography (Hahn et al. 2016; Zayed et al. 2016), indicating binding of large polysaccharides due to higher total charge. Overall, those molecular characteristics of IEX-purified fucoidans are similar to previously reported data suggesting IEX is a reliable method to purify fucoidans from crude extracts.

Glycosidic linkages of purified fucoidans

For structural analysis, we focused on IEX-purified fucoidans from *C. okamuranus*, *F. serratus*, *E. maxima* and *D. potatorum*, and compared these with fucoidan from *F. vesiculosus* as a reference. With methylation analysis, we identified 19 different linkages (Figure 2B). The structurally simplest fucoidan is *C. okamuranus*, since it is mainly composed of 3-linked (57%), 2,3-linked fucose (25%) and minor amounts of terminal fucose (5.1%). All other fucoidans have

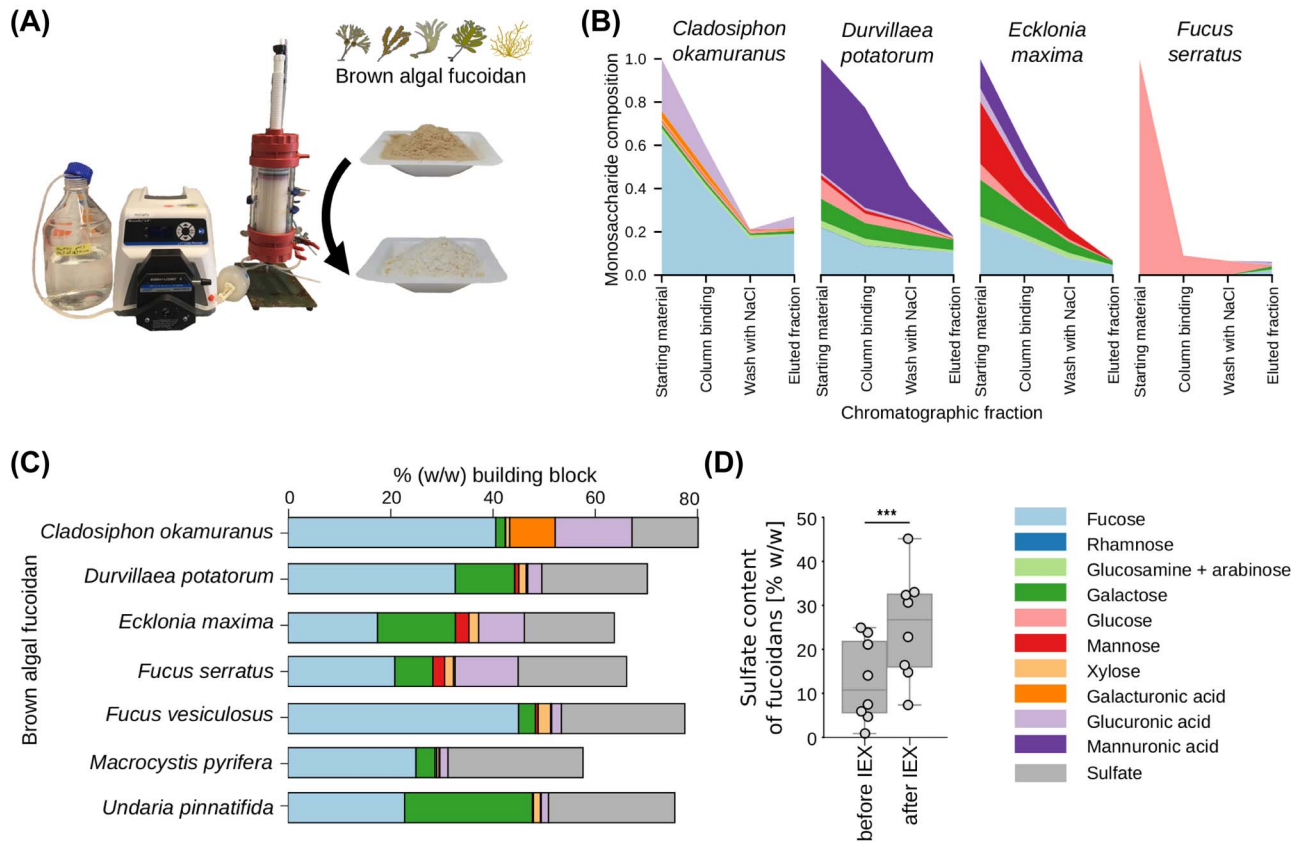
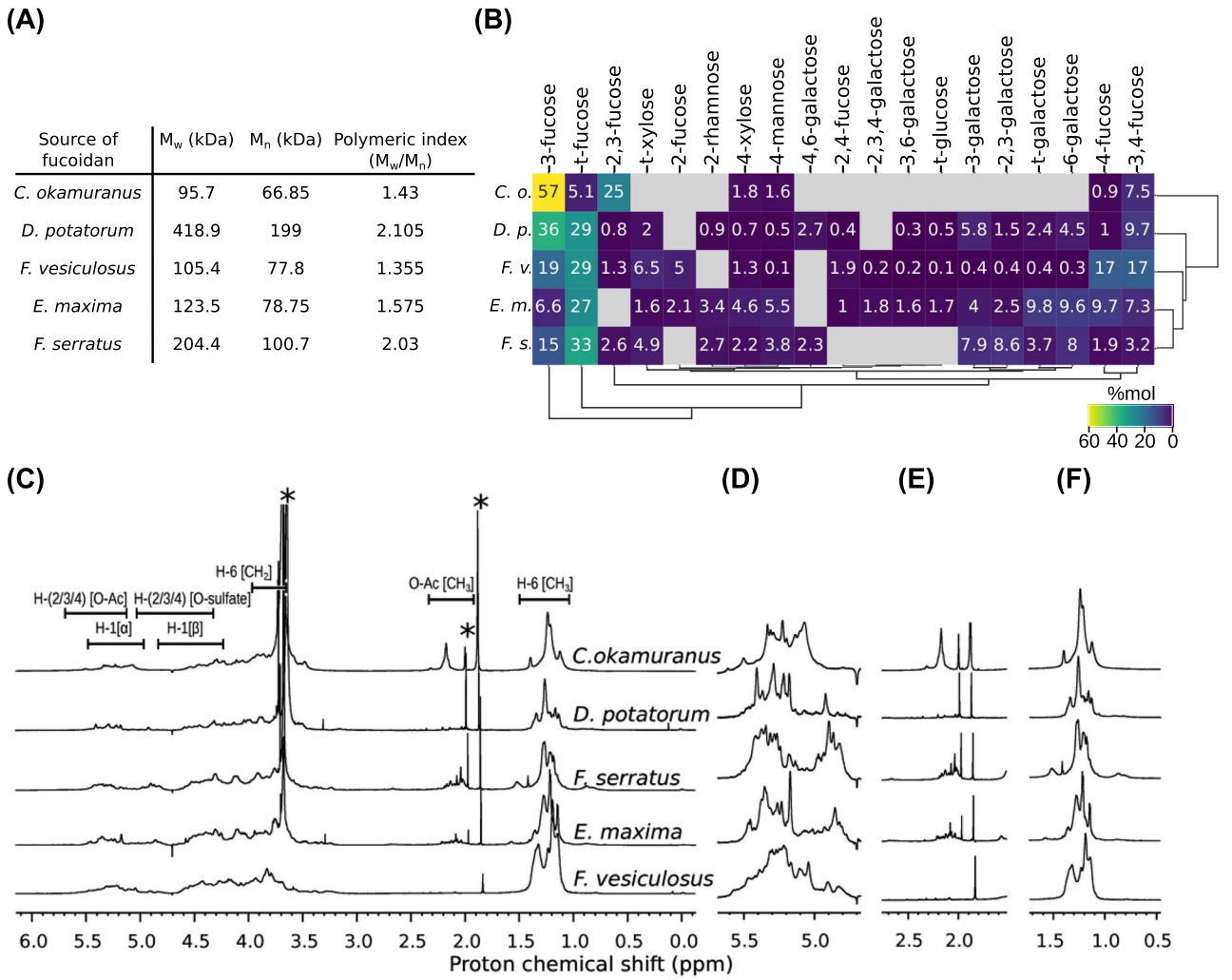


Fig. 1. Ion-exchange purification of fucoidans from different species of brown algae. (A) A scheme of the custom medium-scale setup for the purification of fucoidans used in this study. The peristaltic pump was manually controlled to load, wash or elute fucoidans onto the column packed with ANX-FF resin. The duration of one run, including column wash, was approximately 45 min and yielded up to 350 mg of purified fucoidans per 500 mg starting material. Over the course of the purification, the characteristic brown color of the crude extract changes to a white product due to the removal of proteins and phlorotannins as exemplary shown on the right for fucoidan from *C. okamuranus*. (B) Quantitative glycan profiling during purification of fucoidans from four different brown algae. Changes in absolute amount and relative composition (*y*-axis) of fucoidans per 1 mg of starting material are shown over the course of the purification (*x*-axis). Total carbohydrate content and monosaccharide composition was determined in three independent chromatographic runs ($n = 3$) and their average is shown. (C) Monosaccharide and sulfate content of purified fucoidan from different species of brown algae. The sulfate content and monosaccharide composition was determined in two analytic replicates of the same acid hydrolysis. (D) Comparison of the sulfate content of fucoidans before and after IEX purification. Data points represent the sulfate content before and after purification, and their distribution is summarized in boxplots where the middle line indicates the median, the box designates the interquartile range (IQR) and the whiskers denote 1.5 times the IQR, and *** denote $P < 0.01$ of a paired *t*-test.

a surprisingly high amount of terminal fucose (27–33%), indicating many short fucose branches (Kopplin et al. 2018). *D. potatorum*, *F. vesiculosus* and *F. serratus* are also rich in 3-linked fucose (36, 19 and 15%), whereas fucoidans from *F. vesiculosus* and *E. maxima* are rich in 4-linked fucose (17 and 10%). We found high amount of 3,4 linked fucose in *F. vesiculosus* (17%) and in *D. potatorum* (10%). Additionally, we identified terminal xylose in *F. vesiculosus* and *F. serratus* and a non-negligible amount of 4-linked xylose in *E. maxima* (5%). We identified various galactose linkages in fucoidans from *F. serratus*, *E. maxima* and *D. potatorum*. Overall, the relative abundances of each linkage agree with the monosaccharide composition for those sugars. The linkage analysis suggests all fucoidans are homofucans and possibly have side branches of fucose and other monosaccharides.

Structural characterization of fucoidans with NMR spectroscopy

To verify these structures, fucoidans were analyzed by ^{1}H NMR (Figure 2C–F) and ^{1}H-^{13}C HSQC spectroscopy (Supplementary Figures S2–6). Details are described in the Supplement Information; in short, we found a variety of α-anomeric signals ($\delta_H \sim 5.0$–5.5 and $\delta_C \sim 90$–105) that are mainly α-L-fucose and to a lesser extent, α-hexoses ($\delta_C > 100$) (Chizhov et al. 1999; Grachev et al. 2006; Bilan et al. 2013, 2017). Anomeric signals ($\delta_H \sim 4.25$–5.0 and $\delta_C \sim 100$–110) consistent with β-O-linked hexoses i.e. galactose (Bilan et al. 2013, 2017; Usoltseva et al. 2017; Usoltseva et al. 2018) are present in multiple fucoidans and could be specifically linked to the 4,6- and 6-galactose linkages in case of *F. serratus* fucoidan (δ_H 3.83, 4.09/δ_C 69.1) (Ruthes et al. 2013; Usoltseva et al. 2017). Fucoidan from *F. vesiculosus* has another pair of CH_2 signals (δ_H 3.24, 3.90/δ_C 65.2) attributed to β-linked xylose (Bilan et al. 2002). Signals for O-2, O-3 and/or O-4 sulfation were detected in all fucoidans ($\delta_H \sim 4.5$–5.0/$\delta_C \sim 75$–85). Additionally, fucoidans from *E. maxima*, *F. serratus* and *C. okamuranus* are O-acetylated ($\Delta\delta_H + 1.0$–1.5/$\Delta\delta_C + 1$–4). We identified five positions of O-acetylation in *F. serratus* fucoidan (δ_H/δ_C: 4.88/70.7, 4.97/71.0, 4.98/70.0, 5.09/70.2 and 5.12/71.1) together with two positions in *C. okamuranus* fucoidan (δ_H/δ_C: 5.25/67.4 and 5.50/69.1) (Chizhov et al. 1999; Bilan et al. 2002; Perepelov et al. 2008). Finally, we compared fucoidan from *C. okamuranus* with a characterized oligomer (Supplementary Figure S7)



Source of fucoidan	M_w (kDa)	M_n (kDa)	Polymeric index (M_w/M_n)
C. okamuranus	95.7	66.85	1.43
D. potatorum	418.9	199	2.105
F. vesiculosus	105.4	77.8	1.355
E. maxima	123.5	78.75	1.575
F. serratus	204.4	100.7	2.03

	t-fucose	2,3-fucose	t-xylose	2-fucose	2-rhamnose	4-xylose	4-mannose	4,6-galactose	2,4-fucose	2,3,4-galactose	3,6-galactose	t-glucose	3-galactose	2,3-galactose	t-galactose	6-galactose	4-fucose	3,4-fucose
C. o.	5.1	25				1.8	1.6										0.9	7.5
D. p.	29	0.8	2		0.9	0.7	0.5	2.7	0.4		0.3	0.5	5.8	1.5	2.4	4.5	1	9.7
F. v.	29	1.3	6.5	5		1.3	0.1		1.9	0.2	0.2	0.1	0.4	0.4	0.4	0.3	17	17
E. m.	27		1.6	2.1	3.4	4.6	5.5		1	1.8	1.6	1.7	4	2.5	9.8	9.6	9.7	7.3
F. s.	33	2.6	4.9		2.7	2.2	3.8	2.3					7.9	8.6	3.7	8	1.9	3.2

Fig. 2. Structural characterization of IEX-purified fucoidans and fucoidan from *F. vesiculosus* as control. (A) Macromolecular properties of fucoidans. The molecular mass and derived polymeric index are represented as mean of replicates from two SEC-MALS measurements with a constant Dn/dc of 0.1 mL/g for all fucoidans. (B) Linkage analysis of five different fucoidans. The heatmap show the molecular percentage of different glycosidic linkages derived from their relative peak areas. Rows and columns are ordered by a hierarchical clustering based on the Euclidean distance metric. (C–F) Overlaid ^{1}H NMR spectra comparing fucoidans from five algal species. (C) Overlay of whole spectrum annotated with regions relating to structural moieties. The key for annotated regions is as follows: H-1[α] = α-anomeric protons, H-1[β] = β-anomeric protons, H-(2/3/4)[O-Ac] = protons associated with O-acetylation at positions C2, C3 and/or C4, H-(2/3/4)[O-sulfate] = protons associated with O-sulfation at positions C2, C3 and/or C4, H-6[CH_2] = protons of hexose -CH2OH, O-Ac[CH_3] = acetyl CH_3 protons, H-6[CH_3] = fucose CH_3 protons. Contamination peaks are highlighted with asterisks (*) and likely originate from Tris buffer and/or (poly)ethylene glycol = $\delta \sim 3.7$, acetyl CH_3 signals = $\delta \sim 1.8$, 1.9. (D) Expanded region showing anomeric signals and some residual water signal ($\delta \sim 4.7$). (E) Expanded region showing acetyl CH_3 signals. (F) Expanded region showing C6 CH_3 signals associated with fucose residues.

and found support for α-1,3-linked fucose backbone with 4-O-sulfation and α-1,2-linked glucuronic acid branches (Sakai et al. 2003). Potentially, the structure of fucoidans could be further resolved by NMR of its desulfated derivative or by MS/MS analysis of native oligosaccharides (Yuguchi et al. 2016; Kopplin et al. 2018).

This study shows that IEX efficiently removes contaminations such as proteins and alginates from different fucoidan preparations resulting in pure and structurally defined fucoidans.

Materials and methods

The content, composition, linkage and structure of carbohydrates were analyzed with common laboratory protocols including phenol–sulfuric acid assay, HPAEC-PAD, GC–MS and NMR, which are described in detail in the Supplement Information.

Purification of fucoidans

Fucoidan extracts were purified using a custom medium-scale IEX with a detailed stepwise online protocol (https://www.protocols.io/researchers/andreas-sichert). A MasterFlex L/S peristaltic pump was used to operate an XK50/20 column (GE Healthcare) packed with 200 mL of ANX FF resin (GE Healthcare) at a flow of 50 mL/min. Fucoidans (Supplementary Table SI) were solubilized in 50 mM of Tris pH 7.5 and centrifuged for 30 min at 4000 g. For binding, the supernatant was circulated three times over the column. Next, the column was washed with three column volumes of 50 mM of Tris pH 7.5 and three column volumes of 50 mM Tris pH 7.5 with 500 mM of NaCl. Fucoidans were eluted with 100 mL of 50 mM Tris pH 7.5 with 5 M NaCl. After each run, the column was washed with 50 mM of Tris pH 7.5 with 5 M NaCl, deionized water, 250 mM NaOH pH 13.4 and deionized water. Fucoidans were then dialyzed

(Spectra/Por 6 Dialysis Tubing, 1 kDa MWCO) against ddH_2O and lyophilized. Unless otherwise stated, reagents and equipment were purchased at Thermo fisher scientific (Waltham, MA, USA) Sigma-aldrich (st. Luis, Missouri, USA) Carbosynth.

Supplementary data

Supplementary data for this article are available online at http://glycob.oxfordjournals.org/.

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Author contributions

J.H.H. and A.S. initiated and coordinated the project with H.R. and F.L.A. A.S. purified fucoidans and analyzed their composition. F.L.A. and L.J.K. conducted NMR and SEC-MALS analysis and interpretation. S.L.G. and B.L. conducted linkage analysis and S.L.G. conducted their interpretation. A.S and J.-H.H. prepared the manuscript and received input from all authors.

Conflict of interest statement

The authors declare no competing interests.

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