Carbohydrate Polymers 81 (2010) 41–48

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Structure and antitumour activity of fucoidan isolated from sporophyll of Korean brown seaweed *Undaria pinnatifida*

Andriy Synytsya [a], Woo-Jung Kim [e], Sung-Min Kim [e], Radek Pohl [d], Alla Synytsya [b], František Kvasnička [c], Jana Čopíková [a], Yong Il Park [e,*]

[a] Department of Carbohydrate Chemistry and Technology, Institute of Chemical Technology in Prague, Technická 5, 166 28 Prague 6, Czech Republic
[b] Department of Analytical Chemistry, Institute of Chemical Technology in Prague, Technická 5, 166 28 Prague 6, Czech Republic
[c] Department of Meat Technology and Preservation, Institute of Chemical Technology in Prague, Technická 5, 166 28 Prague 6, Czech Republic
[d] Institute of Organic Chemistry and Biochemistry AS CR, Flemingovo sq. 2, 166 28 Prague 6, Czech Republic
[e] Department of Biotechnology, The Catholic University of Korea, Bucheon-si, Gyeonggi-do 420-743, Republic of Korea

ARTICLE INFO

Article history:
Received 23 November 2009
Received in revised form 16 January 2010
Accepted 22 January 2010
Available online 29 January 2010

Keywords:
Fucoidan from sporophyll *Undaria pinnatifida*
Structure
Antitumour activity

ABSTRACT

Fucoidan from the sporophyll (Miyeokgui) of cultured Korean brown seaweeds *Undaria pinnatifida* (Miyeok) is interesting due to its various biological activities. This polysaccharide was isolated from raw material by dilute acid extraction, ballast alginates were removed by $CaCl_2$ precipitation, and crude extract was purified by chromatography on DEAE–cellulose. Structure and composition of the fucoidan was characterised by various methods (organic elemental analysis, HPLC analysis of neutral sugars, FT-IR, FT-Raman, ^1H and ^{13}C NMR); molecular size and charge density were estimated by GPC and CITP. The polysaccharide was eluted by GPC as a single peak of approximate molecular weight of 1246 kDa. Miyeokgui fucoidan showed lower electrophoretic mobility (RSH ~ 0.127) than carrageenans (0.096–0.100) and chondroitin sulphate A (0.103) but higher than alginate (0.170). Isolated polysaccharide contained α-fucose (50.9 mol%) and β-galactose (44.6 mol%) as main neutral sugar units; no uronic acid was observed. Sulphate (0.97 mol mol^{-1}) and acetate (0.24 mol mol^{-1}) esters were also found. Thus the polysaccharide was defined as *O*-acetylated sulphated galactofucan. The Miyeokgui fucoidan showed antitumour activity against PC-3 (prostate cancer), HeLa (cervical cancer), A549 (alveolar carcinoma), and HepG2 (hepatocellular carcinoma) cells, in a similar pattern to that of commercial fucoidan.

1. Introduction

Fucoidans are highly sulphated cell-wall polysaccharides of brown algae, containing L-fucose as main sugar unit. The main skeleton of fucoidans consist of α-1,3-linked sulphated L-fucose; a repeating sequence of alternating α(1 → 3) and α(1 → 4) glycosidic bonds is also possible (Berteau & Mulloy, 2003; Li, Lu, Wei, & Zhao, 2008). The chemical composition and structure of fucoidans are very diverse (Rioux, Turgeon, & Beaulieu, 2009, 2007) and significantly vary depending on the algae source, place of cultivation and habitat, harvesting time etc. Biological activity and medicinal impact of fucoidans depends strongly on their structural properties. These polysaccharides are known to exhibit a wide range of physiological and biological activities (Cumashi et al., 2007; Li et al., 2008; Shi, Guo, & Wang, 2000), such as anti-inflammatory (Choi et al., 2010), antiviral (Hemmingson, Falshaw, Furneaux, & Thompson, 2006; Hoshino, Hayashi, Hayashi, Lee, & Sankawa, 1998; Lee, Hayashi, Hashimoto, Nakano, & Hayashi, 2004; McClure et al., 1992), anticoagulant (Chevolot et al., 1999), antitumour and antimetastatic (Alekseyenko et al., 2007; Riou et al., 1996), and antiangiogenesis (Hahnenberger & Jakobson, 1991) activities. Due to these activities fucoidans have potential applications in medicine and thus these polysaccharides, their production, structure and properties, have been intensively investigated.

Undaria pinnatifida, commonly called Miyeok in Korea and Wakame in Japan, is common edible brown seaweed plentiful on the shores of the Korean peninsula and Japanese isles and expanding along some regions of the coastlines of Australia and New Zealand. Coastal area of southern Korea peninsula is a perspective region for growth and cultivation of this brown alga. Miyeokgui (Mekabu in Japan), a sporophyll situated at the root part of this alga, is interesting as a plentiful source of specific biologically active fucoidan. It has been reported that this fucoidan has anticoagulant, immunomodulating, antitumour, antiviral and antiprotozoic activities (Chen, Lim, Sohn, Choi, & Han, 2009; Hayashi, Nakano, Hashimoto, Kanekiyo, & Hayashi, 2008; Hemmingson et al., 2006; Kim et al., 2007; Lee et al. 2004; Maruyama, Tamauchi, Hashimoto, & Nakano, 2003; Maruyama, Tamauchi, Hashimoto, & Nakano, 2005; Maruyama, Tamauchi, Iizuka, & Nakano, 2006; Maruyama, Tanaka, Hashimoto, Inoue, & Sasahara, 2007; Yoo et al., 2007). The activated

* Corresponding author. Tel.: +82 2 2164 4512; fax: +82 2 2164 4865.
E-mail address: yongil382@catholic.ac.kr (Y. Il Park).

0144-8617/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2010.01.052

partial thromboplastin time (APTT) assay showed that it induced anticoagulant activity in a dose-dependent manner (Kim et al., 2007). This polysaccharide modulates Th2 responses and thus might be useful for treating allergic inflammation (Maruyama et al., 2005) and mediates tumour destruction through Th1 cell and NK cell responses (Maruyama et al., 2003; Maruyama et al., 2006). Its anticancer activity could be significantly enhanced by partial depolymerisation at mild condition (Yang, Chung, Shin, et al., 2008). This fucoidan is less cytotoxic to immune cells than common fucoidan from *Fucus vesiculosis*, and possesses immunomodulating activity to produce cytokines and chemokines from macrophages and splenocytes (Yoo et al., 2007). Fucoidan from *U. pinnatifida* sporophyll showed potential antiviral activities against herpes simplex viruses (HSV-1, HSV-2) and human cytomegalovirus HCMV (Hemmingson et al., 2006; Lee et al., 2004). This polysaccharide is a selective *in vitro* inhibitor for secretory phospholipase A2-IIA and the phosphorylation of cellular functional basic proteins by A-kinase (Maruyama, Suzuki, Miyai, & Ohtsuki, 2008). It is also able to inhibit the activity of hyaluronidase (IC_{50} = 13.0 μg ml^{-1}) in a dose-dependent manner (Katsube, Yamasaki, Iwamoto, & Oka, 2003). All these reports confirmed that fucoidan from *U. pinnatifida* sporophyll (Miyeokgui, Mekabu) would be promising candidates for pharmacological use.

The structure of Miyeokgui (Mekabu) fucoidan is still poorly investigated. In contrast to common fucoidans, this polysaccharide consists mainly of fucose and galactose, i.e. it is sulphated galactofucan. The ratio between these sugars as well as presence of other monosaccharides including uronic acids significantly varied for different preparations; molecular weight and degrees of substitution (sulphation, *O*-acetylation) are also very variable (Hemmingson et al., 2006). Methylation analysis of several preparations confirmed that this polysaccharide has complex structure with various sugar linkages (1,3-linked fucose, 1,3-, 1,4- and 1.6-linked galactose) and sulphate substitution patterns (2- or 4-position of fucosyl residues, 3- or 6-positions of galactosyl residues) (Hemmingson et al., 2006; Lee et al., 2004).

This study is devoted to structural characterisation of Miyeokgui fucoidan originated from cultured Korean algae using spectroscopic, separation and other analytical methods; and to the estimation of its antitumour activity in comparison with commercial fucoidan from *F. vesiculosus*.

2. Experimental

2.1. Extraction and purification of fucoidan

The sporophyll (Miyeokgui) of cultured Korean *U. pinnatifida* (Miyeok) as a source of polysaccharides used in this study was collected from a southern coastal area of Wando, Korea, and was kindly provided from HarimBio Co., Ltd. (Wando, Korea). The extraction and purification procedures were performed as described by Kim et al. (2007) and illustrated in Fig. 1. Polysaccharide fraction was obtained by acid extraction; ballast alginates were removed by precipitation with $CaCl_2$. Final purification was made using DEAE–cellulose chromatography.

2.2. Elemental analysis

Obtained polysaccharide fraction was analysed for their carbon, hydrogen, nitrogen and sulphur content using the Elementar vario EL III (Elementar Analysensysteme GmbH, Germany). The degree of sulphation *DS* (moles of SO_3^- per mole of sugar units) was calculated based on elemental analysis:

$$DS = \frac{\%S}{\%C} \cdot (6 + 2 \cdot DAc) \cdot \frac{A_C}{A_S},$$

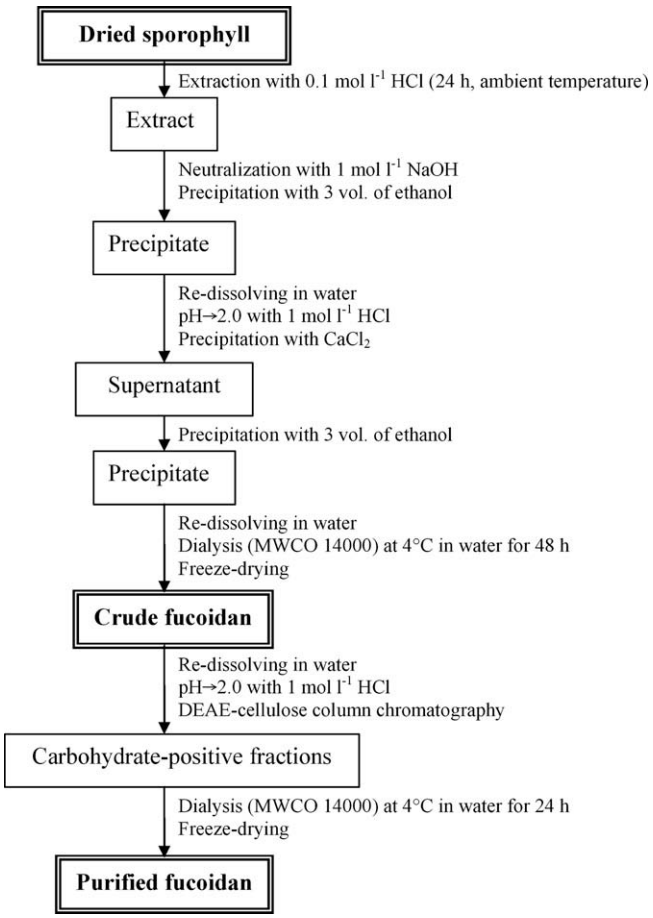

Fig. 1. Isolation and purification of the Miyeokgui fucoidan.

where %S and %C are contents of carbon and sulphur, A_C and A_S are atomic weights of these elements, 6 and 2 are numbers of carbons in the pyranoid ring and in the *O*-acetyl group, *DAc* is the degree of acetylation calculated from ^{1}H NMR.

2.3. Gel permeation chromatography

High-performance gel permeation chromatography (HP-GPC) analysis of the extracts was carried out at room temperature by using a PL aquagel–OH Guard column, followed by PL aquagel–OH Mixed column (Polymer laboratories Ltd., UK), high pressure pump LCP 4100 (ECOM, Czech Republic) and a refractive index detector Shodex RI 71 (Dionex Softron GmbH, Germany). Samples were eluted at a flow rate of 0.5 ml min^{-1} with salt solution (0.05 mol l^{-1} NaH_2PO_4, 0.05 mol l^{-1} Na_2HPO_4, 0.2 mol l^{-1} $NaNO_3$ and 0.02% NaN_3) as a mobile phase.

2.4. Capillary isotachophoresis

Capillary isotachophoresis (CITP) was performed on a column-coupling electrophoretic analyser EA 101 (Villa-Labeco, Slovak Republic) equipped with conductivity and UV (254 nm) detectors. Mixture of 5 mM HCl, 10 mM glycylglycine and 0.05% 2-hydroxyethyl cellulose (HEC) in demineralised water served as leading electrolyte, 10 mM acetic acid as terminating one. Applied driving current was 200 μA and 20 μA in the pre-separation and analytical capillary, respectively. Sample was injected by sample valve with fixed volume of 30 μl. One analysis took 15 min.

2.5. Analysis of neutral sugars

Neutral sugars were released by Saeman hydrolysis (Selvendran, March, & Ring, 1979) and analysed as alditol acetates by GC using a GC 07 chromatograph (Labio, Czech Republic). A 30 m capillary column DB-1 Agilent (J and W) with i.d. 0.25 mm and 0.1 μm film thickness was used. The temperatures of injector and detector were respectively 220 and 230 °C. The oven temperature program was following: 120 °C for 1 min, then rose to 130 °C (2.5 °C/min) for 30 min, and finally rose to 220 °C (5 °C/min). The monosaccharide analysis of TFA-hydrolyzed polysaccharides was performed by HPAEC as described by Lee, Lim, Lee, and Park (2006), using Bio-LC (DX 500 Chromatography System, Dionex Co., USA) system equipped with a pulsed amperometric detector (ED 50, Dionex Co., USA).

2.6. Vibration spectroscopy

FT-IR spectra (spectral region 4000–400 cm^{-1}, resolution 2 cm^{-1}) of the solid samples in the form of KBr tablets were recorded on Nicolet 6700 spectrophotometer (Thermo Scientific, USA) using Omnic 7.0 software. FT-Raman spectra of powder samples were recorded by using Bruker FT-Raman (FRA 106/S, Equinox 55/S) spectrometer equipped with a quartz beam splitter, a liquid nitrogen cooled germanium detector and excitation at 1064 nm from a Nd:YAG laser. The laser power was set at 100 mW, and 256 scans were accumulated with a spectral resolution of 2.0 cm^{-1}. Vibration spectra were 10-point filtered and baseline corrected using Origin 6.0 (Microcal Origin) software. The second derivatives of the spectra were used for wavenumber determination of overlapped bands.

2.7. Nuclear magnetic resonance

^1H and ^{13}C NMR spectra were recorded on Bruker Avance 600 and Bruker Avance 500 in D_2O solutions. Working frequencies were 600.1 MHz and 499.8 MHz for ^1H, 150.9 MHz and 125.7 MHz for ^{13}C, respectively. Signals of 1,4-dioxane (3.75 ppm in ^1H, 69.30 ppm in ^{13}C) were used as a reference. Correlation spectroscopy such as H,H-PFG-COSY, H,C-HSQC and H,C-HMBC were applied for signals assignment. Content of *O*-acetyl groups in fucoidan was estimated as the integrated areas' ratio A_1/A_0 of the methyl hydrogen resonance signals assigned to *O*-acetyls (A_1) and C-6 of fucose (A_0); the degree of acetylation *DAc* (moles of *O*-acetyls per mole of sugar units) was calculated according to the equation:

$$DAc = \frac{A_1}{A_0} \cdot \frac{100}{\%\mathrm{Fuc}},$$

where %Fuc is the content of fucose (mol% of all neutral sugars).

2.8. Cell culture and antitumour activity

HeLa (cervical cancer cell, ATCC No. CCL-2™), PC-3 (prostate cancer cell, ATCC No. CRL-1435™), A549 (carcinomic human alveolar basal epithelial cell, ATCC No. CCL-185™) and HepG2 (hepatocellular carcinoma cell, ATCC No. HB-8065™) cells were grown in Roswell Park Memorial Institute medium (RPMI) 1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% penicillin–streptomycin (GIBCO, USA). The cells were maintained at 37 °C under 5% CO_2 and subcultured twice a week. For antitumour activity of fucoidans, cells were subcultured in 96-well plates at a density of 5×10^4 cells per well. After monolayer cultivation for 24 h in 5% CO_2 at 37 °C, the medium was removed and replaced with 100 μl of the maintenance medium (MM) containing 2% FBS. Cells were then incubated for 24 h with different concentrations (0.1–0.8 mg ml^{-1}) of commercial fucoidan (from *F. vesiculosus*, Sigma, USA) or our fucoidan (from sporophyll of *U. pinnatifida*). The cultures were then re-incubated for an additional 4 h with 20 μl of MTT (5 mg/ml) solution. After removal of the supernatant, 100 μl of DMSO was added to each well to dissolve the crystals completely and then the absorbance was measured at 570 nm using an ELISA Reader (Bio-Rad, USA).

3. Results and discussion

3.1. Chemical composition

Composition of the Miyeokgui fucoidan is summarised in Table 1. The sample contains no nitrogen and significant amount of sulphur. According to these results no proteins was detected (no

Table 1
Elemental analysis and chemical composition of the Miyeokgui fucoidan.

Content of elements (%)				Substitution degrees (mol/mol)		Neutral sugars (mol% of all neutral sugars)			
C	H	N	S	*DS*	*DAc*	Fuc	Gal	Xyl	Man
23.03	3.97	No	9.18	0.97	0.24	50.9	44.6	4.2	0.3

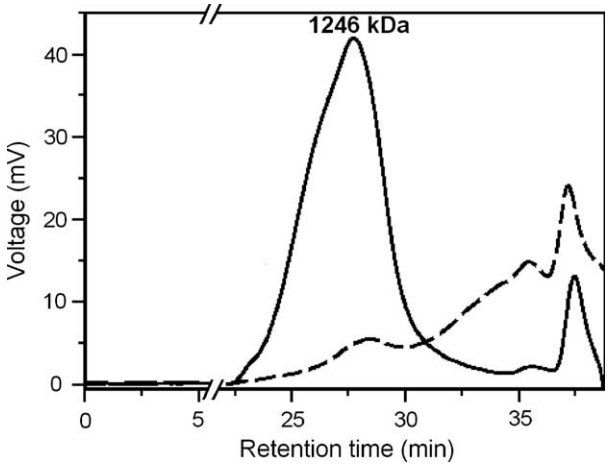

Fig. 2. GPC chromatograms of the Miyeokgui fucoidan: RI (solid) and UV (dash) records.

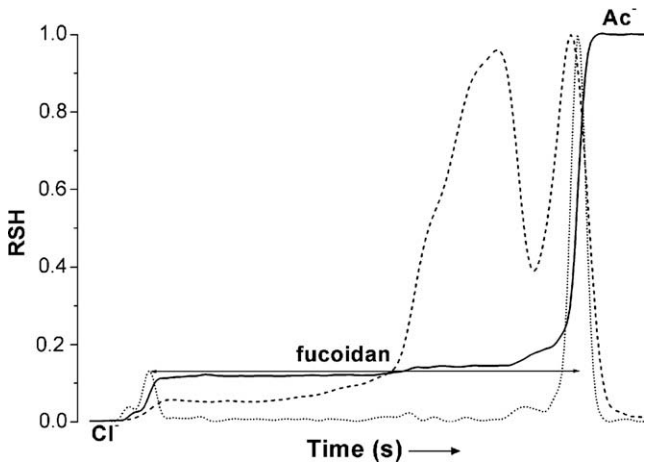

Fig. 3. Isotachopherograms of the Miyeokgui fucoidan: conductivity (solid, 1st derivation – dot) and UV–254 nm (dash) records.

nitrogen), while the presence of sulphur (9.18%) is indicative of sulphated polysaccharides. The content of carbon is relatively low (23.03%) that could be explained by the presence of inorganic compounds like counter ions. Neutral sugar analysis confirmed that fucose (50.9 mol%) and galactose (44.6 mol%) are major neutral sugars of obtained polysaccharide; small amounts of xylose and mannose were also detected. The ratio between fucose and galactose is approximately 1.0: 1.1 that is similar to that reported in literature (Hemmingson et al., 2006; Katsube et al., 2003; Lee et al., 2004).

3.2. GPC and CITP analysis

GPC elution profiles, RI and UV records, of the fucoidan are shown in Fig. 2. The polysaccharide was eluted as a single peak, and its approximate molecular weight was estimated to be 1246 kDa. Corresponding small peak of UV record indicates some aromatics integrated with the polysaccharide. According to the literature (Yang, Chung, & You, 2008; Yang, Chung, Shin, et al., 2008; Hemmingson et al., 2006; Yang et al., 2008), molecular weight of Miyeokgui (Mekabu) fucoidan preparations significantly varied dependently on isolation conditions: mild enzymatic extraction leads to larger polysaccharide macromolecules or aggregates (>10,000 kDa), acidic extraction to smaller ones (710–5,200 kDa). Further treatment (boiling water, microwave heating, $CuAc/H_2O_2$ etc.) led to marked decrease of molecular weight that could improve antitumour activity of this polysaccharide (Yang, Chung, & You, 2008; You, Yang, Lee, & Lee, 2010).

CITP records of the fucoidan (conductivity and UV–254 nm detectors) are demonstrated in Fig. 3. The chloride ion mainly in the aqueous media and acetate ion were used as the leading and terminating electrolytes, respectively. Similar electrolyte system has been successfully used in analysis of algal acidic polysaccharides (Hiraoka, Harada, Uehara, Sekiguchi, & Maeda, 1992). Relative step height (RSH) corresponds to the mobility of analysed polyanions, and the higher RSH the lower mobility. RSH of several anionic polysaccharides were measured at the same CITP conditions. It was found that the Miyeokgui fucoidan showed lower mobility (RSH ~ 0.127) than carrageenans (0.096–0.100) and chondroitin sulphate A (0.103) but higher than alginate (0.170). Mobility of

Table 2
IR and Raman band assignment for the Miyeokgui fucoidan (Pereira et al., 2009; Qiu et al., 2006; Sekkal & Legrand, 1993; Synytsya et al., 2003).

Wavenumber (cm^{-1})		Assignment
FT-IR	FT-Raman	
1740	1739	ν(C=O) – *Ac*
1647		$\delta(H_2O)$
1542		Amide II – proteins
1455	1457	$\delta(CH_2)$ – Gal; $\delta_{as}(CH_3)$ – Fuc, *Ac*
1380	1380sh	$\delta_s(CH_3)$ – Fuc, *Ac*
	1337	CCH, HCO, COH
1256	1269	$\nu_{as}(SO_2)$
1165	1151	ν(COC)
1140	1130	ν(COC)
	1072	$\nu_s(SO_2)$
1058		ν(CO),(CC)
1034		δ_s(OSO), ν(CO)
969	1005	ν_{as}(COS); $\rho(CH_3)$ – Fuc, *Ac*
920		$\rho(CH_3)$ – Fuc, *Ac*
	890	$\nu(C_1H)$ – β-anomers, $\rho(CH_2)$ – Gal
836	839	ν_s(COS)
	788	CO, COH
692	708	τ(CO), γ(COC), γ(OCO)
622sh	600	$\delta_{as}(SO_2)$
583	553	$\delta_s(SO_2)$
	521	γ(CCO), γ(CCC)
	417	$\rho(SO_3)$, γ(CCO), γ(CCC)
	306	γ(CCO), τ(CC)
	256	τ(CO), γ(CCO)
	205	τ(CC), τ(CO)

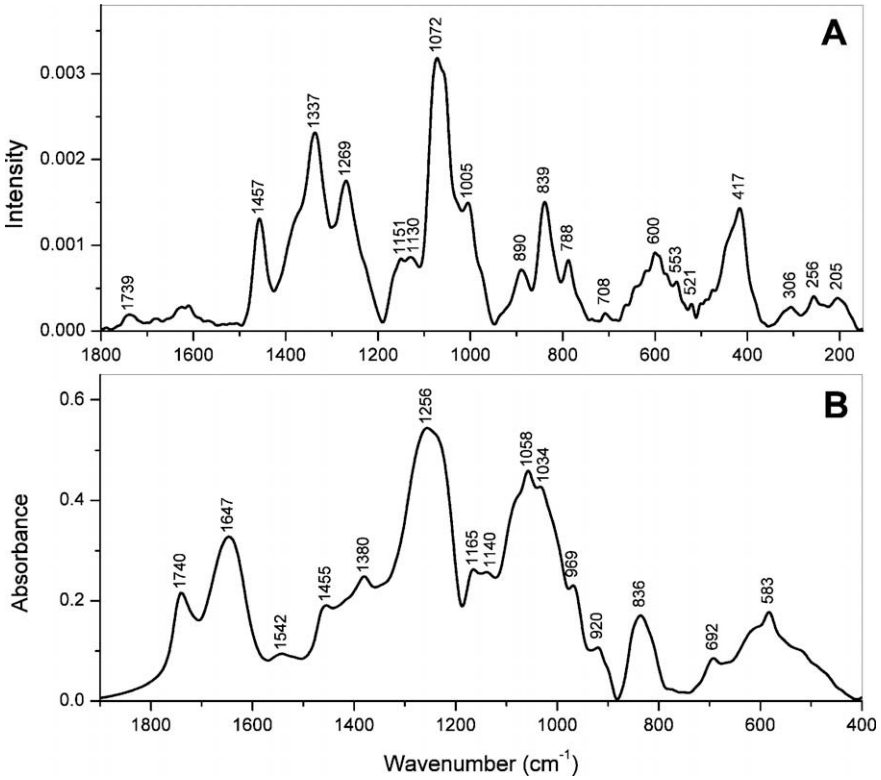

Fig. 4. FT-Raman (A) and FT-IR (B) spectra of the Miyeokgui fucoidan.

fucoidan macromolecules depends on their hydrodynamic size, shape, charge density (distribution of sulphate anions) and presence of other functional groups (*O*-acetyls; CH_3/CH_2OH in the fucose/galactose units). The shape of fucoidan step is not smooth, but slightly rising towards its end (*cf.* Fig. 3). A rapid increase of the UV signal (λ = 254 nm) towards the end of fucoidan step indicating the presence of covalently bound impurities (polyphenols). Such shape of the fucoidan step describes the distribution of polysaccharide macromolecules according to their mobility. Sulphated polysaccharides do not absorb at 254 nm, while aromatics covalently bound to polysaccharide have an evident signal. Thus, fucoidan macromolecules carrying polyphenols demonstrate decreased mobility in electric field.

3.3. Vibration spectroscopy

FT-IR and FT-Raman spectra of obtained fucoidan are shown in Fig. 4. Band at ~1740 cm^{-1} found in both the spectra was assigned to C=O stretching vibration of *O*-acetyl groups (Synytsya, Čopíková, Matějka, & Machovič, 2003). The IR band at 1647 cm^{-1} is attributed to the bending vibration of water. Intense Raman band at 1457 cm^{-1} and corresponding IR band at 1455 cm^{-1} were assigned to scissoring vibration of CH_2 (galactose, xylose) and asymmetric bending vibration of CH_3 (fucose, *O*-acetyls). IR band at 1380 cm^{-1} and Raman shoulder at similar position are originated from symmetric bending vibration of methyls. Raman band at 1337 cm^{-1} were assigned mainly to HCC, HCO and COH vibrations in pyranoid ring. Very intense and broad IR band at 1256 cm^{-1} and corresponding strong Raman band at 1269 cm^{-1} were attributed to asymmetric O=S=O stretching vibration of sulphate esters with some contribution of COH, CC and CO vibrations. Intense Raman band at 1072 cm^{-1} was assigned mainly to symmetric O=S=O stretching vibration of sulphate esters. These bands are characteristic for sulphated polysaccharides (carrageenans, agar etc.) and have been used as marker of sulphation because their intensity increases with the sulphate content (Pereira, Amado, Critchley, van de Velde, & Ribeiro-Claro, 2009) (Table 2). The envelope of strong to medium IR bands at 1200–970 cm^{-1} is caused mainly by CC

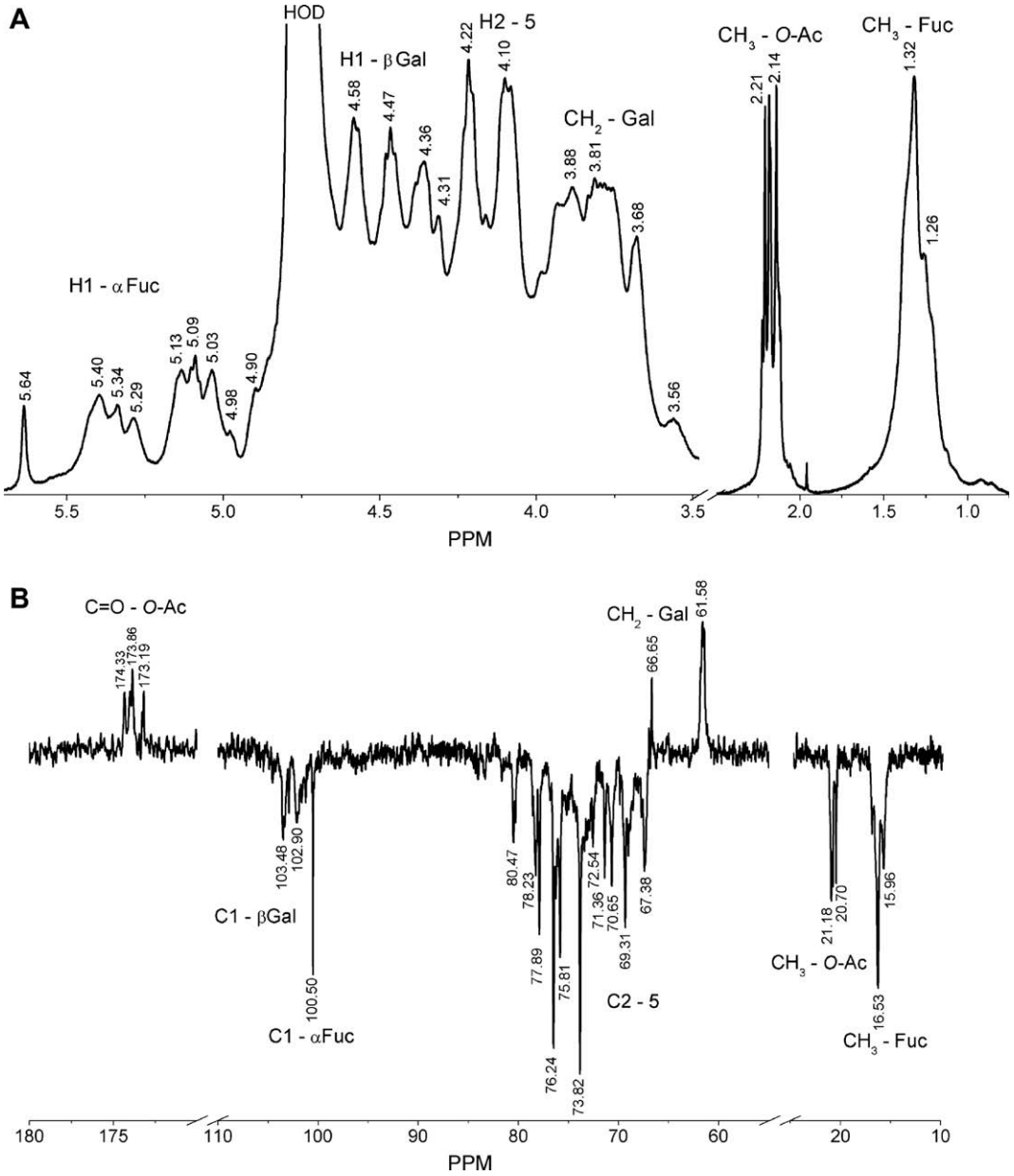

Fig. 5. ^{1}H and ^{13}C NMR spectra (A and B) of the Miyeokgui fucoidan.

and CO stretching in pyranoid ring and COC stretching of glycosidic bonds. Intense absorption at this region is common for all polysaccharides. The IR band at 836 cm^{-1} and corresponding Raman band at 839 cm^{-1} were attributed to COS bending vibration of sulphate substituents at axial C-4 position (Qiu, Amarasekara, & Doctor, 2006). The Raman band at 890 cm^{-1} may indicate the presence of β-glycosidic linkages between monosaccharide units; corresponding band of α-anomers at ~860 cm^{-1} is not detectable owing to overlapping by stronger sulphate band. The IR features at 622 and 583 cm^{-1} (corresponding Raman band at 600 and 553 cm^{-1}) were attributed to the asymmetric and symmetric O=S=O deformation of sulphates (Sekkal & Legrand, 1993).

3.4. NMR spectroscopy

Proton and 13-carbon NMR spectra (D_2O, 20 °C) of purified fucoidan are shown in Fig. 4. The ^1H NMR spectrum (Fig. 5A) contained several intense signals in the α-anomeric (4.9–5.6 ppm) and high-field (1.0–3.5 ppm) regions. The signals of the last region at 1.32 and 1.26 ppm were assigned to C6 methyl protons of L-fucopyranose; several intense and narrow signals at 2.14–2.21 ppm arise from CH_3 protons of *O*-acetyl groups (Bilan, Grachev, Shashkov, Nifantiev, & Usov, 2002; Bilan, Grachev, Shashkov, Nifantiev, & Usov, 2006; Bilan et al., 2004). The molar proportion of fucose and acetate was calculated as 1:0.41 by comparison of integral intensities of the resonance signals of fucose C6 methyl and *O*-acetyl protons. Corresponding molar degree of acetylation was calculated as 0.24. Signals at 5.64–5.03 ppm were assigned mainly to H1 of α-L-fucopyranose residues and to CH protons of *O*-substituted carbons. Two-dimensional $^1H/^1H$ COSY NMR spectrum (not shown) indicates H1/H2 correlations at 5.28/4.84 ppm (2-sulphated fucopyranose) and at 5.33/3.82 ppm (no sulphation at O2) (Chandía & Matsuhiro, 2008). The H6/H5 correlations at 1.32/4.12 ppm and 1.26/4.51 ppm were assigned to 1,3-linked α-L-fucopyranose units.

^{13}C NMR spectrum of fucoidan is shown in Fig. 5B. No C6 carbon signals of uronic acids were found in the spectrum. Several carbonyl carbon signals at 173.2–174.3 ppm and corresponding methyl carbon signals at 20.7–21.2 ppm were assigned to *O*-acetyl groups. Two-dimensional $^1H/^{13}C$ NMR correlation spectra HSQC and HMBC (not shown) confirmed this assignment. The C6 carbon signals of neutral sugar units were found at 16.0–17.1 ppm (CH_3 of α-L-fucopyranose units), 61.6 ppm (CH_2OH of β-D-galactopyranose units) and 66.7 ppm (CH_2OR of β-D-galactopyranose substituted at O6). All the carbon signals mentioned above are splitting into several peaks that confirm the presence of several structural patterns of sugar units dependently on the substitution (glycosylation, sulphation and/or *O*-acetylation). The region ~95–105 ppm contains several resonance signals of anomeric C1 carbons; next region 65–83 ppm contains complex signals of pyranoid ring carbons C2–5. The HSQC cross peaks H1/C1 at 4.71/102.1, 4.96/102.9 and 4.57/103.5 ppm were assigned to β-D-galactopyranose units, 5.30/100.5 ppm to 1,3-linked α-L-fucopyranose-2-sulphate units (Bilan et al., 2002); cross peaks at 5.61/69.4, 5.08/76.0, 5.03/70.6 and 4.89/75.9 to CH of *O*-substituted carbons (Fig. 6).

3.5. Antitumour activity

Antitumour activity of the purified Miyeokgui fucoidan toward PC-3, HeLa, A549 and HepG2 cell lines was investigated, with commercial fucoidan (Sigma, USA) for comparison (Fig. 7A–D). The Miyeokgui fucoidan showed slightly lower activity than the commercial fucoidan against PC-3 prostate cancer cells (Fig. 7A) and A549 carcinomic human alveolar cells (Fig. 7C) by about 20% and 10%, respectively, at the concentrations (0~0.8 mg ml^{-1}) tested. Other than that, both fucoidan samples showed antitumour activity, to some degree, in a similar pattern against these 4 different cancer cell lines, i.e. PC-3, HeLa cell (cervical cancer), A549, and HepG2 cells (hepatocellular carcinoma) (Fig. 7A–D). It was noticeable that both fucoidans showed more significant inhibition of the cell growth against HeLa and A549 cells than PC-3 and HepG2 cells. No cytotoxicity of both fucoidans was observed when Vero cells, which is African green monkey kidney cells (ATCC, USA) and not tumourigenic, were incubated for 24 h in the presence of up to 1 mg ml^{-1} fucoidans (data not shown). These results described that the Miyeokgui fucoidan used in this study has a significant antitumour activity, as reported by other groups about antitumour activities of fucoidans from other sources (Alekseyenko et al., 2007; Riou et al. 1996; Shi et al., 2000). At the moment, our present study is too preliminary to elucidate the relationship between the struc-

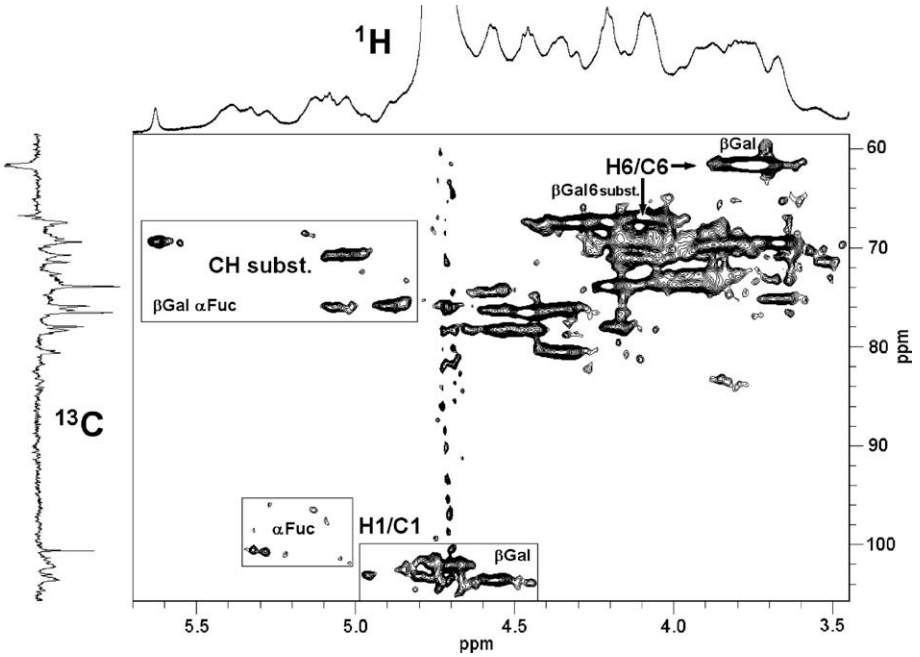

Fig. 6. Partial 2D $^1H/^{13}C$ HSQC spectrum of the Miyeokgui fucoidan.

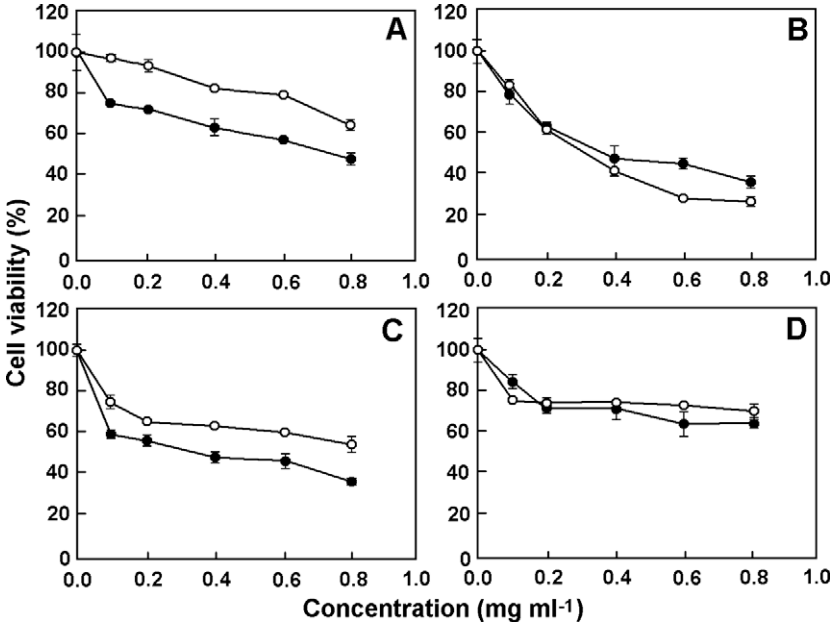

Fig. 7. Antitumour activity of the Miyeokgui fucoidan (open circle) and commercial fucoidan from Sigma, USA (closed circle): PC-3 (A), HeLa (B), A549 (C) and HepG2 (D) cells.

tures of these two different fucoidans and their similar, in some cases, and/or slightly different, in some cases, biological activities.

4. Conclusions

Fucoidan isolated from the sporophyll (Miyeokgui) of Korean seaweed *U. pinatifida* (Miyeok) was characterised by separation (GPC, CITP) and spectroscopic (FT-IR, FT-Raman, NMR) methods. Taking into account the results obtained it may be concluded that this polysaccharide is sulphated galactofucan containing β-D-galactopyranose and α-L-fucopyranose at near equal amounts (44.6 mol% and 50.9 mol%). Xylose (4.2 mol%) and mannose (0.3 mol%) were found as minor sugars, uronic acids were not detected. The fucoidan also contains significant amount of *O*-acetyl groups. Relationship between the galactan and fucan parts in whole polysaccharide as well as the distribution of sulphate and acetate esters are unclear and need more investigation. Specific structural properties of the Miyeokgui fucoidan mentioned above as well as its evident antitumour activity comparable with that of known biologically active commercial fucoidan from *F. vesiculosis* make this polysaccharide interesting for medicinal use.

Acknowledgements

Financial support from the Ministry of Education of the Czech Republic (Projects CEZ: MSM6046137305 and CEZ: MSM6046137307) and from the GRRC program of the Catholic University of Korea supported by Gyeonggi-do province is gratefully acknowledged.

References

Alekseyenko, T. V., Zhanayeva, S. Ya., Venediktova, A. A., Zvyagintseva, T. N., Kuznetsova, T. A., Besednova, N. N., et al. (2007). Antitumor and antimetastatic activity of fucoidan, a sulfated polysaccharide isolated from the Okhotsk Sea *Fucus evanescens* brown alga. *Bulletin of Experimental Biology and Medicine, 143*, 730–732.

Berteau, O., & Mulloy, B. (2003). Sulfated fucans, fresh perspectives: structures, functions, and biological properties of sulfated fucans and an overview of enzymes active toward this class of polysaccharide. *Glycobiology, 13*, 29R–40R.

Bilan, M. I., Grachev, A. A., Shashkov, A. S., Nifantiev, N. E., & Usov, A. I. (2006). Structure of a fucoidan from the brown seaweed *Fucus serratus* L.. *Carbohydrate Research, 341*, 238–245.

Bilan, M. I., Grachev, A. A., Ustuzhanina, N. E., Shashkov, A. S., Nifantiev, N. E., & Usov, A. I. (2002). Structure of a fucoidan from the brown seaweed *Fucus evanescens* C. Ag. *Carbohydrate Research, 337*, 719–730.

Bilan, M. I., Grachev, A. A., Ustuzhanina, N. E., Shashkov, A. S., Nifantiev, N. E., & Usov, A. I. (2004). A highly regular fraction of a fucoidan from the brown seaweed *Fucus distichus* L.. *Carbohydrate Research, 339*, 511–517.

Chandía, N. P., & Matsuhiro, B. (2008). Characterization of a fucoidan from Lessonia vadosa (Phaeophyta) and its anticoagulant and elicitor properties. *International Journal of Biological Macromolecules, 42*, 235–240.

Chen, J.-H., Lim, J.-D., Sohn, E.-H., Choi, Y.-S., & Han, E.-T. (2009). Growth-inhibitory effect of a fucoidan from brown seaweed *Undaria pinnatifida* on *Plasmodium parasites*. *Parasitological Research, 104*, 245–250.

Chevolot, L., Foucault, A., Chaubet, F., Kervarec, N., Sinquin, C., Fisher, A.-M., et al. (1999). Further data on the structure of brown seaweed fucans: Relationships with anticoagulant activity. *Carbohydrate Research, 319*, 154–165.

Choi, J. I., Raghavendran, H. R., Sung, N. Y., Kim, J. H., Chun, B. S., Ahn, D. H., et al. (2010). Effect of fucoidan on aspirin-induced stomach ulceration in rats. *Chemico-Biological Interactions, 183*, 249–254.

Cumashi, A., Ushakova, N. A., Preobrazhenskaya, M. E., D'Incecco, A., Piccoli, A., Totani, L., et al. (2007). A comparative study of the anti-inflammatory, anticoagulant, antiangiogenic, and antiadhesive activities of nine different fucoidans from brown seaweeds. *Glycobiology, 17*, 541–552.

Hahnenberger, R., & Jakobson, A. M. (1991). Antiangiogenic effect of sulphated and non-sulphated glycosaminoglycans and polysaccharides in the chick-embryo chorioallantoic membrane. *Glycoconjugate Journal, 8*, 350–353.

Hayashi, K., Nakano, T., Hashimoto, M., Kanekiyo, K., & Hayashi, T. (2008). Defensive effects of a fucoidan from brown alga *Undaria pinnatifida* against herpes simplex virus infection. *International Immunopharmacology, 8*, 109–116.

Hemmingson, J. A., Falshaw, R., Furneaux, R. H., & Thompson, K. (2006). Structure and antiviral activity of the galactofucan sulfates extracted from *Undaria pinnatifida* (*Phaeophyta*). *Journal of Applied Phycology, 18*, 185–193.

Hiraoka, A., Harada, N., Uehara, T., Sekiguchi, M., & Maeda, M. (1992). Capillary-isotachophoretic analysis of algal acidic polysaccharides and their application to a survey of heparinoid active sulfated polysaccharides in *Chlorophyta*. *Chemical & Pharmaceutical Bulletin, 40*, 783–785.

Hoshino, T., Hayashi, T., Hayashi, J., Lee, J. B., & Sankawa, U. (1998). An antivirally active sulfated polysaccharide from *Sargassum horneri* (Turner) C. Agardh. *Biological & Pharmaceutical Bulletin, 21*, 730–734.

Katsube, T., Yamasaki, Y., Iwamoto, M., & Oka, S. (2003). Hyaluronidase-inhibiting polysaccharide isolated and purified from hot water extract of sporophyll of *Undaria pinnatifida*. *Food Science and Technology Research, 9*, 25–29.

Kim, W. J., Kim, S. M., Kim, H. G., Oh, H. R., Lee, K. B., Lee, Y. K., et al. (2007). Purification and anticoagulant activity of a fucoidan from Korean *Undaria pinnatifida* sporophyll. *Algae, 22*, 247–252.

Lee, J.-B., Hayashi, K., Hashimoto, M., Nakano, T., & Hayashi, T. (2004). Novel Antiviral Fucoidan from Sporophyll of *Undaria pinnatifida* (Mekabu). *Chemical & Pharmaceutical Bulletin, 52*, 1091–1094.

Lee, Y. K., Lim, D. J., Lee, Y. H., & Park, Y. I. (2006). Variation in fucoidan contents and monosaccharide compositions of Korean Undaria pinnatifida (Harvey) suringar (Phaeophyta). *Algae, 21*, 157–160.

Li, B., Lu, F., Wei, X., & Zhao, R. (2008). Fucoidan: Structure and bioactivity. *Molecules, 13*, 1671–1695.

Maruyama, H., Suzuki, K., Miyai, S., & Ohtsuki, K. (2008). Characterization of meFucoidan as a selective inhibitor for secretory phospholipase A2-IIA and the

phosphorylation of meFucoidan-binding proteins by A-kinase *in vitro*. *Biological & Pharmaceutical Bulletin, 31*, 714–718.
Maruyama, H., Tamauchi, H., Hashimoto, M., & Nakano, T. (2003). Antitumor activity and immune response of Mekabu fucoidan extracted from sporophyll of *Undaria pinnatifida*. *In Vivo, 17*, 245–249.
Maruyama, H., Tamauchi, H., Hashimoto, M., & Nakano, T. (2005). Suppression of Th2 immune responses by mekabu fucoidan from *Undaria pinnatifida* sporophylls. *International Archive of Allergy and Immunology, 137*, 289–294.
Maruyama, H., Tamauchi, H., Iizuka, M., & Nakano, T. (2006). The role of NK cells in antitumor activity of dietary fucoidan from *Undoria pinnatifida* sporophylls (Mekabu). *Planta Medica, 72*, 1415–1417.
Maruyama, H., Tanaka, M., Hashimoto, M., Inoue, M., & Sasahara, T. (2007). The suppressive effect of Mekabu fucoidan on an attachment of *Cryptosporidium parvum* oocysts to the intestinal epithelial cells in neonatal mice. *Life Science, 80*, 775–781.
McClure, M. O., Moore, J. P., Blanc, D. F., Scotting, P., Cook, G. M., Keynes, R. J., et al. (1992). Investigations into the mechanism by which sulfated polysaccharides inhibit HIV infection *in vitro*. *AIDS Research and Human Retroviruses, 8*, 19–26.
Pereira, L., Amado, A. M., Critchley, A. T., van de Velde, F., & Ribeiro-Claro, P. J. A. (2009). Identification of selected seaweed polysaccharides (phycocolloids) by vibrational spectroscopy (FTIR–ATR and FT-Raman). *Food Hydrocolloids, 23*, 1903–1909.
Qiu, X., Amarasekara, A., & Doctor, V. (2006). Effect of oversulfation on the chemical and biological properties of fucoidan. *Carbohydrate Polymers, 63*, 224–228.
Riou, D., Colliec Jouault, S., Du Sel, D. P., Bosch, S., Siavoshian, S., Le Bert, V., et al. (1996). Antitumor and antiproliferative effects of a fucan extracted from *Ascophyllum nodosum* against a non-small-cell bronchopulmonary carcinoma line. *Anticanser Research, 16*, 1213–1218.
Rioux, L.-E., Turgeon, S. L., & Beaulieu, M. (2007). Characterization of polysaccharides extracted from brown seaweeds. *Carbohydrate Polymers, 69*, 530–537.
Rioux, L.-E., Turgeon, S. L., & Beaulieu, M. (2009). Effect of season on the composition of bioactive polysaccharides from the brown seaweed *Saccharina longicruris*. *Phytochemistry, 70*, 1069–1075.
Sekkal, M., & Legrand, P. (1993). A spectroscopic investigation of the carrageenans and agar in the 1500–100 cm^{-1} spectral range. *Spectrochimica Acta, 49A*, 209–221.
Selvendran, R. R., March, J. J., & Ring, S. G. (1979). Determination of aldoses and uronic acid content of vegetable fiber. *Analytical Biochemistry, 96*, 282–292.
Shi, Z. Y., Guo, Y. Z., & Wang, Z. (2000). Pharmacological activity of fucoidan from *Laminaria japonica*. *Journal of Shanghai Fish University, 9*, 268–271.
Synytsya, A., Čopíková, J., Matějka, P., & Machovič, V. (2003). Fourier transformed Raman and infrared spectroscopy of pectins. *Carbohydrate Polymers, 54*, 97–106.
Yang, C., Chung, D., Shin, I. S., Lee, H., Kim, J., Lee, Y., et al. (2008). Effects of molecular weight and hydrolysis conditions on anticancer activity of fucoidans from sporophyll of *Undaria pinnatifida*. *International Journal of Biological Macromolecules, 43*, 433–437.
Yang, C., Chung, D., & You, S. G. (2008). Determination of physicochemical properties of sulphated fucans from sporophyll of *Undaria pinnatifida* using light scattering technique. *Food Chemistry, 111*, 503–507.
Yoo, Y.-C., Kim, W.-J., Kim, S.-Y., Kim, S.-M., Chung, M.-K., Park, J.-W., et al. (2007). Immunomodulating activity of a fucoidan isolated from Korean *Undaria pinnatifida* sporophyll. *Algae, 22*, 333–338.
You, S. G., Yang, C., Lee, H. Y., & Lee, B.-Y. (2010). Molecular characteristics of partially hydrolyzed fucoidans from sporophyll of *Undaria pinnatifida* and their in vitro anticancer activity. *Food Chemistry, 119*, 554–559.