Algae
Volume 22(3): 247-252, 2007

Purification and Anticoagulant Activity of a Fucoidan from Korean *Undaria pinnatifida* Sporophyll

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Crude fucoidan was extracted from the sporophyll of Korean *Undaria pinnatifida* collected at a coastal area of Wando, Korea, mainly by dilute acid extraction, ethanol precipitation, $CaCl_2$ precipitation, with an yield of approximately 3.9% in mass. It was further purified by DEAE-cellulose column chromatography and its chemical composition and *in vitro* anticoagulant activity was determined. The average molecular mass of the purified fucoidan was estimated about 2.1 × 103 kDa by size-fractionation HPLC and it consisted of neutral sugar (52.34% in mass), uronic acid (26.2%), and sulfate esters (7.4%). From the HPAEC-PAD analysis, the monosaccharide composition of the purified fucoidan was shown to be fucose, galactose, xylose, and mannose, with a molar ratio of 1, 0.2, 0.02, 0.15, respectively, demonstrating that major monosaccharide was fucose (72.3% in mol percentage) and other sugars, xylose (1.5%), galactose (14.6%), and mannose (10.9%) were present as minor component. The results suggested that this fucoidan is a sulfated, U-type fucoidan. The activated partial thrombloplastin time (APTT) assay of the purified fucoidan showed that the purified fucoidan elicited anticoagulant activity in a dose-dependent manner. Five μg of sporophyll fucoidan delayed the blood clotting time up to 5 times than untreated control and also up to 1.5 times than the same amount of the commercial fucoidan, respectively. Although it is preliminary, these results suggest that the fucoidan of Korean *Undaria pinnatifida* sporophyll would be promising candidates for the development of an anticoagulant.

Key Words: anticoagulant, fucoidan, sporophyll, *Undaria pinnatifida*

INTRODUCTION

Fucoidan is a group of marine sulfated polysaccharides of the cell-wall matrix of brown algae, containing large proportions of L-fucose and sulfate, together with minor amounts of other sugars like xylose, galactose, glucose, mannose, uronic acids, and rhamnose (Berteau and Mulloy 2003; McCandless and Craigie 1979). The main skeleton of fucoidans is α1,3-linked-L-fucose-4-sulfate (Patankar *et al.* 1993), but a repeating structure of alternating α(1->3) and α(1->4) glycosidic bonds is also frequently observed depending on the algal species (Daniel *et al.* 1999; Chevolot *et al.* 1999). These acidic polysaccharides are known to exhibit a wide range of physiological and biological activities, thus medically useful activities (Boisson-Vidal *et al.* 2000), such as anti-inflammatory (Ostergaard *et al.* 2000), antiviral (Beress *et al.* 1993; Hoshino *et al.* 1998; McClure *et al.* 1992), anticoagulant (Nishino and Nagumo 1991; Mourao and Pereira 1999), antitumor (Riou *et al.* 1996; Zhuang *et al.* 1995), and antiangiogenesis (Hahnenberger and Jakobson 1991) activities. All these activities confer to these polysaccharides potential applications in human and veterinary health care and thus production and applications of fucoidans as therapeutic agents have been increasingly important topics of intensive researches.

Many studies on these activities of fucoidan have focused on their effects on blood coagulation (Boisson-Vidal *et al.* 2000). Although heparin exhibits strong anticoagulant activity and thus has been widely used for anticoagulation in hemodialysis patients, many clinical trials have claimed that it shows potentially serious side effects, including hemorrhage, thrombocytopenia, and

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osteoporosis (Fisher 2007). Furthermore, since heparin is produced from mammalian mucosa, it has potential risk of contamination by animal viruses or prions (Boisson-Vidal *et al.* 2000). These reasons have led to consideration of other options for anticoagulation. Anticoagulating activity of fucoidan has been clearly demonstrated by many research groups but it is generally low as compared to heparin (Nishno and Nagumo 1991; Berteau and Mulloy 2003). However, in addition to many biological activities of fucoidan, as mentioned above, being of vegetable origin they are less likely to contain infectious agents, such as animal viruses or prions (Boisson-Vidal *et al.* 2000). Therefore, fucoidans have been proposed as alternatives to the anticoagulant heparin (Nishino and Nagumo 1991; Pereira *et al.* 1999; Chevolot *et al.* 1999; Mourao and Pereira 1999). To date, several different fucoidan preparations from various algal species, including *Fucus versiculosus* (Nishino *et al.* 1994; Mourao and Pereira 1999), *Laminaria brasiliensis* (Mourao and Pereira 1999), *Ecklonia kurome* (Nishino *et al.* 1999), *Ascophyllum nodosum* (Millet *et al.* 1999), *Pelvetia caniculata* (Colliec *et al.* 1994) have been reported for their anticoagulant activity. The structures of fucoidan vary from their algal source species to species and must give rise to variation in the degree of most biological activities, including anticoagulation action (Boisson-Vidal *et al.* 2000).

In this context, we have attempted to elucidate the structure and pharmacological activities of the fucoidan of Korean algal species. As a priminary work, this study report on the purification of a fucoidan from the sporophyll of the Korean *Undaria pinnatifida*, collected at a coastal area of Wando, Korea, and its chemical composition and *in vitro* anticoagulant activity.

MATERIALS AND METHODS

Extraction and purification of fucoidans

The cultured *Undaria pinnatifida* sporophyll as a source of algal fucoidan 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 of fucoidan was performed as described by Kim *et al.* (2004) with minor modification. Briefly, 250 g of the dried sporophyll was cut into small chips (less than 3 × 3 cm) and allowed to stand in 4 L of 0.1 N HCl for 24 h at ambient temperature. The extract was filtered through a typical woman's nylon socks, and the filtrate was neutralized with 1 N NaOH, and fucoidans were precipitated with 3 volumes of ethanol. After centrifugation for 30 min at 6,000 x g, the precipitate was redissolved in water. The pH of the suspension was adjusted to 2.0 with 1 N HCl and to this was $CaCl_2$ added to the final concentration of 4 M. The resulting precipitate was removed through centrifugation and the supernatant was treated with 3 volumes of ethanol. The ethanol precipitation was repeated twice and the precipitate was redissolved in water, dialyzed (MWCO 14,000) at 4°C in water for 48 h and then freeze-dried.

The crude fucoidan thus obtained was further purified by column chromatography. Hundred mg of crude fucoidan was dissolved in 10 ml of water (pH 2.0), applied to a DEAE-cellulose column (3 × 42 cm) pre-equilibrated with water (pH 7.0 adjusted with 0.1 NaOH), and eluted with the same buffer containing increasing concentrations of NaCl (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 M), until no more carbohydrate was detected. Each fraction was assayed for carbohydrates by phenol-sulfuric acid method. The carbohydrate-positive fractions were pooled together, dialyzed (MWCO 14,000) for 24 h in distilled water, and lyophilized. The presence of fucoidan in the fraction was identified by the presence of L-fucose after the monosaccharide composition analysis of each peak by high performance anion-exchange chromatography (HPAEC) as described below.

General methods

The total neutral carbohydrate of the purified fucoidan was determined by the phenol-sulfuric acid method at 490 nm (Dubois *et al.* 1956) using L-fucose as a reference. The amount of sulfate residues was determined by the $BaCl_2$-gelatin method (Loui *et al.* 1982), using Na_2SO_4 as a standard. Uronic acid content was determined by the carbazole reaction (Bitter and Muir 1962), using D-glucuronic acid as a standard. Protein was quantified by Bradford method (Bradford 1976).

Acid hydrolysis of fucoidans and monosaccharide analysis by HPAEC-PAD

To determine the monosaccharide compositions of fucoidans obtained from *Undaria pinnatifida* sporophyll, 10 mg of fucoidans was dissolved in 1 ml of dH_2O, and equal volume of 4 M trifluoroacetic acid (TFA) was added and allowed to stand for 2 h at 100°C under gentle stirring. After the reaction, the mixture was filtered through 0.45 μm syringe filter and vacuum dried using Speed-Vac (Module spin 40, Biotron, Korea). The dried material was re-dissolved in 0.1 ml of dH_2O and dried. The monosaccharide analysis of TFA-hydrolyzed

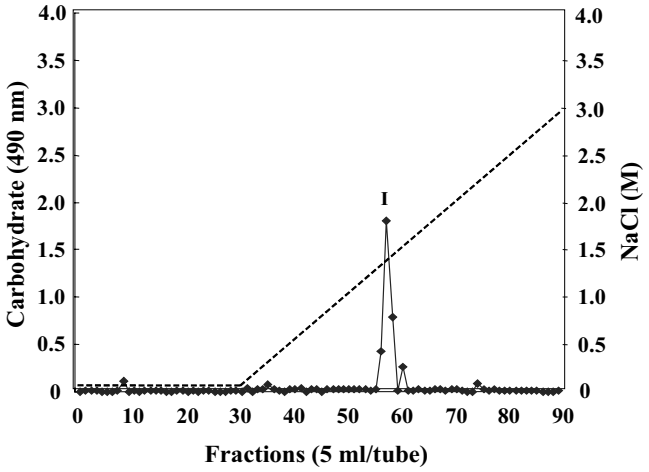

Fig. 1. Fractionation of crude fucoidan by DEAE-cellulose column chromatography. The solid diamonds indicate carbohydrates monitored by phenol-sulfuric acid method at 490 nm. The dashed line indicates the concentration of NaCl.

fucoidans was performed by HPAEC (High Performance Anion-Exchange Chromatography) as described by Lee *et al.* (2006), using Bio-LC (DX 500 Chromatography System, Dionex Co., USA) system equipped with a pulsed amperometric detector (ED 50, Dionex Co., USA).

Estimation of the molecular weight of fucoidan

The average relative molecular mass of the fucoidan was estimated by size-exclusion HPLC (Dionex, USA) using a Shodex OHpak column (SB-806HQ, 8.0 × 300 mm, Showa Denko Co., Japan). Ten μL of 1% fucoidan (in water) was injected, eluted with water at the flow rate of 0.8 ml/min at 60°C and detected with ELSD (Evaporative light scattering detector, Alltech). Dextrans were used as the relative molecular mass standards: 464, 188, 162, 143, 71, 43 kDa (Sigma, St Luis, USA).

Anticoagulant activity

Activated partial thrombloplastin time (APTT) assays were carried out by the method of Andreson *et al.* (1976). Hundred μL of normal human platelet-poor plasma was mixed with 50 μL of a solution of purified fucoidan (0.1, 0.25, 1.0, 5.0 μg) and incubated for 2 min at 37°C. For control, equal volume of dH_2O was added instead of fucoidan solution. For comparison, commercial fucoidan (Sigma, St Luis, USA), which was prepared from *Fucus vesiculosus*, was examined in the same way and its anticoagulant activity was compared to that of our fucoidan. To the reaction mixture, 100 μL of APTT reagent (Sigma, St Luis, USA) was added and incubated for 2 min at 37°C and then 100 μL of 0.35 M $CaCl_2$ was added and the clotting time was recorded on a KC1A coagulometer (Amelung, Lancer 0590, Germany).

Table 1. Monosaccharide composition of the purified fucoidan isolated from *U. pinnatifida* sporophyll

Monomer	Molar Ratio[1)]	Relative Area (%)
L-fucose	1.0	85.7
D-galactose	0.2	9.3
D-xylose	0.02	0.4
D-mannose	0.15	4.6

[1)] The values were obtained from the area of each peak on the HPAEC-PAD chromatogram of acid hydrolysate of the isolated fucoidan.

RESULT AND DISCUSSION

Purification and chemical composition of fucoidan

Fucoidan was extracted from the sporophyll of Korean *Undaria pinnatifida* by dilute acid treatment and the crude preparation was obtained following 75% ethanol precipitation and $CaCl_2$ treatment, with the yield of approximately 3.9% in mass. The crude fucoidan was further purified by collecting the single peak fraction (peak I) eluted from the DEAE-cellulose column (Fig. 1). The fucoidan fraction (peak I) was eluted at approximately 1.4 M NaCl in water (pH 7.0 adjusted with 0.1 NaOH). From the HPAEC-PAD analysis, the monosaccharide composition of the purified fucoidan was shown to be fucose, galactose, xylose, and mannose, with a molar ratio of 1, 0.2, 0.02, 0.15, respectively (Table 1). Therefore, major monosaccharide was fucose (72.3% in mol percentage) and other sugars, xylose (1.5%), galactose (14.6%), and mannose (10.9%) were present as minor component (Table 2 and Fig. 2). As shown in Table 2, the purified fucoidan preparation was shown to be composed of neutral sugars (52.34% in mass), uronic acid (26.2%), and sulfate (7.4%). The negatively charged components (uronic acids and sulfate) of the fucoidan from sprorophyll of *Undaria pinnatifida* were more or less lower than those of other fucoidans but still suggest that biological activities of sprorophyll fucoidan would be, at least, comparable to those of other fucoidans from various algal sources (Koo *et al.* 1995; Berteau and Mulloy 2003; Boisson-Vidal *et al.* 2000).

The average molecular mass of the purified fucoidan was estimated about 2.1×10^3 kDa by size-fractionation HPLC (Fig. 3). The molecular mass of our fucoidan, which was isolated from the sporophyll of *Undaria pinnatifida* collected at Wando, Korea, was much higher

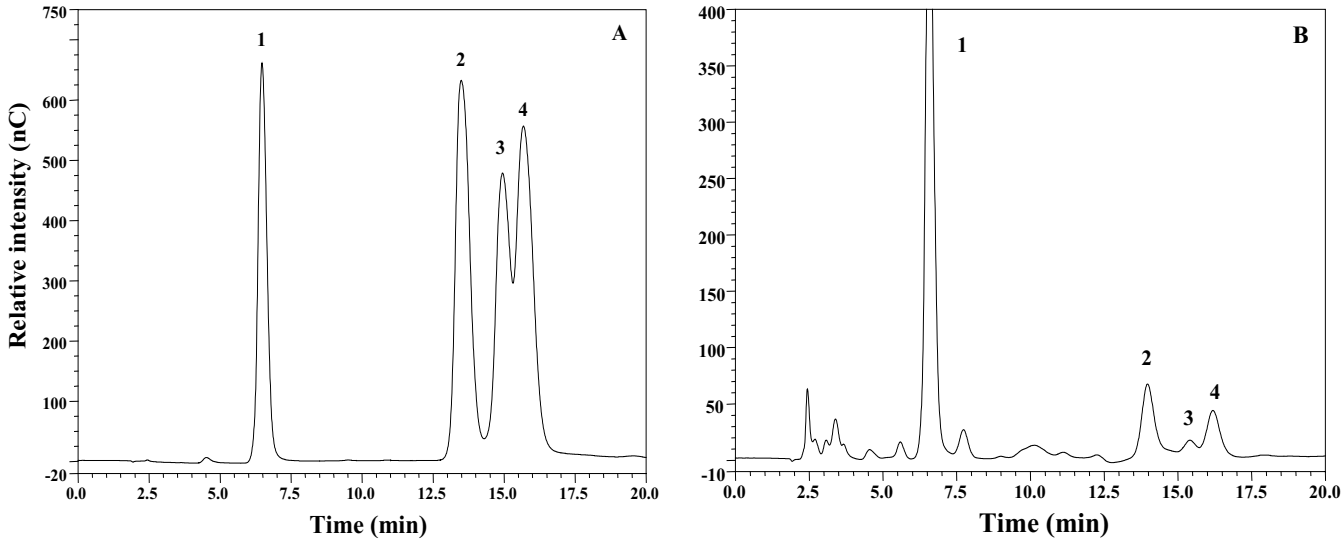

Fig. 2. HPAEC-PAD analysis for monosaccharide composition of the purified fucoidan extracted from *Undaria pinnatifida* sporophyll. A. Chromatogram of standard monosaccharides (Sigma). B. Chromatogram of acid hydrolysates of purified fucoidan. 1, fucose; 2, galactose; 3, xylose; 4, mannose.

Table 2. Chemical composition of the purified fucoidan isolated from *U. pinnatifida* sporophyll

Algal source	Uronic acid (mass %)	Protein (mass %)	Neutral Sugar (mass %)	Sulfate (mass %)	Proportion of monosaccharide (mole %)[1)]			
					Fuc	Gal	Xyl	Man
U. pinnatifida sporophyll	26.2	2.58	52.34	7.4	72.3	14.6	1.5	10.9

1) Values were obtained by setting the sum of each mole number at 100%.
Fuc, fucose; Gal, galactose; Xyl, xylose; Man, mannose

than that (38 kDa) of the fucoidan obtained from the sporophyll of the same species harvested from an aqua-farm at the coastal area of Kijang, Yangsan-gun, Korea, as reported by Koo *et al.* (1995). However, Sakai *et al.* (2003) reported that the molecular mass of fucoidan from *Cladosiphon okamuranus* was 2×10^6 Da, similar to that of our fucoidan. It is now commonly accepted that the sugar composition, degree of sulfation, and molecular mass of fucoidans vary depending on the algal species, their harvesting time and regions of cultivation as well (Mori *et al.* 1982; Sakai *et al.* 2003a). Sakai's group suggested that the large difference in molecular mass may also be caused by the difference of the extraction conditions because the fucoidan is not so stable against heat or acid (Sakai *et al.* 2003). Taken collectively, these data suggest that the fucoidan obtained from the Korean *Undaria pinnatifida* sporophyll collected at the coastal area of Wando, Korea, is a sulfated, U-type fucoidan with high molecular mass.

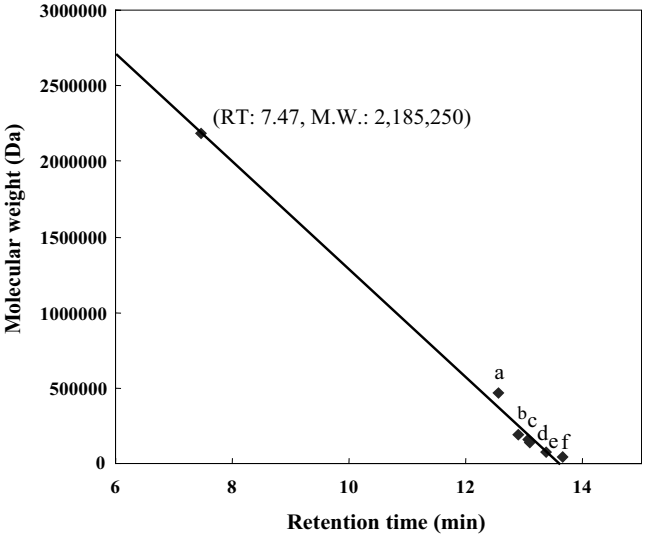

Fig. 3. Estimation of the molecular weight of purified fucoidan by size-exclusion HPLC. The standard size markers were dextrans (Sigma): 464,000 (a), 188,000 (b), 162,000 (c), 143,000 (d), 71,300 (e), 43,000 Da (f). $R^2 = 0.9941$.

Anticoagulant activity

The anticoagulant activity of the purified fucoidan was examined by activated partial thrombloplastin time (APTT) assay. As shown in Fig. 4, the purified fucoidan

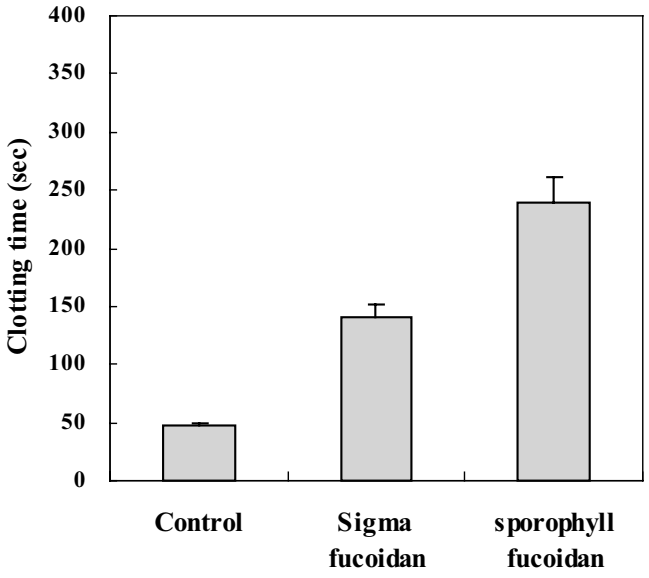

Fig. 4. Anticoagulant activity of the fucoidan purified from the sporophyll of *Undaria pinnatifida*. Activated partial thrombloplastin time (APTT) assays were carried out with 100 μL of normal human platelet-poor plasma and 50 μL of a solution of the purified fucoidan (5.0 μg) or equal volume of dH_2O as a control. Commercial fucoidan (Sigma) was examined in the same way for comparison.

significantly prolonged the clotting time; 5 μg of sporophyll fucoidan delayed the blood clotting time approximately by 5 times than untreated control and also up to 1.5 times than the same amount of the commercial fucoidan (Sigma, St Luis, USA), respectively. The anticoagulant activity of the purified fucoidan increased with increasing concentration (0.1, 0.25, 1.0, 5.0 μg) of the fucoidan (Fig. 5). Although more detailed research is needed, these results suggest that the fucoidan of Korean *Undaria pinnatifida* sporophyll may be promising candidates for the development of an anticoagulant that can replace heparin, which has strong anticoagulant activity but shows potentially serious side effects, including hemorrhage, thrombocytopenia, and osteoporosis (Fisher 2007; Boisson-Vidal *et al.* 2000; Mourao and Pereira 1999; Berteau and Mulloy 2003). Further study on the structure and the molecular mechanism on the anticoagulation activity of this fucoidan are being continued in our lab.

ACKNOWLEDGEMENTS

This work was supported by the Catholic University of Korea Research Fund, 2006. The authors are also grateful to Mr. Jeong-Shik Kim for providing Korean *Undaria pinnatifida* sporophyll.

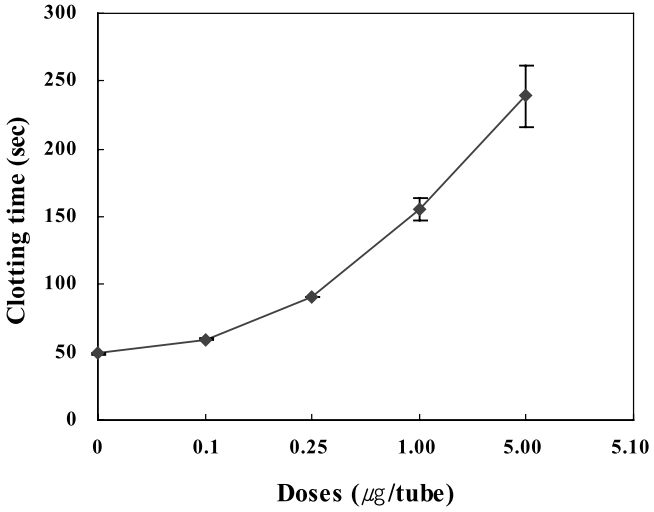

Fig. 5. Dose-dependency of anticoagulent activity of the purified *Undaria pinnatifida* sporophyll fucoidan. Activated partial thrombloplastin time (APTT) assays were carried out as described in the text and in the legend of Fig. 4.

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Received 23 July 2007
Accepted 9 September 2007