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Physicochemical properties and bioactivity of brown seaweed fucoidan prepared by ultra high pressure-assisted enzyme treatment

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Abstract−The effects of ultra-high pressure (UHP, 100 MPa, 40, 24 h) processing and enzyme treatment on the physicochemical properties and anticoagulant activity of fucoidan obtained from *Undaria pinnatifida* sporophyll (UPS) were investigated. Crude UPS fucoidan (F_{UPS}) with an average MW of 877 kDa was slightly depolymerized by UHP-assisted Tunicase treatment, yielding fucoidan with a lower MW (600-800 kDa). UHP-enzyme treatment decreased the sulfate, fucose and galactose contents of F_{UPS} but increased its glucose content. From FT-IR spectrum, UHP-enzyme treatment was found not to cause a structural change on S=O and C-O-S. After UHP-enzyme treatment, the sulfate content and average MW of F_{UPS} decreased with the increase of Tunicase concentration. It was found that among the UHP-enzyme-treated fucoidans, F_{UPS}-T0.3-U (Tunicase 0.3% treatment+UHP treatment) possessed the highest anticoagulant activity. F_{UPS}-T0.3-U appeared to inhibit blood coagulation via intrinsic pathway. With the increase of sulfate content in F_{UPS}, activated partial thromboplastin time (APTT) and thrombin time (TT) showed a tendency of increase. APTT and TT had the highest values in F_{UPS}-T0.3-U in which the sulfate concentration was 24%, but on the contrary decreased at a sulfate concentration of above 24%. This result indicates that there is an optimum sulfate concentration for the anticoagulant activity of fucoidan. Consequently, UHP-assisted enzymatic treatment was found to be helpful for the improvement of anticoagulant activities of fucoidan.

Key words: Fucoidan, Ultra High Pressure Processing, Enzyme Treatment, Sulfate Content, Bioactivity, APTT, TT

INTRODUCTION

Fucoidan, a water-soluble sulfated polysaccharide extracted from marine algae (mainly brown seaweed), has been extensively studied due to its numerous biological activities: anticoagulant [1-3], anticancer [4-6], anti-inflammatory [7], and antiviral [8,9] activities. Fucoidan is composed of fucose, uronic acids, galactose, xylose, and sulfated fucose. Fucoidans are found in numerous brown algae such as *Ascophyllum nodosum* [10], *Fucus vesiculosus* [11], *Laminaria japonica* Aresch. (Laminariales) [12], and *Chorda filum* [13]. *Undaria pinnatifida* ('Miyeok') is common edible brown seaweed in Korea and a good source of fucoidan. Of this brown algae *Undaria pinnatifida,* sporophylls (root part of *Undaria pinnatifida,* 'Miyeokgui') has been reported to have the highest content of fucoidan [14].

This heterogeneous polysaccharide has a high molecular weight (MW) and is highly sulfated [15,16]. Various MWs of fucoidan have been reported in the literature: 200-2,000 kDa. Fucoidans with MWs of above 1,000 kDa show lower water-solubility and bioavailability [17]. Evidence from different studies also suggests that the biological activity of polysaccharides is strongly dependent on the structure of the polymers, i.e., the nature of the sugar components, the links between these sugars along with their molecular weight [18]. Especially, the anticoagulant activity depends on the MW and the chemical composition of fucoidan, particularly the fucose and sulfate contents [19,20]. The inhibitory effects of fucoidans on blood coagulation and cell proliferation are dependent on their sulfation degree [21]. Furthermore, oversulfated fucoidan enhanced its anti-angiogenic and anti-tumor activities [22]. Thus, the sulfate group of fucoidan may play an important role in its biological activities. It has been reported that when fucoidan is treated to form a low-MW polysaccharide, its bioavailability and bioefficacy are enhanced [23,24]. The anti-tumor, anti-HIV, and anticoagulant activities of fucoidan are improved when it is slightly depolymerized to make a lower MW [25-28].

The methods of decreasing the MW of fucoidan include acid hydrolysis [28], radical hydrolysis [29], enzymatic hydrolysis [30], electro-dialysis [17], microbial method [31], and so on. Among them, acid hydrolysis using HCl is normally used, but this method causes the over-desulfation and acid waste problems.

The growing market for natural products has increased the interest in environmentally friendly processes to produce these products. Ultra-high pressure (UHP)-assisted extraction has received world-wide attention in the fields of ceramics, pharmaceutics, metallurgy, plastics making and civil engineering [22]. The application of high pressure to natural materials was initially attempted by Zhang et al. [23] for extraction of polyphenols from green tea. UHP technique using ultra high pressures ranging from 100 to 800 MPa has been reported that it could accelerate the extraction and reach high extraction yields, yet it has no adverse side effects on the activity and structure of the bioactive components [32]. Use of UHP increases mass transfer rates, which enhances cell permeability as well as diffusion of bioactive compounds [33]. UHP treatment is a non-thermal

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process that achieves a maximum retention of the chemical and physicochemical properties of natural products. UHP has been used for extraction of flavonoides from propolis [34], anthocyanins from grapes [21], ginsenosides from *Panax quinquefolium* [35], and flavones and salidroside from *Rhodiola sachalinensis* [34].

Several extraction techniques such as Soxhlet, microwave [36] and ultrasound-assisted extraction [37] have been adopted to extract bioactive polysaccharides from bioresources. Yet there has been no report on the use of UHP-assisted enzymatic hydrolysis for polysaccharide preparation. This is the first trial to use the UHP technique for the production of fucoidan. In the present study, cotreatment of UHP and hydrolytic enzyme (UHP-enzyme treatment) to a crude fucoidan of *Undaria pinnatifida* sporophylls (UPS) was undertaken to obtain a low MW fucoidan.

The objective of this study was to determine the effects of UHP-enzyme treatment on the physicochemical properties and anticoagulant activity of fucoidan. The effects of UHP-enzyme treatment were compared to those of conventional acid hydrolysis to confirm the advantages of UHP-enzyme treatment.

MATERIALS AND METHODS

1. Materials and Equipment

Undaria pinnatifida sporophylls (UPS, a brown seeweed 'Miyeok', harvested in March, 2007) was obtained from Kijang farm (Pusan, Korea) and dried up to a water content of 5%. For comparative study, *Undaria pinnatifida* fucoidan (F_{UP}) and *Fucus vesiculosus* fucoidan (F_{FV}) purchased from Sigma Chemical Co. (St. Louis, MO, USA) were used. Tunicase (β-glucanase), a polysaccharide hydrolytic enzyme originating from *Athrobacter* sp. was obtained from Daiwakasei (Okazaki, Japan). For UHP treatment, an ultra high pressure processing unit DSF-10L (Toyo Koatsu, Hiroshima, Japan) was used. As a positive control for anticoagulant activity assay, heparin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Activated partial prothromboplastin time (APTT) reagent, thrombin time (TT) reagent, prothrombin time (PT) reagent and plasma were obtained from TECO (Neufahrn, Germany).

2. Crude Fucoidan Preparation from UPS

Dried UPS was milled up to a size of less than 20 mm, and then extracted by combining one part of UPS with 40 parts of distilled water (to make a concentration of 2.5%) after pH adjustment to pH 8. The extraction was carried out at 85 °C for 4 h in a 130-L tank, which was agitated at 140 rpm. After hot water extraction, the first supernatant was collected by centrifugation (2,230 ×g for 20 min) and then $CaCl_2$ was added at a concentration of 2% (w/v) to the supernatant. After the solution was placed at room temperature for 1 h, the second supernatant was collected through centrifugation (2,230 ×g for 20 min). The obtained supernatant was concentrated by a rotary evaporator (Eyela, Tokyo, Japan), to make a dry matter content of 25%.

One part of the concentrate was combined with three parts of ethanol (95%) and stored at room temperature for 12 h. The precipitate was collected by centrifugation (2,230 ×g, 20 min), redissolved in a small amount of distilled water, and dialyzed using a dialysis membrane (MWCO 3,500, Biodesign Dialysis, New York, NY, USA) for 12 h. After dialysis, the dialysate was freeze-dried to make crude fucoidan for further analysis and treatment. This crude fucoidan is also denoted as UPS fucoidan (F_{UPS}) in the present study.

3. UHP-enzyme Treatment

The freeze-dried F_{UPS} was dissolved in distilled water (pH was pre-adjusted to 8) at a concentration of 5% (w/v), and placed in a vinyl bag. Tunicase (β-glucanase) was added at a concentration of 0-2% (w/v) to the vinyl bag, which was placed in the UHP unit, DSF-10L. UHP unit was operated at a 100 MPa for 24 h at 40 °C. After UHP treatment, the fucoidan hydrolyzate was freeze-dried.

4. Physicochemical Analyses

The total carbohydrate content of each fucoidan sample was determined by the phenol-sulfuric acid method using fucose as a standard [38]. The sulfate content was analyzed according to the method of Dodgson et al. [39].

For the analysis of saccharide composition of fucoidan, the fucoidan samples (100 mg) were hydrolyzed using 5 ml of 4 M trifluoroacetic acid (TFA) in a sealed vial (Teflon-lined cap) at 100 °C for 4 h. The hydrolyzates were analyzed by HPAEC-PAD (high-pH anion exchange chromatography with pulsed amperometric detection, Bio-LC, Dionex, CA, USA) system with a CarboPac™ PA1 (4×50 mm, Dionex, CA, USA) and a mobile phase of 18 mM NaOH at a flow rate of 1 ml min^{-1} [40,41]. Elemental analysis was conducted by elemental analyzers (EA 1108 and EA 1112 series, CHNS/O mode, Fisions Instruments, Milan, Italy) for analysis of C, H, N and S and for that of O in fucoidan.

The MW distribution of fucoidan was determined by a high-performance size exclusion chromatography (HPSEC) using Summit HPLC system (Dionex, Sunnyvale, CA, USA) with a TSK-GEL column (G5000PW_{XL}, TOSOH Co., Tokyo, Japan) and a refractive index detector (HP 1047A, Hewlett-Packard, CA). For elution, 0.1 M $NaNO_3$ was used at a flow rate of 0.8 ml min^{-1}. Fucoidan (100 mg) was hydrolyzed using 5 ml of 4 M trifluoroacetic acid (TFA) in a sealed vial (teflon-lined cap) at 100 °C for 1 h. After hydrolysis, the sample was analyzed on an OHpac SB-804 HQ column (Shodex, Japan) connected with an OHpac SB-802.5 HQ column (Shodex, Japan). Polysaccharide standards including pullulan, G7 and G8 (Sigma Chemical Co., USA) were used for the MW calibration of fucoidan.

For the identification of sulfate group and sulfur-saccharide linkage pattern, an FT-IR spectrometer (Spectrum GX, Perkin Elmer, USA) was used. The spectra were measured in a frequency range between 4,000-400 cm^{-1}.

5. Anticoagulant Activity Assay

Activated partial thromboplastin time (APTT), which represents the intrinsic pathway of blood coagulation, was assayed as follows: a mixture of 2.5 μl of fucoidan and 22.5 μl of plasma was pre-incubated at 37 °C for 5 min, and then 25 μl of APTT reagent was added to the reaction mixture. APTT was measured after the addition of 20 mM $CaCl_2$ to the mixture using a coagulometer (Coatron M1, TECO, Neufahrn, Germany).

Thrombin time (TT) assay was carried out for the measurement of fucoidan's effect in common pathway of blood coagulation. A mixture of 5 μl of fucoidan (0.25 μg/ml) and 45 μl of plasma (TEControl N, plasma, TECO, Neufahrn, Germany) was pre-incubated at 37 °C for 1 min, and then 50 μl of thrombin reagent was added to the reaction mixture. TT was determined by the coagulometer using 25 μl of plasma as a reference control.

Prothrombin time (PT), which represents the extrinsic pathway

of blood coagulation, was assayed as follows: a mixture of 2.5 μl of fucoidan and 22.5 μl of plasma was pre-incubated at 37 °C for 2 min, and then 25 μl of prothrombin reagent was added to the reaction mixture. PT was determined by the coagulometer.

RESULTS AND DISCUSSSION

1. Physicochemical Properties of UHP-treated Fucoidan

To investigate the effects of acid hydrolysis, enzymatic hydrolysis and UHP-enzyme treatment on sulfate and total sugar content of fucoidan as well as its chemical composition, crude fucoidan (F_{UPS}) was treated under different conditions (Table 1). In our previous study, Tunicase as the suitable enzyme for fucoidan hydrolysis and operating pressure of 100 MPa as the UHP processing condition for UHP unit were established, respectively [42].

In this study, with the same condition of UHP treatment (100 MPa, 40 °C, 24 h), two different concentrations (1% and 0.3%) of Tunicase were selected. In accordance with the research of Bae et al. [43], HCl-treated F_{UPS} in this study showed the lowest sulfate content (7.1%) and total saccharide (TS) content (18%). Bae et al. [43] presented that acid hydrolysis degrades the sulfate groups as well as polysaccharides of fucoidan [43]. This might be due to the acid hydrolysis of sulfate group and sugar residue in fucoidan. Regardless of UHP treatment, 1% Tunicase significantly reduced sulfate content of F_{UPS} (Table 1). When fucoidan was co-treated by UHP and enzyme, the sulfate content of fucoidan decreased as Tunicase concentration increased. This is in good agreement with the report of Alain et al. [24]. This is probably due to enzymatic hydrolysis on sulfate group in fucoidan. The TS content, an important physical property of polysaccharide, increased as a result of the enzyme treatment. These results are similar to those reported by Koo et al. [14].

The elemental analysis for C, H, O and S in F_{UPS} showed that C, H, O content was not significantly changed upon the UHP-enzyme treatment, regardless of enzyme concentration (Table 1). However, sulfur content of F_{UPS} slightly decreased after UHP-enzyme treatment. Acid (1 N HCl)-treated F_{UPS} showed the lowest sulfur content (5.97%), which is in accordance with the experimental result of sulfate content, as shown in Table 1.

From the analysis of saccharide composition, it was shown that *Undaria pinnatifida* fucoidan (F_{UP}) and UPS fucoidan (F_{UPS}) consist of fucose and galactose with higher molar ratio of galactose, while *Fucus vesiculosus* fucoidan (F_{FV}) is composed of fucose only (data not shown). This accords with results reported by Mori et al. [44] in which F_{UPS} had a higher molar content of galactose (fucose : galactose=1 : 1.76, mole based). Additionally, it was found that with the increase of enzyme concentration for the UHP-enzyme treatment, mole% of glucose content increased (data not shown). This result of the present study was similar to that of Kim et al. [3], in which the fucose content of laminarin decreased by the treatment

Table 1. Physicochemical properties of fucoidan prepared by various treatment methods

Group/Origin	Symbols of samples*	Sulfate (%)	TS (%)	Element (%)						
				C	H	O	N	S	Ash	Total
Undaria pinnatifida sporophylls (UPS)	F_{UPS}	31.7±1.6	38±1.9	23.97	4.33	48.66	0.79	9.07	13.18	100
HCl- treated F_{UPS}	F_{UPS}-HCl	7.1±2.8	18±0.2	28.05	4.88	47.13	0.66	5.97	13.31	100
Enzyme treated F_{UPS}	F_{UPS}-T1	20.9±2.4	46±5.1	25.30	4.57	45.89	0.74	8.05	15.45	100
UHP-enzyme treated F_{UPS}	F_{UPS}-T1-U	18.4±0.9	52±4.5	24.97	4.72	47.06	0.75	6.91	15.59	100
	F_{UPS}-T0.3-U	24.5±1.0	37±2.4	23.56	4.21	42.48	0.83	8.49	20.43	100

* Symbols of samples
F_{UPS}-HCl: F_{UPS}+1 N HCl treatment
F_{UPS}-T1: F_{UPS}+Tunicase 1% treatment (without UHP treatment)
F_{UPS}-T1-U: F_{UPS}+Tunicase 1%+UHP treatment
F_{UPS}-T0.3-U: F_{UPS}+Tunicase 0.3%+UHP treatment

Table 2. Molecular weight distribution of fucoidan prepared by various treatment methods

Group	Symbol* of samples	Molecular weight distribution (kDa, %)						Average MW (kDa)
		0.3-3	20-100	100-200	500-600	800-1,000	1,000-1,200	
Crude fucoidan	F_{UPS}	7.6	-	9.5	-	-	82.9	877
HCl- treated F_{UPS}	F_{UPS}-HCl	66.6	33.4	-	-	-	-	15
Enzyme treated F_{UPS}	F_{UPS}-T1	17.3	13.4	-	-	-	69.3	722
UHP-enzyme treated F_{UPS}	F_{UPS}-T1-U	20.7	-	7.8	-	-	63.2	711
UHP-enzyme treated F_{UPS}	F_{UPS}-T0.3-U	20.7	-	10.2	-	69.2	-	687

* Symbols of samples
F_{UPS}-HCl: F_{UPS}+1 N HCl treatment
F_{UPS}-T1: F_{UPS}+Tunicase 1% treatment (without UHP treatment)
F_{UPS}-T1-U: F_{UPS}+Tunicase 1%+UHP treatment
F_{UPS}-T0.3-U: F_{UPS}+Tunicase 0.3%+UHP treatment

using enzyme and acid. Accordingly Tunicase (β-1,3-glucanase) is assumed to break down β-1,3-glycosidic linkage of fucose and galactose in β-1,3-glucan [45,46]. Consequently, the sulfate, fucose and galactose contents of fucoidan decreased, while glucose content increased by UHP-enzyme treatment.

2. MW Distribution by UHP-enzyme Treatment

MW distributions of F_{UPS} and UHP-enzyme-treated F_{UPS} are compared in Table 2. The average MW of F_{UPS} was 877 kDa (0.3-1 kDa : 100-200 kDa : 1,000-1,200 kDa=7.6 : 9.5 : 83.0). Lessonia nigrescens and Ascoseira mirabilis-originated fucoidan was estimated to have an average MW of 670 kDa [47]. The MW of Hizikia fusiforme fucoidan is 42-95 kDa [48], and that of Ecklonia kurome is 21-32 kDa [49]. As shown in Table 2, HCl-treatd F_{UPS} had the lowest MW, while Tunicase-treated F_{UPS} had a slightly lower MW value than crude F_{UPS}. After UHP-enzyme treatment, the average MW decreased with the increase of enzyme concentration, indicating the depolymerization of F_{UPS}.

3. FT-IR Analysis of UHP-enzyme Treated Fucoidan

FT-IR absorption spectra of F_{UPS}, HCl-treated F_{UPS} and UHP-enzyme-treated (100 MPa, 40 °C, 24 h) F_{UPS} are shown in Fig. 1. The signal at 1,240 cm^{-1} was attributed to the asymmetric stretching of S=O. The sulfate bands around 850 cm^{-1} and 820 cm^{-1} were reported to be related to axial and equatorial positions of C-O-S, respectively [50,51]. If the sulfate group is attached on C-2 and C-3 positions of α-L-fucose, it is in equatorial position, and if C-4 position, axial position. As for heparin and F_{UPS}, O-H stretching, bending vibration, stretching vibration and its corresponding bending frequency at 3,600-3,200, 1,100-1,050, 2,950-2,800 and 1,450 cm^{-1} are shown in the FT-IR spectra.

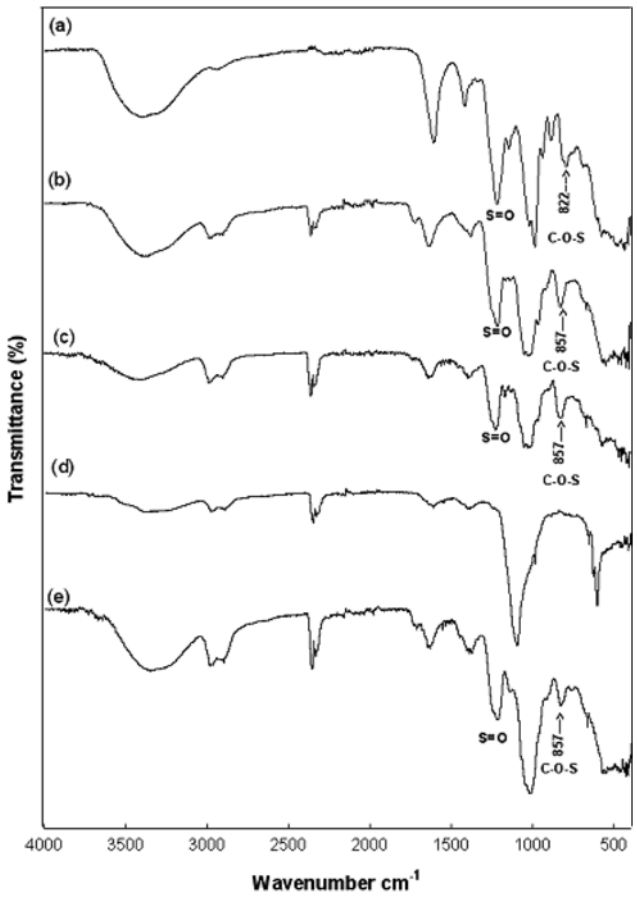

Fig. 1. FT-IR spectrum of fucoidan of various origins and prepared by various treatment methods. (a) heparin; (b) F_{UPS}; (c) F_{FI}; (d) HCl-treated F_{UPS}; (e) UHP-enzyme treated F_{UPS}.

Especially, heparin, F_{FI}, F_{UPS} and UHP-enzyme-treated F_{UPS} showed strong absorption bands around 1,220 cm^{-1}, which indicates the typical S=O linkage of fucoidan. In case of heparin, the sulfate group showed a strong absorption at 822 cm^{-1}, which corresponded to the C-O-S linkage at equatorial position. It was confirmed that most sulfate group is linked on C-2 position [52,53].

On the other hand, the strong band around 850 cm^{-1} in the IR spectra of F_{FI}, F_{UPS} and UHP-enzyme-treated F_{UPS} indicated C-O-S linkages on an axial position (sulfate linkage on C-4). This is in accordance with a report of Lee [54] on IR-spectrum of *Ecklonia stolonifera* fucoidan. It was found that the sulfate group of F_{UPS} is linked on C-4 position of fucose. When crude fucoidan was treated by 1 N HCl for 24 h, a modified absorption spectrum was shown, indicating that S=O and C-O-S links were structurally modified, as shown in Fig. 1. However, in case of UHP-enzyme treatment, structural change on S=O and C-O-S did not occur.

4. Anticoagulant Activity of UHP-enzyme Treated Fucoidan

It was reported that the major antithrombic activity by fucoidan was mediated by heparin cofactor II [55-57]. In the present study, anticoagulant activities of fucoidans prepared by various treatment methods were analyzed (Table 3). TT, APTT and PT for different preparations of fucoidan were measured to investigate their anticoagulant activity and mechanism.

APTT was measured with the fucoidan samples at a concentration of 25 μg/ml, and 4 μg/ml of heparin was used as a positive control. APTT of F_{UPS}, UHP-0.3% enzyme-treated F_{UPS} (F_{UPS}-T0.3-U) and UHP-1.0% enzyme-treated F_{UPS} (F_{UPS}-T1-U) were measured as 125.4 s, 156.8 s and 135.9 s, respectively, indicating that APTT was prolonged by UHP-enzyme treatment. So far, the mechanism of prolongation of APTT was suggested as inhibition of the intrinsic factors such as VIII, IX, XI and XII and/or common pathway. Sulfate content in UHP-enzyme-treated F_{UPS} decreased as the enzyme concentration increased (See Table 1). This is in good agreement with the report of Nishino and Nagumo [58] that the anticoagulant activity of fucoidan decreased as the sulfate content decreased.

TT was also measured with various fucoidan samples at a concentration of 12.5 μg/ml, and 0.4 μg/ml of heparin was used as a positive control. TT of F_{UPS} was measured as 158.1 s, which is comparable to that of heparin (262.1 s), a well-known antithrombin compound. When F_{UPS} was treated by Tunicase only (F_{UPS}-T1), TT slightly decreased (TT=124.3 s), compared to that of crude F_{UPS} (158.1 s). Interestingly, when the crude F_{UPS} was co-treated by Tunicase and UHP, TT significantly increased (181.6 s in F_{UPS}-T1-U and 253.1 s in F_{UPS}-T0.3-U). The prolongation of TT is related to the inhibition of common pathway of blood coagulation. However, the anticoagulant mechanism of UHP-enzyme-treated F_{UPS} is not clarified in the present study. Further study in detail on the mechanism is required.

PT was measured in order to investigate the effect of UHP-enzyme treatment on the anticoagulant activity in extrinsic pathway. As for the PT assay, all tested samples could not significantly prolong the plasma clot time, and the clot time had little relationship to the concentration, like heparin [52]. It was suggested that F_{UPS} did not

Table 3. Anticoagulant activity of fucoidan prepared by various treatment methods

Group	Symbol of samples*	Anticoagulant activity		
		APTT[1] (s)	TT[2] (s)	PT[3] (s)
	Control (plasma)	37.2±1.0	18.5±0.3	14.6±0.1
	Heparin	240.0±10	262.1±3.7	17.5±0.4
Undaria pinnatifida sporophylls (UPS)	F_{UPS}	125.4±9.3	158.1±13.1	12.0±0.2
HCl-treated F_{UPS}	F_{UPS}-HCl	40.2±1.0	12.6±0.3	13.1±0.3
Enzyme- treated F_{UPS}	F_{UPS}-T1	103.8±6.0	124.3±2.4	12.1±0.1
UHP-enzyme treated F_{UPS}	F_{UPS}-T1-U	135.9±2.1	181.6±25.4	11.3±0.1
	F_{UPS}-T0.3-U	156.8±3.1	253.1±10.3	12.0±0.2

* Symbols of samples
F_{UPS}-HCl: F_{UPS}+1 N HCl treatment
F_{UPS}-T1: F_{UPS}+Tunicase 1% treatment (without UHP treatment)
F_{UPS}-T1-U: F_{UPS}+Tunicase 1%+UHP treatment
F_{UPS}-T0.3-U: F_{UPS}+Tunicase 0.3%+UHP treatment
*Heparin concentration: 4 μg/ml (APTT), 0.4 μg/ml (TT), 6 μg/ml (PT)
*Sample concentration: [1]25 μg/ml (APTT), [2]12.5 μg/ml (TT), [3]25 μg/ml (PT)

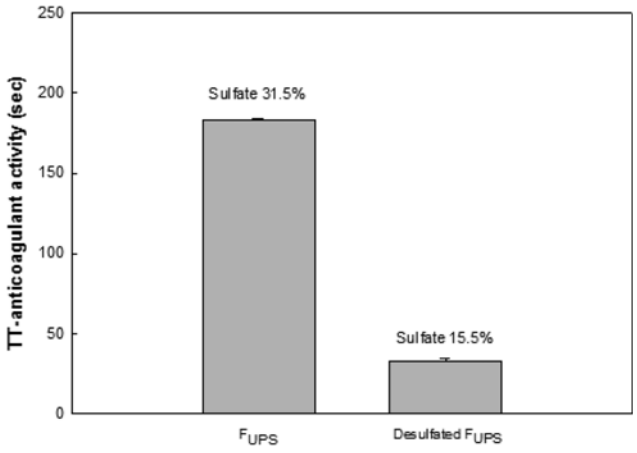

Fig. 2. TT activity of fucoidan (F_{UPS}) and desulfated fucoidan. (TT (plasma): 18.0 s, sample concentration: 25 μg/ml).

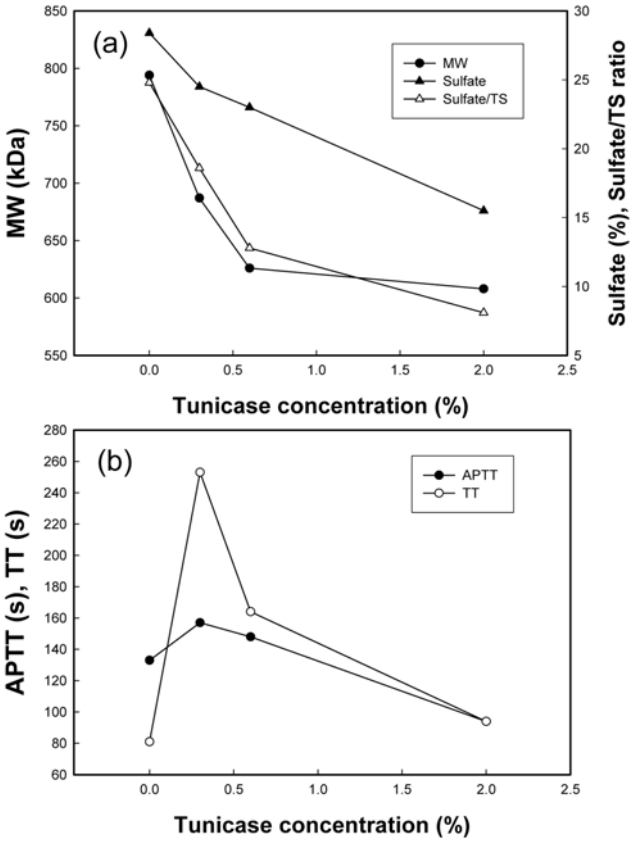

Fig. 3. Effects of Tunicase concentration on (a) MW, sulfate content, sulfate/TS ratio and (b) anticoagulant activities of UHP-enzyme treated UPS fucoidan.

inhibit extrinsic pathway of coagulation. Previous study found sulfated polysaccharide has no effect on the extrinsic mechanism of blood coagulation [52].

Partially desulfated fucoidan with sulfate content below 20% is known to show a significant decrease in both anticoagulant and anticancer activity [59]. To study the relationship between sulfate content and anticoagulant activity of F_{UPS}, the UHP-enzyme-treated F_{UPS} was desulfated by the method of Yuan et al. [60], and the TTs of crude F_{UPS} and the desulfated F_{UPS} were measured (Fig. 2). By the desulfation treatment, the sulfate content decreased from 31.5% to 15.5%, and TT dramatically decreased from 182 s to 32 s. This is in good agreement with Nishino and Nagumo [58] that the anticoagulant activity of fucoidan decreased with the decrease of the sulfate/total sugar ratio. According to Jack et al. [61], the anticoagulant activity of fucoidan is improved by the electrostatic interaction between negative charges of heparin sulfate groups and Lys/Arg residues of antithrombin III. F_{UPS} seemed to show a higher anticoagulant activity by the increased interaction with antithrombin III.

It has been generally accepted that fucoidan with lower MW and higher sulfate group exerted an excellent inhibitory effect against factors responsible for "intrinsic" blood coagulation, thus prolonging the APTT compared to the untreated F_{UPS}. To investigate the effects of Tunicase concentration on MW, sulfate content, sulfate/TS ratio and anticoagulant activities (APTT and TT) of fucoidan, the physicochemical and biological properties of fucoidan prepared

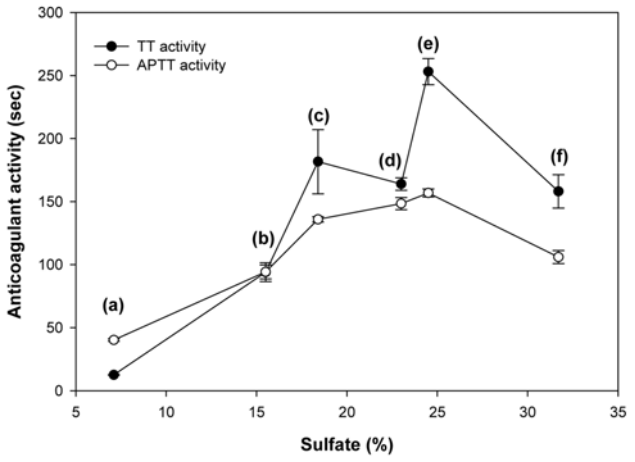

Fig. 4. Interaction between sulfate concentration and anticoagulant activity of fucoidan prepared by different methods (–●–, TT activity; –○–, APTT activity). (a) HCl-treated F_{UPS}; (b) F_{UPS}-T2-U; (c) F_{UPS}-T1-U; (d) F_{UPS}-T0.6-U; (e) F_{UPS}-T0.3-U; (f) F_{UPS}.

by various methods were plotted as shown in Fig. 3. With the same conditions of UHP treatment (100 MPa, 40 °C, 24 h), only Tunicase concentration was varied from 0% to 2%. As the Tunicase concentration increased, average MW and sulfate content of fucoidan decreased. However, there was an optimal Tunicase concentration to achieve the maximum APTT and TT. Among the UHP-enzyme-treated fucoidans prepared using different Tunicase concentrations, F_{UPS}-T0.3-U (0.3% Tunicase treatment) possessed the highest anticoagulant activity (the highest APTT was 156.8 s and the highest TT was 253.1 s). When 0.3% Tunicase was used under UHP condition, the sulfate content of F_{UPS} was 24.5%, which was the highest sulfate content among the UHP-enzyme treatment groups. In Fig. 4, the correlation between anticoagulant activity and sulfate content was investigated. Crude F_{UPS} had the highest sulfate content (31.7%) and the highest MW (877 kDa). Even though crude F_{UPS} had the highest sulfate content, it did not show the highest anticoagulant activity. Instead, F_{UPS}-T0.3-U gave the highest APTT and TT. F_{UPS}-T0.3-U had lower sulfate content (24.5%), and a lower MW of 687 kDa, compared to those of the crude F_{UPS} (Fig. 4). This is suggesting that both sulfate content and MW had some influence on the anticoagulant activity. Nishino and Nagumo [60] reported that the most potent anticoagulant activities of fucoidan were found in the range of the MWs from 10 to 300 kDa. In the present study, the positive effects of UHP treatment on anticoagulant activity of fucoidan are probably due to the effect of UHP hydrolysis on the lowering of MW. The anticoagulant activity of fucoidan was related to MW [49] and sulfate content [62]. According to *in vitro* study of Qiu et al. [62], a sulfated fucoidan highly stimulated GLu-Plg activation by u-PA and by t-PA in comparison to native fucoidan. As shown in Fig. 4, with the increase of sulfate content, APTT and TT showed a tendency to increase. APTT and TT had the highest values when the sulfate concentration was 24% (F_{UPS}-T0.3-U), but on the contrary decreased at a sulfate concentration of above 24%. This result indicates that there is an optimum sulfate concentration for the anticoagulant activity. There might be another correlation between MW and anticoagulant activity. Unfortunately, the mechanism for the anticoagulant activity increase by UHP-enzyme treatment is not clarified in the present study. Even though further detailed study on this is required, we briefly present the effects of UHP-enzyme treatment on fucoidan.

Consequently, in the present study, UHP-assisted enzymatic treatment was found to be helpful for the improvement of anticoagulant activities of fucoidan. This may be the first report that UHP-assisted enzymatic processing could be applied for the modification of algal fucoidan.

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