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Contrasting Correlation in the Inhibition Response of ADP-induced Platelet Aggregation and the Anti-coagulant Activities of Algal fucoidans Derived from *Eisenia bicyclis* and *Undaria pinnatifida sporophylls* (Mekabu)

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Sulfated fucans are known to have both anti-thrombotic and anti-coagulant activities. In this study, the variation in platelet aggregation and anti-coagulant activities was investigated *in vitro* with regard to administered dose, molecular weight distribution, sulfate content, and sugar composition in two algal fucoidans from *Eisenia bicyclis* and *Undaria pinnatifida sporophylls* (Mekabu). The anti-coagulant activity largely correlated with sulfate content and with molecular weight distribution in a dose-dependent manner. However, both fucoidans demonstrated inhibitory responses to ADP-induced platelet aggregation in dose- and structure-dependent manners that contrasted with the anti-coagulant activity. Neither molecular weight distribution nor sulfate content greatly affected platelet-aggregation inhibition (PA-inhibition) by the fucoidan fractions, whereas anti-coagulant activity was sensitive to these structural factors. Interestingly, an *E. bicyclis* fucoidan fraction exhibited almost complete PA-inhibition at a treatment dose of 500 mg/mL while retaining weak anti-coagulant activity. In conclusion, these observations suggest that fucoidan may be a useful anti-thrombotic or anti-platelet agent in various arterial thrombotic disorders, including post-vascular intervention with controlled bleeding complications, due to its anti-coagulant modulating activity.

Key words: Algal fucoidans, Anti-coagulant, Anti-thrombotic, Anti-platelet activity, Inhibition of platelet aggregation, LMW fucoidan.

Introduction

Anti-thrombotic agents with distinct properties are required in a wide variety of medical conditions including extracorporeal circulation and the prevention of venous thromboembolism. Sulfated fucans from brown algae have been investigated as candidate anti-coagulant and anti-thrombotic agents for possible clinical studies. However, a common limitation in the efficacy of these polysaccharides as anti-coagulant and anti-thrombotic agents is their simultaneous effect on bleeding and platelet aggregation. It has been reported that native algal fucoidans induced platelet aggregation *in vitro* (Durig et al., 1997; Mourao et al., 1999). In contrast, a number of *in vivo* studies demonstrated that algal fucoidans from different sources inhibited platelet aggregation effectively or had less hemorrhagic activity than heparin (Alywan et al., 2000; Amano et al., 2005; Trento et al., 2001). Thus, the issue of the platelet aggregation effects of algal fucoidans remains controversial.

Algal sulfated fucans have been tested in *in vivo* models of arterial interface damage. Intravenous administration of the polysaccharide prevented formation of microvascular thrombi induced by endothelial damage in arterioles and venules of mice, with no effect on P- or L-selectin function (Thorlacius et al, 2000). Thus, it was originally suggested that the anti-coagulant effect of fucoidan was primarily responsible for its powerful anti-thrombotic properties *in vivo*, although the correlation between the anti-coagulant and anti-thrombotic activities remains unclear. A low-molecular-weight (LMW) fraction of sulfated fucan from *Ascophyllum nodosum* revealed anti-thrombotic activity when tested on a venous

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thrombosis model in rabbits after intravenous or subcutaneous administration (Millet et al., 1999; Mauray et al., 1995). This activity of LMW fucoidan was attributed to PA-inhibition because it had very week anti-coagulant activity.

Thrombin is a key regulatory point in pathways that leads to blood coagulation and platelet aggregation. Platelet aggregation plays an important role in blood coagulation. Additionally, the enhancement of platelet aggregation accelerates the formation of thrombi, resulting in cerebral and myocardial infarctions and other diseases. Thus, PA-inhibition is important in an anti-coagulant or anti-thrombotic agent, as well as in anti-platelet therapy. Previously, it was suggested that the induction of platelet aggregation *in vitro* was possibly due to the fucoidans' large molecular size. However, in another study, *all* fucoidan fractions of varying molecular weights and sulfate contents induced platelet aggregation *in vitro* (Durig et al., 1997). In this case, low-sulfate fractions were most potent, showing that anti-coagulation and platelet activation depended differently on fucoidan polysaccharide structural factors. A recent comparative study demonstrated that platelet aggregation was highly divergent in various algal fucoidans, depending on the derived species, even in vitro, regardless of molecular weight (Cumashi et al., 2007).

Because the fucoidans are known to have both anti-thrombotic and anti-coagulant activities, in addition to platelet aggregation activation, depending on the algal species, it is necessary to consider their potential use in clinical practice. Indeed, it would be helpful to find fucoidan fractions with *differing* effects on platelet aggregation, depending on algal species, dose, and structure. In this study, we report the contrasting tendencies of anti-coagulant and PA-inhibition activities of algal fucoidans from *Eisenia bicyclis* and Mekabu and the dependence of these properties on structural factors such as sulfate content, molecular weight distribution, and sugar composition.

Materials and Methods

Extraction with acid

E. bicyclis was collected on Woolleung Island, off the east coast of Korea. The dried seaweed was ground in a blender. Isolation and purification of fucoidans from *E. bicyclis* were carried out according to the methods of Usui et al. (1980). The powdered seaweed (500 g) was extracted with 80% ethanol (EtOH, 2.5 L) while stirring overnight. The residual seaweed, recovered by filtration, was immersed overnight in 35% formaldehyde (2.25 L). The formaldehyde was decanted, and the air-dried weed was extracted twice with 2% $CaCl_2$, initially at room temperature for 4 h, and then at 70°C. The extracts were combined, concentrated under reduced pressure at 40°C, and dialyzed against running water for one day and then against distilled water for another day. The concentrated dialyzate was poured into ethanol to purify the fucoidan. The resulting precipitate was dissolved in water and then subjected to freeze-drying. The residue obtained above was further extracted at pH 2.0 and adjusted with hydrochloric acid for 4 h at 70°C. The extraction was repeated twice, and the extracts were combined and treated as above.

Extraction with hot water

Dried *E. bicyclis* (50 g) was extracted with distilled water (500 mL) while heating at 100°C for 2 h, as reported by Nishino et al. (1989). Briefly, the extracted solution (E1) obtained after filtration and the residual precipitates were re-extracted twice, resulting in another extracted solution (E2). Adding a 2× volume of ethanol to the combined E1 and E2 solutions produced a precipitate. Further steps to precipitate alginic acid were followed by treating the precipitate with 320 mL of a solution of 4 M $CaCl_2$. The supernatant obtained after centrifuging was treated with 50 mL of 5% cetyl pyridium chloride (CPC) solution for 24 h at 30°C. The precipitated CPC complex was treated with 3 M $CaCl_2$ solution and a 2× volume of EtOH repeatedly to separate the fucoidan fraction from the CPC complex. Then, the precipitates obtained after centrifuging were pooled and freeze-dried.

Fractionation of fucoidans

Crude Mekabu fucoidans, prepared according to Koo (1997), was obtained from Haewon Biotech, Inc. (a fucoidan manufacturer, Seoul, Korea). Either *E. bicyclis* or Mekabu fucoidan was fractionated by ion-exchange chromatography on a DEAE (diethylaminoethyl) cellulose (2.5×32 cm, XK26, Pharmacia) column and equilibrated with 300 mL of 50 mM sodium acetate (pH 5.0). The column was eluted with a linear gradient of 50 mM sodium acetate, and the same solution plus 5 M NaCl at a flow rate of 0.5 mL/min for 6 h and fractions of 2.5 mL were collected and assayed by the phenol-sulfuric acid reaction (Dubois et al., 1956). The fractions were then pooled and freeze-dried. Further purification was conducted by liquid chromatography on a Teflon hydrophobic column to eliminate phenol-like impurities. The column was eluted with water and then

with 15% aqueous ethanol. Sub-fractionation was performed on a Sepharose CL-6B column (1.5×90 cm, XK16, Pharmacia) with an elution solution of 0.1 M NaCl. Collections in both elutions were monitored with a 226 nm UV detector.

Constituent sugars

The monosaccharide composition of the separated fucoidan was analyzed by a liquid chromatography method using a Bio-LC DX-600 (Dionex, Sunnyvale, CA, USA) system. Fucoidan fractions were dissolved in 2 M trifluoroacetic acid for 4 h at 100°C prior to injection into a CarboPac PA1 column (2.0×250 mm, Dionex, Sunnyvale, CA, USA). The sample was eluted with 10 mM NaOH for 4 min and then eluted with water for 35 min at 0.25 mL/min. A peak was detected with integrated amperometry.

Preparation and analysis of low-molecular-weight fucoidan fragments

LMW fucoidans were obtained by the weak acid hydrolysis method of Murao (2005) from fucoidan extracts of Mekabu and *E. bicyclis*. Fucoidan (50 mg) was dissolved in 1 mL of 0.01 M HCl, and the solution was incubated at 60°C for 1, 4, and 6 h. Thereafter, the mixtures were neutralized with 1.0 mL of 0.01 M NaOH. The partially hydrolyzed fucoidan was applied to a semi-prep scale HPLC column (TSK-GEL G5000PWXL, Tosoh Corp., Japan) or a Sepharose CL-6B column for monitoring fragmentation or collection, respectively. After the column was eluted with the 0.1 M NaCl solution at a flow rate of 0.5 mL/min, 3 mL fractions were collected. The various fractions were pooled, dialyzed against distilled water, and lyophilized.

PAGE

The PAGE method (Melo et al., 2004) was used to estimate the molecular masses of the various fucoidan fractions (F1-7) collected from mild acid hydrolysis and gel filtration chromatography (GFC). In these experiments, 50 μg of each fraction was applied to a 6% 0.75-mm-thick polyacrylamide gel slab in 0.02 M sodium barbital, pH 8.6, and run for 45 min at 100 V. After electrophoresis, the fucoidan fractions were stained with 0.1% toluidine blue in 1% acetic acid and then washed for about 4 h in 1% acetic acid. The molecular masses of the low- molecular-weight fragments from the fucoidan fractions were determined by comparison with the electrophoretic mobility of heparin (~15 kD), used as a standard compound.

Measurement of platelet aggregation

Aggregation in whole blood was assessed by a turbidimetric method with a whole blood aggregometer (Chrono-log, Co. Havertown, PA, USA), according to the method of Born (1962). Reaction tubes containing 450 μL of citrated whole blood were diluted with 450 μL each of one of three concentrations (100, 500, 1,000 μg/mL) of fucoidan fractions or saline. Each tube was pre-warmed to 37°C for 5 min. Then, the reaction was started by the addition of 10 μL agonist solution containing ADP (20 μM). The reaction was allowed to proceed for at least for 10 min, and platelet aggregation was expressed as the increase (%) in light transmittance after ADP addition.

APTT assay

Activated partial thromboplastin time (APTT) clotting assays were carried out by the method of Anderson et al. (1974) using normal human plasma. In these assays, plasma samples (90 μL) were mixed with 10 μL fucoidan solutions containing 0-1,000 μg of fucoidan and incubated for 1 min at 37°C; 100 μL of APTT reagent (APTT-XL; Fisher Diagnostics, Fisher Scientific Company, USA) containing kaolin and bovine phospholipid reagent was added to the fucoidan solution and then incubated for 3 min at 37°C. Prewarmed 0.02 M $CaCl_2$ (100 μL) was then added, and the APTT was recorded as the time for clot formation in a coagulometer (Behnk Elektronik, Germany).

Results

Fractionation and characterization of fucoidans

After an aqueous solution (1%) of the Mekabu fraction had been applied to the column, it was eluted stepwise with 1.25 M (Mekabu FI), 3.5 M (Mekabu FII), and 4.5 M NaCl (Mekabu FIII), successively, until the elutes were free from carbohydrates by the phenol-H_2SO_4 test. Three fractions were eluted from Mekabu fucoidan, as reported previously (Koo, 1997), whereas one fucoidan fraction was derived from *E. bicyclis* fucoidan. The overall yield of *E. bicyclis* fucoidan was 6-10%. Compositional analysis was performed after each fucoidan fraction was completely hydrolyzed, and the results are summarized in Table 1. These results showed that fucose was predominant, and galactose and mannose were minor neutral sugars. This composition profile was quite different from that of the Mekabu fucoidan. The sugar compositions of the two *E. bicyclis* fucoidans that were extracted with hot water or acid were very similar to each other.

Table 1. Sugar composition of various fucoidan fractions (% ratio)

	Fuc	Gal	Glu	Xyl	Man	Rha	Total (g/100 g)
mekabu FI*	74.8	2.6	7.2	12.2	3.2	-	-
mekabu FII*	56.7	10.8	3.3	38.6	7.0	-	-
mekabu FIII*	31.8	57.9	2.2	tra	1.8	-	-
EB I	88.0	8.2	0.6	0.6	2.4	-	31.7
EB II	77.5	11.6	4.2	2.3	3.9	tra	8.8

*, data from reference (Koo 1997); EB I, unfractinated fucoidans extracted with hot water, while EB II is unfractinated fucoidans extracted with acid from EB.

LMW fucoidan and PAGE analysis

Mild acid hydrolysis gradually reduced the molecular size of both *E. bicyclis* and Mekabu fucoidans. Typical HPLC data of a hydrolyzed Mekabu fucoidan are presented in Fig. 1. As the time of hydrolysis proceeded from 1 to 6 h, the proportions of fractions with lower retention times (unfragmented) decreased, whereas the proportions of fractions with longer retention times increased (LMW fucoidans). The decrease in molecular size by mild hydrolysis was not further changed at 12 h of hydrolysis time. Thus, we chose 6 h as a suitable time for mild acid hydrolysis for algal fucoidans.

PAGE revealed that mild acid hydrolysis produced a wide variety of metachromatic bands, but with a few dispersive, broad patterns in the electrophoretic

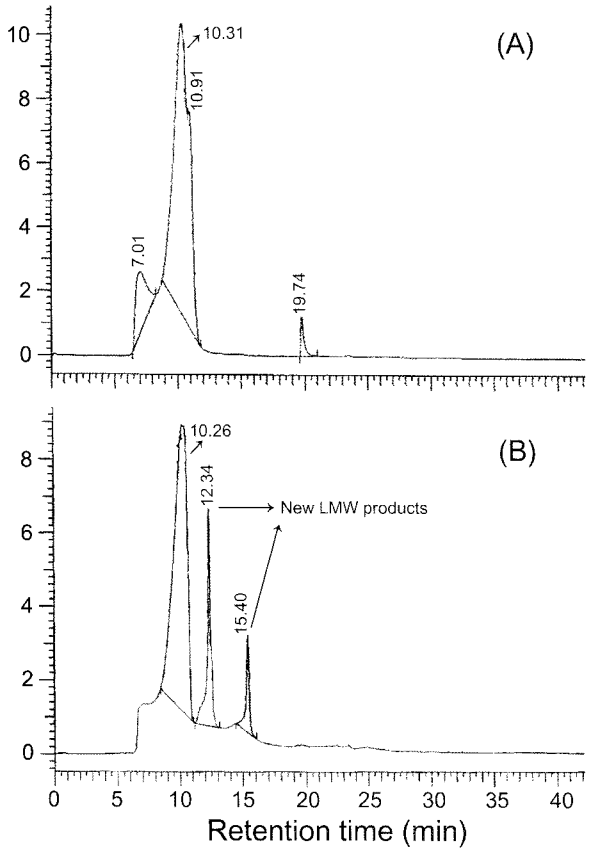

Fig. 1. HPLC elution profiles of intact fucoidans (A) and mild acid hydrolyzed fucoidans (B) of the Mekabu F III. Both intact and partially hydrolyzed fucoidans were applied to a semi-prep scale HPLC column (TSK-GEL G5000PWXL, Tosoh Corp. Japan) and eluted with water at a flow rate of 0.5 mL/min.

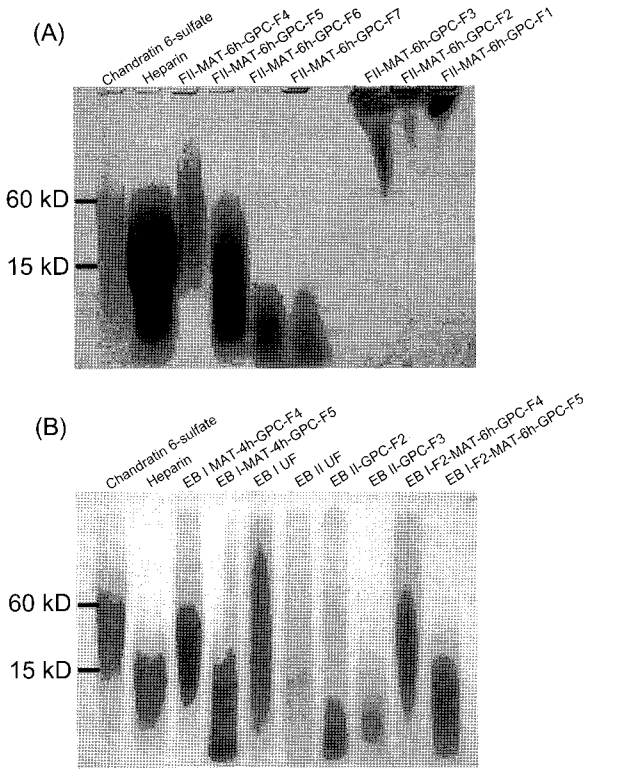

Fig. 2. Various fucoidan fractions (F1-7) from Mekabu FII (A) and E. bicyclis (B) fucoidans before and after mild acid hydrolysis (MAT). These were collected from gel permeation chromatography (GPC), and then analyzed by PAGE, as described under Materials and Methods. The molecular weights (kD) of standard compounds are indicated at the left. These standards are low molecular weight heparin (~15 kD) and chondroitin 6-sulfate (~60 kD). Late eluting fractions in the GFC show their molecular weight distributions less than 15 kD in both Mekabu and *E. bicyclis* fucoidans.

bands. However, fucoidan fractions with molecular weights less than 15 kDa were clearly observed in the hydrolysis of both Mekabu and *E. bicyclis* fucoidans (Fig. 2).

Platelet aggregation response

Evaluation of the platelet aggregation assay revealed that both *E. bicyclis* and Mekabu fucoidan fractions inhibited ADP-induced human platelet aggregation *in vitro*, as indicated by the observation of reduced transmission in the fucoidan-treated group compared with the saline/ADP-only control (Fig. 3). This PA-inhibition occurred in a complex manner depending on dose, sulfate content, molecular weight distribution, and sugar composition of the fucoidan fractions. The results are summarized in Table 2. *E. bicyclis* fucoidan demonstrated the largest inhibition of platelet aggregation, as shown by 0% transmittance, which may represent very little or no platelet aggregation. Mekabu fucoidans showed a markedly variable PA-inhibition depending on the fraction, acid treatment, and dose. Mekabu FII demonstrated the least transmission at a dose of 100 μg/mL, with the transmission increasing with dose. In contrast, a gradually decreasing transmission was observed as dose increased in Mekabu FIII. When the Mekabu fractions were treated with acid, the resultant reactants showed relatively small increases in optical transmission at every dose and fraction compared with the control group. However,

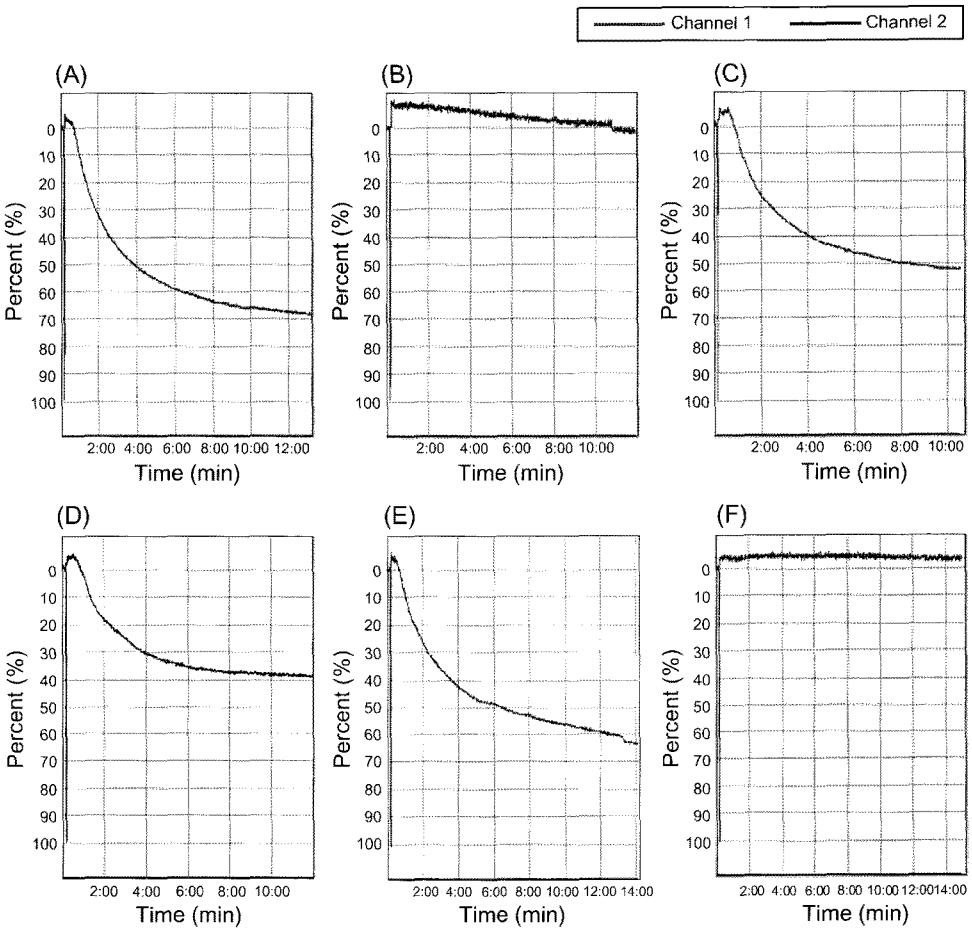

Fig. 3. Increase of light transmittance associated with ADP-induced platelet aggregation detected by aggregometry. The traces show the effects of different doses, molecular weight distributions, sulphate contents, and sugar composition of fucoidans on platelet aggregation. The initial increase in percentage aggregation is due to shape changes of activated platelets. Addition of ADP alone led to 70 % aggregation (A). Addition of 100 μg/mL of Mekabu FII produced only 10% aggregation (B). This represents an inhibition of ADP-induced platelet aggregation by Mekabu FII fucoidan fraction. This inhibition was decreased with the increasing dose of 500 μg/mL (C), and with increasing portions of LMWF in the acid-treated fucoidans for 6 h (D). Addition of 100 or 500 μg/mL of *E. bicyclis* I produced 67% (E), or almost 0% aggregation (F), respectively.

Table 2. Platelet aggregation measured in various fucoidan fractions as transmittance (%)

	100 μg/mL			500 μg/mL			1,000 μg/mL		
	A	B	C	A	B	C	A	B	C
mekFI	37 ± 1.3	63 ± 2.2	63 ± 1.5	52 ± 1.8	52 ± 2.3	53 ± 3.1	50 ± 1.9	46 ± 2.3	70 ± 2.6
mekFII	10 ± 1.0	24 ± 1.3	41 ± 1.2	57 ± 1.9	62 ± 2.2	67 ± 2.7	63 ± 2.1	49 ± 2.3	68 ± 2.8
mekFIII	65 ± 1.7	67 ± 1.5	59 ± 1.2	44 ± 2.3	48 ± 1.2	50 ± 1.6	17 ± 1.1	60 ± 2.2	57 ± 1.8
EB I	67 ± 2.4	69 ± 2.5	70 ± 2.9	0 ± 0.4	12 ± 1.1	16 ± 1.2	8 ± 0.8	23 ± 1.2	33 ± 1.4
EB II	89 ± 2.4	-	-	50 ±2.5	-	-	50 ± 2.6	-	-
Saline	71 ± 3.5								

FI, II, III were obtained by IEX chromatography when mekabu fucoidans were eluted at 1.25M, 3.5 M, 4.5M of NaCl. EB I: unfractinated fucoidans extracted with hot water, while EB II is unfractinated fucoidans extracted with acid from EB. A, B, C is fucoidan fractions without acid treatment, treated with acid for 1 hour, 6 hour, respectively. Data are shown as mean ± SD, n=3.

Table 3. Anticoagulation activities of various fucoidan fractions (APTT sec)

	100 μg/mL			500 μg/mL			1,000 μg/mL		
	A	B	C	A	B	C	A	B	C
mekFI	36 ± 2.2	31 ± 2.6	35 ± 3.1	41 ± 3.4	44 ± 2.5	36 ± 1.2	53 ± 3.6	60 ± 4.1	41 ± 3.3
mekFII	43 ± 4.1	35 ± 1.5	29 ± 2.1	78 ± 2.5	53 ± 3.3	33 ± 2.1	178 ± 8.7	102 ± 3.9	40 ± 3.5
mekFIII	45 ± 1.5	51 ± 2.8	41 ± 2.2	305 ± 4.5	133 ± 4.8	85 ± 3.1	41m ± 1.2m	482 ± 5.1	152 ± 4.6
EB I	52 ± 1.3	47 ± 1.9	41 ± 1.4	149 ± 3.5	91 ± 2.8	78 ± 4.3	16m ± 2.1m	370 ± 4.4	110 ± 3.9
EB II	35 ± 2.1	-	-	43 ± 3.1	-	-	61 ± 2.9	-	-
Saline	35 ± 2.7		1			1			

FI, II, III were obtained by IEX chromatography when mekabu fucoidans were eluted at 1.25M, 3.5 M, 4.5M of NaCl. EB I: unfractinated fucoidans extracted with hot water, while EB II is unfractinated fucoidans extracted with acid from EB. A, B, C is fucoidan fractions without acid treatment, treated with acid for 1 hour, 6 hour, respectively. 41m and 16m indicate 41 minutes and 16 minutes respectively. Data are shown as mean ± SD, n=3.

this increased PA was largest at doses of 100 μg/mL and 1,000 μg/mL for Mekabu FII fucoidan and Mekabu FIII fucoidan, respectively.

Anti-coagulant activity: APTT analysis

The results of anti-coagulant assay are summarized in Table 3. Anti-coagulation activities of the different fucoidan fractions varied in a dose-dependent manner. Both Mekabu and *E. bicyclis* fucoidans showed similar APTT times as doses increased from 100 to 1,000 μg/mL. However, fraction III of Mekabu showed a three-fold longer APTT time compared with the *E. bicyclis* fraction when the dose increased to 1,000 μg/mL. The Mekabu fraction with higher sulfate content showed a much longer APTT time than did the fraction with lower sulfate content. Within the same Mekabu fraction, the APTT times decreased in the fractions obtained from increased hydrolysis times. This reduction was quite marked, as high as 94% in the Mekabu FIII fraction with the highest sulfate content when a dose of 1,000 μg/mL was used.

Discussion

Fucoidans from *E. bicyclis* and *Undaria pinnatifida sporophylls*

The sugar compositions of *E. bicyclis* fucoidans appeared to be markedly different from those of Mekabu fucoidan (Table 1). This difference may reflect different characteristics of species originating from specific growing environments (e.g., water temperature). *E. bicyclis* only grows in an isolated, relatively cold island area of the East Sea, whereas *Undaria pinnatifida sporophylls* are widely distributed along the southern and eastern shores of the Korean Peninsula. Both fucoidans are known to be major components protecting algal cell walls. Undaria fucoidan is known to be released at the beginning of the autumn when the water temperature begins to decrease, resulting in the death of these algae during the autumn and winter. *E. bicyclis* fucoidan remains in the cell wall at the lower temperatures. Thus, *E. bicyclis* is a perennial plant, whereas Undaria is an annual one. This observation is particularly useful for cold-water extraction of fucoidan from annual algae like Undaria (unpublished result).

LMW fucoidan has typically been made either by a radical process using hydrogen peroxide or by strong acid hydrolysis (Nagasawa et al., 1992; Volpi et al., 1992). Recently, Mourao reported that low-molecular-weight fucans were produced site-

specifically using mild acid treatment of linear fucans from echinoderms (Pomin et al., 2005). This technique resulted in the production of more homogeneous LMW fucans in terms of structural distribution than do other radical processes. In this experiment, we degraded algal fucoidans with a multiple branched structure using mild acid treatment. PAGE data demonstrated the production of LMW fucoidans from the branched *E. bicyclis* or Mekabu fucoidan fractions, the molecular weight distributions of which appeared to be around or below 15 kDa. This indicated that mild acid hydrolysis also broke the 2-4 sulfate sites of algal fucoidans specifically, as it did in linear fucoidan (Pomin et al., 2005). In comparison with linear echinoderm fucoidan, the proportion of lower molecular weight material below 10 kDa was smaller in the fragmented algal fucoidans. Additionally, the appearance of eluting PAGE bands was relatively broad and dispersed in our algal species. This observation may result from the branched structure of algal fucoidans, rather than from the linear echinoderm fucoidans.

Anti-coagulant activity and platelet aggregation response

Anti-coagulant activity largely correlated with sulfate content and molecular weight distribution, as reported previously (Pereira et al., 2002). Mekabu FIII showed the largest anti-coagulant activity in a dose-dependent manner, and it markedly decreased as the LMW proportions increased.

For *in vivo* applications, anti-thrombotic effects of the fucoidan and required PA-inhibition need to be considered. Additionally, potential bleeding complications due to associated anti-coagulant activity should be avoided. Thus, to develop a fucoidan anti-thrombotic agent it is preferable to have both a certain range of inhibition of platelet aggregation and the least anti-coagulant activity. Because the latter may be controlled by the molecular weight distribution and the sulfate content of a fucoidan fraction, a desired fraction should retain appropriate PA-inhibition, depending on the specific application.

Originally, a LMW fucoidan was intended to have reduced anti-coagulant activity while retaining its paramount anti-angiogenic characteristics. The inhibition response of ADP-induced platelet aggregation demonstrated markedly different characteristics from anti-coagulant activity, which was correlated in a relatively simple manner with the molecular weight and the sulfate contents. First, PA-inhibition appeared *not* to be proportional to the sulfate content. This was observed at all three applied doses. Second, the PA-inhibiting effects of the Mekabu fucoidan were largest at a dose of 100 μg/mL in a given FII fraction and gradually decreased as the applied dose increased to 1,000 μg/mL. In contrast, the least inhibition occurred at a dose of 100 μg/mL in a given FIII fraction, and it increased with the applied dose to 1,000 μg/mL. When the dose was 500 μg/mL, inhibition responses were distributed in a similar range of transmittance values, regardless of sulfate content or molecular weight distribution. The Mekabu FII fucoidan at a dose of 100 μg/mL demonstrated the largest PA-inhibition with the relatively lowest anti-coagulant activity (an APTT of 43 s) within the Mekabu fucoidan fractions. In contrast, Mekabu FIII fucoidan showed relatively strong PA-inhibition, but the largest anti-coagulant activity (an APTT of as much as 41 min) occurred at a dose of 1,000 μg/mL. This fucoidan fraction showed decreased PA-inhibition but still retained PA-inhibition, compared with the control group, as its molecular weight shifted to LMW. Thus, if it is used in an extracorporeal application, it may work well as a temporary anti-coagulant agent. These data suggest that LMW Mekabu fucoidan may still have PA-inhibition and relatively weak anti-coagulant activities. A similar effect was observed in an *in vivo* anti-thrombotic study (Colliec-Jouault et al., 2003). Thus, the PA-inhibition response did not show a strong dependence on the molecular weight distribution of the fucoidan fraction, as the anti-coagulant activity did at a given dose or in the Mekabu fraction.

This observation of a decoupled correlation of PA-inhibition with molecular weight of Mekabu fucoidan may suggest that PA-inhibition depends more strongly on sugar compositions and their distribution within a fucoidan polymer context than on molecular weight. However, it did not seem to be proportional only to the fucose content of a fucoidan. An *E. bicyclis* fucoidan has predominantly fucose, and its sugar composition is quite different from that of Undaria-derived fucoidans; PA-inhibition was pronounced at 500 μg/mL and was demonstrated to a dose of 1,000 μg/mL. This inhibitory activity was still retained even as the molecular weight distribution shifted to LMW (Table 2). At a dose of 500 μg/mL, the *E. bicyclis* I fucoidan demonstrated the largest inhibition and relatively weak anti-coagulant activity (APTT of 43 s), compared with an APTT of 16 min at a dose of 1,000 μg/mL. Although anticoagulant activity increased 15-fold, PA-inhibition decreased by only 8% with this change in dose. These data suggest that once an administered dose is specifically chosen for a certain anti-thrombotic application, it may

reduce potential bleeding effects due to high anti-coagulant activity. Thus, LMW *E. bicyclis* fucoidan may be a promising candidate as an anti-thrombotic agent with less anti-coagulant activity, possibly eliminating potential bleeding complications.

This point may provide an important advantage for fucoidan agents over other types of anti-thrombotic or anti-platelet agents because potential bleeding complications associated with the anti-coagulant activity may be controllable, either by dose or by modulating various structural factors such as molecular weight, sulfate content, and sugar composition while still retaining PA-inhibition. Molecular weight can be controlled by de-fragmentation, and the other two factors can be adjusted by fractionation of complex fucoidans mixtures. That is, because hemorrhagic complications are the major limitations of new anti-thrombotic or anti-platelet agents, the observed decoupled correlation pattern of PA-inhibition and anti-coagulant activities may be beneficial in determining how to balance the risks and benefits of anti-thrombotic or anti-platelet therapy using a fucoidan agent.

Presently, the ideal dose, route of administration, duration of use, and monitoring tools for these drugs are practically unknown, particularly when used in combination with other anti-coagulants. The presence in a fucoidan of simultaneous PA-inhibition and anti-coagulant properties may make it a sensible candidate for such a drug. In addition, it has been reported that fucoidan inhibited the generation of thrombin from platelets as well as thrombin-induced platelet aggregation (Trento et al., 2001). The participation of platelets in most intravascular thrombotic processes depends on their ability to adhere to an abnormal surface, activate (express precoagulant potential to form the template for clot growth), and aggregate. Thus, current pharmacologic strategies to inhibit platelets have focused on these fundamental properties.

Collectively, these observations of PA-inhibition by fucoidan may suggest that fucoidan can act directly as an inhibitor of the glycoprotein (GP) IIb-IIIa receptor, which mediates platelet aggregation caused by all physiologic agonists, because the GP IIb-IIIa receptor is the final common pathway for aggregation regardless of the agonist. On the other hand, P-selectin expressed in activated platelets participates with GP IIb-IIIa in the initiation of platelet aggregation (Théorêt et al., 2006). Because fucoidan is a well-known P-selectin blocker (Chauvet et al., 1999), the PA-inhibition of fucoidan may be mediated or enhanced by blocking of P-selectin.

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