

Anticoagulating Activities of Low-Molecular Weight Fuco-Oligosaccharides Prepared by Enzymatic Digestion of Fucoidan from the Sporophyll of Korean *Undaria pinnatifida*

Woo Jung Kim¹, Yean-Kyoung Koo², Mi-Kyung Jung³, Hye Ran Moon¹, Sung Min Kim¹, Andriy Synytsya⁴, Hye Sook Yun-Choi², Yeong Shik Kim², Jae Kweon Park¹, and Yong Il Park¹

¹Department of Biotechnology and Biomaterial Engineering Research Center, The Catholic University of Korea, Bucheon 420-743, Korea, ²Natural Products Research Institute, College of Pharmacy, Seoul National University, Seoul 151-742, Korea, ³Hanrib Lifetech Ltd., Bio 21 Center, Jinju 600-844, Korea, and ⁴Department of Carbohydrate Chemistry and Technology, Institute of Chemical Technology in Prague, Technicka 5, 166 28 Prague 6, Czech Republic

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In spite of their potential as biologically active compounds, the high molecular mass and viscous natures of fucoidans have hampered their applications especially as a therapeutic agent. Herein the fucoidan-degrading enzyme activities were partially purified from the cultured cells of *Sphingomonas paucimobilis* PF-1 mainly by ammonium sulfate precipitation. This enzyme preparation degraded fucoidans from the Korean *Undaria pinnatifida* sporophyll into several low-molecular weight fuco-oligosaccharides (LMFOs) with less than 3,749 Da. The FT-IR spectra of intact fucoidan and mixture of LMFOs (1,389~3,749 Da) showed no significant structural difference except for about 10% reduced level of sulfate esters in LMFOs. The LMFOs have exerted strong anticoagulating activities at which the activated partial thromboplastin time (APTT) and thrombin time (TT) were significantly prolonged, although 3~20 times weaker activities were observed than those of intact fucoidan. In addition, unlike intact fucoidan, LMFOs did not affect significantly to the prothrombin time (PT). These results suggest that the partially purified fucoidan-degrading enzyme preparation is valuable for the production of fuco-oligosaccharides having anticoagulating activities, and that the molecular weight and/or sulfate content of the fucoidan from the Korean *Undaria pinnatifida* sporophyll could be important factors for its anticoagulating activity.

Key words: Fucoidan, Low-molecular weight fuco-oligosaccharides (LMFOs), Anticoagulating activity, Activated partial thromboplastin time (APTT) assay, Thrombin time (TT) assay

INTRODUCTION

Although heparins are commonly used to reduce thrombin formation in patients with arterial thrombotic disease, these polysaccharides have shown to cause potentially serious side effects, such as hemorrhage, thrombocytopenia, and osteoporosis (Fischer, 2007; Catherine Boisson-Vidal et al., 2000). The antithrombotic effect of heparins could be improved by increasing the dose, but this would also increase the

hemorrhagic risk. Furthermore, since heparin is obtained from mammalian sources, it has potential risk of contamination by animal pathogens such as viruses (Catherine Boisson-Vidal et al., 2000). These reasons have led to consideration of other options for the development of effective anticoagulant with less side effects.

Fucoidan, a sulfated polysaccharide extracted from brown seaweeds, has anticoagulant and antithrombotic effects mediated by catalysis of thrombin inhibition, mainly by the inhibition of heparin cofactor II (Nardella et al., 1996; Mauray et al., 1995). Anticoagulating activity of fucoidans has been demonstrated by many research groups and fucoidans have thus been proposed as alternatives to the anticoagulant

Correspondence to: Yong Il Park, Department of Biotechnology and Biomaterial Engineering Research Center, The Catholic University of Korea, Bucheon 420-743, Korea
Tel: 82-2-2164-4512, Fax: 82-2-2164-4846
E-mail: yongil382@catholic.ac.kr

heparin, although their activities have been reported to be generally lower than that of heparin (Mourao and Pereira, 1999; Mulloy, 2003). For these reasons, production and applications of fucoidans as therapeutic agents have been increasingly important topics of intensive researches (Mulloy, 2003). However, in spite of their potential as biologically active compounds, the high molecular mass and viscous nature of fucoidan has hampered their application especially as a therapeutic agent. Therefore, a reliable fucoidan-degrading enzyme preparation would be highly desirable to obtain fuco-oligosaccharides and thereby help overcome these problems.

Previously we reported on the isolation and identification of a marine bacterial strain, *Sphingomonas paucimobilis* PF-1, that degrades the fucoidan that we purified from the sporophyll of Korean *Undaria pinnatifida*. The intact cells of this strain could degrade our fucoidan into seven distinct low-molecular weight fuco-oligosaccharides (LMFOs), ranging from 305 to 3,749 Da (Kim et al., 2008). In the present study, we prepared crude enzyme solutions from this strain mainly by sonication and ammonium sulfate precipitation. Subsequently, the LMFOs with molecular weight (1,389 to 3,749 Da) produced by enzymatic digestion were compared to intact fucoidan for any structural differences and their anticoagulating activities.

MATERIALS AND METHODS

Microorganism and culture conditions

The purification of fucoidan from the sporophyll of Korean *Undaria pinnatifida* and the growth of *Sphingomonas paucimobilis* PF-1 (KCTC 11130BP) were performed as previously reported by our group (Kim et al., 2008). The cells were cultured in a minimal medium consisting of 0.2% fucoidan with 2% Bacto peptone in dH₂O (pH 7.8) at 30°C for 4 days on a shaking incubator (180 rpm, JEIO TECH Co.) and then centrifuged at 6,000 rpm, 4°C for 30 min. The cells harvested were stored at -20°C and used for partial purification of fucoidanolytic enzyme activities.

Crude enzyme preparation with fucoidanolytic activity

All operations were done at 4°C unless otherwise stated. The cells (50 g, wet weight) were homogenized on ice using a Sonifier 450 (Branson) for 1 h in 50 mL of 50 mM sodium acetate buffer (pH 5.6). The homogenate was centrifuged at 16,000 rpm for 40 min to remove insoluble materials. The supernatant solution was gradually brought up to 30% (w/v) saturation

with (NH₄)₂SO₄ and then centrifuged at 16,000 rpm for 30 min to remove the precipitates. The supernatant solution was further brought to 60% (w/v) saturation with (NH₄)₂SO₄ and precipitated overnight at 4°C. The precipitate was collected by centrifugation at 16,000 rpm for 30 min, dissolved in 10 mL of 50 mM sodium acetate buffer (pH 5.6), and dialyzed (10 kDa MWCO) extensively against 2 L of the same buffer for 3 days at 4°C and stored at -20°C. Protein obtained was quantified by Bradford method (Bradford, 1976). The resulting protein mixture [30% to 60% (NH₄)₂SO₄ fraction] was then used as the crude enzyme preparation for production of fuco-oligosaccharides.

Effect of pH and temperature on enzyme activity

The fucoidanase activity was assayed by measuring the reducing carbohydrate by Somogyi-Nelson method (Somogyi, 1952) after the enzyme reaction for 72 h in 50 mM sodium acetate buffer (pH 5.6) containing 0.2% of fucoidan. The optimal temperature for fucoidanase activity was determined after the reactions at several different temperatures, ranging from 25 to 42°C. For the determination of the optimal pH, fucoidanase activity of the crude enzyme was examined at 30°C in the following buffers: 50 mM sodium acetate buffer (pH 3 & 5.6) and 50 mM Tris-HCl buffer (pH 7 & 8). The pH stability of the enzyme was examined by measuring the residual activities after pre-incubating the enzyme in the above buffer (pH 3-8) at 30°C for 72 h.

Preparation of fuco-oligosaccharides by enzymatic digestion

For the production of fuco-oligosaccharides by crude enzyme preparation [30% to 60% (NH₄)₂SO₄ fraction], the reaction mixture contained 50 mL of 0.4% fucoidan in 50 mM sodium acetate buffer (pH 5.6) and 50 mL of enzyme solution (733.8 µg of protein). The enzyme reaction was carried out at 30°C for 72 h in a shaking water bath (12 rpm, Dong-A Scientific Co.). The reaction was then stopped by boiling for 10 min. After centrifugation at 12,000 rpm at 4°C for 30 min, the supernatant was taken out and freeze-dried. The dried material was dissolved in 5 mL of distilled water and then precipitated with three volume of 95% ethanol, under gentle shaking for 24 h at room temperature. After centrifugation at 12,000 rpm at 4°C for 30 min, the supernatant was dialyzed (MWCO 1,000) against excess volume of distilled water and then freeze dried. The resulting fuco-oligosaccharides (LMFOs of 1,389~3,749 Da) were used for subsequent experiment.

Analysis of enzyme hydrolysates

The degradation of fucoidan by the crude enzyme preparation was confirmed by size-exclusion HPLC (Dionex) using a Shodex OHPak column (SB-806HQ, 8.0 × 300 mm, Showa Denko Co.). Ten μL of enzyme-hydrolyzed sample was injected, eluted with water at a flow rate of 0.8 mL/min at 60°C and detected with ELSD (Evaporative light scattering detector, Alltech).

Infrared spectroscopy of LMFOs

IR spectra (Nicolet 6700, Thermo Scientific) were recorded as KBr pellets prepared by mixing 100 mg KBr in 1.0 mL of LMFOs solution (15 mg/mL) in distilled water, and stabilized under controlled relative humidity before acquiring the spectrum. IR spectrum was recorded by accumulation of at least 64 scans, with a resolution of 4 cm^{-1} .

Blood anti-coagulant activity

Anticoagulation assays (APTT, PT and TT) were performed using a Fibrometer (BBL Fibrosystem, Fisher Scientific). Human plasma provided from the Blood Bank, Seoul National University Hospital, was stored in a deep-freezer (-70°C). A portion of plasma sample was thawed at room temperature, and directly assayed using the Activated Partial Thromboplastin Time (APTT), Prothrombin Time (PT) and Thrombin Time (TT) assay kits. The APTT clotting assay was carried out by the method of Anderson *et al.* (Andersson, 1976). Hundred μL of normal human platelet-poor plasma (Human plasma from the Blood Bank, Seoul National University Hospital) was mixed with 3 μL of each sample solution samples and incubated for 2 min at 37°C. To the reaction mixture, 100 μL of APTT reagent (STA[®]-PTT A) was added and incubated for 3 min at 37°C and the clotting time was recorded following the addition of 100 μL of 0.025 M CaCl_2 . The TT assay was conducted by the method of Maraganore *et al.* (1989). Briefly, 3 μL sample solution was added into 100 μL normal human plasma and incubated for 3 min at 37°C. Then, 200 μL of Thrombin time reagent (STA[®]-Thrombin) was added and clotting time was recorded. The PT assays were carried out by the method of Matsubara *et al.* (2001). Hundred μL of normal human plasma was mixed with 3 μL of a

solution of sample and incubated for 3 min at 37°C. The clotting times was recorded following the addition of 200 μL of PT assay reagent (Neoplastine[®] CI plus). Values were expressed as the mean of minimum triplicate tests.

RESULTS

Partial purification of fucoidanase

Fucoidanase activity producing LMFOs ranging from 305 to 3,749 Da was partially purified from *Sphingomonas paucimobilis* PF-1. The purification steps were given in Table I. Ammonium sulfate fractionation (30% to 60%, w/v) yielded up to 61.1% of active fraction with specific activity (0.1 U/mg) from the concentrated supernatant of the PF-1 lysates. This active fraction [30% to 60% $(\text{NH}_4)_2\text{SO}_4$ fraction] was used as the partially purified enzyme preparation for degradation of fucoidan.

Effect of pH and temperature on enzyme activity

Fucoidanase activity was assayed at different pH over the range 3.0 to 8.0. The highest enzyme activity was observed at pH 5.6 (Fig. 1A). The enzyme was most active in the range of pH 5-6, but its activity decreased by more than 50% at above pH 7 and below pH 4. The enzyme activity was shown to reach the maximum at 30°C and sharply decreased at over 30°C (Fig. 1B). When the incubation temperature was brought to higher than 37°C, approximately 40-50% of its activity was decreased compared to the maximum activity at 30°C.

Analysis of enzyme hydrolysates

To confirm the degradation of fucoidan by the crude enzyme preparation [30% to 60% $(\text{NH}_4)_2\text{SO}_4$ fraction], aliquots of the hydrolysates produced from the reaction under the optimal conditions (at 30°C and pH 5.6) were analyzed by the size-exclusion HPLC (Fig. 2). Oligosaccharides were resolved into 7 distinct peaks in low molecular mass fractions, peaks 2 to 8. On the basis of their levels estimated by the relative peak areas, the 3 major fractions (peaks 3, 4, 5) represented approximately 76.7% within the total oligomers (Fig.

Table I. Summary of partial purification of fucoidanase

Purification step	Total protein (mg)	Total activity (U)	Specific activity* (U/mg)	Recovery (%)
Crude extract	273.8	19.2	0.07	100
$(\text{NH}_4)_2\text{SO}_4$ precipitation	70.89	7.1	0.1	61.1

*The specific enzyme activity was determined by measuring the reducing sugars by Somogyi-Nelson method. One unit (U) represents μmol of reducing sugars produced per min by 1 mg of crude enzyme protein.

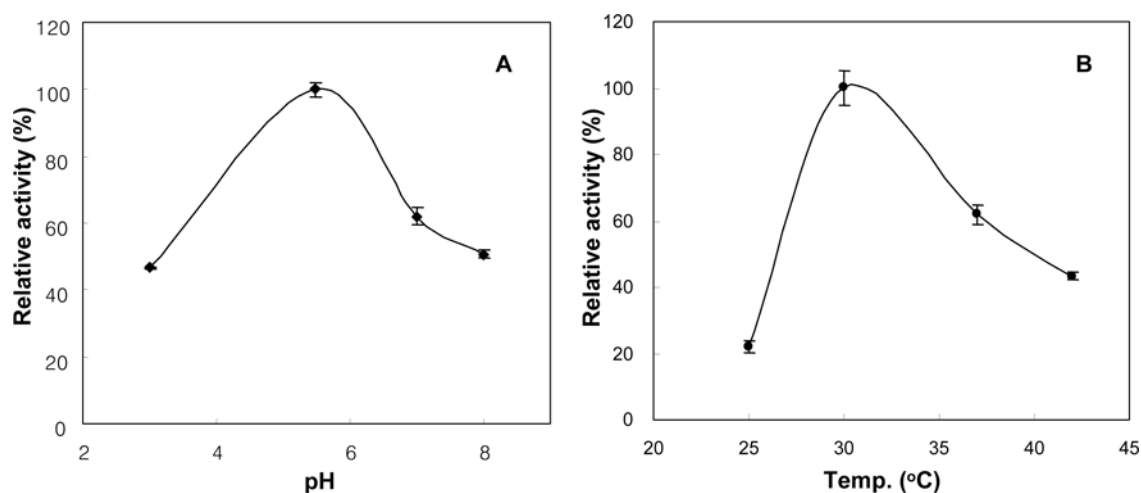


Fig. 1. Effects of pH and temperature on the activity of the partially purified fucoidanase. (A) The effect of pH on enzyme activity. (B) The effect of temperature on enzyme activity. Enzyme activity at each point was expressed as relative percentages when the highest activity was set at 100%.

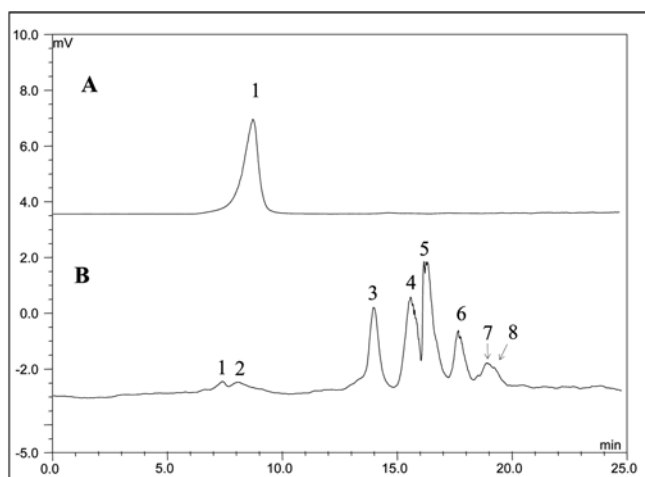


Fig. 2. Size-exclusion HPLC analysis of (A) intact fucoidan and (B) its enzyme hydrolysates

2). Previously, it was shown that, by using the suspension of intact cells of *S. paucimobilis* PF-1 (KCTC 11130BP) as the enzyme source, the fucoidan was depolymerized into 7 low-molecular-mass fucose-containing oligosaccharides ranging from 305 to 3,749 Da (Kim et al., 2008). Thus, the similar pattern of oligosaccharides produced by crude enzyme protein in the present study to those by intact cells, suggested that the crude enzyme protein mixture contain the same fucoidanase enzyme that was on the cell surface and showed an *endo*-type activity which randomly attacks the fucoidan chains (Kim et al., 2008).

Infrared spectroscopy of LMFOs

Intact fucoidan and LMFOs were analyzed by FT-IR to monitor any structural change after enzymatic

hydrolysis of fucoidan (Fig. 3). Although the spectra were very similar to each other, some alterations in minor peak patterns were observed. Broad IR bands observed at $3437\text{--}3456\text{ cm}^{-1}$ correspond to the OH and H_2O stretching vibrations. Several smaller IR bands and shoulders at $2864\text{--}2991\text{ cm}^{-1}$ were assigned to the CH stretching in pyranoid ring and C-6 groups of fucose and galactose units. LMFOs have a peak at 2929 cm^{-1} with a shoulder near 2864 cm^{-1} . These spectral features are typical for CH_2 stretching vibration and were assigned to galactose units. Intact fucoidan showed no pronounced peaks at these positions. Band at 1462 cm^{-1} was assigned to scissoring vibration of CH_2 . This band is more pronounced for LMFOs; spectrum of intact fucoidan has only a small shoulder at this wave number. The differences in CH_2 bands observed for samples intact fucoidan and LMFOs can be explained by the change in Gal/Fuc ratio in fucoidan after enzymatic hydrolysis. The IR band of intact fucoidan at 1738 cm^{-1} was assigned to C=O stretching vibration of *O*-acetyl groups. LMFOs have less pronounced shoulders near 1741 cm^{-1} that is an evidence of smaller acetylation. The IR bands near $1639\text{--}1645\text{ cm}^{-1}$ are commonly attributed to the in-plane bending vibration of water molecules. Peak or shoulder at $1381\text{--}1387\text{ cm}^{-1}$ originated from symmetric bending of methyls (C6 in fucose and *O*-acetyls). Decrease of this band for LMFOs could be due to deacetylation and/or change in Fuc/Gal ratio. The envelope of strong to medium bands at $1200\text{--}970\text{ cm}^{-1}$ is caused mainly by CC and CO stretching in pyranoid ring and COC stretching of glycosidic bonds. These structure sensitive bands are common characteristic of all polysaccharides. The bands and shoulders of this region

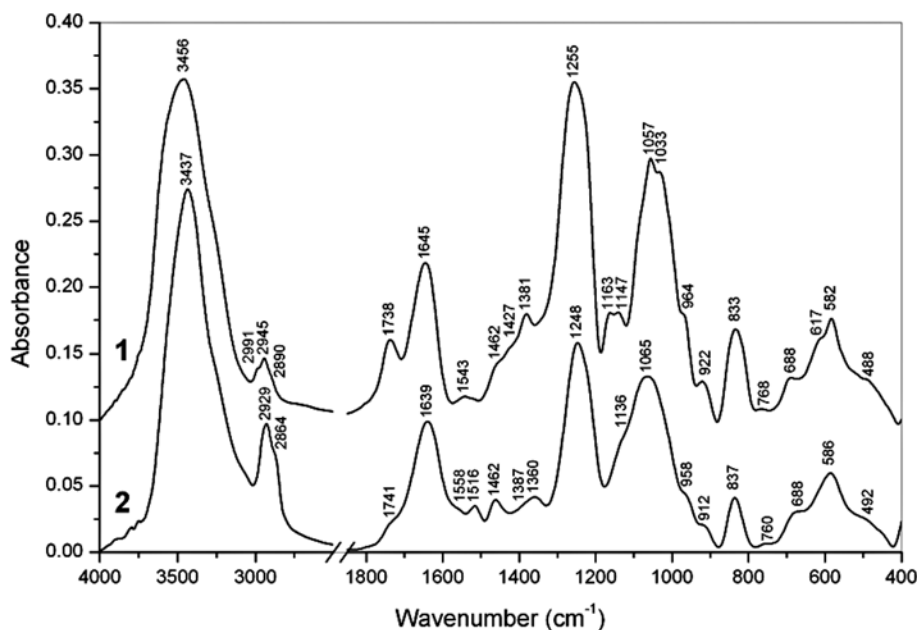


Fig. 3. FT-IR analysis of (1) intact fucoidan and (2) LMFOs.

varied in their position and intensities reflecting some differences in composition and substitution of monomeric units. Very intense and broad IR band at 1248-1255 cm^{-1} was attributed to O=S=O stretching of sulfates, which is a good marker of sulfation. Band centered at 833-837 cm^{-1} was attributed to COS bending of sulfate substituents (Mazumder et al., 2002). Its wavenumber is intermediate between the vibrations of secondary sulfates at equatorial (C-2 or C-3: 820-830 cm^{-1}) and axial (C-4: 845-850 cm^{-1}) positions of fucose or galactose units as well as primary sulfate at (C-6: 867; 815-820 cm^{-1}) of galactose units. The position of COS bending band, however, may depend on other substitutions at the pyranoid ring, such as glycosylation, *O*-acetylation and/or additional sulfation (Chevolot et al., 1999). Band at 582-586 cm^{-1} was attributed to the asymmetric O=S=O deformation of sulfates. Relative ratio between the characteristic sulfate (1248-1255 cm^{-1}) and polysaccharide (1057-1065 cm^{-1}) bands was 1.29 for intact fucoidan and 1.18 for LMFOs. The difference in these ratios can be explained by partial (about 10%) desulfation of intact fucoidan (Fig. 3). Overall, although all the sulphate bands were still pronounced for both samples, enzymatic fragmentation of our fucoidan caused certain level of desulfation. Other than that, the FT-IR spectra of intact fucoidan and LMFOs showed no significant structural variation.

Blood anti-coagulant activity

The anticoagulant activities of intact fucoidan and LMFOs were examined by APTT, TT and PT assays.

Both intact fucoidan and LMFOs significantly prolonged APTT in a dose-dependent manner (Fig. 4A). However, when compared to that of heparin (clotting time 76.3 s at 0.3 $\mu\text{g/mL}$), the intact fucoidan (168.4 s at 10 $\mu\text{g/mL}$) and LMFOs (54.4 s at 10 $\mu\text{g/mL}$) showed about 15- and 46-fold weaker activities, respectively. Although it is still significant, the LMFOs prolonged APTT about 3.1- to 21.4-fold weakly than intact fucoidan at concentrations ranging from 10 to 200 $\mu\text{g/mL}$ LMFOs. Prolongation of APTT suggests the inhibition of the intrinsic and/or common pathway in blood coagulation. As shown in Fig. 4B, both intact fucoidan and LMFOs also strongly prolonged TT in a dose-dependent manner. However, when compared to that of heparin (clotting time 46.4 s, 0.5 $\mu\text{g/mL}$), the intact fucoidan (68.7 s at 10 $\mu\text{g/mL}$) and LMFOs (15.8 s at 10 $\mu\text{g/mL}$) showed about 11.7- and 51.1-fold weaker activities, respectively. The LMFOs prolonged TT about 8.5- to 17.7-fold weakly than intact fucoidan at concentrations ranging from 25 to 100 $\mu\text{g/mL}$ LMFOs. Prolongation of TT indicates the inhibition of thrombin activity or fibrin polymerization. Interestingly however, while intact fucoidan still significantly prolonged the PT, no significant prolongation of PT was observed by LMFOs at the concentration up to 200 $\mu\text{g/mL}$, suggesting that these oligosaccharides did not inhibit extrinsic pathway of blood coagulation (Fig. 4C). These results indicate that intact fucoidan from Korean *Undaria pinnatifida* sporophyll and its low-molecular weight oligosaccharides may exert their anticoagulating activities via different mechanisms.

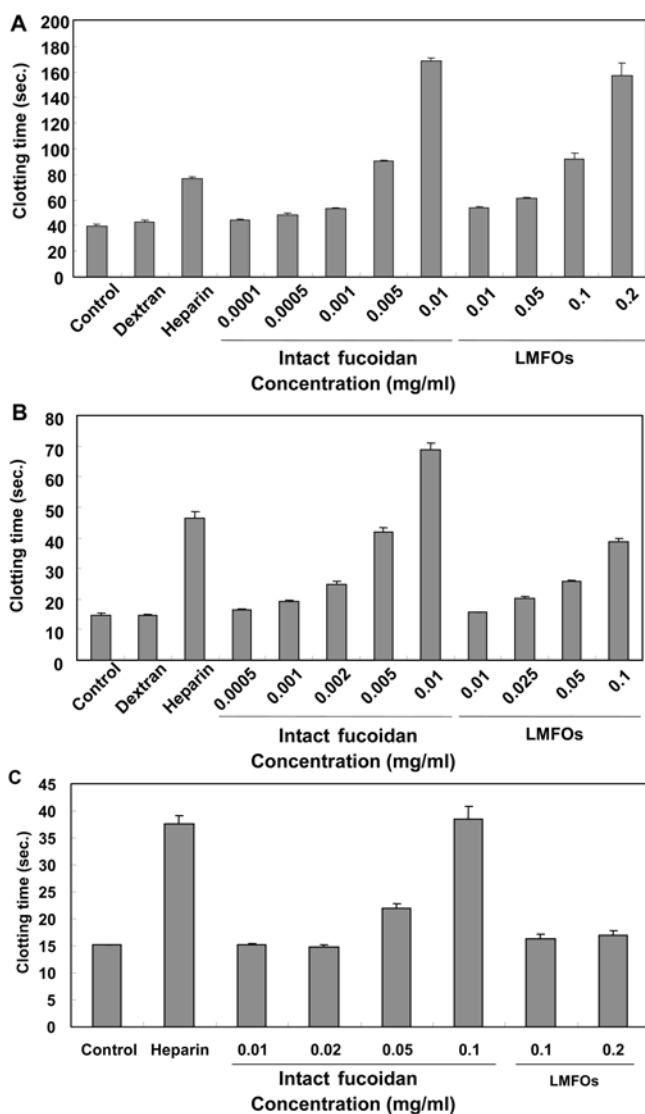


Fig. 4. Anticoagulating activities of intact fucoidan and LMFOs. (A) Activated partial thromboplastin time (APTT) assay. Heparin, positive control (0.3 $\mu\text{g}/\text{mL}$); Control, negative control (D.W.); Dextran, negative control (0.2 mg/mL). (B) Thrombin time (TT) assay. Heparin, 0.5 $\mu\text{g}/\text{mL}$; Control, D.W.; Dextran, 0.2 mg/mL. (C) Prothrombin Time (PT) assay. Heparin, 10 $\mu\text{g}/\text{mL}$; Control, D.W.; Dextran, 0.2 mg/mL.

DISCUSSION

Fucoidans are known to exhibit a wide range of physiological and biological activities, thus medically useful activities, anticoagulating activity being the most prominent (Mourao and Pereira, 1999; Nishino et al., 1991). Since the high molecular mass and viscous nature of these polysaccharides have hampered their application especially as a therapeutic agent, fucoidan-degrading enzyme preparation would be highly desirable to obtain fuco-oligosaccharides. However, no

convenient commercial sources with such activity are available yet for the degradation of these polysaccharides (Mulloy, 2003; Shin-ichi Furukawa, 1992; Urvantseva et al., 2006). Previously, we demonstrated that the intact cell of *Sphingomonas paucimobilis* PF-1 (KCTC 11130BP) digests the fucoidan of Korean *Undaria pinnatifida* sporophyll into fuco-se-containing oligosaccharides in an *endo*-acting manner (Kim et al., 2008). To the best of our knowledge, this was the first report on the fucoidanalytic activity of *Sphingomonas* species and also the first report on the enzymatic degradation of fucoidans from the sporophyll of Korean *Undaria pinnatifida*. The fucoidans of Korean alga may have different structures and therefore different bioactivities from fucoidans of brown alga harvested in other countries. In the present study, we prepared crude enzyme protein solutions [30% to 60% $(\text{NH}_4)_2\text{SO}_4$ fraction] from this strain and produced LMFOs (1,389 to 3,749 Da) by enzymatic digestion of fucoidan and have further investigated the anticoagulant activities of these LMFOs and intact fucoidan. LMFOs produced by this crude enzyme preparation were resolved into 7 distinct oligosaccharides (Fig. 2) and no α -L-fucosidase activity against p-nitrophenyl- α -L-fucoside was observed (data not shown). These results indicate that partially purified fucoidanase cleaves somewhere in the middle of the fucoidan in an *endo*-acting manner. Intact cell (*Sphingomonas paucimobilis* PF-1) and partially purified fucoidanase have shown similar pattern in fucoidan degradation (Kim et al., 2008). LMFOs showed no significant structural differences when compared to intact fucoidan but contained slightly lower level (by 10%) of sulfate.

Anticoagulant effects of intact fucoidan and its LMFOs obtained as enzyme hydrolysates seemed to be addressed via different mechanisms. Although LMFOs still significantly prolonged APTT and TT in a dose-dependent manner, comparing to intact fucoidan, they showed 3~4 times reduced anticoagulating activities in both assays. Furthermore, unlike intact fucoidan, LMFOs did not affect the PT. These results strongly suggest that molecular weight and/or degree of sulfation of fucoidan are important factors for its anticoagulating activity. Overall, the partially purified fucoidan-degrading enzyme preparation can be a valuable tool for the production of fuco-oligosaccharides having anticoagulating activities from the fucoidan of Korean *Undaria pinnatifida* and, although their anticoagulating activities are slightly weaker than intact fucoidan and heparin, the LMFOs produced by this enzyme activity may be promising candidates for the development of an anticoagulant that can replace heparin. Further structural information on LMFOs

and their detailed mechanism of anticoagulant action remain to be resolved for future application of these oligosaccharides as therapeutic agents.

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