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Degradation of fucoidans from *Sargassum fulvellum* and their biological activities

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ABSTRACT

A fucoidan extracted from *Sargassum fulvellum*, was degraded by ultrasound (US) or electron beam (EB) irradiation in the presence of hydrogen peroxide aqueous solution. From the energetic point of view, the most effective method for the fucoidan degradation was found to be EB radiation method in H_2O_2 with a yield of scission G_s 3.310×10^{-8} mol/J at the reaction condition of 1.5% hydrogen peroxide and irradiation 2.5 kGy. The degradation took place by the formation of a reactive hydroxyl radical due to the dissociation of H_2O_2 in the presence of US or EB. The low molecular weight fucoidans (LMWFs) prevented P-selectin binding to Sialyl Lewis X with an IC_{50} (inhibitory concentration 50) of 20 nM as compared to 400 nM for heparin and 25,000 nM for dextran sulfate. The LMWFs showed no hemolytic activity at concentrations up to 950 μg/ml.

1. Introduction

Fucoidan is cell wall polysaccharide composed of variable amounts of fucose, uronic acids, galactose, xylose and sulfate group. This compound exhibits a diversity of biological activities, including anti-inflammatory (Cumashi et al., 2000; Park et al., 2011), anti-oxidant (Chevolot, Foucault, Chaubet, & Kervarec, 1999; Wanga, Zhang, Zhang, Song, & Li, 2010), anti-coagulant/thrombotic (Bachelet et al., 2009; Chevolot et al., 1999; Irhimeh, Fitton, & Lowenthal, 2009) and anti-tumor (Cho, Kim, & Moon, 2013; Kim, Lee, Lee, Kim, & Yang, 2013) effects on numerous diseases. Fucoidans are widely available from various kinds of cheap sources compared with other sulfated polysaccharides, and thus more and more fucoidans have been investigated since the past decades to develop new drugs or functional materials. Because the high molecular mass of polysaccharides, however, caused some problems such as high viscosity, structural heterogeneity and low permeability of the cell membranes for high molecular mass fucoidans as well as low functionality in drug action, several depolymerization methods have been reported to get low molar mass oligosaccharides. The depolymerization has mainly been conducted by acid chemical (Chandía & Matsuhiro, 2008; Colliec, Boisson-Vidal, & Jozefonvicz, 1994; Shi, 1997), oxidative (Tian, Liu, Hu, & Zhao, 2004), enzymatic (Da Costa et al., 2003; Ilyina, Tikhonov, & Varlamov, 2000; Thanassi & Nakada, 1967) or radiative (Choi & Kim, 2013; Choi, 2009; Yue, Yao, Wei, & Mod, 2008) hydrolysis. However, in the acid chemical and enzymatic methods further purification is necessary due to the residues of additives used to initiate reactions and formation of side products. Besides, some substituent on polysaccharides such as sulfate groups can be eliminated during the acid chemical hydrolysis (Yue et al., 2008). Another method for polysaccharide degradation is the sonolysis by ultrasound (US), which can act as a catalyst which causes sonolysis of H_2O_2 molecule to create easily potent oxidizing free radicals, such as $OH^{\bullet}$ and $HOO^{\bullet}$. These hydroxyl and perhydroxyl radicals initiate an oxidative degradation of complex chain of polysaccharides (Chemat, Teunissen, Chemat, & Bartels, 2001).

Energy beam radiation is one of the most popular tools for modification of polysaccharides. During EB irradiation on polymers, the excited states dissipate some of the excess energy by bond scission of the polymer backbone, resulting in massive formation of free radicals. In the presence of oxygen, these free radicals are converted to peroxyl free radicals for polymer chain session (Choi & Kim, 2013; Kang, Dai, Zhang, & Chen, 2007; Pengzhan, 2004; Riesz, 1991; Wasikiewicz, 2005). From time to time, combination of chemical-radiation methods can also be used for effective decreasing the degree of polymerization. US and EB irradiative degradation can be conducted without initiators and side products which result in chemically pure degradation product (Chemat et al., 2001). Advantages of radiation degradation of polymers include ability to control reproducible and quantitative production without special needs

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to adjust for reaction conditions (Choi & Kim, 2013; Pengzhan, 2004; Wasikiewicz, 2005). The radiation process for polysaccharide degradation also gives a biological sterilization of final products that can be used for manufacturing sanitary biomedical materials.

Hydrogen peroxide is an active oxidant which can easily produce hydroxyl radicals and superoxide anion radicals. These free radicals are strong oxidant and have been widely used in the degradation of organic substances (Kang et al., 2007; Kelen, 1983; Montanari, 2001; Riesz, 1991). Due to its ability of decomposition into water and oxygen, there would no production harmful contaminants on the process (Chemat et al., 2001). But this method is not the only proper one because not only the cleavage of glycosidic linkages of polysaccharides is not really specific, but also a wide diversity of oligomers can be obtained. In this line, it is very important to note that Kang et al. (2007) have reported that a novel synergistic effect is able to enhance the formation of hydroxyl radical by the radiolysis of hydrogen peroxide in degradation of chitosan.

For the case of fucoidans, depolymerization of fucoidan by the combined method of US or EB with hydrogen peroxide and their biological activities have rarely shown despite Choi and Kim (2013) have prepared low molecular weight fucoidans by gamma-irradiation without changing the sulfate groups (Choi & Kim, 2013). The combined method using hydrogen peroxide with energy beam or sonication would give a promising way to get a systematic degradation of polysaccharide. Low molecular weight fucoidan (LMWF) is also of potential interest for revascularization in cardiovascular diseases (Deux et al., 2002; Lake et al., 2006; Luyt et al., 2003). Especially, P-selectin functions as a cell adhesion molecule (CAM) on the surfaces of activated endothelial cells, which line the inner surface of blood vessels, and activated platelets.

The chemical compositions and physical properties of fucoidans were known to be strongly dependent on time and area of collection of the seaweed. Mauritius is located at a tropical area in Indian ocean where the weather is even all year round on this island. It should be interested to study about *Sargassum fulvellum* collected in this area. Evidence from different studies suggested that the antioxidant and anticoagulant activities of polysaccharides were strongly dependent on the molar mass and the degree of sulfation. Nevertheless, there are very few reports in the literature on the antioxidant and the anticoagulant capacity of the fractions of the low molar mass fucoidan extracted from *S. fulvellum*.

In this study, the depolymerization of fucoidan extracted from *S. fulvellum* collected from Mauritius with US or EB in the presence of hydrogen peroxide was conducted with explanation of its mechanism in synergistic effect, and the bioavailabilities such as P-selectin binding assay, cell viability and hemolytic activity of the depolymerized fucoidans were demonstrated on the basis of molecular weight effect.

2. Experimental

2.1. Materials and fucoidan extraction

2.1.1. Chemicals

The lyophilized streptavidin–peroxidase conjugate (Buffer, 0.02 M potassium phosphate, 0.15 M sodium chloride, pH 7.2) and bovine serum albumin (BSA) were purchased from Polysciences Inc. (USA). The peroxidase substrate kit ABTS (2, 29-azino-di-[3-ethylbenzthiazoline-6-sulfonic acid]) was purchased from Bio-Rad (Switzerland). SLe^x tetrasaccharide was obtained from Dextra Laboratories (UK). Recombinant Human p-selectin antibody in Laemmli Buffer (25 mM Tris–HCl, pH 6.8, 50 mM DTT, 1% (w/v) SDS, 0.1% (w/v,) bromophenol blue and 2.5% glycerol) was supplied by bio-World Co. The biotinylated polyacrylamide-type glycol-conjugate containing 20% mol SLe^x was obtained from Lectinity Holding Inc. (Moscow, Russia). Human Umbilical Vein Endothelial Cells (HUVEC)-EBM-2 was purchased from Eppendorf AG (Hamburg, Germany).

2.1.2. Seaweed collections and extraction

Brown sea weed, *S. fulvellum*, sample was collected from the coast of Illot Sancho, Mauritius in May, 2011. Epiphytes and sand were removed before use. The algal thalli collected were thoroughly washed in flowing fresh water, air dried in shade, and coarsely powdered and used for the isolation procedure as follows: one hundred grams dry algae were cut and autoclaved in 5000 ml water at 120 °C for 3 h. The hot solution was separated from algae residues by successive filtration through cheesecloth. The solution was concentrated to about 800 ml under reduced pressure, and then 5 g $CaCl_2$ and 300 ml of ethanol (95%) were added to eliminate alginate. The filtrate was fractionated by precipitation with 0.3 volumes of ice-cold acetone under gentle agitation and maintained at 4 °C for 24 h. The precipitate formed was collected by centrifugation (10,000 × *g*, 20 min), vacuum dried and re-suspended in distilled water. The operation was repeated by adding 0.5, 0.7, 1.2, 1.5 and 2.0 volumes of acetone to the supernatant. The precipitate was filtrated through siliceous earth and the solution was dialyzed against tap water for 48 h. Finally, the fucoidan was precipitated by addition of 4000 ml of 95% (v/v) ethanol. The resultant precipitate was washed three times with anhydrous ethanol, and then dried at 80 °C. The final product showed M_n 70,300, M_w 149,564, dispersity ($Đ = M_w/M_n$) 2.127 and η_{inh} 1.87.

2.2. Degradation of fucoidan

2.2.1. H_2O_2

Three grams fucoidan was completely dissolved in 100 ml 0.3% hydrochloric acid solution, then 8 ml H_2O_2 aqueous solutions (the concentrations were 0.0, 1.5, 3.0, 4.5 M) were added. The solutions were stirred and reacted at the desired temperature for 1hr. The resulting solutions were filtered and the filtrate was adjusted to pH 7.0 with NaOH solution and a precipitate was obtained by adding 95% ethanol. The degraded samples were collected after drying the precipitate in vacuum.

2.2.2. US/H_2O_2

US/H_2O_2 degradation of the fucoidan solution was performed using Misonix Sonicator (Misonix, NY, USA) with reactor volume 250 ml and Lauda RM6 circulator used for constant temperature, 30 °C. Frequency of ultrasound generated was 25 kHz with output power 200 W. A suspension for the degradation was prepared by the addition of 2 g fucoidan to 10 ml H_2O_2 aqueous solution with a given concentration (0.0, 1.5, 3.0 and 4.5 M, respectively). The suspension was deaerated by bubbling with pure nitrogen for 1 h to remove oxygen, followed by irradiation with US. Sample of 10 ml was placed in the middle of the reactor, 1 cm above the surface of the transducer. Ultrasound operation time was ranged from 1 up to 40 min in the air atmosphere.

2.2.3. EB/H_2O_2

An EB generator, ELV-8 (EB-Tech, Daejeon, Korea), was used for EB/H_2O_2 degradation of fucoidan. The applied dose was 2.5–20 kGy which can be generated by 2.5 MeV generators. The conveyer belt speed of ELV-8 was 10 m/min. A suspension for the EB degradation was prepared by the addition of 2 g fucoidan to 10 ml H_2O_2 aqueous solution with a given concentration (0.0, 1.5, 3.0 and 4.5 M, respectively). The suspension was deaerated by bubbling with pure nitrogen for 1 h to remove oxygen, followed by irradiation with EB at the absorbed dose with dose rate of 10 kGy/h in a vigorous stirring to avoid low penetration of the beam. After the irradiation degradation, the solutions were filtered and precipitated with 95%

ethanol followed by washing the precipitate twice with distilled water and drying to remove residual H_2O_2.

2.3. Analytical procedure

Gel permeation chromatography (GPC) of the samples was performed on a Wyatt DAWN EOS GPC-MALLS (Wyatt, USA) instrument equipped with Ultrahydrogel Linear column (Waters) and Guard column (Waters), two detectors on line: an RI detector from OPTILAB DSP and a multi angle laser light scattering (MALLS) detector from DAWN EOS. 0.1 N $NaNO_3$ solution was used as a mobile phase at a flow rate of 0.8 ml/min. The columns temperature was maintained at 40 °C. Injection volumes were 100 μl. The raw data were collected and processed to determine M_w and M_n, using Astra software from Wyatt Technology Corp. In order to determine the intrinsic and specific viscosities, 0.5, 0.25, 0.125, 0.0625 g/dl of depolymerized fucoidan solutions in 0.1 N-$NaNO_3$ at 35 °C were measured.

FT-IR spectra were recorded in Nicolet 6700 (Thermo Scientific, USA), with potassium bromide (KBr) disk. Proton NMR spectra were recorded in Advance 400 MHz (Bruker, Germany) in D_2O solution at room temperature. Sulfur content (S%) of the samples was measured by both ICP-AES (Optima 4300 DV, Perkin Elmer, USA) and Elementary analysis (EA; Flash EA 1112 Series; CE Instruments, UK).

2.4. P-selectin assay

P-selectin (10 mg/ml in PBS) was coated onto microtiter plates overnight at 4 °C. The plates were washed with 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4) buffer containing 150 mM NaCl and 1 mM $CaCl_2$ with 3% BSA, and washed again. The samples or anti-human P-selectin (G1 clone) and biotinylated SLe^x-polymer were diluted in the assay buffer and added to the P-selectin-coated wells or the BSA-coated wells (non-specific control) for incubation overnight at 4 °C. The plates were washed with assay buffer. ABTS peroxidase substrate solution was added and the color reaction was stopped after 10 min with 2% oxalic acid. Bound SLe^x-polymer was determined by measuring the optical density at 405 nm using a microplate reader.

Binding of LMWF to P-selectin was characterized by mass spectrometry and surface plasmon resonance (SPR) at Korea Research Institute of Chemical Technology (KRICT). We have analyzed indirectly the formation of complex between P-selectin and the three LMWF.

2.5. Cell viability test

The cell toxicity of samples was determined with Human Umbilical Vein Endothelial Cells (HUVEC)-EBM-2 (Eppendorf AG, Hamburg, Germany) using MTT assay. In brief, 1×10^6 cells were exposed to concentrations ranged from 25 to 200 μg/ml of test samples dissolved in DPBS medium, for a period of 24 h incubated at 37 °C in the presence of 5% CO_2. Cells without test samples served as control. Subsequently, 10 μl of MTT at a concentration of 5 mg/ml was added to each of the incubated cells followed by further incubation for 30 min at 37 °C in the presence of 5% CO_2. After incubation, cells were collected by centrifugation and then suspended in 200 μl of DMSO solvent. Absorbance was measured in a microplate reader at 540 nm.

2.6. Hemolytic activity

The hemolytic activity of samples was determined by a method in which a suspension of erythrocytes cells (RBC) was mixed with equal volume of serial dilutions of the test samples according to World Health Organization (WHO) guidelines (1998). Blood samples were aseptically collected in heparinized sterile tube from healthy volunteers, after getting their consent for experiment. Blood samples were centrifuged at 3000 rpm for 20 min at 4 °C, to remove the cell debris. The resultant pellet was washed (3–4 times) repeatedly with phosphate buffer saline (PBS) at pH 7.4 to obtain erythrocytes cells (RBC). These cells were suspended in phosphate buffer saline containing test solution at a concentration of 100–950 μg/ml and was incubated at room temperature for 10 min in the dark. At the end of incubation, tubes were centrifuged at 6000 rpm for 20 min at 4 °C, in order to separate the intact cell and debris. Sodium dodecyl sulphate (SDS) was served as positive control for hemolytic activity. The amount of released hemoglobin (Hb) in the supernatant was measured spectrophotometrically at 540 nm.

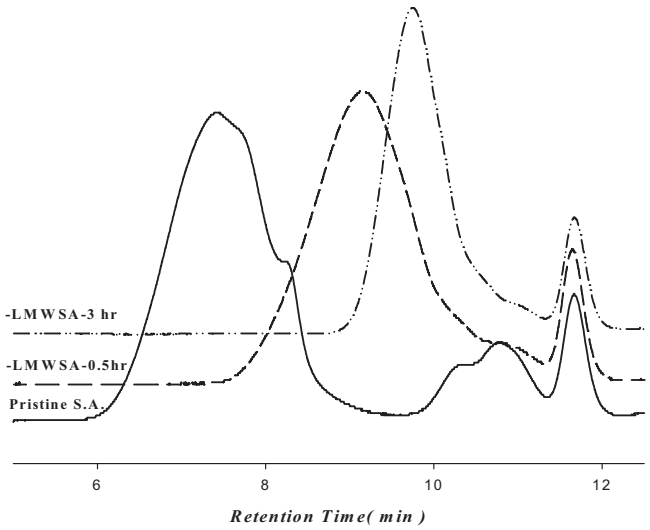

Fig. 1. GPC of degraded fucoidan treated by US/H_2O_2.

2.7. Statistical analysis

All the data shown in biological tests are representative experiments of at least three identical and independent experiments done for each time with $n \geq 6$ samples per conditions. The data were statistically evaluated with SPSS 14.0.1 software. Values were considered significantly different with $p < 0.05$. All the results were expressed as mean ± SE

3. Results and discussion

3.1. Degradation

Degradation of fucoidan by US or EB irradiation in the presence of H_2O_2 were conducted in order to control the rate of degradation and to check the reduction in molecular weight at different reaction conditions. Fig. 1 depicted GPC chromatogram of fucoidans degraded by US irradiation in H_2O_2 which give good separation in size exclusion of the depolymerized samples. Table 1 and Fig. 2

Table 1
Degradation rate of fucoidan by US/H_2O_2.[b]

Deg. rate at t (h)	R_{15}[a]	R_{30}[a]	R_{60}[a]	R_{90}[a]	R_{120}[a]	R_{150}[a]	R_{180}[a]
With ultrasound	76.5	95.9	97.1	98.0	98.8	99.1	99.3
Without ultrasound	55.2	58.9	62.2	65.8	67.9	70.2	72.0

[a] R_t: the degradation rate (R, %/h) between 0 and t in reaction time.
[b] 30 °C, H_2O_2 3 wt%

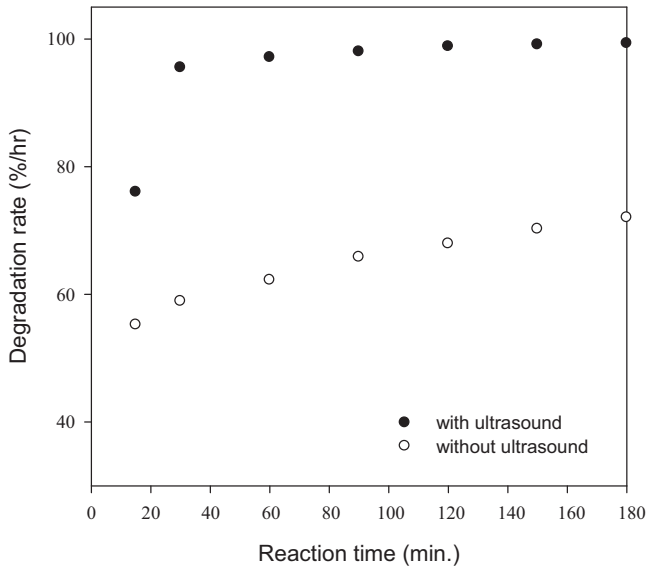

Fig. 2. Degradation rate of fucoidan by US/H_2O_2.

showed degradation rates of fucoidan with or without US in the presence of H_2O_2.

Degradation rate was calculated by Eq. (a) as percentage decrease in molar mass per hour during degradation reaction

$$R_t = (1/t)[1 - (M_t/M_0)] \times 100(\%) \quad \text{(a)}$$

here R_t is degradation rate (%) during degradation reaction at time, t, M_0 is the molar mass of fucoidan at $t = 0$, M_t is the molar mass of the fucoidan degraded after reaction time, t (h).

As shown in Table 1 and Fig. 2, degradation rates by H_2O_2 alone gave 55.2%/h at R_{15}, and slightly increased to 72.0 at R_{180}. On the other hand, degradation rates by US/H_2O_2 gave dramatic increase from 76.5%/h to 99.3%/h under the same condition. For a treatment with H_2O_2 alone, the fucoidan was slowly and weakly depolymerized. On the initiation step of the fucoidan degradation, macroradicals were formed. Subsequent reactions of the macroradicals can be chain scission, hydrogen transfer, inter/intramolecular recombination and disproportionation of macroradicals.

Synergetic degradation uses two or more kinds of degradation techniques together to improve degradation. This combined degradation is more efficient than physical, chemical or biological degradation alone (Weitz-Schmidt, Gong, & Wong, 1999; Yue et al., 2008).

For EB/H_2O_2 degradation, the samples were irradiated with high energy electron beams in the presence of H_2O_2 at a specified dose rate, depending on the extend of degradation of fucoidan. In irradiation processing, dose is defined as the amount of energy absorbed by the target material in unit of gray. The decrease in molar mass of fucoidan in various H_2O_2 concentrations with the irradiation dose is demonstrated in Table 2. A rapid drop of molecular weight was observed at 2.5 kGy, and then the degradation slowed down gradually. This behavior may be attributed to the fact that, the drastic degradation occurs in the initiation step of irradiation, and the slow degradation at higher doses occurred. As shown in Tables 1 and 2, H_2O_2 accelerated considerably the depolymerization rate of fucoidan. The presence of H_2O_2 during irradiation tended to favor chain scission of polysaccharides due to the oxidative action of formed peroxides (Seguchi et al., 1981).

Polymer degradation can be expressed in terms of molecular weight reduction due to polymer chain scission and its efficiency can be estimated by radiation yield of scission G_s (mol/J) (Chemat et al., 2001)

$$G_s = (2c/D \cdot d)[(1/M_w) - (1/M_{w0})] \quad \text{(b)}$$

where G_s is radiation yield of scission (mol/J), D; absorbed dose (Gy), d; solution density (kg/dm^3), c; polymer concentration (g/dm^3), M_w, M_{w0}; weight-average molecular weight of polymer after and before irradiation, respectively. Table 1 shows degradation of fucoidan as an expression of H_2O_2/US degradation. Degradation rate is increasing with decreasing of the reaction time. The most pronounced decrease in molecular weight is in the reaction time range of 15–30 min after US irradiation with H_2O_2, and after that M_w remained almost unchanged value for the oligomer level. In order to evaluate the radiation sensitivity of fucoidan, radiation chemical degradation yield G_s was determined using the Eq. (b). The results are summarized in Table 2. The inherent viscosities of the LMWFs were in the range form 0.78 dl/g for the sample, HP1.5-20, to 1.29 dl/g for the HP3.0-2.5. The G_s values ranged from 1.393×10^{-8} to 3.310×10^{-8} mol/J. The highest G_s value was obtained at the reaction conditions of 1.5% H_2O_2 concentration and 2.5 kGy irradiation.

Table 2
Depolymerization of fucoidan by E-beam (2.5 MeV) with H_2O_2.

Specimens	H_2O_2 (%)	Dose (kGy)	M_w	M_n	$Đ$ (M_w/M_n)	η_{inh}	$G_s \times 10^8$ (mol/J)
Untreated	0	0	149,564	70,300	2.127	1.87	–
HP0-2.5	0	2.5	44,017	22,376	1.967	1.26	2.014
HP0-5.0	0	5.0	33,917	17,261	1.965	1.13	1.543
HP0-10	0	10.0	18,378	9393	1.957	0.93	1.759
HP0-15	0	15.0	12,940	6639	1.949	0.86	1.776
HP0-20	0	20.0	8278	4279	1.935	0.80	2.194
HP1.5-2.5	1.5	2.5	32,363	16,475	1.964	1.11	3.310
HP1.5-5	1.5	5.0	24,594	12,540	1.961	1.01	2.428
HP1.5-10	1.5	10.0	14,494	7426	1.952	0.88	2.336
HP1.5-15	1.5	15.0	9832	5065	1.941	0.82	2.421
HP1.5-20	1.5	20.0	6725	3492	1.926	0.78	2.747
HP3.0-2.5	3.0	2.5	46,348	23,556	1.968	1.29	1.833
HP3.0-5	3.0	5.0	31,586	16,081	1.964	1.10	1.715
HP3.0-10	3.0	10.0	18,378	9393	1.957	0.93	1.759
HP3.0-15	3.0	15.0	13,717	7033	1.950	0.87	1.661
HP3.0-20	3.0	20.0	10,609	5459	1.943	0.83	1.668
HP4.5-2.5	4.5	2.5	46,348	23,556	1.968	1.29	1.833
HP4.5-5	4.5	5.0	36,248	18,442	1.966	1.16	1.393
HP4.5-10	4.5	10.0	14,494	7426	1.952	0.88	2.336
HP4.5-15	4.5	15.0	15,271	7819	1.953	0.89	1.465
HP4.5-20	4.5	20.0	11,386	5852	1.946	0.84	1.541

$\eta_{inh} = \ln [\eta_{rel}/C]$: Inherent viscosities of the solutions were measured at a concentration of 0.5 g/dl in distilled water at 35 °C.

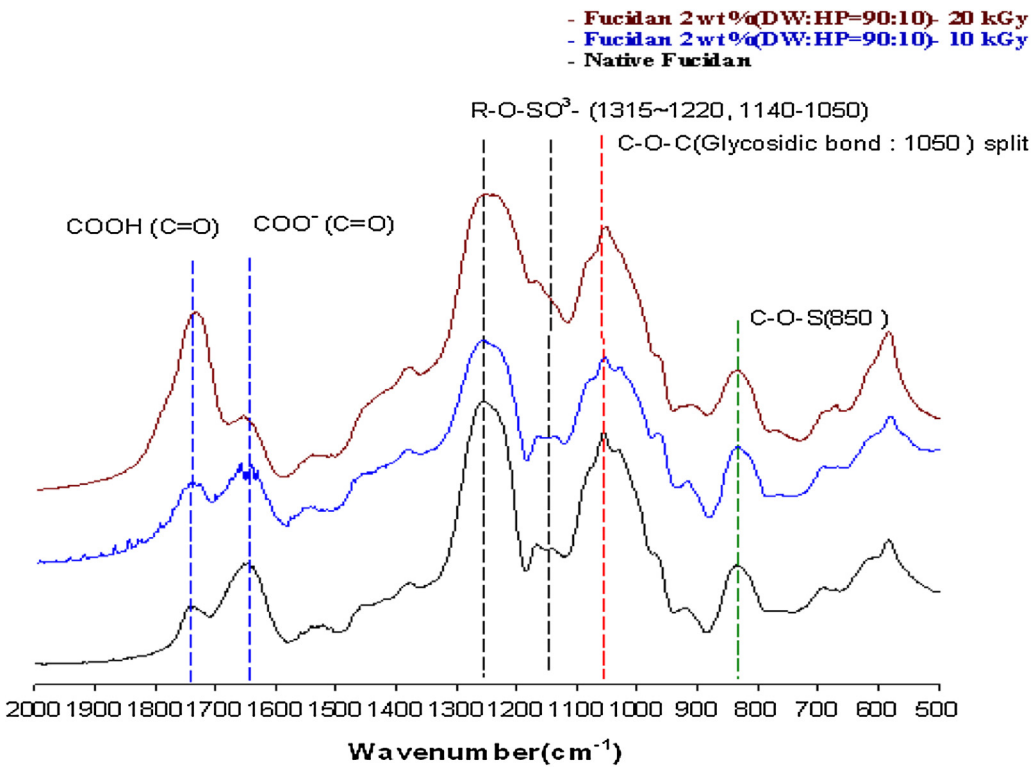

Fig. 3. Infra-red spectra of native and degraded fucoidan.

Irradiation involved the formation of radicals that tended to react more rapidly with oxygen or hydroxyl radical than with molecular chains. Irradiation by EB, which possessed the energy sufficient to cleavage covalent bonds, resulted in formation of free radicals in H_2O_2. Most efficient molecular weight reduction was obtained in 1.5% of H_2O_2 with EB (2.5 MeV) as shown in Table 2. Produced radicals can initiate further reaction such as disproportionation and/or recombination reactions. It can be assumed that the highest ratio of disproportionation/recombination can be formed in the degradation step with EB irradiation in 1.5% H_2O_2 solution. Oligomer formed after irradiation was mainly due to chain depolymerization after irradiative chain scission. Interestingly, most of the dispersity-values of the degraded fucoidans in this study accumulated around $Đ \cong 2$. It can be explained that the cause of ultrasonic or radiative depolymerization was cavitation or shock waves generated during ultrasound wave application. It is known that when a long chain polymer is sonicated or irradiated it preferentially breaks at the middle and long chain radicals result (Wasikiewicz, 2005).

3.2. Chemical structure of degraded fucoidan

The FT-IR spectra of the degraded fucoidan as well as pristine one are shown in Fig. 3. The FT-IR spectra of the degraded fucoidan were similar to that of the initial fucoidan. The untreated and treated samples exhibited characteristic peaks at 1730 cm^{-1} (C=O stretching band of the carbonyl group), 1630 cm^{-1} (COO— antisymmetrical stretching band of the carboxylate group), 1260 cm^{-1} (—S=O stretching band of the sulfate group), 1030 cm^{-1} (C—O—C symmetrical stretching band of glycosidic group), and 830 cm^{-1} (C—O—S stretching band of the sulfate group) (Fig. 3). The IR spectra of the products degraded by EB with H_2O_2 were nearly identical to that of the untreated fucoidan except for the intensity of the peaks, C=O stretching band (1730 cm^{-1}) and COO— antisymmetrical stretching band (1630 cm^{-1}). The peak intensity at 1730 cm^{-1} was increased, that at 1630 cm^{-1} was decreased. There were no changes in the peaks at 1315–1220 cm^{-1} and 1140–1050 cm^{-1} for R—O—SO_3— functional group which meant no removal of sulfate group. Elemental analysis of sulfur was almost the same before and after processing (2.6%). These results support the fact that degradation of fucoidan occurred without any significant side reaction and sulfate group reduction.

Thus the conclusion can be drawn that the whole initial fucoidan's repeating unit still existed in the resulting degraded fucoidan with reduced molecular weight.

Fig. 4 shows the ^{1}H NMR chemical shifts for native and degraded fucoidan. 3.8 ppm (2H) and multiplets at 3.7–3.9 ppm corresponding to the ring methyl protons together with a singlet at 2.0 ppm, which is due to the fucose ring unit protons. In these spectra, the chemical shift of each ^{1}H for treated samples are well corresponded to the chemical shift of native fucoidan, so no significant changes took place in characteristic shifts of depolymerized fucoidan compared with the original fucoidan. In other words, the structure of treated fucoidan remained the same as that of the native fucoidan.

3.3. Antithrombotic activity

P-selectin is a 140-kDa glycoprotein that is typically stored in the secreted granules of platelets and endothelial cells. Secreted P-selectin is thought to play a key role in the adhesion of platelets to monocytes and neutrophils during an inflammatory response (Mitch Revelle, Scott, Kogan, Zheng, & Beck, 1996).

LMWF inhibited the binding of SLe^x to P-selectin (Fig. 5). The binding of SLe^x-polyacrylamide-biotin to immobilized P-selectin was measured in the presence of LMWF in this assay. As shown in Table 3 and Fig. 5, the amount of SLe^x bound to P-selectin decreased with increasing concentrations and decreasing molecular weight of LMWF. An anti-human P-selectin antibody completely blocked the binding of SLe^x to P-selectin. Inhibition of the binding by LMWFs was remarkably pronounced. IC_{50} values of LMWF-36000 (M_w 36,000), LMWF-23000 (M_w 23,000) and LMWF-2000 (M_w 2000)

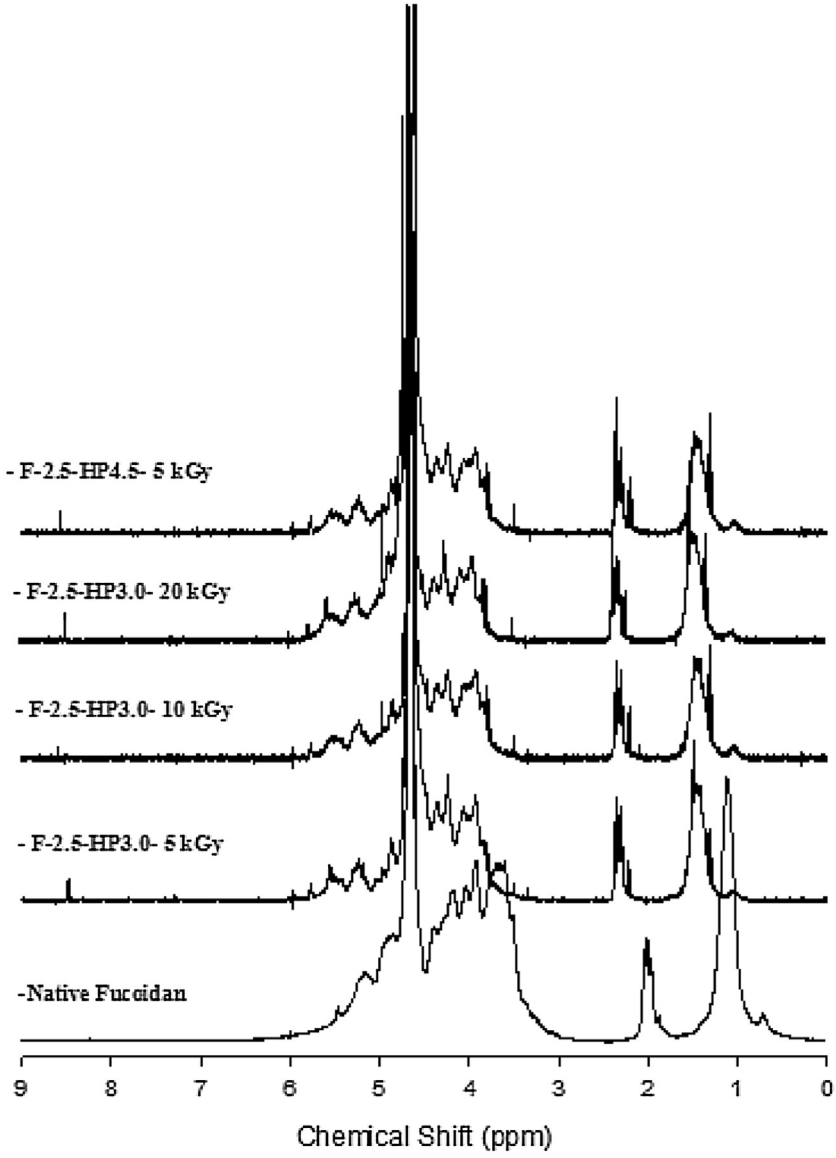

Fig. 4. ^{1}H NMR spectra of the treated or untreated fucoidan by e-beam with H_2O_2.

were 395, 70 and 55 nM, respectively, which were comparable with the values obtained by using fucoidans from *Ascophyllum nodosum* reported by Bachelet et al. (2009). Bachelet et al. reported that IC_{50} value of LMWF with MW 7200 g/mol based on sulfated fucose unit was 20 nM in the same assay. This is assumed that there might be an optimal molecular size of fucoidan to inhibit the p-selectin binding. However, the IC_{50} values we obtained were pretty low compared with heparin and dextran sulfate, with an IC_{50} of 400 nM and >25,000 nM, respectively. It means that LMWFs in this study were much more effective in antithrombotic activity compared with heparin and dextran sulfate.

3.4. Cell viability and haemolytic activity

Cell viability/toxicity of the samples were determined by MTT assay with HUVEC-EBM-2 and tabulated in Table 4. Cytotoxicities of the LMWFs including LMWF-36000, LMWF-23000 and LMWF-2000 were all zero at the concentration of 25 μg/ml, and then

Table 3
Inhibition % of P-selectin binding to Sialyl Lewis X at different fucoidan concentrations.

Specimens	Inhibition % at different concentrations (nM)						IC_{50} (nM)
	10	20	50	100	400	1000	
LMWF-36000	75(6)	65(2)	57(4)	52(4)	51(4)	49(3)	395
LMWF-23000	68(4)	57(3)	50(2)	48(3)	45(3)	45(2)	70
LMWF-2000	54(5)	45(3)	30(4)	32(3)	20(2)	18(2)	55
Heparin							400
Dextran sulfate							25,000

The values in parentheses are standard deviations.

Table 4
Effect of fucoidan on cell viability.

Specimens	Cell viability (%)				Cytotoxicity (%)			
	25 μg/ml	50 μg/ml	100 μg/ml	200 μg/ml	25 μg/ml	50 μg/ml	100 μg/ml	200 μg/ml
LMWF-36000	100	99	96	86	0	1	3	14
LMWF-23000	100	99	96	87	0	1	4	13
LMWF-2000	100	97	95	85	0	3	5	15

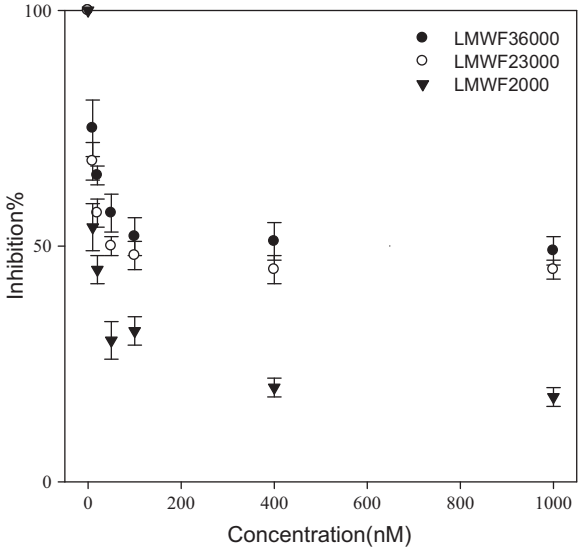

Fig. 5. Inhibition of P-selectin binding to Sialyl Lewis X at different fucoidan concentration.

gradually increased with increasing the concentration. However they were quite weak and low. Thus, it might be justified to state that LMWF is safer than heparin and its constituents in clinical use.

4. Conclusion

Fucoidan extracted from *S. fulvellum* was effectively degraded by US or EB irradiation in the presence of hydrogen peroxide. The existence of synergetic effects of hydrogen peroxide and US or EB on the degradation of fucoidan were found. The combined H_2O_2/US or H_2O_2/EB techniques for potential production of low molar mass fucoidan was based on the formation of a reactive hydroxyl radical due to the dissociation of H_2O_2 in the presence of US or EB. However, the dissociation of H_2O_2 is inefficient when H_2O_2 is used alone. Ultrasonic radiation has been found to be very effective for the dissociation of H_2O_2, which will undoubtedly accelerate the degradation of fucoidan without changing the functional groups such as sulfate in the saccharide unit. The amount of SLe^x bound to P-selectin decreased with increasing concentrations and decreasing molecular weight of LMWF. An anti-human P-selectin antibody completely blocked the binding of SLe^x to P-selectin. Inhibition by LMWFs was remarkably pronounced. The LMWFs showed no haemolytic activity at concentrations up to 950 μg/ml. This study suggested that LMWF could be used in the vascular field as a targeting moiety in biomedical applications.

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