

Structural Effects of Sulfated-Glycoproteins from *Stichopus japonicus* on the Nitric Oxide Secretion Ability of RAW 264.7 Cells

Rong-An Cao^{1,2}, Su-Han Lee³, and SangGuan You²

¹College of Food Science, Heilongjiang Bayi Agricultural University, Daqing 163319, China

²Department of Marine Food Science and Technology, Gangneung-Wonju National University, Gangwon 210-702, Korea

³Department of Food Technology and Service, Eulji University, Gyeonggi 461-713, Korea

ABSTRACT: The effect of various levels of proteins, sulfates, and molecular weight (M_w) of a sulfated-glycoprotein (NF₃) from a sea cucumber, *Stichopus japonicus*, on nitric oxide (NO) releasing capacity from RAW 264.7 cells was investigated. The NF₃ derivatives had various amounts of proteins (4.8~11.2%) and sulfates (6.8~25.2%) as well as different M_w ($640.3 \times 10^3 \sim 109.2 \times 10^3$ g/mol). NF₃ was able to stimulate RAW 264.7 cells to release NO with lower protein contents, indicating that the protein moiety was not an important factor to stimulate macrophages. On the other hand, the NO inducing capacity was significantly reduced with decreased levels of sulfates and M_w , implying that sulfates and M_w played a pivotal role in activating RAW 264.7 cells. It was not clear why sulfates and a certain range of M_w were essential for stimulating macrophages. It appeared that certain levels of sulfates and M_w of sulfated-glycoproteins were required to bind to the surface receptors on RAW 264.7 cells.

Keywords: *Stichopus japonicus*, sulfated-glycoproteins, structure-bioactivity, nitric oxide, RAW 264.7

INTRODUCTION

The sulfated fucans (SF) are water-soluble, complex, and heterogeneous macromolecules, predominantly found in the cell wall of marine brown algae and in the body wall of marine invertebrates (1). They have been reported to exhibit various biological and pharmacological activities, such as anticoagulant, antitumor, and immunomodulating activities (2-4). According to Soeda et al. (5), the SF derivatives with various sulfate contents stimulated tissue plasminogen activator (t-PA)-induced plasma clot lysis and prevented the formation of fibrin polymers, and such activities increased proportionally with the degree of sulfation. It was also reported that the over-sulfated fucans possessed higher macrophage-stimulating activities than native SF, and thus more effectively up-regulated cytokine induction from the macrophage cells (6,7). However, partially desulfated fucans with sulfate contents of less than 20% showed drastic decreases in both anticoagulant and anticancer activities (8). In a study of Nishino et al. (9), the M_w of SF from *Ecklonia kurome* was related to their anti-

coagulant activity. The authors found that SF with M_w ranging from 10×10^3 to 300×10^3 g/mol showed the most potent anticoagulant activities, suggesting that a specific range of sugar-chains and a comfortable conformation were required to bind the thrombin for the potent anticoagulant activity. Therefore, the biological activities of SF appeared to be closely related to their compositional and macromolecular structures. Indeed, a basic understanding of both the primary and secondary structures of SF may lead to successful interpretation of their bioactivities.

Sea cucumbers, known as trepang, beche-de-mer or gamat, are marine invertebrates belonging to Echinodermata, Holothuroidea, and Aspidochirota, and are widely found in the benthic area and deep-seas worldwide (10). They are flexible, elongated, worm-like organisms with a leathery skin and gelatinous body. Sea cucumbers are extensively consumed in China, Japan, Korea and other Asian countries as traditional and nutritional foods and medicinal resources (11). Various components, such as triterpene glycosides, peptides, fatty acids, and SF, are present in the body walls of sea cu-

Received 11 November 2014; Accepted 24 November 2014; Published online 31 December 2014

Correspondence to Su-Han Lee, Tel: +82-31-740-7196, E-mail: shlee@eulji.ac.kr

Copyright © 2014 by The Korean Society of Food Science and Nutrition. All rights Reserved.

© This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

cumbers (10). Among the various constituents, SF are major edible parts of sea cucumbers (12), and have been broadly studied because of their numerous bioactivities including anticoagulant (13), anti-inflammatory (14), antithrombotic (15), antitumor (16), and immunomodulatory (17) activities. Several studies have been performed for the structure analysis of SF from sea cucumbers using various techniques such as surface-enhanced raman scattering, gas chromatography-mass spectrometry (GC-MS), fourier transform infrared spectroscopy (FTIR), and nuclear magnetic resonance (NMR) spectroscopy after some modifications of sulfated polysaccharides (18,19). The reported backbones of SF from sea cucumbers consisted of (1→3)-linked fuco-pyranosides and/or branched (1→3)-linked fuco-pyranosides substituted at C-4 with a fuco-pyranose, and sulfate substitutions occurred at C-2, C-4 or C-2 and C-4 (18,19).

In our previous study, the soluble compounds were extracted from a sea cucumber, *Stichopus japonicus*, followed by the removal of free proteins. Despite the free protein removal, the compounds included large amount of proteins bound to the SF. Actually, the protein-sulfated fucan (PSF) complex is the major ingested compound from sea cucumbers when they are consumed. However, the bio-activity studies of sea cucumbers constituents have been focused on the bio-polymers of either proteins or SF itself and not on the complex form of PSF. Upon the investigation of the PSF from sea cucumbers in our previous studies, the crude and fractionated PSF significantly improved the proliferation of murine macrophage cells (RAW 264.7), and strongly stimulated the RAW 264.7 cells to induce the production of NO and some cytokines such as cyclooxygenase (COX)-2, tumor necrosis factor (TNF)- α , interleukin (IL)-10, and IL-6, suggesting that they were strong immunostimulating agents. Among the crude and fractionated fractions, fraction F₃ having higher SF (76.6%) exhibited relatively strong immunomodulatory activity. Therefore, in the current study, a further investigation regarding a correlation between the molecular structure and the immunomodulatory activity was performed after the sulfate and protein contents as well as the M_w of the F₃ fraction were systematically changed.

MATERIALS AND METHODS

Samples and reagents

The live specimens of sea cucumber *S. japonicus* were collected from the east coast of Korea. The body wall of the sea cucumber was carefully separated from other tissues, washed with tap water, minced, and lyophilized. The dried sample was ground to powder using a grinder, sieved (<0.5 mm), and stored at -20°C until use.

Roswell Park Memorial Institute (RPMI)-1640 medium, penicillin/streptomycin, and fetal bovine serum (FBS) were purchased from Lonza (Walkersville, MD, USA). EZ-Cytox new cell viability assay kit [High sensitive water soluble tetrazolium salt (WST-1)] was purchased from Daeil Lab service Co., Ltd, (Seoul, Korea). Griess reagent (modified) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All of the chemicals and reagents used in this work were of analytical grade.

Isolation and fractionation

The process of extraction and fractionation of the PSF from *S. japonicus* was performed using a previously described method. Briefly, 20 g milled sample of *S. japonicus* was suspended in 200 mL 99% ethanol (EtOH) to remove the pigment at 70°C for 2 h. After the depigmentation, the residue was dissolved in distilled water extracted twice at 80°C for 2 h. The extract solution was concentrated and EtOH was added to precipitate the polysaccharide. The polysaccharide was collected by filtration and washed with EtOH and acetone, then dried at room temperature. The dried polysaccharide was redissolved in distilled water, and the deproteinization was carried out using the method reported by Sevag et al. (20). The crude PSF extract was obtained, which was further fractionated by DEAE Sepharose fast flow column (17-0709-01, GE Healthcare Bio-Science AB, Uppsala, Sweden), which was eluted with distilled water and stepwise NaCl gradient (0.5 to 1.5 M). Four purified fractions were obtained and named as F₁, F₂, F₃, and F₄.

Preparation of fraction F₃ derivatives

The fraction F₃ was modified by deproteinization, desulfation, and hydrolysis to obtain three different levels of protein, sulfate content, and M_w according to the experimental conditions mentioned in Table 1. In the deproteinization experiments, the fraction F₃ (100 mg) was suspended in 100 mL 0.1 M sodium phosphate buffer (pH 7). 5% Flavourzyme (#2384, Novozyme, Tianjin, China) was added to the sample solution, and the reaction was performed at 50°C for 6, 24, and 48 h, respectively. The enzyme was inactivated at 100°C for 10 min and subsequently dialyzed and lyophilized to obtain deproteinized F₃ derivatives. To obtain desulfated derivatives, 100 mg fraction F₃ dissolved in distilled water was applied into a Dowex 50 W resin column (#69011-20-7, Sigma-Aldrich) which was eluted with pyridine. The obtained solution was lyophilized to yield the polysaccharide-pyridinium salt. Then, solvolytic desulfation was carried out under different conditions (Table 1) using dimethyl sulfoxide (DMSO)/methanol (MeOH)/pyridine [89:10:1 (v:v:v)] as the reaction medium. The reaction mixture was dialyzed against distilled water and lyophilized to obtain the desulfated

Table 1. Preparation conditions for deproteinated (DP₁, DP₂, and DP₃), desulfated (DS₁, DS₂, and DS₃), and hydrolyzed (DH₁, DH₂, and DH₃) F₃ from *Stichopus japonicus*

Samples	Temperature (°C)	Reaction time	Reaction medium
Deproteinated F ₃			
DP ₁	50	6 h	5% Flavozymes (phosphate buffer at pH 7.0)
DP ₂	50	24 h	5% Flavozymes (phosphate buffer at pH 7.0)
DP ₃	50	48 h	5% Flavozymes (phosphate buffer at pH 7.0)
Desulfated F ₃			
DS ₁	120	20 min	DMSO/MeOH/Pyridine [89:10:1 (v:v:v)]
DS ₂	120	90 min	DMSO/MeOH/Pyridine [89:10:1 (v:v:v)]
DS ₃	120	120 min	DMSO/MeOH/Pyridine [89:10:1 (v:v:v)]
Hydrolyzed F ₃			
DH ₁	100	2 min	0.01 M HCl (pH 2.3)
DH ₂	100	5 min	0.01 M HCl (pH 2.3)
DH ₃	100	10 min	0.01 M HCl (pH 2.3)

samples with certain sulfate amounts. For hydrolysis, briefly, 100 mg fraction F₃ was dissolved in 30 mL 0.01 M HCl, and the hydrolysis reaction was performed at 100°C for 2, 5, and 10 min, respectively. The samples were neutralized using 0.05 M NaOH after cooling down, dialyzed in a membrane (#3247027, Spectrum Laboratories Inc., Compton, CA, USA) against distilled water and lyophilized to obtain different *M_w* of F₃ derivatives.

Measurement of protein, sulfate content, and molecular weight

Protein content of the samples was estimated by the Lowry method using a DC protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard (Sigma-Aldrich) (21), and the UV chromatogram was recorded by high-performance size-exclusion chromatography (HPSEC) coupled with an UV (HPSEC-UV) system. Determination of sulfate content of the samples was carried out by the bariumchloride-gelatin method using K₂SO₄ as a standard after hydrolysis of the samples with 0.5 M HCl (22), and the FTIR spectra (KBr disc) of the samples were recorded using a Tensor 27 spectrophotometer (Bruker Corporation, Karlsruhe, Germany). For the *M_w* measurement, PSF was dissolved in distilled water (2.5 mg/mL) at 75°C for 15 min, and determined by the HPSEC coupled with an UV, multi-angle laser light-scattering (MALLS) and refractive index (RI) detection (HPSEC-UV-MALLS-RI) system according to our previous method (23).

Assays for macrophage proliferation

For the macrophage proliferation analysis, RAW 264.7 cells (1×10⁶ cells/mL) (100 μL) were under preincubation in a 96-well microplate with RPMI-1640 medium containing 10% FBS, and 100 μL of the samples (10 μg/mL) was added for further incubation at 37°C in a 5% CO₂ incubator. After 72 h, 10% WST-1 reagent (110 μL) was added to the plate after removal of the medium

and incubated at 37°C for another 1.5 h. The 100 μL supernatant was transferred to a 96-well microplate, and the absorbance was measured at 450 nm. The proliferation was expressed using this equation:

$$\text{macrophage proliferation ratio (\%)} = \frac{A_t}{A_c} \times 100$$

where *A_t* and *A_c* are the absorbances of the test group and normal control group, respectively.

Nitric oxide production

To determine NO production, 100 μL RAW 264.7 cells (1×10⁶ cells/mL) were plated in 96-well microplate plates and incubated for 24 h at 37°C in the presence of 5% CO₂. The medium was removed and added 200 μL samples solution (10 μg/mL), medium (as normal control), and 1 μg/mL lipopolysaccharides as a positive control for further incubation 24 h. 100 μL of the cell culture supernatants was mixed with an equal volume of Griess reagent to determine the nitrite concentration according to the method described by Green et al. (24). After incubation at room temperature for 10 min, the absorbance was measured at 540 nm with an EL-800 microplate reader (BioTek instruments Inc., Winooski, VT, USA), and the NO concentration was calculated using sodium nitrite as a standard.

Statistical analyses

All experiments were performed in triplicate (n=3). Data were presented as the mean values with the standard deviation. All statistical analyses were performed using SAS (SAS Institute, Cary, NC, USA). Significant differences were tested by a Student's *t*-test, a one-way analysis of variance (ANOVA), and Duncan's multiple-range test. The critical *P* value was set at 0.01, and a probability value of *P*<0.01 was considered to be statistically significant.

RESULTS AND DISCUSSION

Characterization of fraction F₃ derivatives

In our previous study, PSF was extracted from the body wall of sea cucumber, *S. japonicus*, subsequently fractionated by ion-exchange chromatography, which yielded four fractions (F₁, F₂, F₃, and F₄). Among these fractions, the F₃ fraction mainly consisted of carbohydrates (44.6%) and sulfates (32.0%) with relatively lower protein contents (14.1%) compared to F₁ and F₂, and exhibited strong immunomodulatory activities, producing high level of NO and cytokines from RAW 264.7 cells. Monosaccharide composition analysis showed that carbohydrates of F₃ were mainly composed of fucose (94.8%), and minor amounts of galactose (4.3%), glucose (0.6%), and mannose (0.3%). Methylation and NMR analysis revealed that the structure of F₃ was mainly composed of a backbone of (1→3)- α -L-linked fucosyl residues with sulfation at C-2 and/or C-4.

In order to investigate a correlation between the molecular structure and the immunomodulatory activity, the fraction F₃ was deproteinated, desulfated, and hydrolyzed under the conditions shown in Table 1. The proximate composition of native F₃ (NF₃) and deproteinated F₃ (DP₁₋₃) is presented in Table 2. The enzyme treatment (6 h) led to a marked decrease in the protein con-

tent of F₃ fraction from 14.1% to 11.2%, significantly decreased to 8.1% with the increase in reaction time to 24 h, and again drastically decreased to 4.8% after 48 h treatment. The HPSEC-UV chromatograms of DP₁₋₃ are shown in Fig. 1. Compared with NF₃, DP₁₋₃ exhibited considerably lower UV peak levels, corresponding to their protein contents, indicating that the enzyme treatment effectively removed the proteins from NF₃. Table 2 also shows that the enzyme treatment significantly decreased the M_w of NF₃ from 982.0×10^3 g/mol to 932.5×10^3 , 921.2×10^3 , and 901.8×10^3 g/mol for DP₁, DP₂, and DP₃, respectively, while maintaining similar levels of sulfate contents. It was unclear why the M_w of NF₃ significantly decreased after the flavourzyme treatment because flavourzyme is not a carbohydrate-hydrolyzing enzyme. It is likely that the removal of protein from NF₃ might be related to its decrease in M_w . Wada et al. (25) also reported a mass reduction of a glycoprotein after completely removing its proteins, suggesting that the protein moiety considerably contributed to the M_w of the glycoprotein.

For the desulfated samples (DS₁₋₃), a considerable desulfation (21.2%) occurred after the reaction for 20 min (DS₁). Increased reaction for 90 and 120 min led to further removal of sulfate esters, 48.1% (DS₂) and 78.8% (DS₃), respectively, which was also indicated in the FTIR

Table 2. Chemical composition and M_w of NF₃ from *Stichopus japonicus* and its derivatives

Derivatives	Protein content (%)	Sulfate content (%)	M_w (10^3 g/mol)
NF ₃	14.1±0.1	32.0±1.0	982.0±15.1
DP ₁	11.2±0.1	27.5±0.5	932.5±20.1
DP ₂	8.1±0.1	23.1±0.3	921.2±19.4
DP ₃	4.8±0.1	30.1±0.4	901.8±14.7
DS ₁	13.6±0.2	25.2±0.3	680.3±10.2
DS ₂	12.8±0.1	16.6±0.2	402.2±8.6
DS ₃	13.0±0.2	6.8±0.3	212.5±12.0
DH ₁	13.0±0.3	31.1±0.4	640.3±18.2
DH ₂	13.2±0.2	28.3±0.3	340.4±12.7
DH ₃	12.3±0.4	30.6±0.4	109.2±13.6

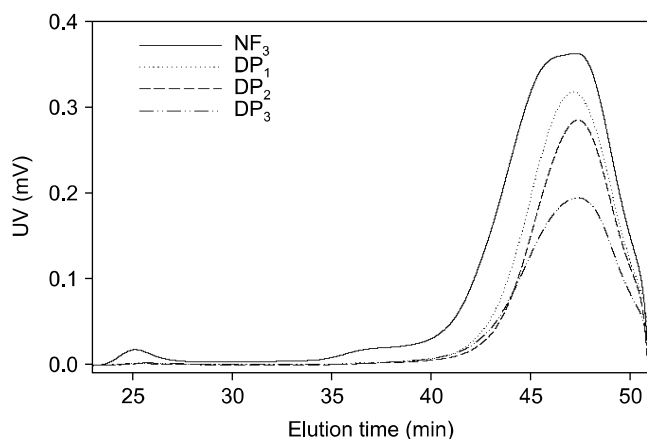


Fig. 1. UV chromatograms of NF₃ and deproteinated F₃, DP₁, DP₂, and DP₃.

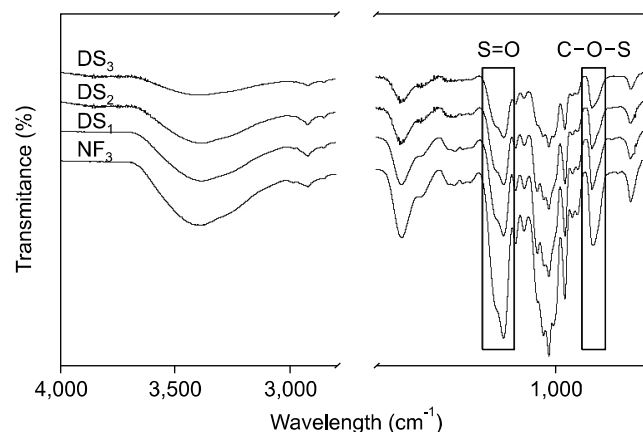


Fig. 2. FTIR spectra of NF₃ and desulfated F₃ at 120°C for 20 min (DS₁), for 90 min (DS₂), and for 120 min (DS₃).

spectra as shown in Fig. 2. Compared to the NF₃, two characteristic absorption bands at 1240 cm⁻¹ and 820 cm⁻¹ in the DS₁, DS₂, and DS₃ spectra successively decreased with increasing desulfation time. These results indicate that the sulfate esters are successfully removed from NF₃ by the reaction. However, the desulfation reaction also led to considerable decrease in the *M_w* of NF₃ from 982.0×10³ g/mol to 680.3×10³, 402.2×10³, and 212.5×10³ g/mol after 20, 90, and 120 min reactions, respectively. The occurrence of molecular degradation seemed to be inevitable due to the heat treatment (120°C) during the solvolytic reaction. It was also reported by Kawashima et al. (7) that the polymeric degradation simultaneously occurred in the desulfation process of the sulfated fucans.

Fig. 3 shows the HPSEC chromatograms of NF₃ and partially hydrolyzed NF₃ (DH₁₋₃). Such hydrolysis times (2, 5, and 10 min) were selected to obtain derivatives having similar *M_w* ranges of DP₁₋₃ and DS₁₋₃ to exclude the *M_w* effect in those samples on NO production from RAW 264.7 cells. As shown in the HPSEC chromatograms, NF₃ was eluted from the SEC columns between

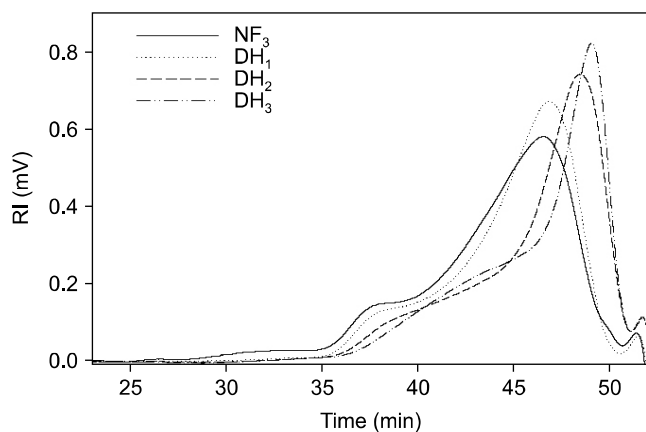


Fig. 3. The HPSEC chromatograms of NF₃ and hydrolyzed F₃ at 100°C for 2 min (DH₁), for 5 min (DH₂), and for 10 min (DH₃).

the elution times of 35~51 min. The acid and heat treatment in boiling water for 2 min slightly altered the elution time and the shape of the elution profile. However, when heated for 5 and 10 min, the elution times of the sample molecules were significantly changed with peak shifts in the chromatograms, implying the occurrence of polymeric degradation. The molecular degradation is also shown in Table 2. The *M_w* of NF₃ calculated from the ASTRA software decreased from 982.0×10³ g/mol to 640.3×10³ g/mol after 2 min of heating in boiling water, markedly decreased to 340.4×10³ g/mol with the increase of heating time to 5 min, and significantly decreased to 109.2×10³ g/mol after 10 min of heating. These results showed that the *M_w* values of DH₁₋₃ were in the similar *M_w* ranges of DP₁₋₃ and DS₁₋₃. Despite the significant decreases in the *M_w*, the hydrolysis appeared to be mild because the protein and sulfate contents of DH₁₋₃ were relatively similar to those of NF₃. The above results reveal that it is possible to obtain NF₃ derivatives having different amounts of proteins and sulfates as well as different *M_w*. Therefore, these modified derivatives enable us to investigate the effects of various proteins, sulfates and *M_w* on the NO releasing capacity from RAW 264.7 cells.

Proliferation and Nitric oxide production from RAW 264.7 cells

Upon stimulation by various external factors, macrophages release some chemokines such as NO, IL-1β, TNF-α, reactive oxygen intermediates, and other substances to remove pathogens and/or cells including cancer cells. Especially, the released NO from macrophages is a highly reactive and an important molecule in the control of the immune system (26). Fig. 4A shows the effect of NF₃ and its derivatives at concentration of 10 μg/mL on the proliferation of macrophage cells, RAW 264.7. The presence of NF₃ and its derivatives considerably improved cell proliferation (*P*<0.01), suggesting

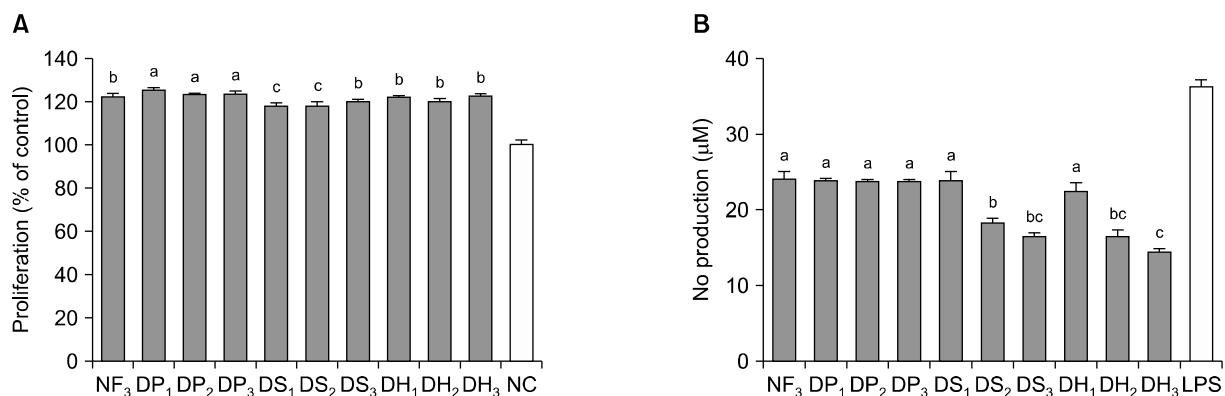


Fig. 4. Macrophage proliferation (A) and NO production (B) of RAW 264.7 cells following treatment with NF₃, deproteinated F₃ (DP₁₋₃), desulfated F₃ (DS₁₋₃), and hydrolyzed F₃ (DH₁₋₃). RAW 264.7 cells (1×10⁵ cells/well) were treated with 10 μg/mL samples or 1 μg/mL LPS (positive control). Values were presented as mean±standard deviation (n=3). The letters (a-c) indicated a significant difference (*P*<0.01) between the native F₃ and different derivatives.

that these samples were not toxic to RAW 264.7 cells at the concentration tested in this study.

The effect of the molecular structures of NF₃ and its derivatives on the immunomodulating activity was monitored on the basis of their NO releasing capacity from RAW 264.7 cells (Fig. 4B). The level of NO produced from RAW 264.7 cells by NF₃ (10 µg/mL) was found to be high (>24 µM/mL), indicating its potent immuno-stimulatory activity. No significant difference on the NO production was found on the DP₁₋₃, indicating that protein moiety in NF₃ was not a major factor on the stimulation of RAW 264.7 cells. However, significantly lower levels of NO production were observed by DS₂, DS₃, DH₂, and DH₃, suggesting that the sulfates and M_w played an important role on the RAW 264.7 cell stimulation.

It was reported by Chen et al. (27) that a sulfated-glycoprotein from *Lycium barbarum* L. stimulated peritoneal macrophages, markedly up-regulating the expression of CD40, CD80, CD86, and MHC class II molecules, in which the protein moiety of the glycoproteins was directly correlated with the macrophage stimulation. The results suggested that the protein moiety was critical for the interaction with the macrophage cells, probably through the regulation of the binding ability of the glycoproteins to the cell receptor. Such trend, however, was not observed in the current study. Instead, in the current study, the sulfates and M_w of NF₃ were found to be directly related to the NO releasing capacity from RAW 264.7 cells. With lower levels of sulfates and M_w, the NF₃ was unable to stimulate the RAW 264.7 cells, suggesting that the sulfates and M_w played a pivotal role in the binding of NF₃ on the receptor of RAW 264.7 cells. According to Jiang et al. (6), the sulfate content in the sulfated-fucans was correlated with the up-regulation of cytokine responses from RAW 264.7 cells, indicating that the sulfates were essential for the stimulation of the macrophages. In addition, other reports demonstrated that the sulfates of the sulfated-fucans played a pivotal role in its anticancer (28), antiviral (29), and anticoagulant (8) activities. Furthermore, the M_w of sulfated-fucans was an important structural element responsible for its antiviral (30), anticoagulant (31), and antitumor activities (32). However, in other sulfated-fucans from *Undaria pinnatifida*, the decrease of M_w was not responsible for the activation of the macrophages and the cytokine production (7). Therefore, it was suggested that the active component of the sulfated-glycoproteins could be varied and be a unique characteristic depending on their origins.

In the current study, the effects of sulfate and protein contents as well as M_w of NF₃ obtained from *S. japonicus* on the immunomodulation were systematically investigated through the NO production from RAW 264.7

cells. NF₃ could not stimulate the RAW 264.7 cells to produce NO with decreased levels of sulfates and M_w, which appeared to be critical to activate the macrophages. However, it was unclear why sulfates and a certain range of M_w were essential for stimulating macrophages. Further investigation on the complex formation of the sulfated-glycoproteins and cell surface receptors is required, because it appeared that certain levels of sulfates and M_w of sulfated-glycoproteins are necessary to bind to the surface receptors on RAW 264.7 cells. Therefore, more research on the binding mechanism between the sulfated-glycoprotein and cell surface receptors would lead to better understanding of the relationship between its molecular structures and bioactivities.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

REFERENCES

- Pomin VH, Pereira MS, Valente AP, Tollefsen DM, Pavão MS, Mourão PA. 2005. Selective cleavage and anticoagulant activity of a sulfated fucan: stereospecific removal of a 2-sulfate ester from the polysaccharide by mild acid hydrolysis, preparation of oligosaccharides, and heparin cofactor II-dependent anticoagulant activity. *Glycobiology* 15: 369-381.
- Mao WJ, Fang F, Li HY, Qi XH, Sun HH, Chen Y, Guo SD. 2008. Heparinoid-active two sulfated polysaccharides isolated from marine green algae *Monostroma nitidum*. *Carbohydr Polym* 74: 834-839.
- Riou D, Collic-Jouault S, Pinczon du Sel D, Bosch S, Siavoshian S, Le Bert V, Tomasoni C, Siquin C, Durand P, Roussakis C. 1996. Antitumor and antiproliferative effects of a fucan extracted from *Ascophyllum nodosum* against a non-small-cell bronchopulmonary carcinoma line. *Anticancer Res* 16: 1213-1218.
- Yoo YC, Kim WJ, Kim SY, Kim SM, Chung MK, Park JW, Suh HH, Lee KB, Park YI. 2007. Immunomodulating activity of a fucoidan isolated from Korean *Undaria pinnatifida* sporophyll. *Algae* 22: 333-338.
- Soeda S, Kozako T, Iwata K, Shimeno H. 2000. Oversulfated fucoidan inhibits the basic fibroblast growth factor-induced tube formation by human umbilical vein endothelial cells: its possible mechanism of action. *Biochim Biophys Acta* 1497: 127-134.
- Jiang Z, Ueno M, Nishiguchi T, Abu R, Isaka S, Okimura T, Yamaguchi K, Oda T. 2013. Importance of sulfate groups for the macrophage-stimulating activities of ascophyllan isolated from the brown alga *Ascophyllum nodosum*. *Carbohydr Res* 380: 124-129.
- Kawashima T, Murakami K, Nishimura I, Nakano T, Obata A. 2012. A sulfated polysaccharide, fucoidan, enhances the immunomodulatory effects of lactic acid bacteria. *Int J Mol Med* 29: 447-453.
- Haroun-Bouhedja F, Ellouali M, Siquin C, Boisson-Vidal C. 2000. Relationship between sulfate groups and biological activities of fucans. *Thromb Res* 100: 453-459.

9. Nishino T, Aizu Y, Nagumo T. 1991. The influence of sulfate content and molecular weight of a fucan sulfate from the brown seaweed *Ecklonia kurome* on its antithrombin activity. *Thromb Res* 64: 723-731.
10. Bordbar S, Anwar F, Saari N. 2011. High-value components and bioactives from sea cucumbers for functional foods—a review. *Mar Drugs* 9: 1761-1805.
11. Yu L, Ge L, Xue C, Chang Y, Zhang C, Xu X, Wang Y. 2014. Structural study of fucoidan from sea cucumber *Acaudina molpadioides*: a fucoidan containing novel tetrafucofucose repeating unit. *Food Chem* 142: 197-200.
12. Mourão PA, Pereira MS, Pavão MS, Mulloy B, Tollefsen DM, Mowinckel MC, Abildgaard U. 1996. Structure and anticoagulant activity of a fucosylated chondroitin sulfate from echinoderm sulfated fucose branches on the polysaccharide account for its high anticoagulant action. *J Biol Chem* 271: 23973-23984.
13. Chen S, Xue C, Yin LA, Tang Q, Yu G, Chai W. 2011. Comparison of structures and anticoagulant activities of fucosylated chondroitin sulfates from different sea cucumbers. *Carbohydr Polym* 83: 688-696.
14. Guérard F, Decourcelle N, Sabourin C, Floch-Laizet C, Le Grel L, Le Floc'H PL, Gourlay F, Le Delezir RL, Jaouen P, Bourseau P. 2010. Recent developments of marine ingredients for food and nutraceutical applications: a review. *J Sci Hal Aquat* 2: 21-27.
15. Liu X, Sun Z, Zhang M, Meng X, Xia X, Yuan W, Xue F, Liu C. 2012. Antioxidant and antihyperlipidemic activities of polysaccharides from sea cucumber *Apostichopus japonicus*. *Carbohydr Polym* 90: 1664-1670.
16. Jin JO, Shastina VV, Shin SW, Xu Q, Park JI, Rasskazov VA, Avilov SA, Fedorov SN, Stonik VA, Kwak JY. 2009. Differential effects of triterpene glycosides, frondoside A and cucumarioside A₂-2 isolated from sea cucumbers on caspase activation and apoptosis of human leukemia cells. *FEBS Lett* 583: 697-702.
17. Zhang Y, Song S, Song D, Liang H, Wang W, Ji A. 2010. Proliferative effects on neural stem/progenitor cells of a sulfated polysaccharide purified from the sea cucumber *Stichopus japonicus*. *J Biosci Bioeng* 109: 67-72.
18. Kariya Y, Mulloy B, Imai K, Tominaga A, Kaneko T, Asari A, Suzuki K, Masuda H, Kyogashima M, Ishii T. 2004. Isolation and partial characterization of fucan sulfates from the body wall of sea cucumber *Stichopus japonicus* and their ability to inhibit osteoclastogenesis. *Carbohydr Res* 339: 1339-1346.
19. Chen S, Hu Y, Ye X, Li G, Yu G, Xue C, Chai W. 2012. Sequence determination and anticoagulant and antithrombotic activities of a novel sulfated fucan isolated from the sea cucumber *Isostichopus badionotus*. *Biochim Biophys Acta* 1820: 989-1000.
20. Sevag MG, Lackman DB, Smolens J. 1938. The isolation of the components of streptococcal nucleoproteins in serologically active form. *J Biol Chem* 124: 425-436.
21. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin-Phenol reagents. *J Biol Chem* 193: 265-275.
22. Dodgson KS, Price RG. 1962. A note on the determination of the ester sulphate content of sulphated polysaccharides. *Biochem J* 84: 106-110.
23. Bao HH, You SG. 2011. Molecular characteristics of water-soluble extracts from *Hypsizygus marmoreus* and their *in vitro* growth inhibition of various cancer cell lines and immunomodulatory function in RAW 264.7 cells. *Biosci Biotechnol Biochem* 75: 891-898.
24. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. 1982. Analysis of nitrate, nitrite, and [¹⁵N]nitrate in biological fluids. *Anal Biochem* 126: 131-136.
25. Wada Y, Gu J, Okamoto N, Inui K. 1994. Diagnosis of carbohydrate-deficient glycoprotein syndrome by matrix-assisted laser desorption time-of-flight mass spectrometry. *Biol Mass Spectrom* 23: 108-109.
26. Jiao L, Li X, Li T, Jiang P, Zhang L, Wu M, Zhang L. 2009. Characterization and anti-tumor activity of alkali-extracted polysaccharide from *Enteromorpha intestinalis*. *Int Immunopharmacol* 9: 324-329.
27. Chen Z, Soo MY, Srinivasan N, Tan BK, Chan SH. 2009. Activation of macrophages by polysaccharide-protein complex from *Lycium barbarum* L. *Phytother Res* 23: 1116-1122.
28. Ji CF, Ji YB, Meng DY. 2013. Sulfated modification and anti-tumor activity of laminarin. *Exp Ther Med* 6: 1259-1264.
29. Adhikari U, Mateu CG, Chattopadhyay K, Pujol CA, Damonte EB, Ray B. 2006. Structure and antiviral activity of sulfated fucans from *Stoechospermum marginatum*. *Phytochemistry* 67: 2474-2482.
30. Witvrouw M, De Clercq E. 1997. Sulfated polysaccharides extracted from sea algae as potential antiviral drugs. *Gen Pharmacol* 29: 497-511.
31. Pereira MS, Mulloy B, Mourão PA. 1999. Structure and anticoagulant activity of sulfated fucans comparison between the regular, repetitive, and linear fucans from echinoderms with the more heterogeneous and branched polymers from brown algae. *J Biol Chem* 274: 7656-7667.
32. Yang C, Chung DH, Shin IS, Lee HY, Kim JC, Lee YJ, You SG. 2008. Effects of molecular weight and hydrolysis conditions on anticancer activity of fucoidans from sporophyll of *Undaria pinnatifida*. *Int J Biol Macromol* 43: 433-437.