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Structural characteristics of polysaccharides extracted from *Cladophora glomerata* Kützinger affecting nitric oxide releasing capacity of RAW 264.7 cells

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ABSTRACT

The effect of molecular structures of sulfated polysaccharide (NF₁) isolated from *Cladophora glomerata* Kützinger on the NO releasing capacity was investigated. It was possible to obtain the NF₁ derivatives having various amounts of proteins (2.55–8.50%) and sulfates (6.88–13.1%) as well as different M_w ($141\text{--}610 \times 10^3$ g/mol), which enabled to investigate the effects of various proteins, sulfates and M_w of NF₁ on the NO releasing capacity from RAW 264.7 cells. The activity was lost after desulfation of the polysaccharides and moreover slightly increased NO releasing capacity after oversulfation of NF₁ polysaccharides with the degree of sulfation (DS, 3.70). Suggesting that the sulfate groups were essential to activate NF₁ polysaccharides are key factors to regulate the immunomodulatory activities.

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1. Introduction

Green algae belonging to the Chlorophyta division are ubiquitously found species both in marine and freshwater. The bright green color of green algae is largely attributed to chlorophyll a and b as well as the accessory pigments of β -carotene and xanthophylls (Yang & Zhang, 2009). Green algae are a rich source of sulfated polysaccharides (SPs), called ulvan, which are soluble, heterogeneous and bioactive anionic macromolecules. The SPs are known to possess a number of biological and pharmacological activities, such as anticoagulation, anticancer, antiviral, anti-hyperlipidemia and immunomodulation (Mao et al., 2008; de Sousa et al., 2007; Zhou et al., 2004). Unique structural features have been reported for the SPs of green algae, which mainly contained rhamnose and uronic acid with major repeating disaccharide units of α -L-Rhap-(1 \rightarrow 4)-D-xylp and (\rightarrow 4)- β -D-GlcpA-(1 \rightarrow 4)- α -L-Rhap (Lahaye & Ray, 1996; Ray, 2006). Their glycosidic linkage patterns are closely related between species of green algae (Leiro, Castro, Arranz, & Lamas, 2007; Liang, Mao, Peng, & Tang, 2014; Ma et al., 2013). Sulfate groups were found at O-2, O-3 or O-4 positions of the SP chains from *Ulva rigida*, *Enteromorpha compressa* and *Gayralia oxysperma* (Cassolato et al., 2008; Lahaye & Ray, 1996;

Ray, 2006; Chattopadhyay et al., 2007), indicating their species-specific dependences.

The biological activity of the SPs from green algae was closely involved with their structural features such as sulfate content and molecular weight (M_w). Qi et al. (2005) reported that oversulfated SPs were more effective superoxide and hydroxyl radical scavenging agents than their native SPs (Qi et al., 2005), however, partially desulfated SPs with sulfate contents of less than 20% showed drastic decreases in anticoagulant and anticancer activities, indicating the pivotal roles of sulfate groups on antioxidation, anticoagulant and anticancer activities (Haroun-Bouhedja, Ellouali, Siquin, & Boisson-Vidal, 2000). The SPs from *U. lactuca* with relatively lower M_w ($< 5 \times 10^3$ g/mol) exhibited a strong inhibition on the proliferation and differentiation of human cancer cells (Kaeffer, Benard, Lahaye, Blottiere, & Cherbut, 1999). On the other hand, the SPs from brown seaweed *Ecklonia kurome* 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 was required for the potent anticoagulant activity (Nishino, Aizu, & Nagumo, 1991). As reported in these studies, the structure and physico-chemical properties are the major factors affecting the bioactivities of the SPs (Tabarsa, Lee, & You, 2012). Therefore, a basic understanding of both the primary and secondary structures for the SPs may lead to the successful interpretation of their bioactivities. To date, numerous studies have been performed on

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the structural effects of the SPs on the bioactivities; however, the researches have been focused on the effect of only one factor either the molecular weight or the sulfate content without consideration of other factors (Tabarsa et al., 2015).

Cladophora glomerata Kützinger is a macro-green algae belonging to the Division Chlorophyta and the genus *Cladophora*, which consists of filamentous branches with multinucleate cells, reticulate chloroplasts and thick cell walls without mucilaginous sheaths (Leliart et al., 2009). It is widely distributed in freshwater of northern Thailand, especially in Nan province, and is known as a nutritious food because of its high levels of sterols, triterpenoids and volatile oils (Kuniyoshi, Yamada, & Higa, 1985). It has been also consumed as a traditional medicine to relieve peptic ulcers, dyspepsia, rheumatoid arthritis and hypertension (Peerapornpisal, Amornletpison, Rujjanawate, Ruangrit, & Kanjanapothi, 2006). Such biological activities might be attributed to various components of *C. glomerata* Kützinger (Laungsuwon and Chulalaksanankul, 2013). Among its various components, the aqueous extracts included large amount of SPs. In our previous study, it was found that the SPs from *C. glomerata* Kützinger were potent immuno-stimulators (Surayot et al., 2015). The activity was mainly associated with F₁ fraction that was obtained by fractionating the crude SPs using an anion exchange chromatography. In the present study, therefore, a further investigation regarding a correlation between the molecular structure and the immuno-stimulatory activity was carried out after the sulfate and protein contents as well as the molecular weights of F₁ fraction were systematically changed.

2. Material and methods

2.1. Materials

C. glomerata Kützinger was collected from Nan River at Tha Wang Pha District, Nan Province, Thailand. The RPMI-1640 medium, fetal bovine serum (FBS), penicillin and streptomycin 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 Daeillab service Co., Ltd, Korea. Griess reagent (modified) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals used in this work were analytical grade.

2.2. Isolation and fractionation of polysaccharide

The extraction and purification of polysaccharides from *C. glomerata* Kützinger were carried out using methods described previously (Surayot et al., 2015). Briefly, to obtain the sulfated polysaccharides, the polysaccharide sample was extracted into distilled water at 98 °C for 1 h. The crude sample was then recovered by the

addition of ethanol (99%). The sample was fractionated using an ion exchange chromatography equipped with a DEAE-Sepharose fast flow column (17-0709-01, GE Healthcare Bio-Science AB, Uppsala, Sweden). The chromatography yielded two fractions (F₁ and F₂). The most immunostimulating fraction (F₁) was chosen for further analyses to examine the structure-bioactivity relationship.

2.3. Preparation of different polysaccharide derivatives

Various polysaccharide derivatives comprised of three different levels of protein moieties, sulfate esters and molecular weight were provide under the experimental conditions summarized in the Table 1. Briefly, F₁ fraction was partially deproteinated in three levels using Flavourzyme (#2384, Novozyme, Tianjin, China) and subsequently dialyzed and lyophilized. To obtain desulfated derivatives, F₁ fraction was primary converted to polysaccharide-pyridinium salts after passing through Dowex 50 W resin column (X-8, H⁺, 1 × 15 cm). Then, solvolytic desulfation under different conditions (Table 1) was implemented to produce polysaccharide bearing certain sulfate amounts (Tabarsa et al., 2012). F₁ fraction, also underwent different mild acid hydrolysis using 0.01 M HCL (Table 1) to reach polysaccharide chain having different molecular weights. Once reaction mixture was cooled, the sample neutralized using 0.05 M NaOH, dialyzed in membrane (#3247027, Spectrum Laboratories, Compton, CA, USA) against distilled water and eventually lyophilized.

2.4. Oversulfation

The purified F₁ fraction was oversulfated according to the method Soeda et al. (Soeda, Sakaguchi, Shimeno, & Nagamatsu, 1992). Briefly, the purified F₁ fraction was further sulfated in a mixture of dimethylformamide and sulfite trioxide-trimethylamine complex and allowed to react at 50 °C for 24 h. After cooling, the reaction mixture was mixed with saturated solution of sodium acetate in ethanol, and poured into cold ethanol. The precipitated formed (highly sulfated polysaccharide) was collected by centrifugation. The obtained sulfated-polysaccharide, classified as OS₁ was re-dissolved in distilled water, dialyzed and lyophilized. The degree of substitution (DS) was calculated from the sulfur content on the basis of Schoniger's formular (Schöniger, 1956).

$$DS = \frac{1.62 \times S\%}{(32 - 1.02 \times S\%)}$$

The FT-IR spectra (KBr disc) of the polysaccharides were recorded by using a Tensor 27 spectrophotometer (Bruker, Germany).

Table 1

Preparation conditions for deproteinated (DP₁, DP₂ and DP₃), desulfated (DS₁, DS₂ and DS₃) and hydrolyzed (DH₁, DH₂ and DH₃) F₁ polysaccharides obtained from *Cladophora* sp.

Sample	Temperature (°C)	Reaction time	Reaction medium
Deproteinated F ₁ polysaccharids			
DP ₁	50	6 h	5% Flavozyms (phosphate buffer at pH) 7.0)
DP ₂	50	12 h	5% Flavozyms (phosphate buffer at pH) 7.0)
DP ₃	50	18 h	5% Flavozyms (phosphate buffer at pH) 7.0)
Desulfated F ₁ polysaccharids			
DS ₁	120	10 min	DMSO/MeOH/Pyridine (89;10;1(v:v:v))
DS ₂	120	30 min	DMSO/MeOH/Pyridine (89;10;1(v:v:v))
DS ₃	120	45 min	DMSO/MeOH/Pyridine (89;10;1(v:v:v))
Hydrolyzed F ₁ polysaccharids			
DH ₁	100	5 min	0.01 M HCL (pH 2.3)
DH ₂	100	20 min	0.01 M HCL (pH 2.3)
DH ₃	100	35 min	0.01 M HCL (pH 2.3)

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