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Hyaluronidase-inhibiting Polysaccharide from *Caulerpa lentillifera*

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Abstract: Algal sulfated polysaccharides are known to be effective hyaluronidase inhibitors. We evaluated hyaluronidase inhibitory activity of sulfated polysaccharide (SP) from *Caulerpa lentillifera.* Results showed that SP with IC_{50} of 163 μg/mL appears to allosterically inhibit the hyaluronidase activity. Main sugar composition and sulfate content of SP was estimated to be Gal, Glc, Xyl, Man, uronic acids, and sulfate in the weight percent of 27.7: 28.9: 14.6: 22.5: 3.4: 21.7. We modified the SP by desulfation and partial hydrolysis with trifluoroacetic acid (TFA) to investigate the effect of sulfate content and molecular weight on inhibition. Hyaluronidase inhibitory activity of desulfated SP, 0.1 M TFA-hydrolyzed SP and 0.5 M TFA-hydrolyzed SP were significantly lower than that of native SP, revealing that sulfate content or molecular weight is important for hyaluronidase inhibition.

Key words: *Caulerpa lentillifera*, algal polysaccharides, hyaluronidase inhibition, sulfated polysaccharide

INTRODUCTION

Among 100 *Caulerpa* spp., *Caulerpa lentillifera* and *C. racemosa* are the two most popular edible green algae in the Asia-Pacific Region. *Caulerpa lentillifera* is one of the main sea products cultivated on a large scale in Okinawa, Japan, where it is known as Umi-budou.[1)2)3)] Polysaccharide is the most abundant compound in *Caulerpa* spp., containing up to 83.2 % of algal dry weight.[3)] Rather than role of basic nutrition, algal polysaccharide have been studied for wide range of physiological and biological activities, so that they play key roles in the fields of pharmaceutical, nutraceutical, cosmeceutical and functional foods.[4)] In recent years, bioactivities of polysaccharides, extracted from *C. lentillifera* have been reported including antioxidant, anticoagulant and anticancer,[5)6)] immune-stimulatory,[2)7)] anti-inflammatory,[8)] anti-tumor, and therapeutic microbial effects.[2)] Hyaluronidase inhibitory activity is another important activity, reported for algal polysaccharides. Furthermore, inhibitory activity of some enzymes such as dipeptidyl peptidase-IV and α-glucosidase were observed in *C. lentillifera* extract previously.[9)] Therefore, in this study, we focused on the inhibitory activity of hyaluronidase with polysaccharide from *C. lentillifera.*

Hyaluronic acid is a principal component of the extracellular matrix in connective tissue, distributed through organs and body fluids in human body.[10)] Since hyaluronic acid is extremely hydrophilic, it plays a vital role in maintaining skin smoothness and moistness, and reducing wrinkles, while involves in many fundamental physiological and pathological processes.[11)] Hyaluronidase, an enzyme for hyaluronic acid hydrolysis involves in development of inflammatory diseases, tumor invasiveness, metastasis, invasive breast adenocarcinoma, metastatic human melanoma, colon carcinoma, and glioblastoma cell lines.[12)] Therefore, hyaluronidase inhibitors are vital to overcome phenomena accompanied by abnormal decomposition of hyaluronic acid such as above mentioned diseases, deregulation of skin homeostasis and wounds. Particularly, hyaluronidase inhibitors likely to become increasingly important as therapeutic agent in pharmaceutical industry and antiaging agents in the cosmetic industry. Despite reporting several types of hyaluronidase inhibitors, up to date, algal acidic polysaccharides have found to be a promising potential inhibitor, for example, sulfated polysaccharide from *Cladosiphon okamuranus,*[13)] *Lessonia nigrescens*, *Laminaria angustata,*[14)] *Undaria pinnatifida*,[12)] *Padina pavonica*,[15)] *Fucus vesiculosus*,[16)] *Monostroma nitidum,*[17)] and *Porphy-*

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Abbreviations: AIR, alcohol insoluble residue; CPC, cetylpyridinium chloride; CPC-P, cetylpyridinium chloride precipitation; CPC-S, cetylpyridinium chloride supernatant; SP, sulfated polysaccharide from *C. lentillifera*; DSP, desulfated SP; HWE, hot water extraction; $SP_{0.1}$, partially hydrolyzed SP by 0.1 M TFA; $SP_{0.5}$, partially hydrolyzed SP by 0.5 M TFA.

ridium purpureum.[18] However, little is known about hyaluronidase inhibiting polysaccharide from *C. lentillifera,* which has already been reported to contain sulfated polysaccharide.[2)7)8)]

Although many studies measure the hyaluronidase inhibitory activity from algal sulfated polysaccharide, the mechanism and influencing factors were not widely understood. Li *et al*. suggested that sulfate content and molecular weight of the bioactive polysaccharide significantly affect their biological activities.[19] Sulfated polysaccharide from green algae *Monostroma nitidum* and brown algae *Cladosiphon okamuranus* explained a strong positive relationship between sulfate content and hyaluronidase inhibitory activity.[13)17)] Despite lack of previous data about the effect of molecular weight of algal sulfated polysaccharide on hyaluronidase inhibitory activity, some compound such as outer layer of green coffee bean and phlorotannin from Fucales showed a positive relationship between hyaluronidase inhibitory activity and molecular weight.[20)21)] In contrast, Asada *et al*. demonstrated that in despite of hyaluronidase inhibitory activity of alginate from *Lessonia nigrescens* increased with increasing molecular weight up to some extent, the highest molecular weight compound (388 kDa) had decreased inhibitory activity.[14] Therefore, it is clear that sulfate content and molecular weight can affect diversely on hyaluronidase inhibitory activity. So far, the effect of both sulfate content and molecular weight of sulfated polysaccharides on hyaluronidase inhibitory activity were not investigated together. Here, we investigated the hyaluronidase inhibitory activity of sulfated polysaccharides from *C. lentillifera* and evaluate the impact of highly influential factors: sulfate contents and molecular weight of the polysaccharides on its activity.

MATERIALS AND METHODS

Algae materials. *Caulerpa lentillifera* purchased from the local market of Ishigaki island, Okinawa, in 2007, was used in this study. Alcohol insoluble residue (AIR) from *C. lentillifera* was obtained in the same manner as previously reported and kept frozen at − 30 ˚C until start of the experiment.[22] Briefly, fresh seaweed was washed with tap water, lyophilized and powdered. The algal powder was sequentially treated with 80 % ethanol, chloroform/methanol (1:1, v/v) and acetone. The residue was collected as AIR after filtration through filter paper.

Extraction and purification of polysaccharides from C. lentillifera. Polysaccharides were extracted as described by Konishi *et al.*[22] In brief, AIR was stirred in water, and heated at 80 ˚C for 1 h. Supernatants from two consecutive extractions were pooled after centrifugation and named hot water extraction (HWE). Purification of acidic polysaccharides from HWE was based on cetylpyridinium chloride (CPC) precipitation as described by Tako *et al.*[23] In brief, HWE was kept at 37 ˚C for 16 h after adding 2 % of CPC solution and collected CPC supernatant and CPC precipitation by centrifugation. The CPC supernatant was precipitated by the addition of 2 volume of ethanol and the CPC precipitation was re-precipitated with a three-fold volume of ethanol after dissolving in 4 M $CaCl_2$. Then, both ethanol precipitations were stirred overnight with distilled water and freeze dried after dialysis. The final resultants from CPC supernatant and CPC precipitation were named CPC-S and CPC-P respectively.

Analysis of chemical composition. Total sugar and uronic acid (UA) contents were determined by the phenol–sulfuric acid method[24] using glucose (Glc) as the standard, and *m*-hydroxybiphenyl method[25] using galacturonic acid as the standard, respectively. To estimate sulfate content and sugar composition, polysaccharides were hydrolyzed in 2 M trifluoroacetic acid (TFA) at 121 ˚C for 1 h. The hydrolysate was dried to remove TFA and dissolved in distilled water. The hydrolysate was subjected to high-performance liquid chromatography with an AS4A-SC column (4 mm × 250 mm, Dionex Co., Tokyo, Japan) to measure sulfate content. The column was eluted at 1 mL/min at room temperature with buffer containing 1.7 mM $NaHCO_3$ and 1.8 mM Na_2CO_3.[22] The weight of sulfate in the sample was calculated from a calibration curve using Na_2SO_4 as a standard based on the molecular weight of HSO_3. The content of sulfate was calculated using the following equation.

$$\text{Sulfate}(\%)=\frac{Weight\ of\ Sulfate}{Dry\ weight\ of\ the\ sample}\times 100\,\%$$

Monosaccharides in the hydrolysate were analyzed by high-performance anion-exchange chromatography coupled with a pulsed amperometric detector (HPAEC-PAD) using Carbo Pac PA1 column (4 mm × 250 mm, Dionex, ICS-5000). The eluent flow was 1 mL/min at room temperature with buffer containing 0.5 M NaOH and 0.5 M CH_3COONa/0.1 M NaOH.[22]

Hyaluronidase inhibitory activity. Hyaluronidase inhibitory activity of the extracted polysaccharides (HWE, CPC-S and CPC-P) was estimated following procedures outlined in previous reports with a slight modification.[12)14)] Hyaluronic acid sodium salt from rooster comb was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and molecular weight was estimated to be 2.38×10^6 by size-exclusion chromatography by using a column of TSKgel G5000 PWXL (7.8 mm × 300 mm, Tosoh Co., Kyoto, Japan) as the method described in this study (Data not shown). Hyaluronidase type I-S from bovine testis (451 U/mg) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and a enzyme unit (U) is defined as the amount of enzyme that liberates one micromole of *N*-acetylglucosamine from hyaluronic acid per minute at 37 ˚C and pH 4.0.[26] First, the reaction mixture containing 100 µL of hyaluronidase (5 mg/1.5 mL) in 50 mM sodium acetate buffer (pH 4.5) and 200 µL of polysaccharide sample was incubated at 37 ˚C for 15 min. Then, 700 µL of hyaluronic acid (1 mg/mL) as a substrate, containing 100 µL 1.5 M NaCl and 18 µL of 1 M sodium acetate buffer (pH 4.0) was added and further incubated at 37 ˚C for 15 min. The reaction was stopped by boiling at 100 ˚C for 10 min. Quantitative analysis of *N*-acetyl-amino sugar (product) was determined by modified Morgan–Elson method.[27] Percentage inhibition was calculated as follows:

$$\text{Inhibition}(\%)=100\times\{1-(S-B)/(C-B)\},$$

where B is the absorbance at 585 nm in the absence of an enzyme, C is the absorbance at 585 nm in the absence of an inhibitor, and S is the absorbance at 585 nm in the presence

of an inhibitor.[12)]

In order to evaluate the dose-dependent manner of inhibitor, inhibition rate was measured at different concentrations of CPC-P (0−339 μg/mL), and also of fucoidan (0−358 μg/mL) extracted from Okinawa mozuku (*Cladosiphon okamuranus*) as described by Tako *et al.*[23)] to compare the mechanism of inhibition with known hyaluronidase inhibitory polysaccharide. Furthermore, the mechanism of action of hyaluronidase inhibition was characterized using two different strategies – desulfation and partial hydrolysis – to examine the effect of sulfate content and molecular weight.

Chemical desulfation of CPC-P was performed using the method of Shiroma *et al.*[28)] Sample (100 mg) was dissolved in distilled water (20 mL) and passed through DOWEX 50W × 8 (H^+, 100 mesh) resin purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). After neutralization with pyridine, the solution was lyophilized. The lyophilized pyridinium salt was dissolved in DMSO:MeOH (9:1; v/v, 20 mL). The mixture was heated at 80 °C for 4 h and then desulfated product was dialyzed against distilled water and lyophilized to obtain desulfated CPC-P (DSP).

Determination of molecular weight. Extracted CPC-P was partially hydrolyzed using 0.1 M or 0.5 M TFA at 70 °C for 3 h to obtain low molecular weight CPC-P. After removing TFA by drying, the hydrolysates were dissolved in distilled water. Partially hydrolyzed CPC-P in 0.1 M and 0.5 M TFA were named $SP_{0.1}$ and $SP_{0.5}$, respectively. Molecular weights of CPC-P, DSP, $SP_{0.1}$ and $SP_{0.5}$ were determined by size-exclusion chromatography (LC-6A; Shimadzu Co., Kyoto, Japan) equipped with a column of TSKgel G5000 PWXL (7.8 mm × 300 mm, Tosoh Co., Kyoto, Japan) and a refractive index detector RID-10A at 35 °C.[28)] The column was eluted by water at a flow rate of 1 mL/min. Pullulan P-10 (molecular weight = 0.96×10^4), P-50 (4.71×10^4), P-200 (20.0×10^4) and P-800 (70.8×10^4) (Showa Denko Co., Tokyo, Japan) were used as the molecular weight standards.

RESULTS

Preparation and characterization of C. lentillifera polysaccharides.

The yield and chemical composition of polysaccharides extracted from *C. lentillifera* are shown in Table 1. Polysaccharides were initially extracted from 5 g of AIR of *C. lentillifera* by hot water treatment and HWE consisted of mainly galactose (Gal), Glc, xylose (Xyl), mannose (Man) together with UA, and a small amount of fucose (Fuc), rhamnose (Rha), arabinose (Ara) residues. After purifying the polysaccharide with CPC, yields of CPC-S and CPC-P were 275 mg and 463 mg, respectively. Sugar composition analysis showed that CPC-S contained 91.4 % Glc, but UA, Gal, Xyl, and Man represented less than 5 %. The CPC-P contained 21.7 % sulfate and 53 % total sugar, including mainly Gal, Glc, Xyl, and Man. The molar ratio of sugar in CPC-P was estimated to be Gal, Glc, Xyl, and Man in the ratio of 0.2: 0.2: 0.12: 0.16 (Table 2). These data indicate that CPC-P is SP.

Hyaluronidase inhibitory activity of polysaccharides extracted from C. lentillifera.

The hyaluronidase inhibitory activities of HWE, CPC-S and CPC-P (SP) were 32.3, 24.7 and 90.4 %, respectively, at a polysaccharide concentration of 339 μg/mL (Fig. 1). It was clear that there was a low percentage of inhibition (0−2 %) at low SP concentration (0−40 μg/mL) but higher SP concentration (200−339 μg/mL) required to inhibit reaction at a higher rate (70−92 %) (Fig. 2a). Thus, the inhibition curve was distinctly sigmoid in shape and IC_{50} of SP was 163 μg/mL. We compared the mode of hyaluronidase inhibition of SP with known hyaluronidase inhibitors such as fucoidan and it showed a hyperbolic curve as shown in Fig. 2b.

Effect of sulfate content of SP on hyaluronidase inhibitory activity.

We prepared DSP to investigate the effects of sulfate content in the polysaccharide on hyaluronidase inhibition.

Table 1. Yield and chemical composition of polysaccharides from *Caulerpa lentillifera.*

Sample	Yield (mg)	Total sugar in mg (%)	Monosaccharide (wt %)								SO_3^- (%)
			Fuc	Rha	Ara	Gal	Glc	Xyl	Man	UA	
HWE	1942	753 (39%)	0.3	0.2	0.3	21.6	39.8	20.4	14.1	3.3	11.7
CPC-S	275	247 (90%)	0.1	n.d.	n.d.	2.5	91.4	0.7	0.7	4.6	6.6
CPC-P	463	244 (53%)	0.6	0.3	0.8	27.7	28.9	14.6	22.5	3.4	21.7

n.d.: less than 0.1. (in 5 g AIR)

Table 2. Comparison of the hyaluronidase inhibition, composition and molecular weight of sulfated polysaccharides extracted from different algae types.

Algae	IC_{50} (μg/mL)	Molar ratio of monosaccharide									SO_3^- (%)	Molecular weight	References
		Fuc	Ara	Gal	Glc	Xyl	Man	Rha	Rib	UA			
Caulerpa lentillifera	163.3	n.d.	0.02	0.20	0.20	0.12	0.16	n.d.	−	0.03	21.7	169×10^4	This study
Undaria pinnatifida	13.0	0.14	−	0.14	−	−	−	−	−	n.d	0.71*	6×10^4	Katsube *et al.,* 2003[12)]
Cladosiphon okamuranus	25.6	0.75	−	−	−	0.04	−	−	−	0.21	13.2	−	Tako and Minami, 2008[13)]
Fucus vesiculosus	2.9	0.49	0.02	0.03	0.08	0.04	−	−	−	0.34	27.0	735 kDa	Pozharitskaya *et al.*, 2020[16)]
Porphyridium purpureum	210.0	−	−	0.31	0.14	0.43	−	−	0.03	0.09	4.5	500 kDa	Mase *et al*., 2013[18)]
Monostroma nitidum	145.0	−	−	n.d.	0.08	0.02	−	0.75	−	0.15	20.0	70×10^4	Yamamoto *et al.*, 2016[35)]

*Molar ratio, n.d.: less than 0.01, Rib: Ribose.

After desulfation, sulfate content of DSP decreased to 1.6 %. Results highlighted that desulfation of SP decreased to −0.9 % of inhibitory activity, which showed no inhibition of hyaluronidase (Fig. 3). Profiles of the molecular weight distribution of SP and DSP (Fig. 4a, d) indicated approximately similar molecular weight distribution with a retention time at 4.5−5.5 min, suggesting that the desulfation reaction didn't affect any depolymerization or unfavorable chemical changes in the polysaccharide molecules. The peak molecular weight of SP and DSP were 169×10^4 and 155×10^4 respectively.

Effect of molecular weight of SP on hyaluronidase inhibitory activity.

In order to examine the effect of molecular weight on the inhibitory activity, we partially hydrolyzed the SP using 0.1 or 0.5 M TFA to obtain a SP of lower molecular weight than native SP. The native SP showed molecular weight of about 169×10^4 with one main peak using size-exclusion chromatography (Fig. 4a). Approximately, 30 % of $SP_{0.1}$ was composed of polymers of low molecular weight range of 42.2 to 0.4×10^4 (Fig. 4b) and 70 % of $SP_{0.5}$ contained low molecular weight polymers of range 23.7 to 0.4×10^4 (Fig. 4c). Increasing the concentration of TFA in hydrolysis clearly increased production of low molecular weight compounds, and inhibitory activities of low molecular weight $SP_{0.1}$ and $SP_{0.5}$ were 7.7 and 2.9 %, respectively (Fig. 3).

DISCUSSION

The water-soluble polysaccharides derived from *Caulerpa* spp. mainly contain glucans and sulfated polysaccharide such as sulfated xyloarabinogalactans,[29] and xylogalactomannans.[30] Among these, previous studies revealed that sulfated polysaccharides were found to be a highly bioactive compound. This time we found hyaluronidase inhibitory activity with sulfated polysaccharide from *C. lentillifera* in terms of the effect of sulfate content and molecular weight. Our result confirmed that purified sulfated polysaccharide show significantly high activity of 90.4 % in 339 µg/mL of SP compared to other polysaccharide fractions. Chaiklahan *et al*. suggested that sulfated polysaccharide from *Caulerpa* spp. generally contained approximately 8−23 % sulfate.[1] Presence of 21.7 % of sulfate in CPC-P confirmed that it was a sulfated polysaccharide, mainly composed of Gal, Glc, Xyl, Man, and a minor amount of UA (Table 3). In past years, several sulfated polysaccharides were extracted and purified from *Caulerpa* spp. as listed in Table 3. Comparison of monosaccharide composition with preceding studies found that Gal, Glc, Xyl and Man are generally present in sulfated polysaccharide in *Caulerpa* spp.[2)30)31)32] In addition to Gal, Glc, Xyl, and Man a considerable amount of Ara contains in sulfated heteroglycan extracted from *C. filiformis*,[28] and *C. racemosa*.[32] Although sulfated polysaccharide derived from *C. lentillifera* consisted mainly of Gal, Glc,

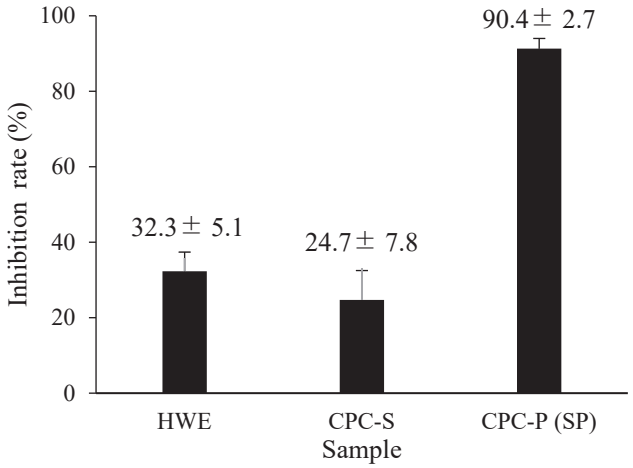

Fig. 1. Hyaluronidase inhibitory activity of polysaccharides extracted and purified from *C. lentillifera* at a polysaccharide concentration of 339 µg/mL.
Data are shown as mean ± SD.

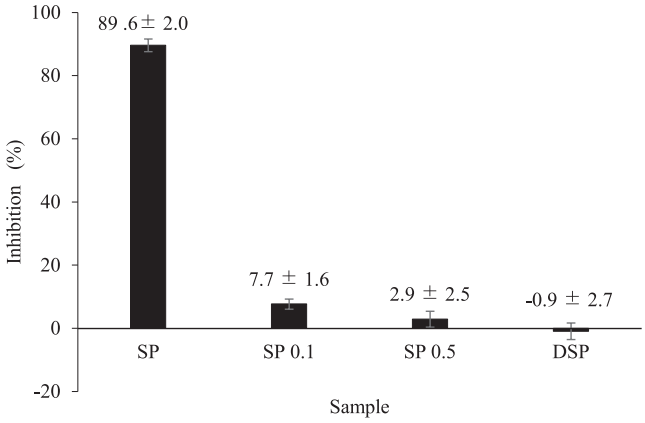

Fig. 3. Hyaluronidase inhibitory activity of SP, $SP_{0.1}$, $SP_{0.5}$, and DSP at a polysaccharide concentration of 339 µg/mL.
Data are shown as mean ± SD.

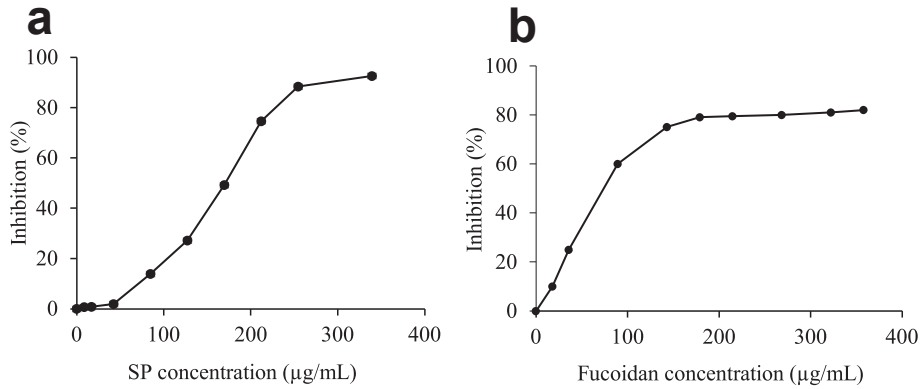

Fig. 2. Hyaluronidase inhibitory activity of (a) SP and (b) fucoidan.

Xyl, and Man, their composition and molecular weight may differ due to different source material, habitat, environmental parameters, and processes of extraction and purification as shown in Table 3.[33]

It was reported that IC_{50} value of hyaluronidase inhibitors derived from sulfated polysaccharide of seaweeds were ranging from 2.9 to 210 µg/mL as shown in Table 2. We also examined the substantial hyaluronidase inhibition of SP with IC_{50} of 163 µg/mL. Yamamoto *et al.* revealed that 100 µg/mL of natural sulfated polysaccharides such as porphyrin, rhamnan sulfate, κ-carrageenan, fucoidan and λ-carrageenan showed 25, 40, 35, 70, and 80 % of hyaluronidase inhibitory activity, respectively[17], while our study showed 20 % of activity at the same SP concentration (Fig. 2a). Furthermore, mode of inhibition of SP was predicted to be allosteric due to the sigmoid shape in dose-dependent curve, but it was hyperbolic shape in fucoidan. Different shapes of the hyaluronidase inhibitory curves such as sigmoid and hyperbolic were also recorded in phlorotannin from brown seaweeds *Cystoseira nodicaulis* and *Fucus spiralis* respectively.[21] Furthermore, sigmoid dose-dependent curve was reported for pectic acid and further clarified its hyaluronidase inhibitory mechanism as non-competitive.[34] Moreover, mode of hyaluronidase inhibition of SP derived from *Monostroma nitidum*[17] was found to be competitive but inhibition was of mixed uncompetitive–noncompetitive type in *Undaria pinnatifida.*[12] Taken all together, it became possible to state that different shapes of the dose-dependent inhibition curve might be due to various inhibition patterns of the inhibitors. However, further enzyme kinetic analysis is required to elucidate the exact mechanism of SP in hyaluronidase inhibition.

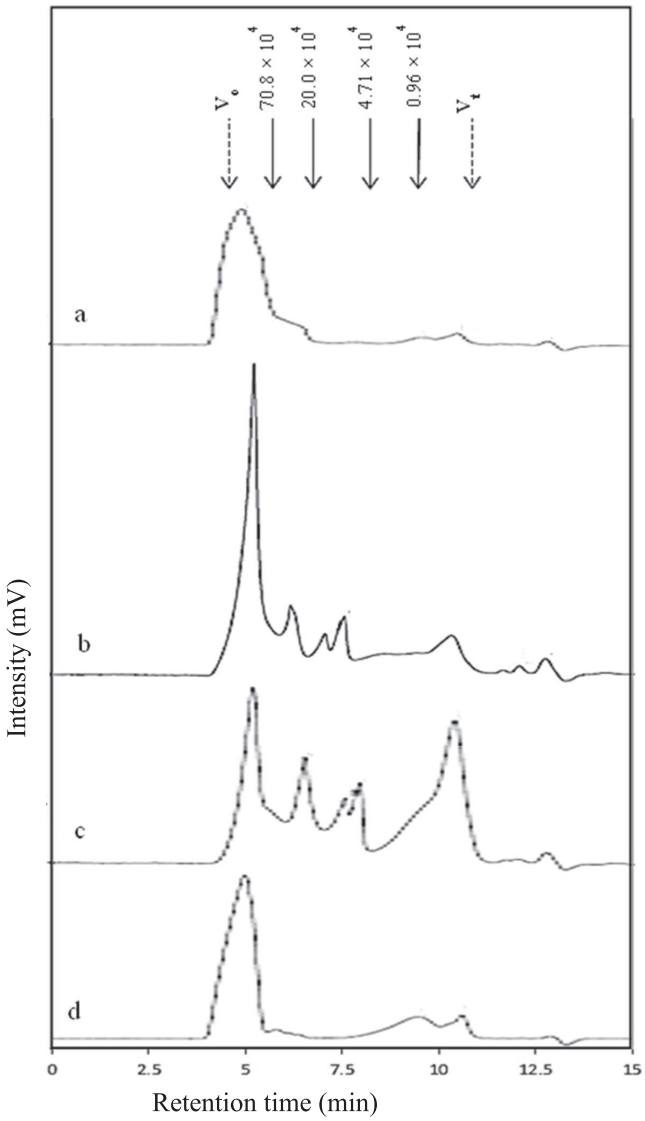

Fig. 4. Size-exclusion chromatography of (a) native SP, (b) $SP_{0.1}$, (c) $SP_{0.5}$ and (d) DSP.

Solid arrows indicate the elution positions of size standards of pullulan with molecular weight of highest peak. Dashed arrows indicate the void volume (V_o) and total column volume (V_t).

Regarding influencing factors of hyaluronidase activity, Li *et al*. suggested that sulfate content and molecular weight of the polysaccharide significantly affect their biological activities.[19] Therefore, we modified SP by desulfation to determine the effect of sulfate content and used TFA hydrolysis to assess the impact of molecular weight on the inhibition. It was found that existence and spatial positioning of sulfate groups on polysaccharides play key roles in their biological activity profile. Sun *et al* found that sulfate groups can substituted at C-3 positions of xylose and mannose, and C-6 positions of galactose of xylogalactomannan from *C. lentillifera*.[30] In our study, inhibitory activity of native SP was significantly decreased −0.9 % after desulfation as shown in Fig. 3, suggested that the sulfate group in the inhibitor remarkably influenced hyaluronidase inhibitory activity. The present finding agreed with the finding of Yamamoto *et al*. of a strong positive relationship between sulfate content and hyaluronidase inhibitory activity in rhamnan sulfate from *Monostroma nitidum* with different sulfate contents (0.5, 7, 20, and 41 %) in different polysaccharide concentrations (0.1, 0.12, 0.16, 0.18, and 0.2 mg/mL).[17][35] Moreover, Toida *et al.* found that chemically over-sulfated glycosaminoglycans (chondroitin sulfate, dermatan sulfate, heparan sulfate, heparin and hyaluronan) showed stronger hyaluronidase inhibitory activity (IC_{50}: 1.35, 1.33, 0.78, 1.28, and 1.14 µg/mL, respectively) than

Table 3. Comparison of the chemical composition and molecular weight of sulfated polysaccharides extracted from *Caulerpa* spp.

Algae	Habitat	Purification method	Monosaccharide (wt%)						SO_3^- (%)	Molecular weight	References
			Gal	Glc	Xyl	Man	Ara	UA			
C. lentillifera	Ishigaki, Okinawa	CPC precipitation	27.7	28.9	14.6	22.5	0.8	3.4	21.7	1.69×10^6	This study
C. lentillifera	Onna village, Okinawa	Superdex column	44.2*	2.2*	49.3*	–	–	4.3*	21.5	>100 kDa	Maeda *et al.*, 2012[2]
C. lentillifera	Changai island, China	DEAE column	43.2	–	–	38.7	–	2.3	21.3	3878 kDa	Sun *et al.*, 2018[30]
C. filliformis	Cape town, South Africa	Remove glucan by α-amylase	5†	n.d	2†	2†	1†	–	17.6	–	Macki and Percival, 1960[31]
C. rasemosa	Gujarat, India	DEAE column	43.0	2.0	27.0	n.d	28.0	–	12.0	80 kDa	Chattopadhyay *et al.*, 2007[32]

n.d.: less than 0.1, *Molar %, †Molar ratio.

the original glycosaminoglycans.[36] Collectively, the present results provide support for the well-known fact that hyaluronidase inhibitory activity greatly depends on presence of the sulfate group.

Several reports have demonstrated a correlation between the molecular weight of inhibitor and hyaluronidase inhibitory activity.[37][38][39] Although significantly stronger inhibitory activity was reported by high molecular weight native SP in our study, it was significantly low in $SP_{0.1}$ and $SP_{0.5}$ (Fig. 3), suggesting that decreasing of molecular weight may affect reduction of inhibitory activity. This reduction may also be due to the partial removal of the sulfate groups from the polysaccharide during hydrolysis. However, previous studies evident that occurrence of desulfation is negligible during the partial hydrolysis of sulfated polysaccharide with diluted TFA (0.1−0.75 M) at slightly elevated temperatures (60−100 ˚C) for several hours (1−2 h).[40][41][42] Therefore, decreasing of molecular weight seems to cause reduction of inhibitory activity in our study. Similar results were reported by Asada *et al*. who demonstrated that hyaluronidase inhibitory activity of alginate from *Lessonia nigrescens* increased with increasing of the inhibitor molecular weight within the range of 150−370 kDa.[14] In contrast, Asada *et al.* found that polysaccharides with the highest molecular weight (388 kDa), generally had decreased inhibitory activity.[14] Furusawa *et al*. also discovered that higher molecular weight acidic polysaccharide from outer layer of green coffee bean contributed most to the hyaluronidase inhibition.[20] All these results suggest that long chains of polysaccharide with high molecular weight are required for hyaluronidase inhibitory activity.

Nevertheless, the sulfate content of SP appeared to contribute more than molecular weight did to the inhibition, because DSP showed a significantly lower inhibitory activity despite having a high molecular weight similar to SP (Fig. 4a, d). Consistently, Furusawa *et al*. revealed that UA content of an acidic polysaccharide contributed more to the hyaluronidase inhibitory activity than its molecular weight.[20] The present results indicate that hyaluronidase inhibitory activity of acidic polysaccharides mainly depended on action of the acidic group in the inhibitor compared to other factors. Beside sulfate content and molecular weight, hyaluronidase inhibitory activity and the mechanisms of action of sulfated polysaccharide are influenced by a number of factors, including type of polysaccharide backbones and glycosidic linkages present in the polysaccharide.[39] This hypothesis could be further confirmed by comparison of inhibitory activity with sugar composition, sulfate content and molecular weight of SP and other sulfated polysaccharides reported in many studies (Table 2). Based on the result and references, it can be suggested that hyaluronidase inhibitory activity might be affected by more than single feature of the inhibitor molecule.

CONCLUSIONS

In the present study, SP, mainly consisted of Gal, Glc, Xyl, and Man found to effectively inhibit hyaluronidase activity. This activity correlated with sulfate content and molecular weight of SP. However, molecular weight alone was not likely sufficient, and the sulfate group was essential to inhibit hyaluronidase activity. It can be suggested that hyaluronidase inhibitory activity by SP might be affected by more than single feature of the inhibitor molecule.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest

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