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ORIGINAL ARTICLE

Chemistry and Biochemistry

The composition and anti-inflammatory effect of polysaccharides from the red alga *Chondrus verrucosus*

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Abstract
Polysaccharides prepared from the red alga *Chondrus verrucosus* (Rhodophyta, Gigartinales) were subjected to anion-exchange column chromatography, and the chemical characteristics of the fractions (designated CV1, CV2 and CV3 in the order of elution) were examined based on carbohydrate and sulfate contents and monosaccharide composition. Furthermore, the anti-inflammatory effect of the fractions on the degranulation in RBL-2H3 cells stimulated by A23187 was investigated. The results showed that the major monosaccharide component was galactose. The CV1 and CV2 fractions showed higher anti-inflammatory activity against RBL-2H3 cells than the CV3 fraction. The difference in activity may be related to the sulfate contents, namely, the contents of CV1, CV2 and CV3 fractions were 25.3 ± 3.3%, 28.1 ± 1.1%, and 7.4 ± 1.8%, respectively.

Keywords Red alga · Polysaccharide · *Chondrus verrucosus* · Anti-inflammatory activity

Introduction

Allergy is an overreaction of the immune system as a self-defense strategy (Kay 2000), causing unfavorable symptoms such as dermatitis, rhinitis and rheumatoid arthritis, etc. Regarding the mechanism involved, hyaluronidase inhibitors and mast cell degranulation have been referred to. The former might serve as anti-allergic or anti-inflammation agents, and thus contribute to the maintenance of hyaluronic acid homeostasis by suppressing the decomposition of hyaluronic acid (Furusawa et al. 2011). Regarding the latter, hyaluronidase-inhibitory activity of polysaccharides from snailfish *Liparis tessellatus* eggs and marine alga *Porphyridium purpureum* have been reported to suppress the degranulation of rat basophilic leukemia RBL-2H3 cells through calcium ionophore (A23187)-stimulated cells (Ticar et al. 2017). Various mediators, such as β-hexosaminidase and histamine, are subsequently released from the mast cells. It is thus a suitable marker for the determination of the granules formed in the mast cells (Guo et al. 2009).

Many kinds of seaweeds are consumed in Japan, Korea, China and parts of Europe. Not only the nutrients but also bioactive components in seaweeds have been found to be beneficial for human health (Rajapakse and Kim 2011). Polysaccharides, especially, have recently been attracting attention as medical materials which are involved in apoptosis induction, antioxidant and anti-allergic activities, and as having an antitumor effect, and various biological activities of polysaccharides in seaweeds have been found (Ye et al. 2008). Because of these health promoting effects, seaweed polysaccharides have attracted more and more attention as food additives (Cui et al. 2018). The polysaccharides exhibit a variety of chemical and biological functions, and many of these functions strongly depend on the structures, such as the presence of sulfuric acid groups. They are thus beneficial for developing new drugs (Zhang et al. 2003). However, information on the anti-inflammatory activity of seaweed-derived sulfated polysaccharides is still fragmentary (Wijesekara et al. 2011).

The red alga *Chondrus verrucosus* is distributed along the central Pacific coast of Japan (Bellgrove and Aoki 2008). The distribution of life history phases of *C. crispus* from the same genus has been reported in detail (Garbary et al. 2011). Those from the genus *Chondrus* can be regarded as

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economically important species for the materials for carrageenan, but *C. verrucosus* per se has not been utilized so far. Shirota et al. (2008) purified chitinase from this species and found its strong activity. However, to the best of our knowledge, there has been no information available about the polysaccharides from *C. verrucosus.* Therefore, the aim of this study was to estimate whether the polysaccharides from this species have the potential for prevention and treatment of allergies based on their chemical characteristics and β-hexosaminidase inhibitory assay on degranulated RBL-2H3 cell activity.

Material and methods

Materials

The specimens of *C. verrucosus* were collected on the coast of Kesennuma City, Miyagi Pref., Japan, in May 2016. After rinsing with seawater, the whole algal body was freeze dried, powdered in a mortar with a pestle, and stored at − 30 °C until used.

Cell Counting Kit-8 (PubChem CID: 9833444) was purchased from Dojindo Laboratories (Kumamoto, Japan). Rat basophilic leukemia cell-derived line (RBL-2H3) was purchased from RIKEN Cell Bank (Tsukuba Science City, Ibaraki, Japan). Eagle's minimal essential medium (EMEM), RPMI 1640 medium (Roswell Park Memorial Institute Media, Hampshire, United Kingdom), fetal bovine serum (FBS, Lot No. S00000S1820, Biowest, Nuaille, France), penicillin (PubChem CID: 6869), and streptomycin (PubChem CID: 19649) were purchased from GE Healthcare Life Sciences (Buckinghamshire, England). Calcium ionophore (A23187) (PubChem CID: 24277964) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA). All the other reagents were of analytical grade.

Extraction of polysaccharides

The dried powder of the algal body (6.3 g) was soaked in 250 ml of methanol/chloroform (1:1, v/v) for 3 days at 30 °C in the dark in order to remove pigments and lipophilic substances, and subsequently washed with distilled water. The residue (3.0 g) was extracted 3 times with 9 ml of 0.17 M HCl (final pH 2) at 65 − 70 °C for 1 h. The extracts were pooled, neutralized with 2 M NaOH, and evaporated to dryness in vacuo. The dried material was dissolved in 250 ml of distilled water, and then the polysaccharides were precipitated with four volumes of absolute ethanol. The precipitate including polysaccharides was washed with absolute ethanol and freeze dried according to Anno et al. (1966).

Separation of polysaccharides by ion-exchange column chromatography

The crude polysaccharide fraction (2.0 g) was dissolved in 20 ml of distilled water, and was loaded to a Toyopearl DEAE-650 column (Cl^- form, 3Φ × 21 cm, Tosoh, Tokyo, Japan) equilibrated with 0.04 M HCl. The column was washed with 80 ml of the same solution, and was subjected to elution with a linear gradient of 0–2.5 M NaCl with a total volume of 216 ml. Aliquots of 1 ml of 5% phenol solution (w/v) and 5 ml of 18 M sulfuric acid were added to 1.0 ml of each fraction to develop a color reaction according to Dubois et al. (1956). The absorbance at 490 nm was used to obtain the elution pattern. The obtained fractions of polysaccharides were intensively dialyzed against distilled water and then freeze dried according to Sulkowska-Ziaja et al. (2011).

Chemical analysis

Total sugar content of the polysaccharides was determined by the phenol–sulfuric acid method (Dubois et al. 1956). The sulfate content was determined by using a $BaCl_2$-gelatin turbidimetry method using sulfuric acid as a standard (Saito et al. 1990). Briefly, an equal volume of 8 M trifluoroacetic acid was added to 1 ml of the polysaccharide solution (1 mg/ml) and brought to hydrolysis at 100 °C for 3 h, followed by evaporation to dryness. After dissolving the obtained solid in 1 ml of distilled water, 0.2 ml of this aqueous solution was taken into a test tube, and 3.8 ml of 4% trichloroacetic acid (w/v) and 1 ml each of $BaCl_2$-gelatin aqueous solution (0.5% $BaCl_2$ and 0.5% gelatin, w/v) were added. The mixture was allowed to stand at room temperature for 20 min, and the absorbance at 360 nm was measured.

The monosaccharide composition was analyzed by gas chromatography (GC), which provides high resolution comparable to high performance liquid chromatography, according to Milo et al. (2002). Galactose, xylose and glucose were used as standards. Briefly, 1 mg of polysaccharide was dissolved in 1 ml of 0.5 M HCl in methanol, followed by complete hydrolysis at 100 °C for 16 h. An aliquot (500 µl) of *N*-trimethylsilylimidazole in anhydrous pyridine reagent (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) was added to 500 ml of 10% monosaccharide solution, and the mixture was shaken for 30 s, followed by incubation at 60 °C. An aliquot (1 µl) of the mixture (trimethylsilyl derivatives) was subjected to GC analysis. The conditions of GC were as follows: the column, DB17 (Agilent, Creek Blvd, Santa Clara, CA, USA) (0.25 mm ID × 30 m, 0.25 µm film), column temperature in the range of 150–250 °C (at elevating rate of 2 °C/min), injection at 230 °C, carrier gas (N_2) with a flow rate of 1 ml/min, and FID detection.

Anti-hyaluronidase activity assay

The assay was performed using a modified Morgan–Elson method (Muckenschnabel et al. 1998). Half maximal inhibitory concentration (IC_{50}) was defined as the amount of polysaccharide that inhibited 50% of hyaluronidase activity. IC_{50} was expressed as the mean of three measurements from each of the five different concentrations for all the fractions, including 1-hexadecanepyridinium chloride, which is generally used as an anti-allergic medicine, as a positive control (PC). All the reactions were performed in triplicate.

Cell culture

RBL-2H3 cells were cultured in EMEM supplemented with 10% FBS, 100 U/ml of penicillin and 100 µg/ml of streptomycin under an atmosphere of 5% CO_2 at 37 °C.

Cell viability analysis

Cell proliferation was determined using a Cell Counting Kit-8, according to the manufacturer's instructions (Hou et al. 2007). In brief, cells were seeded in 96-well plates and cultured for 12 h. After incubation with various concentrations of the polysaccharide fractions for 24 h, 10 µl of CCK-8 dyes was added to each well, and the cells were incubated at 37 °C for 4 h. Then the absorbance was measured at 450 nm. The assay was carried out in triplicate.

β-Hexosaminidase inhibitory activity on RBL-2H3 cells

The inhibitory activity was measured by A23187-stimulated assay (Awane et al. 2016). RBL-2H3 cells suspended in EMEM containing 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 10% FBS, were seeded onto a 96-well culture plate at 4.0×10^4 cells per well, and cultured at 37 °C for 24 h under humidified 5% CO_2. RBL-2H3 cells were inoculated and treated with various concentrations of each fraction. Compound 48/80 was used as PC. After rinsing the cells with modified Tyrode's (MT) buffer (20 mM HEPES, 13 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, and 0.05% BSA, pH 7.4), they were treated with 80 µl of MT buffer containing various concentrations of the fractions. An aliquot (20 µl) of 3 µM A23187 diluted in MT buffer was added to each well, and the cells were incubated at 37 °C for 30 min. The supernatant was subsequently collected from each well, and the cells were sonicated in 100 µl of lysis buffer (Tris–EDTA buffer consisting of 50 mM Tris and 20 mM EDTA, pH 8.0) for 5 s on ice. Both supernatant and cell lysate (50 µl of each per well) were transferred into a new 96-well microplate and incubated for 5 min at 37 °C. An aliquot (50 µl) of 2 mM 4-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside (Wako Pure Chemical Industries, Osaka, Japan) dissolved in 0.4 M citrate buffer (pH 4.5) was then added to each well and incubated at 37 °C for 25 min. The enzyme reaction was terminated by adding 100 µl of 0.2 mM borate buffer (pH 9.8), and the absorbance was measured at 405 nm using a Multiskan FC microplate reader (Thermo Scientific, Vantaa, Finland). The release rate (%) of β-hexosaminidase was calculated as follows:

$$\text{Release rate } (\%) = \frac{A_{\text{supernatant}} - A_{\text{blank of supernatant}}}{(A_{\text{supernatant}} - A_{\text{blank of supernatant}}) + A_{\text{lysate}} - A_{\text{blank of lysate}}} \times 100,$$

where "*A*" is the absorbance at 405 nm.

Statistical analysis

The data were shown as the mean ± SD (standard deviation) of three to five determinations. Statistical analyses were performed using ANOVA (one way of analysis variance) and Tukey–Kramer. The values of a significance level lower than 0.05 were considered to be statistically significant.

Results

Separation of polysaccharides

The crude extract of polysaccharides from *C. verrucosus* was applied to a Toyopearl DEAE-650 anion-exchange column chromatography using a linear gradient NaCl (0–2.5 M). The three major fractions containing polysaccharides (designated CV1, CV2 and CV3 in the order of elution from the column) were obtained at the salt concentrations of around 0.69 M, 0.96 M, and 1.69 M NaCl, respectively. These fractions were subjected to further analysis for anti-inflammatory activity (Fig. 1). The three fractions were separately pooled, extensively dialyzed against distilled water for 3 days at 4 °C, and then lyophilized. The yields (dry weight/total polysaccharide dry weight) of CV1, CV2 and CV3 were 39.2%, 29.8% and 10.4%, respectively (Table 1).

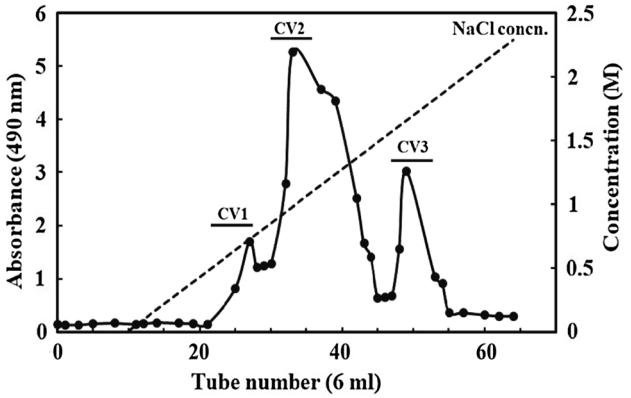

Fig. 1 Elution pattern of polysaccharides from a Toyopearl DEAE-650 column. The salt concentration is given from the start of the elution from the column. The three major fractions (designated CV1, CV2, and CV3 in the order of elution) are indicated by the *horizontal lines*

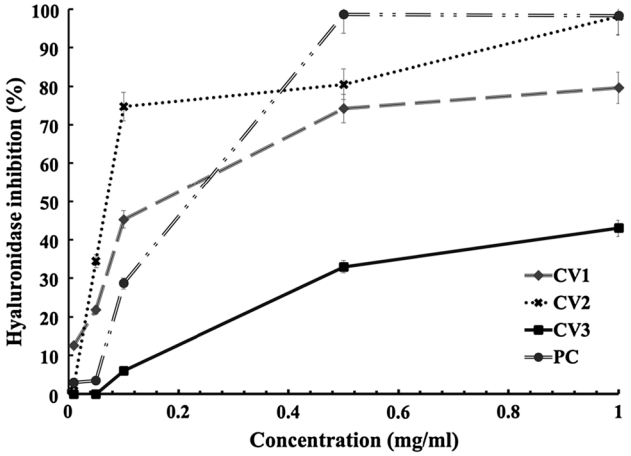

Fig. 2 Anti-hyaluronidase activity of the polysaccharide fractions. 1-Hexadecanepyridinium chloride was used for the positive control (PC). Each value represents the mean ± SD of three independent experiments

Chemical profiles of the polysaccharide fractions

Carbohydrate contents were found to be 65.9 ± 3.4%, 60.2 ± 3.4% and 46.2 ± 4.4% for CV1, CV2 and CV3, respectively (Table 1). When the contents of monosaccharides, namely, galactose, xylose and glucose in the obtained polysaccharide fractions were analyzed, galactose was found to be the major monosaccharide in the sulfated polysaccharide fractions, namely, 86.5%, 78.3% and 45.2% for CV1, CV2 and CV3, respectively. The other monosaccharides were also found in smaller amounts, namely, 2.1%, 5.8% and 7.2% of glucose for CV1, CV2 and CV3, respectively, and 3.2%, 5.5% and 9.4% of xylose, respectively. On the other hand, the sulfate contents were 25.3 ± 3.3, 28.1 ± 1.1, and 7.4 ± 1.8 for CV1, CV2 and CV3, respectively (Table 1).

Hyaluronidase inhibitory activity

The effects of the three fractions on the hyaluronidase activity are shown in Fig. 2. The results showed that they inhibited the activity in a dose-dependent manner, and the inhibitory effect of CV2 with higher sulfate content was the strongest among the three. The IC_{50} values of CV1 and CV2 fractions were 0.11 mg/ml and 0.07 mg/ml, respectively.

Effect on degranulation of RBL-2H3 cells

The polysaccharide fractions showed no measurable cytotoxic effect on RBL-2H3 cells over the concentration range of 100–400 μg/ml (data not shown), though A23187 was used to sensitize the cells. Then, the release of β-hexosaminidase in RBL-2H3 cells was measured. The effects of the polysaccharide fractions on the degranulation of β-hexosaminidase from rat RBL-2H3 basophils are shown in Fig. 3. The *C. verrucosus* polysaccharide fractions inhibited the degranulation of RBL-2H3 cells stimulated by A23187. Under the culture conditions, however, neither polysaccharide fractions nor a compound 48/80 (used as PC) gave rise to any statistically significant release of β-hexosaminidase from the cell. When the enzymatic activity was plotted against the sulfate concentration (Fig. 4), the activity seemed to tend to increase dependent on the sulfate content.

Table 1 The chemical and monosaccharide compositions of polysaccharide fractions by ion exchange chromatography

Fraction	Yield (%)[a]	Carbohydrate content (%)[b]	Sulfate content (%)[b]	Monosaccharide composition (%)		
				Gal	Glc	Xyl
CV1	39.2	65.9 ± 3.4	25.3 ± 3.3	86.5	2.1	3.2
CV2	29.8	60.2 ± 3.4	28.1 ± 1.1	78.3	5.8	5.5
CV3	10.4	46.2 ± 4.4	7.4 ± 1.8	45.2	7.2	9.4

[a]% of total crude polysaccharides weight

[b]% of sample dry weight

Gal galactose, *Glc* glucose, *Xyl* xylose

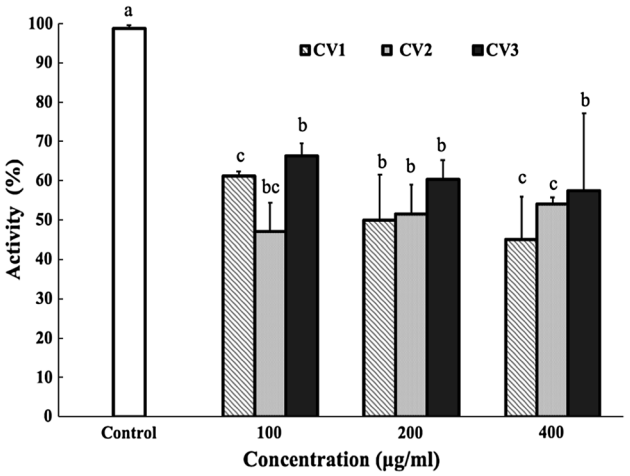

Fig. 3 Effects of *C. verrucosus* polysaccharides on A23187-stimulated degranulation of RBL-2H3 cells. Cells were individually pretreated with different concentrations (100, 200 and 400 μg/ml) of the polysaccharide fractions for 4 h, and then stimulated with A23187 for 30 min, followed by β-hexosaminidase release assays. *Different letters* indicate significant differences ($p < 0.05$) by Tukey–Kramer test. Each datum represents the mean ± SD of five independent experiments

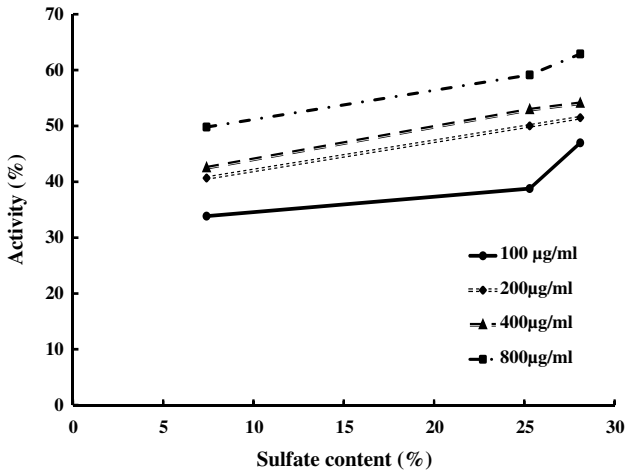

Fig. 4 Relation between β-hexosaminidase activity and sulfate concentration based on the polysaccharide fractions. The data for the fractions CV1, CV2 and CV3 as obtained in Fig. 1 and Table 1 were used

Discussion

A number of synthetic and natural products have been utilized to solve various problems related with human health. Plants, which provide a wide structural diversity of natural compounds have been used for the development of many medicines (Manigandan et al. 2015). Seaweeds also can give rise to a wide range of functional molecules which are useful for biomedical applications. Therefore, the seaweeds have attracted the interest of researchers to explore novel lead compounds for the pharmaceutical industry (Palanisamy et al. 2018).

Sulfate contents in the fractions obtained by ion-exchange chromatography (Fig. 1) were in the range from 7.4 to 28.1% (Table 1). These values are higher compared with that (3.75%) of the red alga *Gracilaria corticata* whose sulfated polysaccharide showed high antibacterial activity against human pathogens (Seedevi et al. 2017). The sulfate content seems to be related to the biological functions of polysaccharides, because it has been reported that desulfated polysaccharide from *Ulva rigida* showed lower immunostimulatory activity (Leiro et al. 2007). However, further investigations are needed to establish the relationship between the sulfate content and the immunostimulatory function, because the structural requirements for the function have not been elucidated in detail (Jiao et al. 2011).

CV1 and CV2 fractions showed higher values also in the yield and amount of total sugar than CV3. It has been reported that the yield of polysaccharides differs depending on different extraction methods, and also on the seasonal variations, environmental conditions and physiological factors of the specimens (Armisen 1995). The galactose content of the sulfated polysaccharides from the red alga *G. birdiae* was reported to be 51% (Souza et al. 2012). The value was also lower than the sum of CV1 and CV2 fractions. Since the sulfated polysaccharides were extracted at high temperature (70 °C) in the present study, compared with 25 °C in their report, the content of monosaccharide composing the extracted polysaccharides may have resulted in higher values. This was in accordance with a previous study by Gómez-Ordóñez et al. (2014) reporting a galactose content of 95.2%. The results obtained in the present study also coincided with those in an earlier report on the polysaccharide from the red alga *Ahnfeltiopsis flabelliformis* with a high content (56.9%) of galactose and a smaller content (3.2%) of other monosaccharides (Kravchenko et al. 2014). The polysaccharides extracted from different kinds of seaweeds should have different compositions of monosaccharides, which may be due to the differences in phylogeny of the specimens used, extraction methods and their habitats (Li et al. 2006).

It has been established that hyaluronidase is involved in inflammatory and allergic reactions (Sakamoto et al. 1980). Hyaluronic acid, consisting of *N*-acetylglucosamine and glucuronic acid, is one of the glucosaminoglycans in the connective tissues of mammals. Degradation of hyaluronic acid has been observed in chronic rheumatism cases (Gotoh et al. 1988). In this study, the inhibitory activity of hyaluronidase was estimated by the IC_{50} values. The IC_{50} of 1-hexadecanepyridinium chloride (PC), which is used as an anti-allergic medicine, was 0.17 mg/ml. The value

of CV1 fraction (0.11 mg/ml) was found to be comparable to that of this substance. CV1 and CV2 fractions showed higher hyaluronidase inhibitory activity compared with that of *Undaria pinnatifida* sporophyll. It was fucoidan which was found to be responsible for suppression of IgE production in atopic dermatitis patients (Iwamoto et al. 2011). The other anti-allergic substances obtained from brown algae were identified to be phlorotannins (Gupta and Abu-Ghannam 2011). On the other hand, the IC_{50} value for CV3 could not be detected, suggesting CV3 alone did not show any hyaluronidase inhibitory activity.

The RBL-2H3 cells possess antigen-specific antibody IgE and high-affinity fragment crystallizable (Fc) receptors specific to IgE. When IgE is bound to mast cells via the Fc receptors, the mast cells are activated, followed by degranulation. Subsequently, chemical mediators such as cytokines are secreted, leading to immune responses (Smith et al. 1997). Mast cell granules and allergy chemical mediators (such as histamine and β-hexosaminidase) are easily detected throughout the degranulation, and thus are excellent markers for degranulation of mast cells (Tang et al. 2015). The calcium ionophore A23187, which was used in this study, can directly transport extracellular Ca^{2+} into the cells and induces degranulation (Okazaki et al. 1999). The increase in intracellular Ca^{2+} ionophores through the extracellular Ca^{2+} influx is essential for the activation of mast cells and thereby evokes degranulation in mast cells (Fowler et al. 2003; Passante and Frankish 2009; Cho et al. 2004).

In this study, *C. verrucosus* polysaccharide fractions were added to the culture medium of A23187-stimulated RBL-2H3 cells at various concentrations, and the extent of degranulation was assayed by β-hexosaminidase release. The effects of polysaccharide fractions on release of β-hexosaminidase (Fig. 3) and the apparent dependence on sulfate concentration (Fig. 4) could be explained as follows: the polysaccharides could not be incorporated into the cells, but suppressed the influx of Ca^{2+} into the cells by chelating extracellular Ca^{2+} (Sakai et al. 2011). Another possibility is that the polysaccharide was incorporated into the cells and inhibited A23187-induced activation of intracellular signaling molecules, such as the phosphorylation of kinases. It is also likely that IgE directly inhibited the upstream pathway of the Ca^{2+} influx. However, the mechanism involved seems to be very complicated as inferred by the studies on the effect of fucoidan on apoptosis (Kim et al. 2010).

Based on the results obtained in this study, it is suggested that the polysaccharides from *C. verrucosus* have anti-inflammatory activity which is closely related to anti-allergic activity, although antigen–antibody reaction based examination has not been performed.

In summary, the polysaccharide fractions from *C. verrucosus* were demonstrated to inhibit the A23187-induced degranulation of mast cells, as observed by the decrease in release of β-hexosaminidase. From the results in this study, the polysaccharides might be useful as a material for therapeutic agents for allergic inflammation.

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