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Inhibitory effect of chlorophyll c2 from brown algae, *Sargassum horneri*, on degranulation of RBL-2H3 cells

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ABSTRACT

The anti-allergic effect of alcohol extracts from edible seaweeds was evaluated by the degranulation of a rat basophilic leukemia cell line (RBL-2H3). Extracts prepared from the brown algae *Sargassum horneri*, *Ecklonia cava* and *Ecklonia kurome*, inhibited the degranulation of RBL-2H3 cells, whereas those from green algae and red algae did not. The active substance in *S. horneri* was purified and identified as chlorophyll c2, a major pigment in brown algae. The purified chlorophyll c2 preparation also suppressed the degranulation of RBL-2H3 cells. In contrast, chlorophyll a and b did not affect cell degranulation. Therefore, chlorophyll c2 from brown algae might be useful for the prevention or mitigation of allergic symptoms.

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1. Introduction

Allergic diseases, such as seasonal allergic rhinitis, atopic dermatitis, asthma and food allergy, are a rapidly increasing chronic health problem in Japan.

According to a 2005 Nasal Allergy Medical Care Guideline in Japan, approximately 16% of Japanese people suffer from pollinosis.

The Epidemiological Survey on Allergic Diseases in Japan from 1992 to 1994 showed that one-third of the Japanese population suffers from an allergy.

Allergic diseases and symptoms cause major discomfort, decreasing the quality of life of sufferers.

Mast cells and basophils are known as critical participants in allergic diseases. These cells express high affinity receptors for immunoglobulin E (IgE) (FcεRI) on the cell membrane. The interaction between multivalent antigens and IgE-bound

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Abbreviations: AcCN, acetonitrile; ANOVA, analysis of variance; Chl-c2, Chlorophyll c2; DMEM, Dulbecco's modified Eagle's medium; EtOH, ethanol; FBS, fetal bovine serum; HPLC, high-performance liquid chromatography; Ig, immunoglobulin; IT, ion trap; LC, liquid chromatography; LHC, light harvesting complex; MeOH, methanol; MS, mass spectrometry; MT buffer, modified Tyrode buffer; Mw, molecular weight; OD, optical density; PBS, phosphate-buffered saline; RT, retention time; SD, standard deviation; TOF, time-of-flight 1756-4646/\$ - see front matter © 2012 Elsevier Ltd. All rights reserved.

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FcεRI on the cell membrane induces the release of granules containing chemical mediators, such as histamine, prostaglandin, and leukotriene. In addition, cytokines released by mast cells and basophils activate chemotaxis and phagocytosis, as well as induce tissue inflammation.

Various foodstuffs have been reported to reduce the symptoms of allergic diseases. There are many reports concerning the anti-allergic effects of natural resources such as apple polyphenol (Kanda et al., 1998), isocoumarins (Kurume et al., 2008), coumarin and flavone derivatives from estragon and thyme (Watanabe, Shinmoto, & Tsushida, 2005). In addition to these compounds, carotenoids derived from edible seaweeds, such as fucoxanthin in *Undaria pinnatifida* (Sakai, Sugawara, Matsubara, & Hirata, 2009), porphyrin in *Porphyra tenera* and *Porphyra yezoensis* (Ishihara, Oyamada, Matsushima, Murata, & Muraoka, 2005), and alginic acids in *Lessonia nigrescens* (Asada et al., 1997), have been reported to have anti-allergic effects.

Recently, Toyohara of Kyoto University established the marine extract library, thereby contributing to the screening and development of novel functional substances.

This library contains numerous marine organisms, such as sea stars, sea cucumbers, shellfishes, and seaweeds, collected from all over Japan. The substances in these marine organisms were extracted in alcohol and collected in this library.

We evaluated the anti-allergic effects of alcohol extracts from the edible seaweeds in this library in an effort to find novel materials for use in health care.

2. Materials and methods

2.1. Preparation of extracts

The extracts were provided by Toyohara of Kyoto University, containing *Sargassum horneri*, *Ecklonia cava*, *Ecklonia kurome*, *Ulva pertusa* Kjellman, *Gelidium amansii*, and *P. yezoensis*.

The seaweeds were soaked in fresh water three times to remove salts, and then dried at 25 °C for 12 h. Each dried seaweed sample was powdered with Sample Mill (Foss, Hillerød, Denmark) and extracted in 100% MeOH, 0%, 20%, 40%, 60%, 80% and 100% of aqueous ethanol for 15 h at 25 °C.

After extraction, the extract solutions were filtrated by filter paper (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) and dried by evaporation. The dried extracts were stored at –80 °C until use.

2.2. Fractionation of the extracts from *S. horneri*

The brown algae *S. horneri* was harvested from Matsushima Bay, Miyagi, Japan and Yamada Bay, Iwate, Japan. *S. horneri* was soaked in fresh water three times to remove salts, and then dehydrated at 25 °C for 12 h. Four hundreds grams of dried *S. horneri* were extracted with 6 L of 80% aqueous ethanol and the dried extract (29 g) was partitioned between 1:1 (v/v) of 1-butanol and water.

The 1-butanol phase was dried, and the weight of the dried materials was 26 g. Next, the extract was partitioned between 1:1 (v/v) of 90% aqueous methanol and hexane.

After evaporation of the 90% aqueous methanol phase, 13.4 g of residual substances remained. The residue, which

showed a significant suppressive effect on the degranulation of RBL-2H3 cells, was then subjected to column chromatography on a silica gel PSQ100B column (i.d. 80 mm × length 300 mm) (Fuji Silysia Chemical, Aichi, Japan).

The adsorbed substances were eluted stepwise with each 7 L of the following methanol/chloroform (M/C) mixtures: 5/95, 10/90, 25/75, 50/50, and 100/0. These 5 fractions were represented as Fr. 1–Fr. 5, respectively. Fr. 1 showed a significant suppressive effect on cell degranulation and was further purified using silica gel column chromatography with a Hi-Flash TM silica gel column (i.d. 46 mm × length 130 mm) (Yamazen, Osaka, Japan). The adsorbed substances were eluted stepwise with the following M/C mixtures: 0/100 (Fr. a), 5/95 (Fr. b), and 100/0 (Fr. c).

Since Fr. c showed a significant suppressive effect on cell degranulation, further fractionation using high-performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan) was undertaken. Purification was performed at 25 °C on an XTerra prep MS C18 HPLC column (i.d. 10 mm × length 250 mm) (Waters, Milford, MA, USA). The adsorbed substances were eluted using a gradient of acetonitrile (AcCN) as following: from 0 to 8 min: 45%, from 8 to 18 min: 75%, from 18 to 28 min: 90%, and from 28 to 35 min: 100% at 3 mL/min.

As shown in Fig. 1, the HPLC chromatogram (OD 254 nm) of Fr. c (12.5 mg) showed one major peak at the retention time (RT) of 31 min. This main peak was again subjected to HPLC under the same condition, and fractionated into the following four fractions: Fr. A (RT: 0–27 min, 3 mg), Fr. B (RT: 27–30 min, 2.6 mg), Fr. C (RT: 30–32 min, 2.8 mg), and Fr. D (RT: 32–35 min, 1.3 mg).

Respectively, these 4 fractions were added to MT buffer and their effects on RBL-2H3 cell degranulation were assessed.

2.3. LC–MS analysis

A Prominence HPLC system coupled to LCMS-IT-TOF, equipped with an electrospray ionization interface (Shimadzu, Kyoto, Japan), was used.

Separations were performed using an XTerra MS C18 HPLC column (i.d. 2.1 mm × length 100 mm) (Waters). Eluents were A: 0.1% formic acid and B: AcCN. A liner gradient from 10% to 90% B was supplied for 40 min, 90% B for 5 min, from 90% to 10% during the next 5 min, followed by an isocratic hold at 10% B for an additional 10 min, at a flow rate of 0.2 mL/min.

The MS was operated under the following conditions: probe voltage of 4.50 kV, CDL temperature of 200 °C, block heater temperature of 200 °C, nebulizer gas flow of 1.5 L/min, ion accumulation time of 50 ms, MS range of m/z 200–1200.

2.4. RBL-2H3 cells

RBL-2H3 cells (JCRB0023) were obtained from the Health Science Research Resources Bank (Osaka, Japan). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; SAFC Biosciences, St. Louis, MO, USA), 100 unit/mL of penicillin G (Wako Pure Chemical Industries, Osaka, Japan)

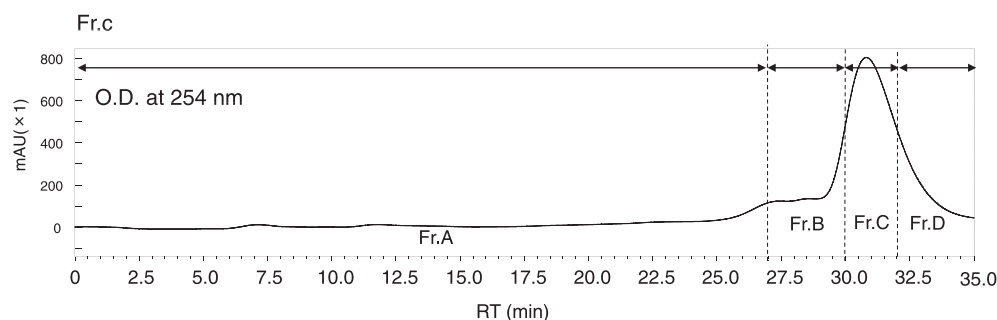


Fig. 1 – HPLC chromatogram of Fr. c prepared from *S. horneri*. Fraction c from *S. horneri* was subjected to HPLC chromatography using an XTerra prep MS C18 HPLC column. The OD of the extracts was monitored at 254 nm.

and 100 µg/mL of streptomycin (Wako) at 37 °C under humidified 5% CO₂–95% air.

2.5. β-Hexosaminidase release assay

Antigen-induced degranulation of basophilic cells was determined using the β-hexosaminidase release assay (Horigome et al., 2008). RBL-2H3 cells were inoculated in a 24-well culture plate at 2.5×10^5 cells/well, and cultured for 12 h at 37 °C. After washing with phosphate-buffered saline (PBS), the cells were sensitized with 25 ng/well of anti-DNP IgE (Sigma) for 2 h. After IgE sensitization, the cells were washed with modified Tyrode buffer (MT buffer) composed of 137 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 20 mM HEPES, and 0.1% bovine serum albumin twice to remove free anti-DNP IgE. Next, MT buffer containing the seaweed extract was added to each well, and incubated for 10 min. After 10 min, 100 ng/well of DNP-HSA (Sigma) was added to the wells, and incubated for 30 min. Then, the culture plate was placed on ice to terminate antigen stimulation, and the culture supernatant was collected from each well. Cell lysis buffer (Sigma) was then added to each well, and the cells were disrupted by sonication to prepare the cell lysates.

The culture supernatant or the cell lysate was mixed with 0.1 M citrate buffer (pH 4.5) containing 3.3 mM *p*-nitrophenyl-2-acetoamide-β-D-glucopyranoside, and the mixture was stored at 37 °C for 25 min. The enzymatic reaction was terminated by the addition of 2 M glycine buffer (pH 10.4). The absorbance at 405 nm of each reaction mixture was measured. β-Hexosaminidase release was calculated using the following formula:

$$\beta\text{-hexosaminidase release (\%)} = ((S - SB) / ((S - SB) + (C - CB))) \times 100 \quad (1)$$

Where S is the absorbance of the culture supernatant, SB is the blank absorbance of the culture supernatant, C is the absorbance of the cell lysate, and CB is the blank absorbance of the cell lysate.

2.6. Cell viability assay

RBL-2H3 cells cultured in a 96-well plate at 37 °C were washed with PBS, and MT buffer containing 0, 20, 100, 500 µg/mL of the seaweed extract was added to each well. After incubation

for 40 min, WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt) solution (Dojindo Laboratories, Kumamoto, Japan) was added to each well at 10 v/v, % of MT buffer. After incubation at 37 °C for 30 min, the culture plate was placed at room temperature for 10 min, and absorbance at 450 nm was measured.

2.7. Statistical analysis

All results are expressed as the mean ± standard deviation (SD). Statistical differences among groups were evaluated by one-way analysis of variance (ANOVA) with Dunnett's test.

The statistical analyses were conducted by using StatLight #4 (Yukms, Tokyo, Japan). Values at **p* < 0.05 and ***p* < 0.01 were considered to be statistically significant.

3. Results

3.1. Inhibitory effect of seaweed extracts on RBL-2H3 degranulation

The inhibitory effects of seaweed extracts on the degranulation of RBL-2H3 cells were examined. Only brown algae extracts (*S. horneri*, *E. cava*, and *E. kurome*) significantly inhibited the degranulation of RBL-2H3 cells (Fig. 2). Moreover, *S. horneri* extract showed a more potent inhibitory effect compared with *E. cava* and *E. kurome*. In contrast to *E. cava* or *E. kurome*, *S. horneri* can be harvested in large amounts along the coast of Japan. Therefore, in this study, we focused on *S. horneri* as a potential novel anti-allergic foodstuff.

3.2. Optimization of the extraction conditions

As we were attempting to develop a functional food from *S. horneri*, it was necessary to change the extraction solvent from MeOH to EtOH. To optimize the extraction conditions, *S. horneri* was extracted using various concentrations of aqueous ethanol (0%, 20%, 40%, 60%, 80% and 100%). Each extract was added to MT buffer at 100 or 500 µg/mL, and their effects on RBL-2H3 cells were assessed.

As shown in Fig. 3, the active substance in *S. horneri* was extracted between 60% and 100% aqueous ethanol. Specifically, 80% aqueous ethanol was the most effective extraction solvent. Hence, *S. horneri* extract was prepared using 80% aqueous ethanol for further experiments.

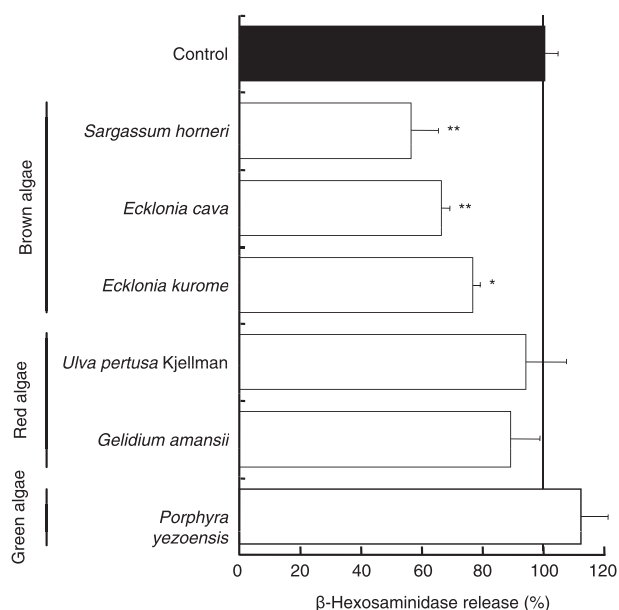


Fig. 2 – Inhibitory effect of edible seaweed extracts on the degranulation of RBL-2H3 cells. RBL-2H3 cells (2.5×10^5 cells/well) were sensitized with 25 ng/well anti-DNP IgE, incubated for 2 h, samples were added and incubated for 10 min. The cells were then stimulated with 100 ng/well DNP-HSA for 30 min. β -Hexosaminidase activity was measured in the culture supernatant and the cell lysate. The data are shown as β -hexosaminidase activity relative to control, and results are presented as the means $\pm$ SD ($n = 4$). Statistically significant differences compared to control, analyzed using Dunnett's test, are represented as * $p < 0.05$, ** $p < 0.01$.

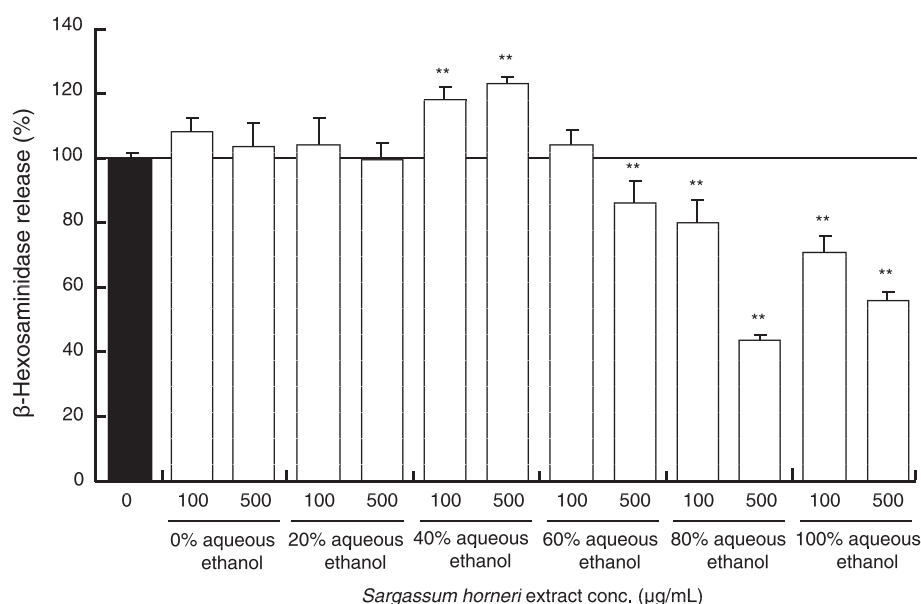


Fig. 3 – Optimization of EtOH concentration (v/v, %) for the extraction of the active substance from *S. horneri*. *S. horneri* was extracted using various concentrations of aqueous ethanol. RBL-2H3 cells sensitized with IgE were incubated with various concentrations of aqueous ethanol, and cell degranulation was assessed. Results are expressed as the means $\pm$ SD ($n = 4$). Statistically significant differences compared to control, analyzed using Dunnett's test, are represented as ** $p < 0.01$.

3.3. Dose effects of *S. horneri* extract on the suppression of RBL-2H3 cell degranulation

S. horneri extract in 80% aqueous ethanol was added to MT buffer at various concentrations (0, 20, 100 and 500 μ g/mL), and the dose effects were examined. As a result, the extract inhibited the degranulation of RBL-2H3 cells in a dose-dependent manner (Fig. 4A). As shown in Fig 4B, RBL-2H3 cell viability was not affected by the extract at any dose. This fact indicates that *S. horneri* extract inhibits the degranulation of RBL-2H3 cells without cytotoxic effects.

3.4. Purification of *S. horneri* extract

To determine the active compound in *S. horneri*, the 80% aqueous ethanol extract of *S. horneri* was fractionated, and the inhibitory effect of each fraction was examined. As a result, Fr. c significantly inhibited the degranulation of RBL-2H3 cells (data not shown). Subsequently, Fr. c was purified by HPLC, and 4 fractions were obtained.

These fractions were examined the inhibitory effects, and Fr. C significantly suppressed the degranulation of RBL-2H3 cells (data not shown). As shown in Fig. 1, Fr. C contains the main suppressive components in Fr. c. Therefore, Fr. C was subjected to molecular weight analysis using LC-MS and spectrophotometric analysis.

3.5. Characterization of the active substance in Fr. C

Fig. 5A shows the HPLC chromatogram (OD 450 nm) of Fr. C and the visible absorption spectrum. The HPLC chromatogram showed two peaks at 35.3 and 42.1 min. The substances eluted

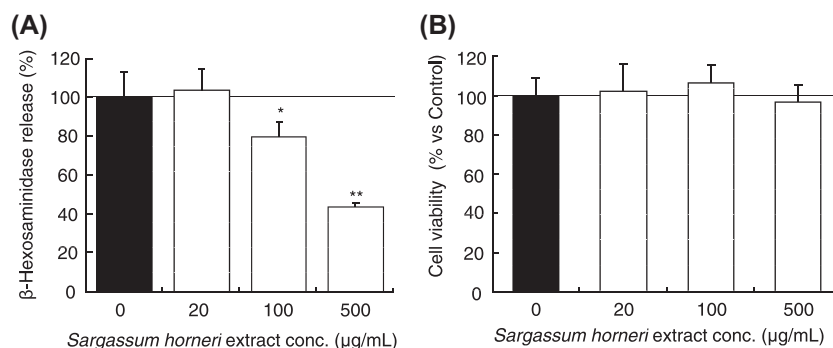
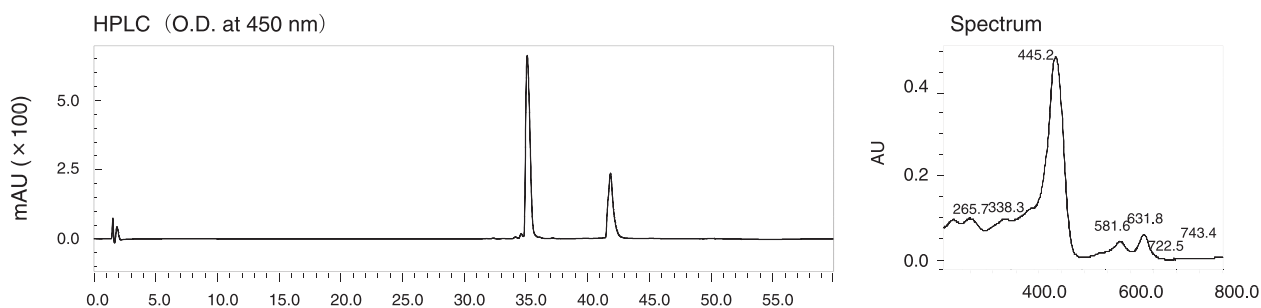


Fig. 4 – Dose effects of *S. horneri* extract on the degranulation (A) and the cell viability (B) of RBL-2H3 cells. (A) RBL-2H3 cells sensitized with IgE were incubated with various concentrations of *S. horneri* extract in 80% aqueous ethanol. (B) After a 40-min incubation with various concentrations of the 80% aqueous ethanol extract, WST-1 solution was added to MT buffer, and RBL-2H3 cells were incubated for 30 min. Finally, the absorbance at 450 nm was measured. Results are expressed as the means $\pm$ SD ($n = 4$). Statistically significant differences compared to control, analyzed by Dunnett's test, are represented as * $p < 0.05$, ** $p < 0.01$.

at 35.3 and 42.1 min gave intense mass spectral signals at m/z : 609.1 and 587.2, respectively. The former mass spectral signal corresponded to the quasi-molecular ion peak $[M+H]^+$ of chlorophyll c2 (Chl-c2), the main photosynthetic pigment in brown algae (the molecular weight (Mw) of Chl-c2 is 608.1). The latter signal corresponded to the quasi-molecular ion peak $[M+H]^+$ of pheophytin c2, a chlorophyll-related compound lacking a central Mg^{2+} (Mw: 586.2). In addition, the visible absorption spectrum of Fr. C showed the position of maxima at 445.2 nm, 581.6 nm and 631.8 nm. Because the major

component in Fr. C was determined to be Chl-c2, purified Chl-c2 was subjected to the same analysis as Fr. C. Fig. 5B shows the HPLC chromatogram and the visible absorption spectrum of the purified Chl-c2 (Wako). The HPLC chromatogram of Chl-c2 also showed two peaks at 35.6 and 42.4 min, almost the same as those of Fr. C. The former peak gave an intense mass spectral signal at m/z 609.1, corresponding to the quasi-molecular ion peak $[M+H]^+$ of Chl-c2, while the latter peak at m/z 587.2 corresponded to the quasi-molecular ion peak $[M+H]^+$ of pheophytin c2. Furthermore, the spectral

(A) Fr. C



(B) Chlorophyll c2

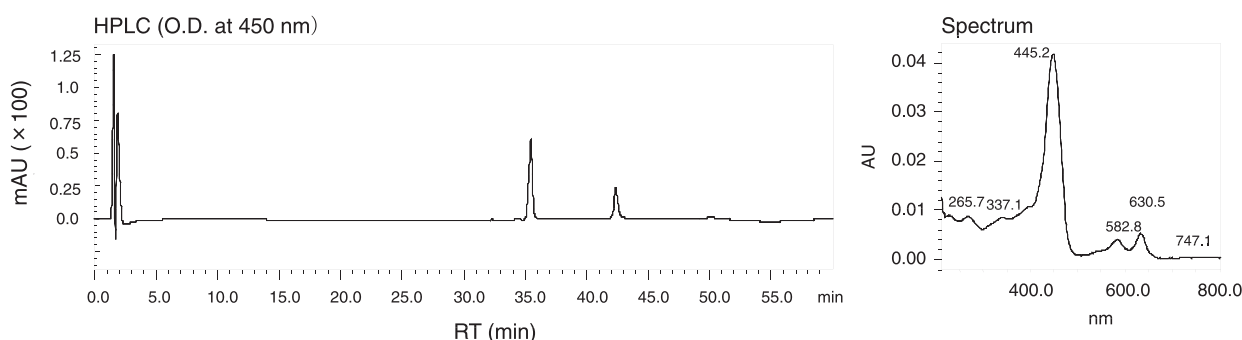


Fig. 5 – Characterization of the active substance in Fr. C from *S. horneri*. (A) HPLC chromatogram of Fr. C monitored at 450 nm and the visible absorption spectrum of Fr. C. (B) HPLC chromatogram of purified Chl-c2 monitored at 450 nm and the visible absorption spectrum of Chl-c2. Peak wavelengths are indicated on the absorption spectra.

character of Chl-c2 was almost the same as that of Fr. C. This suggests that the major component in Fr. C is Chl-c2.

3.6. Inhibitory effect of chlorophylls on RBL-2H3 cell degranulation

As indicated in Fig. 5, the active substance in *S. horneri* was determined to be Chl-c2. Next, the effects of three purified chlorophylls, Chl-a, Chl-b, and Chl-c2 (Wako), on cell degranulation were examined. As shown in Fig. 6, Chl-c2 significantly inhibited the degranulation of RBL-2H3 cells in a dose-dependent manner. In addition, Chl-c2 did not affect RBL-2H3 cell viability at any dose (data not shown). In contrast, Chl-a and Chl-b did not suppress cell degranulation. These results suggest that the active substance in *S. horneri* is Chl-c2.

4. Discussion

We screened several edible seaweeds from the marine extract library at Kyoto University for anti-degranulation effects. The extracts prepared from the brown algae *S. horneri*, *E. cava*, and *E. kurome* inhibited the degranulation of RBL-2H3 cells. Specifically, *S. horneri* extract showed potent inhibitory effects on RBL-2H3 cell degranulation without cytotoxicity. Compared with *E. cava* and *E. kurome*, *S. horneri* can be obtained at a low cost and in large amounts in Japan. Hence, we focused on *S. horneri* as an anti-allergic foodstuff candidate.

To optimize the extraction conditions, *S. horneri* was extracted using various aqueous ethanol concentrations. As a result, 80% aqueous ethanol most effectively solubilized the active substance in *S. horneri*; therefore, extracts were prepared using 80% aqueous ethanol for further experiments, and the active substance was identified. As a result of purification, the active substance in *S. horneri* was identified as Chl-c2, one of the major pigments in brown algae.

Sakai reported that fucoxanthin, a major pigment and carotenoid of brown algae, suppressed the degranulation of

mast cells when the cells were treated with fucoxanthin 4 h prior to antigen stimulation (Sakai et al., 2009).

Fucoxanthin was assumed to modulate the function of lipid rafts and inhibit the translocation of the high affinity IgE receptor (FcεRI) to lipid rafts. Therefore, the active compound in *S. horneri* was expected to be fucoxanthin, and we assessed the suppressive effect of purified fucoxanthin using our assay system.

As a result, fucoxanthin had no effect on the degranulation of RBL-2H3 cells (data not shown). Sakai et al. (2009) also reported that fucoxanthin did not inhibit the degranulation of RBL-2H3 cells when the cells were treated with antigen and fucoxanthin simultaneously.

The duration of fucoxanthin treatment required to suppress degranulation was at least 4 h prior to antigen stimulation (Sakai et al., 2009). In contrast, in our assay, Chl-c2 treatment 10 min before antigen stimulation inhibited the degranulation of RBL-2H3 cells.

These facts suggest that *S. horneri* contains at least two substances, Chl-c2 and fucoxanthin, which contribute to the suppression of degranulation by different mechanisms of action. Currently, the mode of action of Chl-c2 is under investigation. The inhibitory effects of three purified chlorophylls, Chl-a, Chl-b, and Chl-c2, were evaluated. Chl-c2 was not cytotoxic, and was the only compound to significantly inhibit the degranulation of RBL-2H3 cells, suggesting that the active substance in *S. horneri* is Chl-c2. Chlorophylls are photosynthetic pigments contained in certain plant tissues. Chl-a is found in almost all plants, while Chl-b is found in green plants. On the other hand, the Chl-c family, mainly Chl-c1, Chl-c2 and Chl-c3, is distributed in the Chromista, which includes brown algae and diatoms. Therefore, brown algae, such as *S. horneri*, *E. cava*, and *E. kurome*, showed inhibitory effects on RBL-2H3 cell degranulation (Fig. 2).

In the Chromista and diatoms, Chl-c2 exists with others from the Chl-c family, such as Chl-c1 or/and Chl-c3 (Bernhard, Robert, Wolfhart, & Hugo, 2006). In this study, while other Chl-c family members were not identified as active compounds,

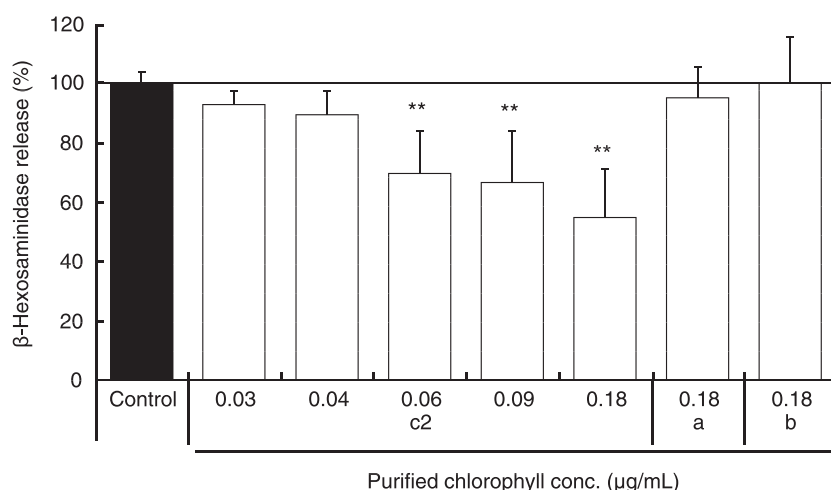


Fig. 6 – Inhibitory effect of chlorophylls on the degranulation of RBL-2H3 cells. Following stimulation with 25 ng/well IgE, RBL-2H3 cells were incubated with purified chlorophylls (Chl-a, Chl-b, Chl-c2), and the effect on cell degranulation was assessed. Results are expressed as the means $\pm$ SD ($n = 4$). Statistically significant differences compared to control, analyzed by Dunnett's test, are represented as ** $p < 0.01$.

they might show suppressive effects on the degranulation of RBL-2H3 cells.

The molecular structure of the Chl-c family differs from other chlorophylls. The Chl-c family contains Mg-phytytoporphyrins, rather than Mg-chlorins of Chl-a and Chl-b. In addition, the molecules of the Chl-c family contain a *trans* acrylic acid at C-17, instead of the propionic acid side chain of Chl-a and b. The difference in molecular structure between the Chl-c family and other chlorophylls might contribute the suppressive effect of Chl-c2. Sakai, Komura, Nishimura, Sugawara, and Hirata (2011) reported that phycoerythrin and phycoerythrobilin inhibited the calcium ionophore-induced degranulation of RBL-2H3 cells.

Phycoerythrobilin also suppressed the antigen-dependent degranulation of RBL-2H3 cells. Phycoerythrobilin is the major light-harvesting pigment in red algae, while phycoerythrin is a water-soluble chromoprotein and binds to phycoerythrobilin through cross-linking of its cysteine residues. Sakai also postulated that phycoerythrin might suppress the influx of Ca^{2+} into cells by chelating extracellular Ca^{2+} . On the other hand, phycoerythrobilin may inhibit the antigen-dependent activation of intracellular signaling of the degranulation.

Chl-c2 also combines with accessory pigments such as carotenoids and light harvesting complex (LHC) proteins in plants. Therefore, Chl-c2 and a Chl-c2 protein complex might have a similar suppressive effect, but act via different mechanisms. However, the mode of action of Chl-c2 in suppressing the degranulation of RBL-2H3 cells is unknown, necessitating further studies. In addition, studies on the effects of other Chl-c family members, such as Chl-c1 and c3, on cell degranulation are now in progress.

In conclusion, we demonstrated that Chl-c2 in brown algae significantly suppressed the degranulation of RBL-2H3 cells. Thus, Chl-c2 from brown algae may be useful for the prevention or mitigation of allergic symptoms.

Acknowledgments

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producing coasts for *S. horneri*, from the 2011 Tohoku earthquake and tsunami.

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