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Note

Inhibition of Mast Cell Degranulation by Phycoerythrin and Its Pigment Moiety Phycoerythrobilin, Prepared from *Porphyra yezoensis*

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Phycoerythrin from the red alga *Porphyra yezoensis* has been demonstrated to possess antioxidative activity and is expected to have anti-inflammatory activity. In this study, we examined the effects of phycoerythrin and its pigment moiety, phycoerythrobilin, on the degranulation of mast cells. Phycoerythrin (0.01 mg/mL) and phycoerythrobilin (1 μ mol/L) significantly inhibited calcium ionophore (A23187) induced release of β -hexosaminidase from rat basophilic leukemia (RBL-2H3) cells (approximately 50% and 40%, respectively). Phycoerythrobilin significantly suppressed IgE-antigen-stimulated degranulation of RBL-2H3 cells, while phycoerythrin had no effect. A23187-induced degranulation of peritoneal exudate cells (PEC) prepared from rats treated orally with phycoerythrin (4 mg/kg) or phycoerythrobilin (1 μ mol/kg) was significantly reduced compared to control PEC. These results suggest that phycoerythrin and phycoerythrobilin exhibit anti-inflammatory activities through the suppression of mast cells degranulation *in vivo*.

Keywords: *Porphyra yezoensis*, nori, phycoerythrin, phycoerythrobilin, anti-inflammation, mast cell, degranulation

Introduction

Porphyra yezoensis is a red alga that is abundantly cultivated in eastern Asia. In Japan, *P. yezoensis* is generally used to prepare nori, which is a traditional marine food. Dried nori is well known to contain many nutritional components such as proteins, polyunsaturated fatty acids, carotenoids and dietary fibers. Phycoerythrin, the major light-harvesting pigment protein in red algae (including *P. yezoensis*), is a water-soluble chromoprotein and binds to phycoerythrobilin through cross-linking of its cysteine residues. Phycobiliproteins, such as phycoerythrin, in phycobilisomes can transfer light energy to chlorophyll a of the type II photosystem at a reaction center in the photosynthetic membrane (Endo *et al.*, 1984). We previously reported that phycoerythrin inhibits the 2,2'-azobis-(2-amidinopropane)-dihydrochloride (AAPH) induced peroxidation of linoleic acid (Hirata *et al.*, 2002). Phycoerythrin has also been reported to show antioxidative activity and a hepatoprotective effect (Hirata *et al.*, 1998; Soni *et al.*, 2009). Thus, we hypothesized that phycoerythrin

possesses anti-inflammatory activity.

Mast cells play important roles in type I allergic reactions. In mast cells, the cross-linking of IgE-bound high affinity IgE receptors (Fc ϵ RI) by an antigen initiates an intracellular biochemical cascade followed by degranulation (Metcalfe *et al.*, 1997; Church and Levi-Schaffer, 1997; Galli *et al.*, 2005). Degranulation induces the release of histamine and many kinds of inflammatory mediators from mast cells. Therefore, inhibition of degranulation by food components and medicinal compounds leads to the relief of allergenic responses. The rat basophilic leukemia cell line (RBL-2H3), a tumor analog and a model for mast cells, has the phenotypic characteristics of mucosal mast cells and is an excellent tool for studying the modulation of mast cell activation. Peritoneal exudate cells (PEC), which contain peritoneal mast cells and basophilic granulocytes, have been widely used to study the release of allergic mediators (Sullivan *et al.*, 1976; Swieter *et al.*, 1989; Schmutzler *et al.*, 1995).

In the present study, we investigated the effects of phycoerythrin and its pigment moiety, phycoerythrobilin, on the degranulation of mast cells to evaluate their anti-inflammatory activity.

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Materials and Methods

Chemicals Anti-DNP IgE, calcium ionophore A23187, *p*-nitrophenyl-*N*-acetyl- β -*D*-glucosaminide, Triton X-100 and RPMI-1640 medium were purchased from Sigma (St. Louis, MO, USA). DNP-BSA was obtained from Molecular Probes (Eugene, OR, USA).

Preparation of phycoerythrin and phycoerythrobilin

Freshly harvested *P. yezoensis* was stored at -80°C until use. Phycoerythrin was isolated from lyophilized *P. yezoensis* (Bermejo *et al.*, 2001) and the visible absorption spectrum of the fraction containing phycoerythrin was measured using a spectrophotometer (UV-2400, Shimadzu, Kyoto, Japan). The extinction coefficient of phycoerythrin at 565 nm ($E^{1\%}_{1\text{cm}} = 80.2$) was used for quantification. Phycoerythrobilin was obtained by methanolysis of phycoerythrin (Romay *et al.*, 1998a). In brief, 5 mg of phycoerythrin was dissolved in 0.1 mL water and 1.9 mL methanol, and was then heated at 90°C . After 3 h, 2 mL water and 4 mL chloroform were added and the mixture was centrifuged at $750 \times g$ for 10 min, after which phycoerythrobilin was obtained from the chloroform layer. The extinction coefficient of phycoerythrobilin at 591 nm ($E^{1\%}_{1\text{cm}} = 25,200$) was used for quantification. Phycoerythrobilin was analyzed using a high performance liquid chromatography (HPLC) system with photodiodearray detector (PDA). An ODS-80Ts column (250×4.6 mm, Tosoh, Tokyo, Japan) was used with a mixture of methanol/water/acetic acid (50:50:1, v/v/v) as the mobile phase at a flow rate of 1.0 mL/min. The column temperature was maintained at 40°C .

Cell culture Rat basophilic leukemia (RBL-2H3) cells (Health Science Resources Bank, Osaka, Japan) were cultured in RPMI-1640 medium containing 10% FBS and antibiotics (100 units/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin; Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified atmosphere in the presence of 5% CO_2 .

β -Hexosaminidase release assay The degree of degranulation of RBL-2H3 cells was determined using the β -hexosaminidase release assay (Nakano *et al.*, 2005). β -Hexosaminidase is stored in secretory granules of mast cells and is released concurrently with histamine during degranulation (Cho *et al.*, 2004; Nakano *et al.*, 2005). Thus, the β -hexosaminidase activity released into the medium is used as a marker of mast cell degranulation. RBL-2H3 cells were seeded in 96-well plates (5.0×10^4 cells/well), and after overnight incubation, those RBL-2H3 cells were treated with the indicated concentration of phycoerythrin or phycoerythrobilin dissolved in serum-free RPMI-1640 medium for 90 min. Phycoerythrobilin was dissolved in DMSO and the final concentration of DMSO in the medium was 0.1%. After washing twice with Tyrode's buffer (1.17 mmol/L NaCl,

5.4 mmol/L KCl, 2.0 mmol/L CaCl_2 , 1.0 mmol/L MgCl_2 , 5.6 mmol/L glucose, 25 mmol/L HEPES, 0.1% BSA, pH 7.7), the cells were stimulated with 120 μL Tyrode's buffer containing 1 $\mu\text{mol}/\text{L}$ calcium ionophore A23187 for 20 min. The supernatants were collected, and cell lysates were obtained using Tyrode's buffer containing 0.1% Triton X-100. Aliquots (50 μL) of the supernatants and cell lysates were incubated with 50 μL 5 mmol/L *p*-nitrophenyl-*N*-acetyl- β -*D*-glucosaminide in 0.1 mol/L citrate buffer (pH 4.5) at 37°C for 1 h. The reaction was terminated by the addition of 0.1 mol/L $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$ (pH 10.0). *p*-Nitrophenol, which is the product of the reaction, was detected by measuring optical absorbance at 405 nm.

In the case of stimulation with antigen, RBL-2H3 cells were seeded in 96-well plates (5.0×10^4 cells/well) with 0.45 $\mu\text{g}/\text{mL}$ anti-DNP IgE. After overnight incubation, the sensitized RBL-2H3 cells were treated with the indicated concentration of phycoerythrin or phycoerythrobilin for 90 min. After washing twice with Tyrode's buffer, the cells were stimulated with 10 $\mu\text{g}/\text{mL}$ DNP-BSA for 30 min. Released β -hexosaminidase was measured as described above.

The percentage of β -hexosaminidase release was calculated as follows: β -hexosaminidase release (%) = supernatant OD value of the stimulated cells / (the cell lysate OD value + supernatant OD value of the stimulated cells).

Isolation of PEC from rats treated with phycoerythrin or phycoerythrobilin Nine-week-old male Sprague-Dawley rats (Japan SLC Inc., Shizuoka, Japan) were treated orally with phycoerythrin (4 mg/kg body weight) or phycoerythrobilin (1 $\mu\text{mol}/\text{kg}$ body weight). Control rats were treated orally with water only. Ninety min after treatment, PEC were isolated under diethyl ether anesthesia as previously described (Zaima *et al.*, 2005). In brief, 20 mL of Tyrode's buffer containing 0.1% D-glucose and 0.1% fish gelatin was injected into the peritoneal cavity, and the abdomen was gently massaged for 2 min. Then, the peritoneal cavity was opened, and fluid containing the PEC was collected. After centrifugation at $200 \times g$ for 5 min, the cell pellets were resuspended in a modified ammonium chloride buffer (155 mmol/L NH_4Cl , 1 mmol/L KHCO_3 , 10 mmol/L EDTA-2Na, pH 7.4) and then incubated for 5 min on ice. The cell suspension was centrifuged at $200 \times g$ for 5 min and the cell pellets were resuspended in the RPMI-1640 medium. Cell viability was measured by trypan blue staining. The total number of PEC from each rat was approximately $2-8 \times 10^6$ cells. PEC were then seeded in 96-well plates at 1.0×10^4 cells/well in RPMI-1640 medium supplemented with 10% FBS, 100 units/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin at 37°C in a humidified atmosphere in the presence of 5% CO_2 . After stimulation with 1 $\mu\text{mol}/\text{L}$ A23187 for 20 min,

the release of β -hexosaminidase from PEC was measured as described above. Animal experiments were performed in accordance with the guidelines of Kyoto University for the use and care of laboratory animals.

Statistical analysis Data are reported as means \pm SD. Statistical analyses were performed by one-way analysis of variance (ANOVA) with Fisher's PLSD to identify levels of significance between groups.

Results and Discussion

Purification of phycoerythrin and phycoerythrobilin

The visible absorption spectrum of phycoerythrin prepared from *P. yezoensis* is shown in Fig. 1A. The purified phycoerythrin did not contain phycocyanin because there was no absorption peak at 615 nm. Phycoerythrobilin, prepared by methanolysis of phycoerythrin, was analyzed by HPLC-PDA (Fig. 1B, 1C and 1D). According to their spectra and retention times, 2 peaks corresponding to 3E- and 3Z-phycoerythrobilin were identified (Beale and Cornejo, 1991). The abundances of 3E-phycoerythrobilin and 3Z-phycoerythrobilin were approximately 66% and 33%, respectively.

Effects of phycoerythrin and phycoerythrobilin on the degranulation of RBL-2H3 cells In the case of stimulation with A23187, which directly evokes degranulation, approximately 50% of intracellular β -hexosaminidase was released from RBL-2H3 cells via degranulation. Pretreatment with phycoerythrin or phycoerythrobilin for 90 min significantly suppressed the release of β -hexosaminidase from cells in a dose-dependent manner (Fig. 2). The inhibition ratios of 0.01 mg/mL phycoerythrin and of 1 μ mol/L phycoerythrobilin were approximately 50 and 40%, respectively.

The effects of phycoerythrin and of phycoerythrobilin on the degranulation of DNP-IgE sensitized RBL-2H3 cells stimulated with DNP-BSA as an antigen were also evaluated. Approximately 25% of the intracellular β -hexosaminidase was released by the antigen-antibody reaction. This experimental condition closely mimics the intravital degranulation of mast cells caused by the antigen-antibody reaction. Preincubation with 0.01–1 μ mol/L phycoerythrobilin for 90 min significantly inhibited the degranulation of RBL-2H3 cells while phycoerythrin had no effect (Fig. 3). Phycoerythrin and phycoerythrobilin had no direct effect on

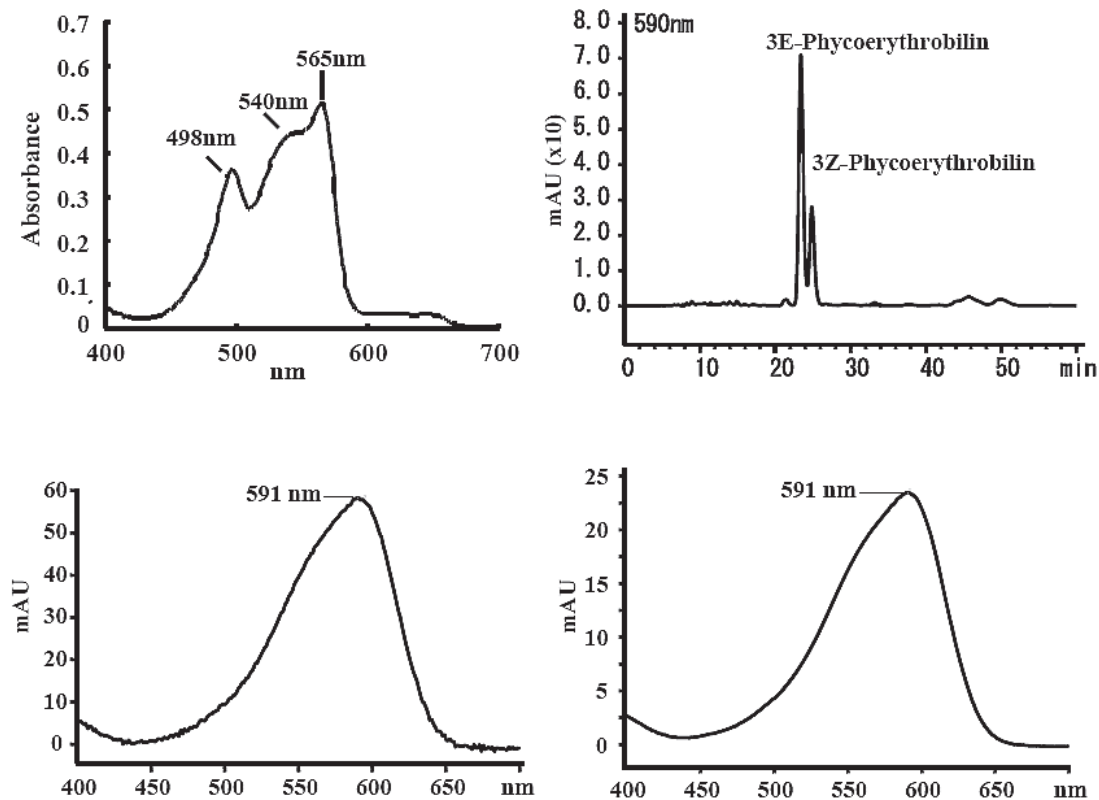


Fig. 1. Characterization of purified phycoerythrin and phycoerythrobilin.

(A) Visible absorption spectra of phycoerythrin prepared from *P. yezoensis*. (B) Phycoerythrobilin prepared by methanolysis of phycoerythrin was analyzed by HPLC with PDA. (C, D) Absorption spectra of 3E-phycoerythrobilin (C) and 3Z-phycoerythrobilin (D).

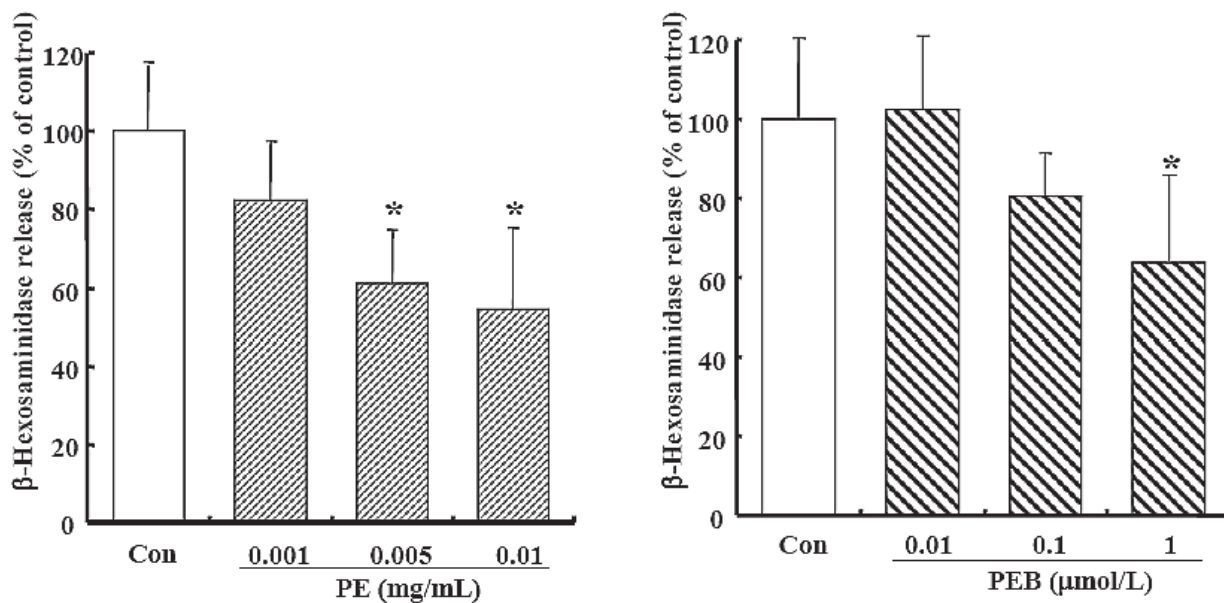


Fig. 2. Effect of phycoerythrin and phycoerythrobilin on A23187-induced degranulation of RBL-2H3 cells.

RBL-2H3 cells were incubated with the indicated concentrations of phycoerythrin (A, PE) or phycoerythrobilin (B, PEB) for 90 min and were then stimulated with 1 μmol/L A23187 for 20 min. Released β-hexosaminidase was measured. Values are means ± SD, $n = 4$. * Significantly different from control (Con) stimulation, $P < 0.05$.

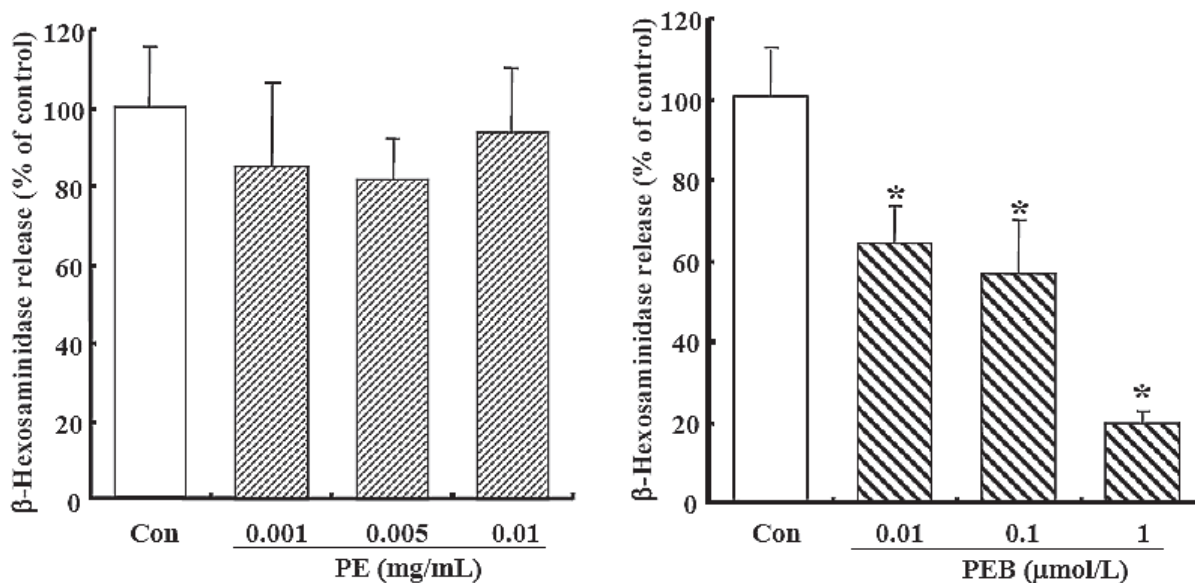


Fig. 3. Effects of phycoerythrin and phycoerythrobilin on the DNP-BSA-induced degranulation of RBL-2H3 cells.

RBL-2H3 cells were sensitized with DNP-IgE for 24 h. Sensitized cells were incubated with the indicated concentrations of phycoerythrin (A) or phycoerythrobilin (B) for 90 min and were then stimulated with 10 μg/mL DNP-BSA as antigen for 30 min. Released β-hexosaminidase was measured. Values are means ± SD, $n = 4$. * Significantly different from control, $P < 0.05$. Abbreviations are the same as those given for Fig. 2.

β -hexosaminidase enzyme activity (data not shown).

It is well known that elevation of the intracellular Ca^{2+} concentration is critical for the degranulation of mast cells (Beaven *et al.*, 1984; Parekh and Penner, 1997; Cho *et al.*, 2004). The calcium ionophore A23187 can directly transport extracellular Ca^{2+} into mast cells, thereby inducing degranulation (Lee and Oliver, 1995; Okazaki *et al.*, 1999). In this study, phycoerythrin only inhibited A23187-induced degranulation, whereas phycoerythrobilin suppressed degranulation induced by both A23187 and the IgE-antigen reaction. Phycoerythrin is not readily incorporated into cells, because it is a chromoprotein with a molecular weight of $> 268,000$ (Alan and Alexander, 1984). However, phycoerythrin may suppress the influx of Ca^{2+} into cells by chelating extracellular Ca^{2+} . On the other hand, phycoerythrobilin might be incorporated into cells and inhibit IgE-antigen-induced activation of intracellular signaling molecules, such as the phosphorylation of kinases (Sakai *et al.*, 2009).

Evaluation of the degranulation of PEC from rats treated orally with phycoerythrin or phycoerythrobilin It was reported that PEC contain approximately 10–15% mast cells, and both PEC and purified mast cells from PEC released similar amounts of histamine upon stimulation with A23187 (Inagaki *et al.*, 1994). In this study, approximately 20% of

the β -hexosaminidase was released from PEC by the stimulation with A23187. Oral treatment with phycoerythrin or with phycoerythrobilin significantly suppressed A23187-induced PEC degranulation (Fig. 4). PEC degranulation was inhibited by approximately 25% and 20% in rats treated orally with phycoerythrin or phycoerythrobilin, respectively. Orally ingested phycoerythrin is assumed to be digested in the gastrointestinal tract and the covalently bound phycoerythrobilin may be released (Yabuta *et al.*, 2010). We speculate that the activity of phycoerythrin *in vivo* might be due to the absorbed phycoerythrobilin, which is released from phycoerythrin in the digestive tract.

Many studies have demonstrated that phycocyanin, a major chromoprotein in blue-green algae, has antioxidant and anti-inflammatory activities (Romay *et al.*, 1998b; Romay *et al.*, 1998c; Gonzales *et al.*, 1999; Hirata *et al.*, 2000; Hirata *et al.*, 2002; Romay *et al.*, 2003). It has also been reported that phycocyanin has hepatoprotective, anti-inflammatory and anti-arthritis properties due to its suppression of the activation of cyclooxygenase-2 (COX-2) and its inhibition of lipid peroxidation from free radicals (Bhat *et al.*, 2001; Reddy *et al.*, 2000). Phycocyanin enhances the biological defense activity against infectious diseases by sustaining immune system function and inhibits allergic inflammation by

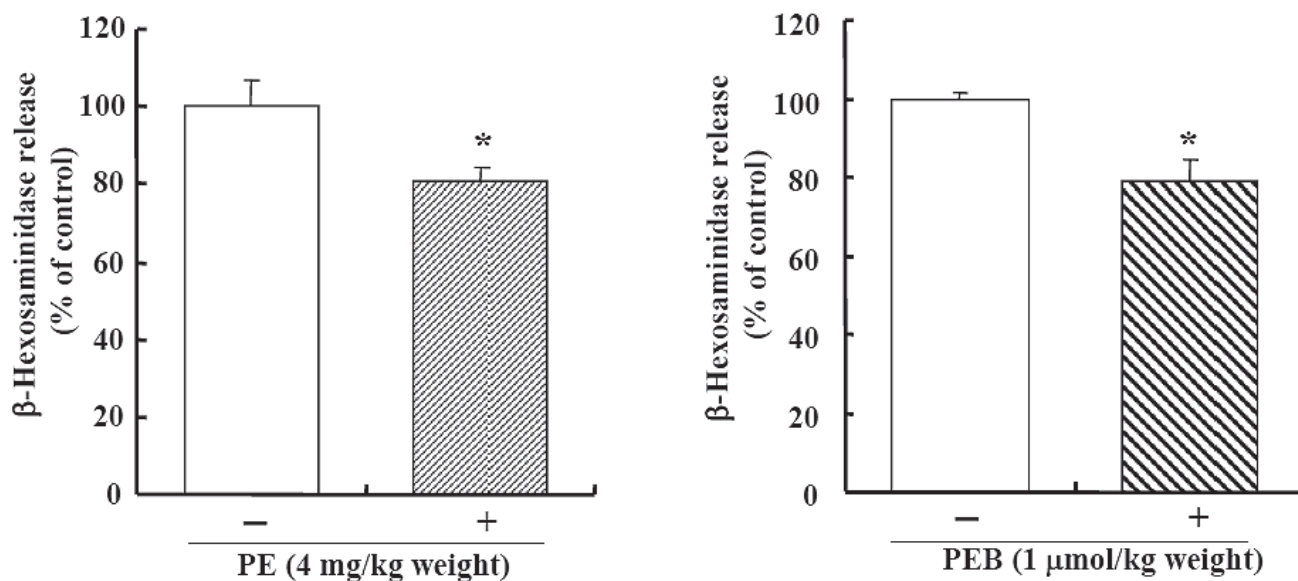


Fig. 4. Evaluation of PEC degranulation from rats treated orally with phycoerythrin or phycoerythrobilin.

Rats were treated orally with 4 mg/kg phycoerythrin (A) or 1 $\mu\text{mol/kg}$ phycoerythrobilin (B). Ninety min after treatment, PEC were isolated and then stimulated with 1 $\mu\text{mol/L}$ A23187 for 20 min. Released β -hexosaminidase was measured. Values are means \pm SD, $n = 3$. *Significantly different from control, $P < 0.05$. Abbreviations are the same as those given for Fig. 2.

suppressing the production of antigen-specific IgE (Nemoto-Kawamura *et al.*, 2004). However, there is little information about food functions of phycoerythrin from red algae. In this study, we demonstrated that phycoerythrin and phycoerythrobilin significantly suppress the degranulation of mast cells. This is the first report that demonstrates the anti-inflammatory effects of phycoerythrin and phycoerythrobilin.

Porphyra spp. generally contain approximately 3–5 g phycoerythrin per 100 g dry weight. Twenty-five phycoerythrobilin molecules are bound together to form a phycoerythrin molecule (MW 268,000) (Alan and Alexander, 1984). The doses of phycoerythrin and phycoerythrobilin in the present study were equivalent to the intake of 5–12 g dried nori, prepared from *P. yezoensis*, for humans. Thus, the daily consumption of nori might be effective in preventing allergy.

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