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Anti-inflammatory effects of dulse (*Palmaria palmata*) resulting from the simultaneous water-extraction of phycobiliproteins and chlorophyll *a*

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ABSTRACT

The use of dulse (*Palmaria palmata*) as a source of edible anti-inflammatory products was evaluated in this study. Phycobiliproteins and chlorophyll *a* were simultaneously extracted from lyophilized dulse leaves *via* water-extraction, and subjected to thermolysin digestion to produce thermolysin-digested water-extract (*d*-DWE). *d*-DWE significantly reduced tumor necrosis factor-α, interleukin-6, and nitric oxide in LPS-stimulated murine macrophages (RAW 264.7 cells), and orally administered *d*-DWE mitigated acute inflammation in carrageenan-induced paw edema of mice. Mass spectrometry revealed *d*-DWE contained peptide LRDGEIILRY (derived from phycoerythrin β-chain) and chlorophyll *a* decomposition products, and they individually reduced the secretion of the proinflammatory mediators in LPS-stimulated RAW 264.7 cells. These results indicate the anti-inflammatory activity could be from a combined effect of phycobiliprotein and chlorophyll *a* decomposition products prepared from the water-extract of dulse. Thus, inexpensive and safe water-extraction method is effective for the extraction of anti-inflammatory components from dulse.

1. Introduction

Inflammation is an important host defense reaction by the immune system, which protects the body from harmful stimuli such as damaged cells, irritants, and invasive pathogens. On the other hand, recent medical findings strongly suggest the importance of regulating excessive inflammation for human health, as excessive or chronic inflammation often leads to inflammatory disorders and provokes age-related diseases (Iwalewa, McGaw, Naidoo, & Eloff, 2007). Recently, there is evidence that diet can play an important role in reducing inflammation (Galland, 2010; Wu & Schauss, 2012), and various food materials have been screened for anti-inflammatory effects and various kinds of anti-inflammatory food compounds have been identified (Lee & Pan, 2013).

Seaweed is recognized as an important biomass for supplying bioactive compounds, as well as being an important seafood resource. For example, brown seaweeds rich in polysaccharides have attracted a lot of attention (Gupta & Abu-Ghannam, 2011) as a source of functional dietary fiber having immunomodulating (Katayama, Nishio, Kishimura, & Saeki, 2012), anti-tumor (Sheng et al., 2007), and anti-oxidant activity (Chandini, Ganesan, & Bhaskar, 2008). Focusing on the study of anti-inflammatory function, polysaccharides (Hwang et al., 2011) and phycobiliproteins (Sakai, Komura, Nishimura, Sugawara, & Hirata, 2011) derived from seaweed have been shown to have anti-inflammatory effects. Moreover, recent research has indicated that chlorophylls and their degradation products have inhibitory effects on the production of proinflammatory mediators in immune cells (Islam et al., 2013). These findings are encouraging a novel utilization of seaweeds as an edible anti-inflammatory material.

Dulse (*Palmaria palmata*), a red algae, grows in high latitude zones of the Atlantic and Pacific Oceans and is a popular edible seaweed in Ireland, Brittany (France), Maine (USA), and Nova Scotia (Canada) (Mouritsen et al., 2013). However, dulse is an under-utilized seaweed in Northern Asia, and in particular it is rarely consumed in Japan. In kelp aquaculture, one of the most important marine industries in Northern Japan, dulse is considered problematic because it often overgrows at the surface of kelp seedling ropes and hinders seedling growth. On the other hand, dulse is beneficial as a biomaterial, with fast growth and a high protein content reaching > 30% of dry weight (Mouritsen et al., 2013). The major constituents of dulse proteins are phycobiliproteins, phycoerythrin and phycocyanin (Gantt, Lipschultz, Grabowski, & Zimmerman, 1979). They are light-harvesting pigment-proteins and exist as a water-soluble protein complex called a phycobilisome, which is connected to Photosystem II containing chlorophylls (Gantt, 1996; Zilinskas & Greenwald, 1986). Since phycobiliproteins and chlorophylls have been shown to possess anti-inflammatory activity, as described above, effective utilization of these biologically active compounds may contribute to developing value-added

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applications of dulse resources.

The objective of this study was to evaluate the use of dulse as a source of edible anti-inflammatory products. Water-extraction, a conventional food processing method, was used to prepare an anti-inflammatory extract from dulse, and the anti-inflammatory activity of dulse water-soluble components was evaluated using *in vitro* and *in vivo* models of inflammation. In addition, the anti-inflammatory compounds contained in the water-extract were identified using MALDI-TOF-MS analysis.

2. Materials and methods

2.1. Preparation of water-soluble components of dulse

Dulse was collected at the coast of Hakodate city, Hokkaido, the Nothern island of Japan. The collected dulse leaves were gently washed with tap water and lyophilized using a laboratory freeze-dryer (FDU-506, Tokyo Rikakikai Co. Ltd., Tokyo, Japan). The lyophilized leaves were ground into powder using a wonder blender (WB-1, Osaka Chemical Co. Led., Osaka, Japan) and passed through a sieve with a mesh size of 500 μm, and the powder was suspended 20-fold (w/v) with cold distilled water. After gently stirring at 4 °C overnight, the suspension was centrifuged at 10,000*g* for 15 min and the supernatant was collected as the first-step-water-extract (FWE). Saturated ammonium sulfate was added to FWE to a final concentration of 70% and followed by centrifuging at 15,000*g* for 15 min to remove mycosporine-like amino acids and polysaccharides. After being resuspended with 70%-saturated ammonium sulfate three times, the precipitate collected by the centrifugation was dissolved in distilled water and subjected to dialysis with 10-fold volume of cold distilled water to remove ammonium sulfate. The final water-soluble fraction was collected after centrifugation at 15,000*g* for 15 min and the supernatant was used as the dulse water-extract (DWE) for the anti-inflammatory assays. DWE was stored at − 60 °C until the thermolysin-digestion described below. The yield of DWE prepared from the washed and lyophilized leaves was approximately 20% (*w*/w).

2.2. UV–visible absorption spectroscopy of water-soluble fractions

Absorption spectra of FWE and DWE were measured in the range of 200–700 nm using a spectrophotometer (U-1800, Hitachi High-Technologies Corporation, Tokyo, Japan). Samples were diluted with distilled water to adjust the absorption maximum value to < 1.0. The pH of the water-extract was 6.5, and was adjusted to 11.0 by the addition of 1.0 M NaOH.

2.3. Quantitative analysis of phycobiliprotein and chlorophyll a in DWE

The protein concentration of the DWE was measured by the Bradford method(Bradford, 1976) using a Bio-Rad protein assay kit (Bio-Rad, Richmond, CA, USA). Chlorophyll *a* was extracted from the DWE using 80% acetone and the concentration (nmol/mL) was calculated using the following equation(Porra, Thompson, & Kriedemann, 1989): $12.25 \times A^{663.6} – 2.55 \times A^{646.6}$, where $A^{663.6}$ and $A^{646.6}$ were the absorbances at 663.6 and 646.6 nm obtained using a 1-cm cuvette. The concentrations of phycobiliprotein and chlorophyll *a* determined in the DWE were used to calculate the yields from the lyophilized leaves.

2.4. Enzymatic digestion of DWE

DWE was dissolved in distilled water (10 mg/mL) and digested with thermolysin (from *Bacillus thermoproteolyticus rokko*, Wako Pure Chemical Industries, Osaka, Japan) at 70 °C for 3 h (enzyme:DWE = 1:100 [w/w]). The pH of the reaction mixture was adjusted to 8.0 during the digestion process to keep the optimum condition for the enzymatic reaction, and the enzymatic reaction was terminated by boiling samples for 15 min (no insoluble matter was generated). The thermolysin-digested DWE (*d*-DWE) thus obtained was lyophilized, and stored at − 60 °C until use.

2.5. Preparation of phycobilin fraction from DWE

Phycobilin (pigment moiety) was cleaved from phycobiliproteins by hot alcohol treatment and collected according to the method of Chapman et al. (Chapman, Cole, & Siegelman, 1968) with slight modification. Briefly, DWE was lyophilized and refluxed with 99.5% ethanol at a final concentration of 10 mg/mL at 100 °C for 1 h. After cooling to room temperature and centrifuged at 15,000 × *g* for 10 min, the supernatant was evaporated to remove the ethanol. The dried residue was dissolved in distilled water and the waters-soluble fraction was obtained as phycobilin fraction. The resulting phycobilin fraction was stored at − 60 °C until use.

2.6. SDS-PAGE analysis of DWE digestion

Digestion of DWE was analyzed by Tricine SDS-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) (Schägger & Jagow, 1987). After electrophoresis, the gel was soaked in 40% methanol and 10% acetic acid for 30 min to fix the protein fragments. To observe the behavior of phycobiliproteins in *d*-DWE, fluorescence from phycobilin was detected. Fluorescence imaging was carried out using a gel documentation system equipped with LED illuminator (Light Capture II, ATTO, Tokyo, Japan). The gel was then stained with Bio-Safe CBB G-250 Stain (Bio-Rad) and destained using distilled water to observe protein subunit component.

2.7. Assessment of anti-inflammatory activity using lipopolysaccharide (LPS)-stimulated macrophages

RAW 264.7 murine macrophage cell line (American Type Culture Collection) was used to assess anti-inflammatory activity by measuring nitric oxide (NO), anti-murine tumor necrosis factor-α (TNF-α), anti-murine interleukin-6 (IL-6). The RAW 264.7 cells were maintained and cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 0.1 mM non-essential amino acids (10% FBS-DMEM) at 37 °C in a 5% CO_2 humidified atmosphere. The culture reagents were purchased from Life Technologies (Carlsbad, California, USA). *d*-DWE from dulse was dissolved in phosphate buffered saline (PBS, pH 6.8) and filtered through a 0.22-μm membrane. In the preliminary cytotoxicity assessment using WST-1 (Saigusa, Nishizawa, Shimizu, & Saeki, 2015), *d*-DWE used in this study showed no negative effect on cell proliferation of RAW 264.7 cells at a final concentration of 100–1000 μg/mL. In the analysis of NO production, RAW264.7 cells (200 μL, 2×10^5 cells/well) were seeded in 96-well plates, allowed to adhere for 2 h, and washed twice with phosphate-buffered saline prior to sample-treatment. Cells were cultured in 200 μL of fresh 10% FBS-DMEM (without phenol red) containing *d*-DWE (0–500 μg/mL), 0.5 ng/mL of Interferon-γ (IFN-γ; Peprotech, Inc., Rocky Hill, NJ, USA), and 2.5 ng/mL of LPS (from *Salmonella typhimurium*, Sigma-Aldrich, Missouri, USA) for 24 h. IFN-γ (2.5 ng/mL), and LPS (5 ng/mL) were used only when analyzing the effect of phycobillin. The secretion of NO was directly measured in the culture supernatants using the Griess method (Baer, Schmidt, Mayer, & Kukovetz, 1995) with sodium nitrite as a standard. In the analysis of TNF-α and IL-6 production, *d*-DWE (0–500 μg/mL) and LPS (2.5 ng/mL) were added to RAW264.7 cells seeded in 96-well plates and cultured in fresh 10% FBS-DMEM as described above. After cultivation for 24 h, the culture supernatants were analyzed by sandwich enzyme-linked immunosorbent assay (ELISA) to measure TNF-α and IL-6 concentrations (Nishizawa, Saigusa, & Saeki, 2016).