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In vitro and *In vivo* Effects of *Gelidium amansii* on Intestinal Immune System

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Abstract Purified compound with intestinal immune system-modulating properties, GWE-2c, was isolated from methanol extract of *Gelidium amansii* by sequential procedures with silica gel column, LH-20 Sephadex gel column, and thin-layer chromatographies. In the presence of GWE-2c, strong immunoactivity in Peyers patch cell-mediated bone marrow cells was observed *in vitro*. *In vivo* intestinal immune-modulating activity was also enhanced by crude phenolic compound (GWE) of *G. amansii* in a dose-dependent manner. Investigation of production of several cytokines in Peyer's patch cells upon stimulation with GWE *in vivo* revealed the levels of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-6 increased. Results suggest that the phenolic compound from *G. amansii* represents immunopotentiator and biological response modifier at *in vitro* and *in vivo* levels.

Keywords: *Gelidium amansii*, intestinal immune system, Peyer's patches, GM-CSF, IL-6, immunomodulator

Introduction

Gelidium amansii, a well-known red algae, is widely used in the agar industry due to its high gelling strength and low sulfate content. It is employed to can foods as a gelling agent, and in beer, coffee, and ice cream as a clouding agent. In some Asian countries such as Korea, Japan, China, and Indonesia, *G. amansii* is also consumed freshly or in dried or pickled forms (1, 2). Recently, it has been used as an ingredient for diet food because it is high in dietary fiber. However, little is known about the biologically active functions and related compounds of *G. amansii* as a medicinal source.

The mucosal epithelial layer of gut associated lymphoid tissue (GALT) forms the interface between the external and internal environments in the gastrointestinal tract (3). The GALT is composed of Peyer's patches, intraepithelial lymphocytes, mesenteric lymph nods, lamina propria, and thoracic duct. The intestinal immune system largely contributes to the defense system of mucosa and the regulation of systemic immune system such as systemic inflammatory response syndrome and sepsis. Peyers patches, which have a follicular structure with T- and B-lymphocytes, macrophages, and dendritic cells, are the main inductive sites of the intestinal immune system (4). In this inductive site, B and T cells are primed against intestinal antigens, resulting in the activation and alternation of the lymphocyte-homing receptors (5). Upon existing Peyer's patches, sensitized IgA-committed B cells migrate through the mesenteric lymph nodes and spleen, possibly encountering additional differentiation signals before their arrival at distant mucosal sites. Therefore, Peyer's patches essentially appear to be a factory producing activated precursor plasma cells, which, through their special characteristics and homing capabilities, return and generate immune responses within the gut tissues (6).

In recent years, search for the natural immunomodulators of intestinal immune system has received a great deal of attention due to their efficacy in the suppression of allergic reactions and autoimmune diseases with reduced side effects (7). In the large intestine, dietary fiber is fermented into short-chain fatty acids by intestinal bacteria, which, in turn, exerts stimulatory intestinal immune functions such as elevated mesenteric lymph node antibody concentrations and increased amount of immunoglobulin A (8). Some plant-derived polysaccharides modulate the Peyers patches in the intestinal immune system, leading to an increase in the immune response (9). However, most attempts on these researches have mainly focused on the dietary fiber and plant-derived polysaccharides. Therefore, it is necessary to evaluate the action of algae on the immune systems of intestine and identify their active small molecular components.

In the present study, we isolated the intestinal immunomodulator from *G. amansii* and investigated its *in vitro* and *in vivo* stimulatory effects in the intestinal immune system. In addition, the secretion of cytokines (granulocyte-macrophage colony-stimulating factor and interleukin-6) upon the *in vivo* induction of the intestinal immunomodulator was monitored.

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Materials and Methods

Sample and chemicals *G. amansii* cultivated in Jeju

Island, Korea was obtained from Chung-Boo market (Seoul, Korea), and was authenticated by Dr. Sangin Shim at Gyeongsang National University. The voucher specimen was deposited at the same institute. RPMI-1640 medium, fetal bovine serum (FBS), and penicillin-streptomycin were purchased from GIBCO (Grand Island, NY, USA). Hank's balanced salt solution (HBSS) and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Alamar Blue™ was the product of Alamar Bio Sciences Inc. (Sacramento, CA, USA). Silca gel, and analytical and preparative TLC were from Merck Co. (Darmstadt, Germany). Rat anti-mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-6 mAb (clone MP1-22E9 and MP5-20F3, respectively) were purchased from PharMingen (San Diego, CA, USA). All other chemicals and solvents were of analytical grade.

Extraction and isolation The plant material was homogenized in an Ultra Turrax homogenizer (7,000 rpm, 20 min). After centrifuging the homogenate (5,000×*g*, 30 min), the supernatant was dried in an evaporator under reduced pressure. The crude extract was then suspended in water and re-extracted with hexane, acetone, methanol, and water to give the respective fractions. After the methanol-soluble fraction was partitioned with water, chloroform, ethyl acetate, and butanol, the ethyl acetate solubles were applied with chloroform to silica gel column containing increasing amounts of methanol as an eluent. Fractions eluted with chloroform-methanol (60:40) were further purified by column chromatography on Sephadex LH-20 with 50% methanol as an eluent, resulting in two subfractions, GWE-1 and GWE-2. The GWE-2 fraction was developed on a preparative thin layer chromatogram with a mixture of chloroform and methanol (9:1) as mobile phase, yielding five subfractions.

HPLC analysis HPLC was performed using a Young-Lin M-930 instrument equipped with an M-720 UV detector and a reversed-phase C-18 column (Lichrospher, 150-4, Waters). A combination of acetonitrile, methanol, and water (1:3:96) was used as mobile phase at a flow rate of 0.5 mL/min. The UV-absorbing substances were detected at 290 nm.

Intestinal immune system-modulating activity The intestinal immune system-modulating activities of the crude extracts and compounds were determined according to the procedure of Hong *et al.* (10). Peyer's patches were isolated from the small intestines of the C3H/He mice (female, Daihan-Biolink Co., Chungbuk, Korea) and suspended in RPMI 1640 medium containing 5% FBS, penicillin (100 U/mL), and streptomycin (100 μL/mL). The cell suspension (180 μL, 2.2×10^6 cells/mL) was cultured with 20 μL distilled water (control) or tested samples in 96-well flat bottom plates at 37°C for 5 days under a humidified atmosphere of 95% air and 5% CO_2. Resulting culture supernatant (50 μL) was incubated with a bone marrow cell suspension (2.5×10^5 cells/mL) from C3H/He mice under the same conditions described above. At day 6, the number of proliferated bone marrow cells was determined by the Alamar Blue™ reduction assay (11). The intestinal immune system-modulating activity was expressed as the stimulation of cell growth of bone marrow cells compared with that of the control (Peyers patch cells incubated with distilled water).

For the *in vivo* assay, sample was fed to specific pathogen-free C3H/He mice for 14 consecutive days. The Peyer's patch cells were then prepared from the small intestine of mice. Subsequent procedures were identically performed as described above.

ELISA for the production of GM-CSF and IL-6 The level of GM-CSF in the culture of Peyer's patches was determined by enzyme-linked immunosolvent assay (ELISA). For the preparation of Peyer's patches, tested samples were orally administered into specific pathogen-free C3H/He mice at 0.25, 0.50, 0.75, and 1.00 g/kg/day. Control mice received distilled water instead of the sample solution. After the preparation of Peyers patch cells at day 14, the cells were incubated for 5 days at 37°C under a humidified atmosphere of 95% air and 5% CO_2. A solution (10 μg/well) of purified anti-mouse GM-CSF mAb (clone MP1-22E9) in 50 μL bicarbonate buffer (pH 8.5) was added to the microtiter plates for ELISA (MaxiSorp™ F96, Nunc, Roskide, Denmark) at 4°C overnight. Unbound antibody was removed by washing with PBS containing 0.05% Tween 20 (PBS-Tween). The plates were further incubated with 3% BSA in PBS at room temperature for 2 hr (blocking). Samples containing GM-CSF or conditioned medium of Peyer's patch cell were added to the first antibody-coated well at 100 μL/well, and the plates were further incubated at room temperature for 1 hr. After the plates were washed four times with PBS-Tween, 100 μL biotinylated anti-mouse GM-CSF (clone MP1-31G6, PharMingen) in PBS containing 10% fetal calf serum (FCS) was added to the wells, and the plates were incubated for 4 hr at room temperature. The plates were then washed six times with PBS containing 0.05% Tween 20. Alkaline phosphatase-labeled streptavidin diluted with PBS-Tween containing 10% fetal calf serum (1:2000) was added to each well, and the plates were then incubated at room temperature for 1 hr. After washing the wells with PBS-Tween eight times, each well was incubated with 150 μL chromogenic substrate solution (1 mg *p*-nitrophenyl-phosphate disodium salt in 1 mL of 1.0 M diethanolamine buffer, pH 9.8). The absorbance was measured at 405 nm using a microplate reader.

For quantitative measurement of IL-6 in the supernatant of Peyer's patch cultures, ELISA employing the multiple antibody sandwich principle was used. A solution (2 μg/well) of purified anti-mouse IL-6 mAb (clone MP5-20F3) in 50 μL PBS was added to the microtiter plate, which was kept at 4°C overnight. Unbound antibody was removed by washing with PBS-Tween. The subsequent procedures were performed as described above, except for using biotinylated anti-mouse IL-6 (clone MP5-32C11, PharMingen) as the 2nd antibody.

Statistical analysis Data were analyzed by the difference between means, and statistical significance was calculated from Fisher's least significant difference (LSD) or Student's t-test.

Results and Discussion

Isolation of intestinal immune system modulator from *Gelidium amansii* Food constituents possess not only the nutritional values but also exert physiological effects by modulating the immune, nervous, and endocrine systems (12). Therefore, natural medicines have been frequently used in the treatment of several diseases such as autoimmune diseases, inflammations, and allergies, which are difficult to cure using western medicines. In the present investigation, we isolated the immunopotentiating compound from *G. amansii*. The methanol extractives (10 g) of *G. amansii*, which exhibited a strong intestinal immune system-modulating activity (1.3-fold of control) assayed by enhancing the bone marrow cell-proliferative cytokines from Peyers patch cells, were divided into water-, chloroform-, ethyl acetate-, and butanol-solubles, among which the ethyl acetate fraction showed the highest intestinal immuno-stimulatory activity (Fig. 1). Further fractionation was performed using the sequential procedures with silica gel column, Sephadex LH-20 gel column, and thin-layer chromatographies. Relatively high intestinal immune system-modulating activity was found in the spot (GWE-2c; 46 mg) showing an R_f value of 0.25 among the five subfractions obtained by thin layer chromatography (Table 1). The purified intestinal immuno-stimulatory compound, GWE-2c (UV l_{max} value, 290 nm), showed a single peak on HPLC, indicating its high purity (Fig. 2). The purified compound was reactive with Folin-Ciocalteu's reagent, H_2SO_4, and 1N NaOH, while no reaction was observed with anthrone, molish, ninhydrin, ehrlich, and ferric chloride. These results implied that intestinal immune system modulator from *G. amansii* might be the phenolic compound. This is somewhat interesting, because the intestinal immune-modulating action of plant constituents has been found to be largely related to polysaccharides (8, 9).

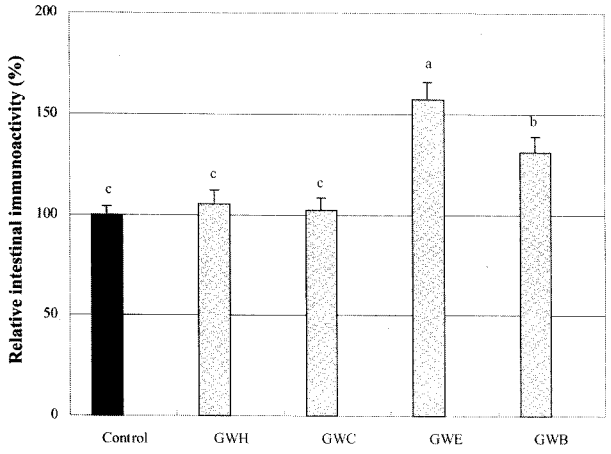

Fig. 1. Intestinal immune system modulating activities of various solvent fractions from the methanolic extract of *Gelidium amansii*. The intestinal immune system modulating activity was calculated as the percentage of the growth of Peyer's patch cell-mediated bone marrow cells of a sample to a control. Peyer's patch cells from C3H/He mice were incubated with each sample at a concentration of 100 μg/mL. The GWH, GWC, GWE and GWB represent water-, chloroform-, ethyl acetate- and butanol-solubles, respectively. Values are the mean±S.D. of three replicates. Different letters above the bar are statistically different by the analysis of Fisher's least significant difference ($p < 0.01$).

Table 1. Intestinal immune system modulating activities of subfractions from *Gelidium amansii* by thin layer chromatography

Subfractions	Relative intestinal immunoactivity (%)
Control	100±3.8 d
GWE-2a	123±4.6 c
GWE-2b	109±4.9 cd
GWE-2c	179±6.7 a
GWE-2d	144±3.6 b
GWE-2e	119±5.8 c

The intestinal immune system modulating activity was calculated as the percentage of the growth of Peyer's patch cell-mediated bone marrow cells of a sample to a control. Peyer's patch cells from C3H/He mice were incubated with each sample at a concentration of 100 μg/ml. Data represent the mean±S.D. of three replicates. GWE-2a = R_f value of 0.06, GWE-2b = R_f value of 0.19, GWE-2c = R_f value of 0.25, GWE-2d = R_f value of 0.44, and GWE-2e = R_f value of 0.88. Values in a column indicate with different letters are significantly different ($p< 0.01$).

In vivo* intestinal immune system-modulating effect by *G. amansii The effects of crude phenolic compound (GWE; ethyl acetate solubles of methanol extract) from *G. amansii* on Peyers patch cell-mediated bone marrow cell proliferation were investigated as measures of *in vivo* immune responses. Peyers patches, which were obtained from C3H/He mice fed with GWE at 0.25, 0.50, 0.75, and 1.00 g/kg/day for 14 days, were cultured for 5 days. Subsequently, the cultures were added to the bone marrow cells for the determination of intestinal immune system-modulating activity. The stimulatory effect for the proliferation of bone marrow cells was exhibited in the crude phenolic compound from *G. amansii* (Fig. 3). Up to 0.75 g/kg/day, GWE incited the intestinal immune system-modulating activity in a dose-dependent manner, while no difference in the activity was found in the 0.75 and 1.00 g/kg/day samples. The stimulatory responses of bone marrow cell proliferation is likely to occur through Peyer's patches, which are important lymphoid organs of the intestine. A close correlation was observed between an increase in the number of Peyer's patch lymphocytes and an elevated mucosal defense (13). Various immuno-

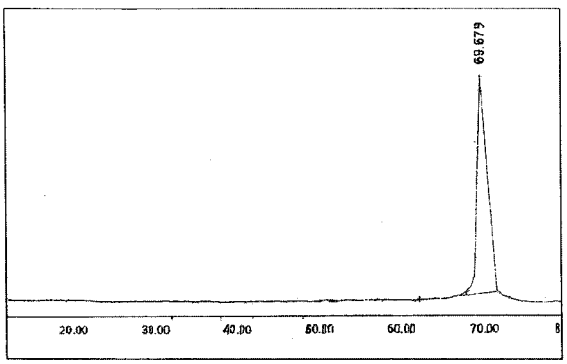

Fig. 2. HPLC profile of intestinal immune system modulating compound from *Gelidium amansii*. The HPLC analysis was performed on a Young-Lin M-930 instrument, using a reversed-phase C-18 column (3.9 × 150 mm) column and an M-720 UV detector at 290 nm. A mixture of acetonitrile, methanol and water (1:3:96, v/v/v) was used as mobile phase with a flow rate of 0.5 ml/min.

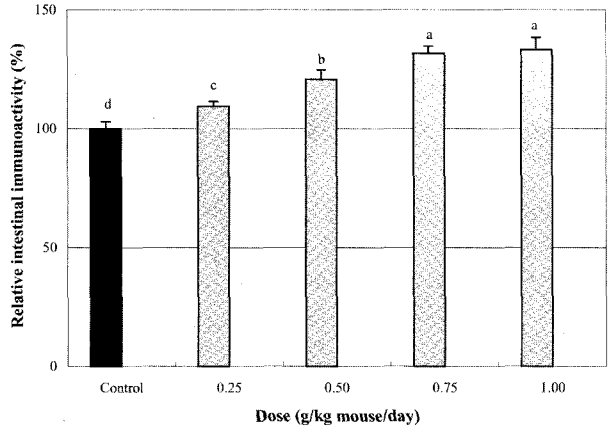

Fig. 3. Effects of orally administered gwe obtained from *Gelidium amansii* on peyer's patch cell-mediated bone marrow cell proliferation. Peyer's patches from C3H/He mice fed with the sample (GWE) at various doses for 14 days, were cultured for 5 days. The intestinal immune system modulating activity was calculated as the percentage of the growth of Peyer's patch cell-mediated bone marrow cells of a sample to a control. The GWE represents ethyl acetate-solubles from the methanolic extract of *Gelidium amansii*. Values are the mean±S.D. of four replicates. Different letters above the bar are statistically different by the analysis of Fishers least significant difference ($p < 0.01$).

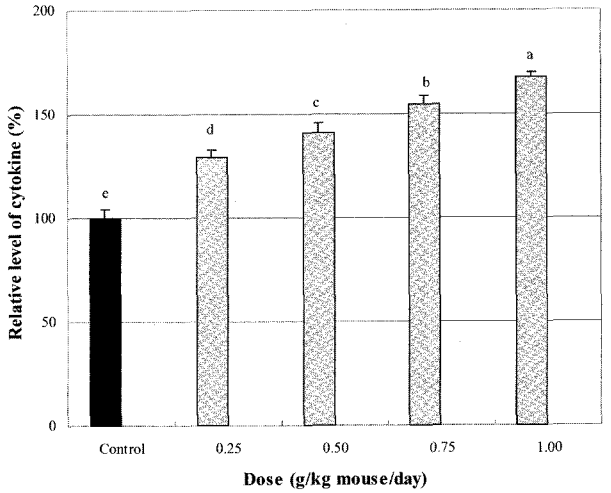

Fig. 4. Stimulation of murine Peyer's patch cells for the production of GM-CSF by GWE obtained from *Gelidium amansii*. The sample (GWE) was orally administered into C3H/He mice at the various doses. At day 14, Peyer's patches were cultured from mice. After the incubation for 5 days, the resulting cell-free supernatants were subjected to ELISA for GM-CSF as described in Materials and Methods. The GWE represents ethyl acetate-solubles from the methanolic extract of *Gelidium amansii*. Values are the mean±S.D. of four replicates. Different letters above the bar are statistically different by the analysis of Fishers least significant difference ($p < 0.01$).

modualting substances could be effective simulators of Peyer's patch cells. Generally, macromolecules such as polysaccharides, rather than low-molecular-weight substances, have drawn attention as potent immunological compounds (14, 15). In the present investigation, we demonstrated that oral administration of phenolic compound from *G. amansii* could stimulate the murine intestinal immune system. In spite of the great interests in the physiological and pharmacological effects of phenolic compounds (16, 17), few studies are available concerning the influence of phenolic compounds on the intestinal immune system.

Production of GM-CSF and IL-6 by *G. amansii in vivo*

Figures 4 and 5 show the changes in the secretion of cytokines by GWE from *G. amansii* in the Peyer's patch cells. The levels of GM-CSF in the conditioned medium of Peyer's patch cells were significantly increased compared to the control (Fig. 4). The production of GM-CSF was gradually increased by the administration of up to 1.00 g/kg/day of GWE to the mice, indicating the high correlation between the dose of GWE and the level of GM-CSF. In the case of IL-6 production, a drastic increase in the level was observed at 0.75 g/kg/day of GWE, while no difference was found between the doses of 0.75 and 1.00 g/kg/day (Fig. 5). These results suggest that GM-CSF and IL-6 may, at least in part, contribute to the proliferation of Peyer's patch cell-mediated bone marrow cells.

Lymphocytes, typically activated T cells, are known to secrete growth factors such as GM-CSF and IL-6 (18). These growth factors stimulate the proliferation of hematopoietic cells, followed by the differentiation into granulocytes or macrophages (19, 20). In the present study, we demonstrated that the oral administration of crude phenolic compound from *G. amansii* to mice enhanced the secretion of hematopoietic growth factors from Peyer's patch cells. T cells are known as the sources of CSF's and

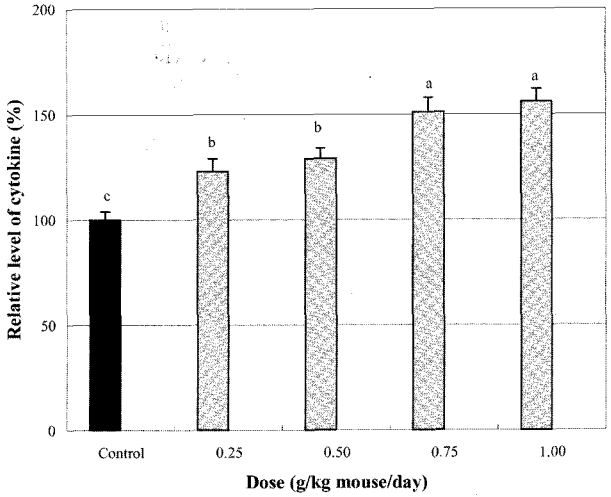

Fig. 5. Stimulation of murine Peyer's patch cells for the production of IL-6 by GWE obtained from *Gelidium amansii*. The sample (GWE) was orally administered into C3H/He mice at the various doses. At day 14, Peyer's patches were cultured from mice. After the incubation for 5 days, the resulting cell-free supernatants were subjected to ELISA for IL-6 as described in Materials and Methods. The GWE represents ethyl acetate-solubles from the methanolic extract of *Gelidium amansii*. Values are the mean±S.D. of four replicates. Different letters above the bar are statistically different by the analysis of Fisher's least significant difference ($p < 0.01$).

various cytokines (21). Therefore, T cell activation, which is caused by oral administration of GWE, may contribute to the secretion of GM-CSF and IL-6 from Peyer's patch cells. Cytokines such as GM-CSF and IL-6 have been reported to act on the cells participating in the systemic immune response (7).

The regulatory molecules of the intestinal immune system have potential as new immunomodulators of mucosal and systemic immune systems. Results of the present study showed that the phenolic compound from *G. amansii* exhibit stimulatory effects on the intestinal immune system. Strong intestinal immune system-modulating activity was observed both *in vitro* and *in vivo*. Also, the production of such cytokines as GM-CSF and IL-6 appears to be significantly induced by the phenolic compound from *G. amansii* in the Peyers patch cells *in vivo*. The presence of these cytokines may aid the overall activation of intestinal immune system. Research is underway to characterize the structural identification of the intestinal immune system-modulating compound from *G. amansii*, and to elucidate the mechanisms of intestinal immune responses in its presence.

Acknowledgments

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