

RESEARCH ARTICLE

The role of peptides derived from *Spirulina maxima* in downregulation of FcεRI-mediated allergic responses

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Scope: *Spirulina* has been found suitable for use as a bioactive additive. It is an excellent source of protein that can be hydrolyzed into bioactive peptides. Two peptides LDAVNR (P1) and MMLDF (P2) purified from enzymatic hydrolysate of *Spirulina maxima* have been reported to be effective against early atherosclerotic responses. In this study, the intracellular mechanism involved in the downregulation of these peptides on high-affinity IgE receptor-mediated allergic reaction was further investigated.

Methods and results: RBL-2H3 mast cells were pretreated with P1 or P2 and sensitized with dinitrophenyl-specific IgE antibody before stimulation of antigen dinitrophenyl-BSA. It was revealed that P1 and P2 exhibited significant inhibition on mast-cell degranulation via decreasing histamine release and intracellular Ca²⁺ elevation. The inhibitory activity of P1 was found due to blockade of calcium- and microtubule-dependent signaling pathways. Meanwhile, the inhibition of P2 was involved in suppression of phospholipase Cγ activation and reactive oxygen species production. Moreover, the suppressive effects of P1 and P2 on generation of IL-4 were evidenced via depression of nuclear factor-κB translocation.

Conclusion: These findings indicate that peptides P1 and P2 from *S. maxima* may be promising candidates of antiallergic therapeutics, contributing to development of bioactive food ingredients for amelioration of allergic diseases.

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1 Introduction

Allergy is known as a hypersensitivity reaction of the body's immune system toward normally innocuous environmental substances [1, 2]. Notably, mast cells play a central role in triggering IgE-mediated allergic reactions [3]. Activation of mast cells can be initiated by allergen-induced cross-linking of IgE bound by the high-affinity IgE receptors (FcεRI) on the

cells [4]. Mast-cell activation triggers a cascade of intracellular events, including activation of tyrosine kinases such as Syk and Fyn, phospholipid enzyme phospholipase Cγ (PLCγ), Grb2-associated binder 2 (Gab2), and nuclear factor (NF)-κB; production of reactive oxygen species (ROS), increase of intracellular calcium ion (Ca²⁺) level; polymerization of microtubule; degranulation; and cytokine generation [5]. Mast cells contain numerous prominent cytoplasmic granules staining metachromatically with cationic dyes. Evidently, mast-cell activation causes the process of degranulation that is involved in the movement of cytoplasmic granules to plasma membrane, the fusion of the cytoplasmic granule membranes with the plasma membrane, and the subsequent release of granule-associated stored mediators. Notably, the movement of granules is based on the polymerization and rearrangement of microtubule while the fusion is dependent on the elevation of intracellular calcium. Mast-cell degranulation is

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Abbreviations: DNP, dinitrophenyl; Gab2, Grb2-associated binder 2; NF, nuclear factor; PLCγ, phospholipid enzyme phospholipase Cγ; ROS, reactive oxygen species

considered to be one of the critical steps in allergic responses, causing the release of various preformed mediators such as histamine, proteases, inflammatory cytokines, and lipid mediators [6]. These mediators are the origination of various pathophysiologic events in acute allergic reactions including airway constriction, mucous production, and recruitment of other inflammatory cells [1]. Therefore, various antiallergic drugs have been developed so far, which inhibit degranulation of mast cells.

Spirulina is an unbranched, helicoidal, filamentous blue-green algae or cyanobacterium that has a long history of use as food [7]. *Spirulina* is one of the most concentrated natural sources of nutrition and suitable for use as a bioactive additive. Recently, *Spirulina* is gaining more attention from the medical scientists due to its potential as nutraceutical and pharmaceutical. A number of published studies have reported various biological activities and significant therapeutic effects of *Spirulina* [8, 9]. Notably, *Spirulina*-based dietary supplement can provide protective effect against allergic responses [10, 11]. Therefore, *Spirulina* is considered as a promising agent for allergy treatment. However, the intracellular mechanism underlying the inhibitory effects of *Spirulina* on allergic responses in FcεRI-mediated mast cells remains to be elucidated.

Recently, peptides from protein hydrolysates have received much attention due to their health beneficial effects [12–14]. They can be used as versatile raw materials for producing nutraceuticals and pharmaceuticals for humans. The bioactive peptides have been obtained from various raw materials that contain high protein concentration. Notably, *Spirulina* has been known to be an excellent source of proteins (60–70% of its dry weight) that can be hydrolyzed into bioactive peptides. Prompted by this background, the present study was designed to isolate bioactive peptides from gastric enzymatic hydrolysate of *S. maxima* and evaluated their protective effects against allergic reactions in mast cells mediated allergic model.

2 Materials and methods

2.1 Reagents and materials

S. maxima was kindly donated by the Korea Institute of Ocean Science and Technology (Ansan, Korea). Dinitrophenyl-BSA (DNP-BSA), DNP-specific IgE, and FITC-labeled mouse monoclonal anti-α-tubulin antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). Specific antibodies used for Western blot analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Cell Signaling Technology (Danvers, MA, USA). Calcium-specific fluorescence probe (Fura-3/AM) was purchased from Santa Cruz Biotechnology. All primers using in this study were obtained from Bioneer (Daedeak-gu, Daejeon, South Korea).

Preparation of enzymatic hydrolysate and purification of bioactive peptides have been determined according to Vo et al.

[15]. Peptides were identified as LDAVNR (mass: 686.37, pI: 6.42, net charge: 0) and MMLDF (mass: 655.27, pI: 3.02, net charge: –1).

2.2 Cell culture and cell viability

RBL-2H3 mast cells were purchased from the Korean Cell Line Bank (Seoul, Korea). Cells culture and cell viability were performed as described by Vo et al. [16]. The different concentrations of peptides including 50, 100, and 200 μM are used for 3-(4,5-dimethyl-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. The results were expressed as percentage of surviving cells over blank cells (no addition of peptide).

2.3 Histamine release assay

RBL-2H3 cells were seeded into 24-well plates (2×10^5 cells/mL). Cells were treated with different concentrations of peptides (50, 100, or 200 μM) for 6 h before being sensitized overnight with 1 μg/mL of DNP-specific IgE antibody. The sensitized cells were washed two times with Tyrode buffer and stimulated with 1 μg/mL of DNP-BSA for 60 min. Histamine release in the supernatants was determined as previously described [16]. Histamine release levels were calculated as a percentage compared to control: Release ratio (%) = $(T - B)/(C - B) \times 100$, where B is the group without stimulation as well as sample treatment, C is the stimulated group without treatment of the tested sample, and T is the stimulated group with presence of the tested sample.

2.4 Measurement of the intracellular Ca²⁺ level

RBL-2H3 cells were seeded into black 96-well plates (2×10^5 cells/mL) and treated with peptides (200 μM) for 6 h before being sensitized overnight with 1 μg/mL of DNP-specific IgE antibody. The cells were then incubated with 2 μM of Fura-3/AM for 1 h. Subsequently, the cells were washed with Tyrode buffer and stimulated with 1 μg/mL of DNP-BSA. The Fura-3/AM fluorescence intensity was measured after 2 min of stimulation at an excitation wavelength of 360 nm and an emission wavelength of 528 nm by using a microplate reader (GENios Tecan Austria, Grodig/Salzburg, Austria). In addition, fluorescence images were visualized and photographed under fluorescence microscope (CTR 6000, Leica, Wetzlar, Germany) after addition of 3% paraformaldehyde.

2.5 Fluorescence staining assay for microtubule detection

RBL-2H3 cells were seeded into 12-well plates (1×10^4 cells/mL) and treated with peptides (200 μM)

for 6 h before being sensitized overnight with 1 $\mu\text{g/mL}$ of DNP-specific IgE antibody. Subsequently, the cells were washed with Tyrode buffer and stimulated with DNP-BSA for 30 min. The medium was then removed and 3% paraformaldehyde was added for 10 min. Afterward, the cells were permeabilized with 0.1% Triton X-100 for 15 s and incubated with an FITC-labeled anti- α -tubulin antibody at a 1:100 dilution for 60 min at 37°C. The images were captured under fluorescence microscope.

2.6 Measurement of ROS production

RBL-2H3 cells were seeded into 12-well plates (1×10^4 cells/mL) and treated with peptides (200 μM) for 6 h before being sensitized overnight with 1 $\mu\text{g/mL}$ of DNP-specific IgE antibody. The cells were then incubated with dihydroethidium at the concentration of 5 μM for 1 h at 37°C. For fluorescence staining assay, the cells were washed with Tyrode buffer and stimulated with 1 $\mu\text{g/mL}$ of DNP-BSA for 1 h. The medium was then removed and 3% paraformaldehyde was added for 10 min. Fluorescence images were visualized and photographed under fluorescence microscope. For flow cytometry assay, the cells were washed, harvested, and resuspended into PBS buffer containing 1 $\mu\text{g/mL}$ of DNP-BSA for 1 h at 37°C. Finally, the cells were analyzed by flow cytometry (BD Diagnostic Systems, Cockeysville, MD, USA). The numbers in histograms indicate the percentage of ROS production.

2.7 Molecular docking

Docking calculations were performed as described by Himaya et al. [17]. They were carried out on 1rpq-MEMBRANE and 3h4 w-HYDROLASE protein model, using Docking Server (<http://www.dockingserver.com>).

2.8 Measurement of cytokine production

RBL-2H3 cells were seeded into 24-well plates (2×10^4 cells/mL) and treated with various concentrations of peptides (50, 100, or 200 μM) for 6 h before being sensitized overnight with 1 $\mu\text{g/mL}$ of DNP-specific IgE antibody. The cells were then washed and stimulated with 1 $\mu\text{g/mL}$ of DNP-BSA for 24 h. The supernatants were collected, and the production of IL-4 was quantified by sandwich immunoassays following the protocol of R&D systems.

2.9 Reverse transcription-PCR (RT-PCR) analysis

RBL-2H3 cells were treated with different concentrations of peptides (50, 100, or 200 μM) for 6 h and sensitized overnight

with 1 $\mu\text{g/mL}$ of DNP-specific IgE antibody. The sensitized cells were washed and stimulated with 1 $\mu\text{g/mL}$ of DNP-BSA for 24 h. Total cellular RNA was isolated using Trizol reagent according to the manufacturer's instructions. The cDNA and DNA were synthesized as described by Vo and Kim [18]. Primer sequences used in this study were as follows: for IL-4, sense 5'-TGA-TGG-GTC-TCA-GCC-CCC-ACC-TTG-C-3' and antisense 5'-CTT-TCA-GTG-TTG-TGA-GCG-TGG-ACT-C-3'; for GAPDH, sense 5'-TTC-ACC-ACC-ATG-GAG-AAG-GC-3' and antisense 5'-GGC-ATG-GAC-TGT-GGT-CAT-GA-3'. The samples were visualized by UV light using AlphaEase gel image analysis software (Alpha Innotech, San Leandro, CA, USA).

2.10 Western blot analysis

RBL-2H3 cells were treated with different concentrations of peptides (50, 100, or 200 μM) for 6 h and sensitized overnight with 1 $\mu\text{g/mL}$ of DNP-specific IgE antibody. The sensitized cells were washed and stimulated with 1 $\mu\text{g/mL}$ of DNP-BSA. The cells were lysed in RIPA lysis buffer. The detection of protein was performed as described by Vo and Kim [18]. In a parallel experiment, nuclear protein was prepared using nuclear extraction reagents according to the manufacturer's protocol (Sigma).

2.11 Statistical analysis

Data were analyzed using the analysis of variance (ANOVA) test of statistical package for the social sciences (SPSS). The statistical differences among groups were assessed by using Duncan's multiple range tests. Differences were considered significant at $p < 0.05$.

3 Results

3.1 Effect of P1 and P2 on degranulation in antigen-stimulated RBL-2H3 cells

We first evaluated the effect of the pre-incubation of P1 and P2 on mast-cell degranulation via measuring histamine release. It was found that P1 and P2 reduced histamine release from the activated mast cells in a dose-dependent manner (Fig. 1A). Histamine release levels upon treatment with 200 μM of P1 and P2 were 32 and 40% as compared to control group stimulated with DNP-BSA alone, respectively. In a parallel experiment, the inhibitory effect of peptides on mast-cell degranulation was confirmed via cell morphological changes. As shown in Fig. 1B, the normal cells in blank group were generally branch-shaped with clear membranes, whereas the activated cells in control group were observed to be round-shaped, reduced cell size, disrupted boundaries, and irregular surfaces. However, the pretreated cells with P1 or P2

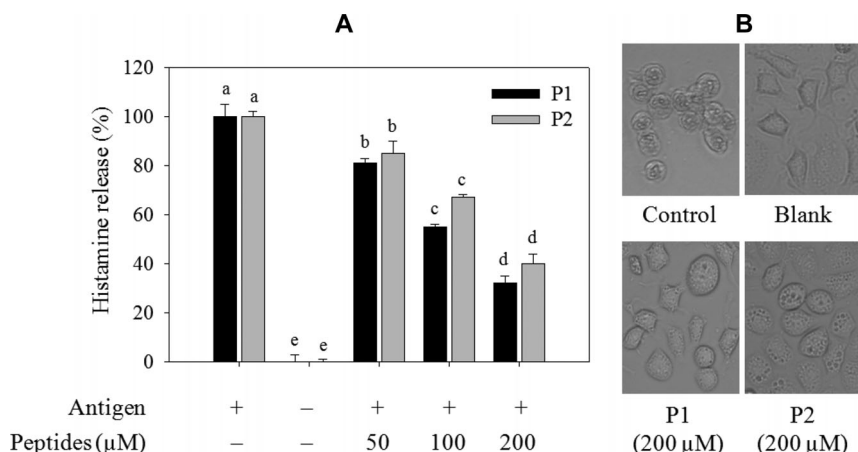


Figure 1. Effect of P1 and P2 on degranulation in antigen-stimulated RBL-2H3 mast cells. The cells were treated with peptides P1 or P2 for 6 h and sensitized overnight with DNP-specific IgE antibody before stimulation of antigen DNP-BSA for 60 min. (A) The levels of histamine release were measured via a spectrofluorometric assay. Each determination was made in triplicate, and the data are shown as means $\pm$ SD. Different letters a–e indicate significant difference among groups ($p < 0.05$) by Duncan's multiple-range test. (B) The representative images of the cells were assessed by using light microscopy (magnification, $\times 20$). Blank: -DNP-BSA-peptide; Control: +DNP-BSA-peptide.

before being exposed to antigen DNP-BSA exhibited protective effect against the morphological changes. Obviously, the inhibitory activity of P1 was higher than that of P2. Especially, the inhibitory activities of P1 and P2 on mast-cell degranulation were not due to any cytotoxicity (Supporting Information Fig. 1).

3.2 Effect of P1 and P2 on microtubule polymerization in antigen-stimulated RBL-2H3 cells

To determine the inhibitory activities of P1 and P2 on mast-cell degranulation, signaling pathway related to microtubule polymerization was analyzed via immunofluorescence staining and Western blot assays. Pronouncedly, the cells exposed to antigen (control group) exhibited high density of microtubule polymerization as compared to the blank group (Fig. 2A). Notably, P1 treatment was able to decrease the level of microtubule polymerization, whereas P2 treatment did not show a significant inhibition. Moreover, Western blot assay revealed that the increase in phosphorylation levels of protein-tyrosine kinases Fyn and adaptor protein Gab2 in the activated mast cells were significantly reduced by P1 in a dose-dependent manner. Conversely, P2 was observed to be not effective in suppression of the activation of Fyn/Gab2-mediated pathway (Fig. 2B).

3.3 Effect of P1 and P2 on intracellular Ca^{2+} elevation in antigen-stimulated RBL-2H3 cells

Furthermore, the inhibitory activities of P1 and P2 on mast-cell degranulation were also demonstrated via investigating signaling pathway related to intracellular Ca^{2+} elevation. As shown in Fig. 3, the activation of mast cells increased phosphorylation of Syk and PLC γ (Fig. 4C), and thus causing elevation of intracellular Ca^{2+} levels (Fig. 4A and B) after the cells were exposed to antigen alone (control groups). Conversely, these increases were remarkably alleviated in the cells

pretreated with P1 or P2 (200 μM) before stimulating with antigen DNP-BSA. It was observed that P1 effectively suppressed the activation of both signaling molecules including Syk and PLC γ while P2 inhibited the activation of PLC γ but not of Syk.

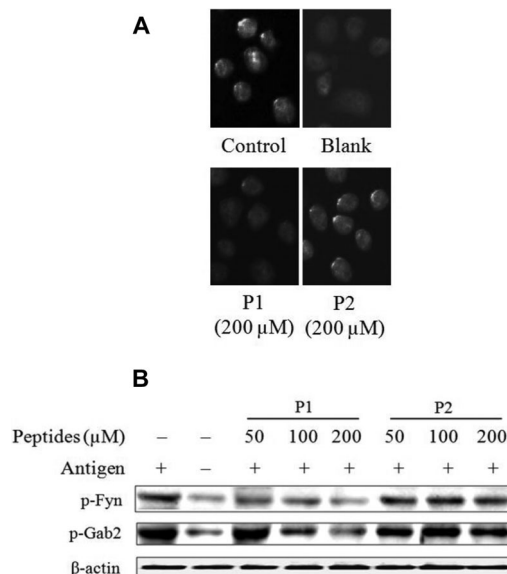


Figure 2. Effect of P1 and P2 on microtubule polymerization in antigen-stimulated RBL-2H3 mast cells. The cells were treated with peptides P1 or P2 for 6 h and sensitized overnight with DNP-specific IgE antibody before stimulation of antigen DNP-BSA for 30 min. (A) The cells were permeabilized with 0.1% Triton X-100 for 15 s and incubated with an FITC-labeled anti- α -tubulin antibody for 60 min. The levels of microtubule polymerization were assessed by using light microscopy (magnification, $\times 20$). Blank: -DNP-BSA-peptide; Control: +DNP-BSA-peptide. (B) The expression levels of microtubule-dependent signaling molecules were assessed by Western blotting analysis. β -actin was used as an internal control. The phosphorylation of Fyn and Gab2 were abbreviated by p-Fyn and p-Gab2, respectively.

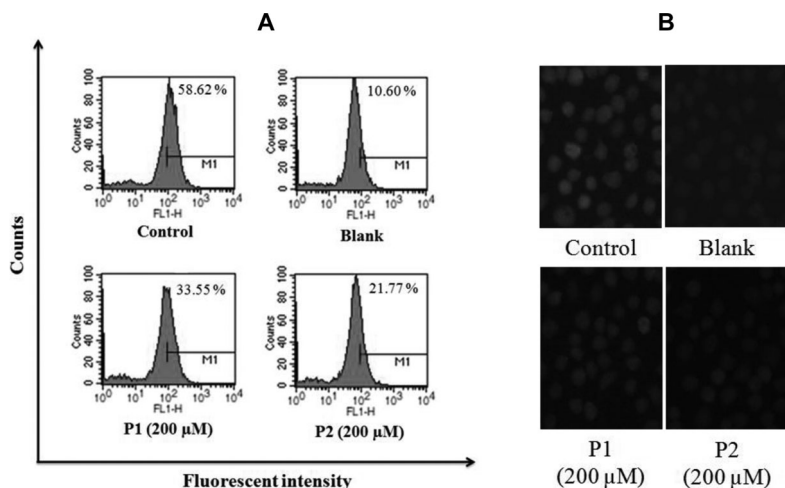


Figure 3. Effects of P1 and P2 on ROS production in antigen-stimulated RBL-2H3 mast cells. The cells were treated with peptides P1 or P2 for 6 h and sensitized overnight with DNP-specific IgE antibody. The cells were then incubated with dihydroethidium for 1 h before stimulated with antigen DNP-BSA for 1 h. The level of ROS production was monitored by flow cytometry (A) and light microscope with 20 $\times$ magnification (B). Blank: –DNP-BSA–peptide; Control: +DNP-BSA–peptide.

3.4 Docking results showing peptide–protein interaction

Peptide P1 suppressed both calcium- and microtubule-dependent signaling pathways, suggesting the blockade of Fc ϵ RI. Thus, P1 was examined to molecular docking studies on Fc ϵ RI- α chain protein model (RCSB ID: 1rpq). The interacting site and hydrogen bond plot between P1 and α chain of Fc ϵ RI receptor were shown in Supporting Information Fig. 2a and b. Peptide P1 bound to target protein with binding energy

+0.52 kcal/mol, hydrogen bond energy –3.03 kcal/mol, electrostatic energy –0.39 kcal/mol, total intermolecular energy –3.42 kcal/mol, and interacted surface 651.078. Moreover, P2 was found to inhibit enzyme PLC specially. Therefore, molecular docking studies of P2 on enzyme PLC protein model (RCSB ID: 3h4 w) was also investigated. Similarly, the interacting site and hydrogen bond plot between PLC domain and P2 was shown in Supporting Information Fig. 2c and d. According to the results, P2 was shown to bind to target protein with binding energy +1.20e+03 kcal/mol, hydrogen

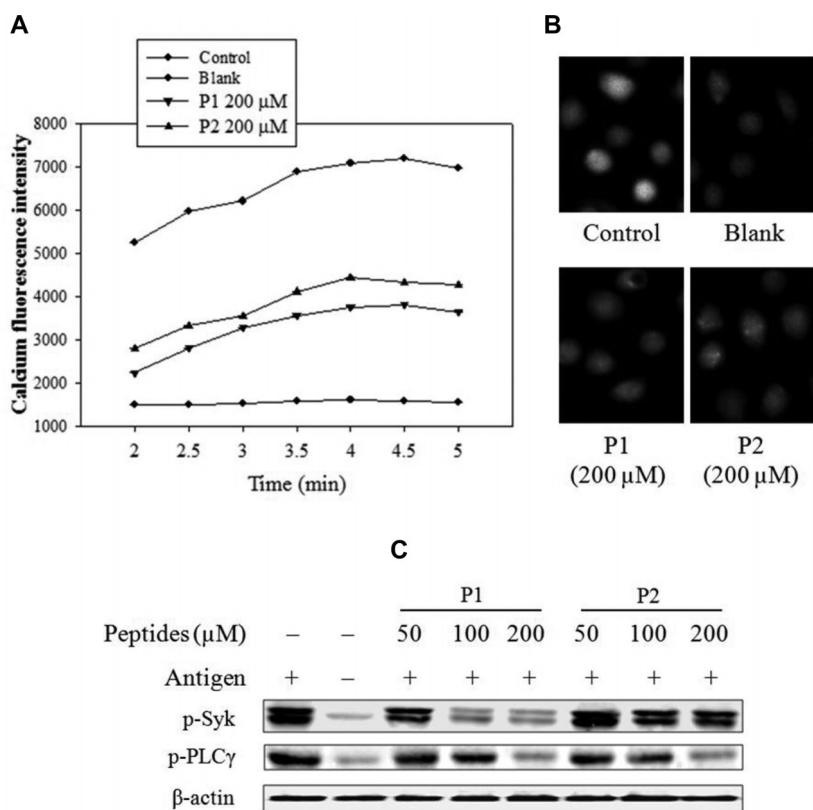


Figure 4. Effect of P1 and P2 on intracellular Ca²⁺ elevation in antigen-stimulated RBL-2H3 mast cells. (A and B) The cells were treated with peptides P1 or P2 (200 μ M) for 6 h and sensitized overnight with DNP-specific IgE antibody. The cells were then incubated with Fura-3/AM for 1 h before stimulated with antigen DNP-BSA. The levels of intracellular Ca²⁺ elevation were monitored by a spectrofluorometric assay (A) and light microscopy with 20 $\times$ magnification (B). Each determination was made in triplicate, and the data are showed as means $\pm$ SD. The results were considered significant at a value of $p < 0.05$. Blank: –DNP-BSA–peptide; Control: +DNP-BSA–peptide. (C) The expression levels of Ca²⁺-dependent signaling molecules were assessed by Western blotting analysis. β -actin was used as an internal control. The phosphorylation of Syk and PLC γ were abbreviated by p-Syk and p-PLC γ , respectively.

bond energy $+1.19 \times 10^3$ kcal/mol, electrostatic energy -0.05 kcal/mol, total intermolecular energy $+1.19 \times 10^3$ kcal/mol, and interacted surface 926.094.

3.5 Effects of P1 and P2 on ROS production in antigen-stimulated RBL-2H3 cells

We next attempted to determine whether P1 and P2 attenuate ROS production in Fc ϵ RI-mediated mast-cell activation using dihydroethidium fluorescence indicator. The flow cytometry assay showed that the cells stimulated with antigen increase amount of ROS from 10.60% of blank group (without stimulation) to 58.62%. However, ROS production was markedly decreased by P1 or P2 treatments at the concentration of 200 μ M. The amount of ROS production was reduced from 58.62 to 33.55% or 21.77% in the cells pretreated with P1 or P2, respectively (Fig. 3A). Similarly, light microscope assay has confirmed that P1 and P2 were effective in suppression of ROS production (Fig. 3B). Notably, the suppressive effect of P2 was observed to be stronger than that of P1.

3.6 Effects of P1 and P2 on IL-4 generation in antigen-stimulated RBL-2H3 cells

On the other hand, we also examined the inhibitory effect of P1 and P2 on IL-4 expression and production in the activated mast cells. In this sense, we have revealed that P1 and P2 induced a dose-dependent suppression on protein (Fig. 5A) and mRNA (Fig. 5B) expression of IL-4 in antigen-stimulated RBL-2H3 cells. In a parallel experiment, we have confirmed the effect of P1 and P2 on the expression of IL-4 through measuring the production of this cytokine in culture supernatants using enzyme-linked immunosorbent assay. It was observed that the levels of cytokine production were increased in the culture supernatants of antigen-stimulated RBL-2H3 cells (Fig. 5C). The amounts of IL-4 in the stimulated cells was 110 pg/mL, whereas the correlative amount of this cytokine in the nonstimulated cells was 14 pg/mL. Meanwhile, these increases were considerably diminished in a concentration-dependent manner by peptide treatments. At the concentration of 200 μ M, P1 and P2 reduced IL-4 level to 44 and 57 pg/mL, respectively.

3.7 Effects of P1 and P2 on NF- κ B activation in antigen-stimulated RBL-2H3 cells

In order to further examine the suppression of P1 and P2 on IL-4 generation, the level of antigen-induced NF- κ B activation was analyzed via Western blot assay. Indeed, RBL-2H3 cells stimulated by antigen DNP-BSA induced a remarkable degradation of I κ B- α in the cytoplasm and subsequent nuclear translocation of p50 and p65 subunits into nucleus (Fig. 6). However, the nuclear translocation of p50/p65 subunits was

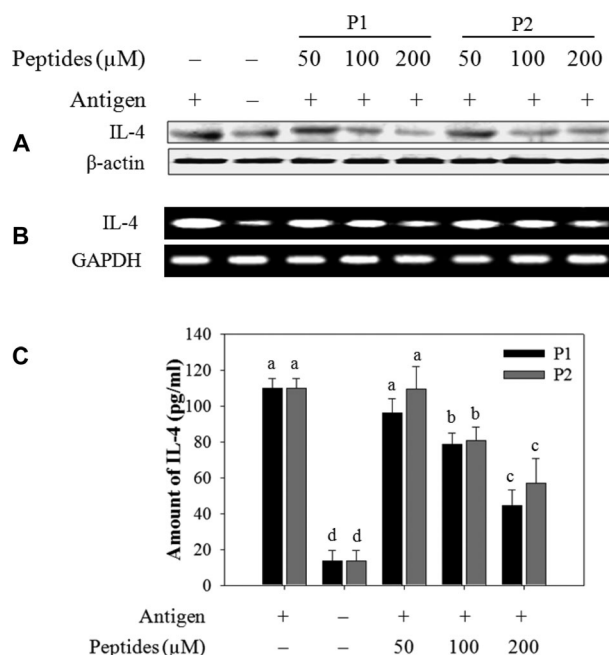


Figure 5. Effects of P1 and P2 on IL-4 expression and production in antigen-stimulated RBL-2H3 mast cells. The cells were treated with different concentration of peptides P1 or P2 for 6 h and sensitized overnight with DNP-specific IgE antibody before stimulation of antigen DNP-BSA for 24 h. The protein levels of IL-4 were assessed by Western blotting (A) while the mRNA transcription levels were determined via RT-PCR (B). β -actin and GAPDH were used as internal controls for Western blot and RT-PCR analysis, respectively. The production levels of IL-4 (C) were quantified in culture media using commercial ELISA kits. Each determination was made in triplicate, and the data are showed as means $\pm$ SD. Different letters a–e indicate significant difference among groups ($p < 0.05$) by Duncan's multiple-range test.

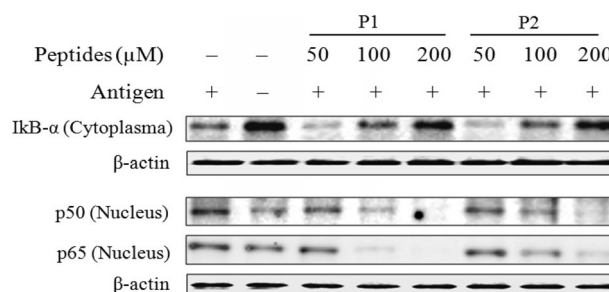


Figure 6. Effects of P1 and P2 on NF- κ B activation in antigen-stimulated RBL-2H3 mast cells. The cells were treated with different concentration of peptides P1 or P2 for 6 h and sensitized overnight with DNP-specific IgE antibody before stimulation of antigen DNP-BSA for 30 min. Cytosolic and nuclear protein extracts were prepared by commercial kit from Sigma. The levels of p50/p65 subunits in nuclear protein extract and I κ B- α in cytosolic protein extract were determined by a Western blot analysis. β -actin was used as an internal control.

decreased in RBL-2H3 cells exposed to peptide P1 or P2. In addition, P1 and P2 blocked antigen-induced $\text{I}\kappa\text{B-}\alpha$ degradation in a concentration-dependent manner. Obviously, P1 and P2 inactivated NF- κB via blocking the degradation of $\text{I}\kappa\text{B-}\alpha$ protein and inhibiting the nuclear translocation of p50/p65 subunits.

4 Discussion

Mast-cell degranulation is significantly involved in pathogenesis of allergic diseases such as bronchial asthma, atopic dermatitis, and hay fever by releasing various mediators [19]. Among them, histamine is considered as the major mediator of the acute inflammatory and immediate hypersensitivity responses. Its effects are mediated through histamine receptors (H1, H2), causing angioedema, vascular hyperpermeability, vasodilatation, bronchoconstriction, mucus production, and hypothermia [20]. Thus, histamine is the major target for potential antiallergic drugs and often used as a marker of mast-cell degranulation. Interestingly, peptides P1 and P2 have been found to be able to inhibit mast-cell degranulation via attenuating antigen-induced histamine release in a concentration-dependent manner in RBL-2H3 cells. Indeed, mast-cell degranulation results in the fusion of the cytoplasmic granule membranes with the plasma membrane. Thus, the cell morphology is changed due to the release of granule-associated stored mediators. Meanwhile, P1 and P2 exhibited protective effect against the morphological changes. These results indicated that P1 and P2 possess inhibitory effect on mast-cell degranulation, and thus reducing the level of histamine release from the activated mast cells. Herein, the inhibitory activities of P1 and P2 on mast-cell degranulation were suggested to be due to suppressing polymerization of microtubule and elevation of intracellular Ca^{2+} level in the activated mast cells.

It has been evidenced that cytoskeletal rearrangements involve in mast-cell degranulation [21]. According to Smith et al. [22], antigen-mediated movement of granules is dependent on microtubules in the mast cell line. Moreover, the inhibition of tubulin polymerization can suppress antigen-induced degranulation [23]. These studies indicate that microtubule polymerization plays an important role in mast-cell degranulation. Thus, we attempt to examine whether P1 and P2 inhibit the microtubule polymerization in antigen-stimulated RBL-2H3 mast cells. We have found that peptide P1 was effective in suppression of microtubule polymerization that was not observed on peptide P2. Substantially, Fyn/Gab2-mediated pathway has a critical role in the polymerization of microtubule and translocation of granules to the plasma membrane [21]. Microtubule polymerization is associated with phosphorylation of Gab2 in a Fyn-dependent manner. In this sense, P1 treatment caused significant inhibition on phosphorylation of signaling molecules, Fyn and Gab2. Conversely, P2 treatment has no inhibition on the activation of Fyn/Gab2-mediated pathway. Accordingly, it

indicated that P1 suppressed Fyn/Gab2-mediated pathway, causing inhibition of microtubule polymerization. As the result, P1 blocked translocation of granules to the plasma membrane, and thus inhibiting the degranulation process in the activated mast cells. On the contrary, the inhibitory activity of P2 on Fyn/Gab2-mediated microtubule polymerization was not observed. Therefore, the inhibitory activity of P2 may be suggested to involve in suppression of calcium-dependent signaling pathway.

The process of mast-cell degranulation requires not only the polymerization of microtubule but also the elevation of intracellular Ca^{2+} . The microtubule polymerization facilitates translocation of granules to the plasma membrane, while the intracellular Ca^{2+} elevation is important in the regulation of granule–plasma membrane fusion [21, 24]. The increase in intracellular Ca^{2+} concentration is a necessary and sufficient stimulus for mast-cell degranulation [25]. Thus, we also examined whether P1 and P2 alleviate the intracellular Ca^{2+} level in the antigen-stimulated RBL-2H3 mast cells. Notably, both P1 and P2 caused remarkable downregulation on intracellular Ca^{2+} elevation. As the result, the alleviative effects of P1 and P2 on intracellular Ca^{2+} elevation resulted in inhibition of granule–plasma membrane fusion, and thus reducing histamine release from the activated mast cells. It is supported by previous reports that some antiallergic drugs inhibit histamine release via inhibition of intracellular Ca^{2+} elevation [26, 27]. Substantially, intracellular Ca^{2+} elevation in mast cells has been known as a downstream signaling of Syk-dependent $\text{PLC}\gamma$ activation [28]. Aggregation of $\text{Fc}\epsilon\text{RI}$ sequentially activates antigen-mediated protein–tyrosine kinases such as Syk and subsequent tyrosine phosphorylation of cellular proteins such as $\text{PLC}\gamma$. The activated $\text{PLC-}\gamma$ subsequently hydrolyzes phosphatidylinositol-3,4-bisphosphate into the two second messengers, inositol-1,4,5 trisphosphate (IP3) and diacylglycerol (DAG). IP3 then binds to specific receptors on the ER, causing release of calcium ions from ER internal stores [28]. Obviously, the tyrosine phosphorylation of $\text{PLC}\gamma$ are essential to generate signals for a sustained Ca^{2+} elevation. In this regard, we elucidated the effect of P1 and P2 on the Syk/ $\text{PLC}\gamma$ phosphorylation. It was revealed that P1 suppressed the phosphorylation of Syk and $\text{PLC}\gamma$, meanwhile P2 specially inhibited the activation of $\text{PLC}\gamma$ but not of Syk. These results suggested that P2 may directly inhibit $\text{PLC}\gamma$ activation, causing alleviation of intracellular Ca^{2+} elevation. However, the alleviative effect of P1 on intracellular Ca^{2+} elevation may relate to suppression of upstream signaling of $\text{PLC}\gamma$ such as Syk. Collectively, it was assumed that P1 inhibited mast-cell degranulation via suppressing both calcium-dependent pathway and microtubule-dependent pathway, suggesting inhibitory activity of P1 at early event of activation, such as blockade of receptor $\text{Fc}\epsilon\text{RI}$ activation. Herein, molecular docking studies have further performed to understand the molecular interaction of P1 to $\text{Fc}\epsilon\text{RI}$. The results have shown that P1 bound to α -chain domain of $\text{Fc}\epsilon\text{RI}$ with binding energy +0.52 kcal/mol. This interaction may cause the obstacle of the formation of IgE- $\text{Fc}\epsilon\text{RI}$ complex,

thus suppressing the activation of IgE-FcεRI-dependent signaling pathways. Meanwhile, inhibitory activity of P2 on mast-cell degranulation appeared to be associated with suppression of PLCγ activation, an inducer of Ca²⁺ elevation in mast cells. In this sense, molecular docking studies have shown that P2 bound to enzyme PLC domain with binding energy +1.20e+03 kcal/mol. This interaction may inactivate the activity of PLC enzyme, causing diminution of intracellular Ca²⁺ elevation and subsequent inhibition of mast cell degranulation.

In addition, the role of ROS as an inducer of Ca²⁺ elevation and degranulation has been also reported in numerous recent studies [29, 30]. Meanwhile, the blockade of ROS production by DPI or antioxidants such as (-)-epigallocatechin gallate suppresses IgE-mediated histamine release [31, 32]. Therefore, inhibition of ROS production is considered to be important to suppress mast-cell degranulation. According to our results, P1 and P2 effectively diminished ROS production in antigen-activated mast cells. Notably, the inhibitory activity of P2 was observed to be stronger than P1. Evidently, methionine is the sulfur-containing amino acid in proteins/peptides that play an important role in regulating free-radical processes. Many studies have reported that the presence of thiols such as methionine and glutathione in their structures is responsible for the appearance of antioxidant properties [33, 34]. Meanwhile, P2 contains two methionine amino acids in its sequence, which may be responsible for its high ROS scavenging activity. These results suggested that the effective antioxidant activity of P2 significantly contributes to the depression of mast-cell degranulation.

Besides degranulation, the activation of mast cells also leads to generation of several cytokines such as IL-1β, IL-4, IL-6, IL-8, and TNF-α [1, 35]. Especially, IL-4 plays a crucial role in the pathogenesis of allergic diseases, such as the induction of IgE synthesis and the development of mast cells [36]. Therefore, modulation of production and expression of IL-4 in mast cells can provide a useful therapeutic strategy for allergic inflammatory disease. Likewise, the treatment of P1 or P2 induced a dose-dependent suppression on IL-4 generation in antigen-activated RBL-2H3 cells. The suppressive effects of P1 and P2 on IL-4 generation were suggested to be due to inactivation of NF-κB. The production and expression of allergic mediators such as cytokines are regulated by several intracellular signaling pathways, especially NF-κB. The transcription factor NF-κB plays a central role in the induction of gene expression, and is considered as a promising target for the treatment of diseases [37]. Conversely, the blockade of NF-κB transcriptional activity leads to inhibition of expression and production of inflammatory mediators [38]. Obviously, P1 and P2 inactivated NF-κB via blocking the degradation of IκB-α protein and inhibiting the nuclear translocation of p50/p65 subunits, which may lead to depression of IL-4 production and expression. Substantially, ROS are well known to involve in activation of NF-κB, causing production and expression of inflammatory cytokines [39]. Evidently, P1 and P2 have been shown to attenuate ROS production in antigen-

stimulated RBL-2H3 mast cells. Therefore, the inhibitory activities of P1 and P2 on NF-κB activation could be explained, at least in part, via diminishing ROS production in the activated cells.

In conclusion, we have determined that peptides P1 and P2 purified from enzymatic hydrolysate of the edible microalgae *S. maxima* possess inhibitory activities on FcεRI-mediated allergic responses in RBL-2H3 mast cells via suppression of degranulation, intracellular Ca²⁺ elevation, and cytokine generation. Notably, the inhibitory effect of P1 on degranulation was found due to down-regulation of calcium-dependent signaling pathway and microtubule-dependent signaling pathway. Meanwhile, P2 depressed PLCγ activation and intracellular ROS production, thus inhibiting degranulation (Supporting Information Fig. 3). Our results indicate that peptides P1 and P2 can contribute to suppression of allergic reactions and might be potential candidates for novel inhibitor of allergic reactions.

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