



Anti-allergic activity of R-phycoerythrin from *Porphyra haitanensis* in antigen-sensitized mice and mast cells



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ABSTRACT

The prevalence of food allergy has increased in Asian countries. Marine algae have been proposed as the potential resource for anti-allergic therapeutics. The present study was aimed at isolating R-phycoerythrin (RPE) from *Porphyra haitanensis* and determining the anti-allergy potential of RPE in antigen-sensitized mice and mast cells. In animal experiments, RPE could effectively reduce tropomyosin (TM)-specific immunoglobulin E (IgE) and histamine levels, alleviate allergy symptoms and jejunum tissue inflammation in mice, and inhibit the expression and release of cytokines (interleukin-4 (IL-4) and IL-13) in peritoneal lavage fluid. In spleen lymphocyte experiments, high purity of RPE skewed the immunological function of CD4⁺ T cells towards Th1 activity. A higher expression of interferon (IFN)- γ was induced by a synergistic effect of TM and RPE. Through the Jun N-terminal kinase and Janus kinase 2 signaling pathways, IFN- γ synthesis was induced by RPE in combination with TM. Anti-allergic effect of RPE was evaluated in IgE-mediated rat mast RBL-2H3 cells. The results demonstrated that RPE inhibited allergy markers, including the release of β -hexosaminidase, histamine and ROS in antigen-sensitized RBL-2H3 cells. RPE also suppressed the production of pro-inflammatory factors (IL-4 and tumor necrosis factor- α). In conclusion, RPE decreased allergic sensitization against TM by blocking Th2 cell polarization as well as suppressed the release of allergic-mediators in antigen-stimulated mast cells. It may be used as a functional food component or active pharmaceutical ingredient for allergic patients.

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1. Introduction

Food allergy is an important public health problem which can result in a series of symptoms mostly, involving skin and gastrointestinal tract. As a kind of food allergies, shellfish allergy among adults and children in the Asia-Pacific is fairly prevalent and ranks as one of the most common foods causing allergy [1]. Tropomyosin (TM) has been identified as the major allergen of shellfish, and it causes adverse hypersensitivity in allergic individuals [2]. The results of *in vivo* and *in vitro* experiments showed that allergic reaction caused by TM usually accompanied the promotion of serum specific immunoglobulin E (IgE), interleukin (IL)-4, IL-13, and IL-5, which were the signs of a type I hypersensitivity reaction and Th2 cell polarization [3,4]. In view of the increasing prevalence of food allergy, there is a pressing need to develop effective precautions or therapies. So far, several allergen-nonspecific strategies have been investigated [5], which are generally safe and well tolerated in

patients and may provide a pathway for hypersensitivity treatment. However, these kinds of active substances are rarely found and applied.

Generally, the anti-allergic activities of these bio-activators are tested in animal models and cell models *in vitro*. Mast cells are secretory cells that are central to specific and innate immunity, allergy response, and inflammation [6]. The RBL-2H3 cell line has been widely used as a mast cell model for IgE-mediated cell study [7]. After stimulation with antigen, mast cells release β -hexosaminidase, a marker of mast cell degranulation, and various allergic mediators such as histamine and cytokines [8].

In China, laver is popular with consumers because of its great taste and abundant nutrients. *Porphyra haitanensis* is cultivated on a commercial scale in southern areas of China, mainly for the domestic market. Some immunoregulatory active substances have been purified from *P. haitanensis* and identified as phlorotannins, polysaccharides, and phycobiliproteins [9–11].

Our previous study has reported that sulphated polysaccharides from *P. haitanensis* could suppress the Th2 immune responses in the TM-sensitized mice [12]. During this study, R-phycoerythrin (RPE) extracted from *P. haitanensis* was found to have the similar efficacy. RPE, the light-harvesting photosynthetic accessory protein, is a member of the phycobiliprotein family in *P. haitanensis*. Chang et al. [13] reported

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that RPC extracted from *Bangia atropurpurea* exhibited pharmaceutical functions in alleviating allergic airway inflammation, whereas the anti-allergy activity of RPC extracted from *P. haitanensis* has not been identified in the antigen-sensitized mice and mast cells.

In this study, the anti-allergy activity of RPC was evaluated by measuring the levels of the TM-specific serum IgE, histamine and relevant cytokines in the TM-sensitized mice, and the inhibitory effects of RPC on the IgE-mediated allergic response have been further investigated in the mast cells.

2. Materials and methods

2.1. Purification and identification of TM and RPC

The purification of TM from mud crab (*Scylla paramamosain*) was carried out as described previously [14]. RPC was purified from *P. haitanensis* according to the method of Lin et al. [15,16]. The purified TM was identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western-blotting. Moreover, RPC was characterized by SDS-PAGE, native-PAGE, two-dimensional electrophoresis map (2-DE map) and ultraviolet-visible absorption spectroscopy.

2.2. Animals and immunizing protocol

2.2.1. Animals

Female BALB/c mice, 6–8 weeks of age, were obtained from the Animal Center of Xiamen University. Mice were housed in an SPF environment maintained at $22 \pm 1^\circ\text{C}$ with a relative humidity of $55 \pm 10\%$, and experiments were performed in conformity with the laws and regulations for treatment of live animals in Jimei University, SCXK 2012-0005.

2.2.2. Immunization of mice

Highly purified TM and RPC were diluted in phosphate buffer solution (PBS). Mice were immunized according to the method described before [17] with slight modifications. Briefly, the mice of the TM-treated group were immunized intraperitoneally with 0.2 mL PBS containing TM (0.1 mg/mouse) and Alum adjuvant (Pierce, Rockford, USA) on days 0 and 14. The mice of the TM + RPC-treated group were immunized intraperitoneally with TM (0.1 mg/mouse) plus RPC (0.1 mg/mouse) and adjuvant. The negative control mice were given intraperitoneal injections of PBS and adjuvant (PBS group). On days 28, 30 and 32, each mouse from the TM group and TM + RPC group was challenged 3 times by gavages of 5 mg TM in a total volume of 500 μL , and 1 h before each gavage, mice from the TM + RPC group were treated with RPC (5 mg/mouse). The negative group was treated with an equal volume of PBS. The venous blood of mice was bled from the tails on days -1, 1, 15, and 33. Feces were collected 24 h after the last challenge (day 33) (Fig. 1). The whole experiment was repeated three times with 6 mice per group.

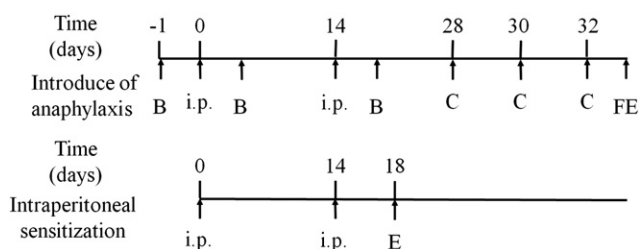


Fig. 1. Animal experimental design. The time line for the experiments and the given treatments. B, blood sampling; F, feces sampling; C, allergen challenge; E, exsanguination; i.p., intraperitoneal injection.

2.3. Murine model experiments

2.3.1. Assessment of clinical anaphylactic reactions and TM-specific serum IgE

Clinical anaphylactic reactions were evaluated for 30–40 min after challenge (day 32), using a scoring system as described by Li et al. [18]. The levels of the TM-specific serum IgE were determined by indirect ELISA with the protocol described previously [12]. All assays were performed in triplicate. The TM-specific serum IgE levels were also detected by Western-blotting analysis according to the method of Huang et al. [19].

2.3.2. Histological analysis of jejunum tissues

The jejunum tissues of mice were obtained and fixed with 10% neutralized buffered formalin for histological analysis. The jejunum sections (5 μm thick) were hydrated through an ethanol gradient to water, stained with hematoxylin and eosin, and examined by light microscopy (Leica RM2235, Solms, Germany).

2.3.3. Measurement of histamine in feces and sera

The minced feces (1 g) were homogenized with 2 mL of 5% trichloroacetic acid (TCA) for 2 min. After being centrifuged at $3000 \times g$ for 10 min (4°C), the supernatant was collected. The residue was extracted again with 2 mL 5% TCA. Both supernatants were combined, and adjusted to a final volume of 4 mL with 5% TCA. The following steps of high performance liquid chromatography (HPLC) analysis were performed as described before [20].

Mice venous bloods were obtained 24 h after the last immunization (day 15). Sera were collected for the measurement of histamine according to the manual (IBL, Hamburg, Germany).

2.4. Measurement of cytokines

2.4.1. Preparation of peritoneal lavage fluid (PLF) and splenic lymphocyte from mice

Mice were sacrificed 24 h after the last immunization (day 15). Hanks solution (4 mL) was injected into the peritoneal cavity. The washouts pooled from mice were centrifuged at $120 \times g$ for 10 min and the supernatants were collected for cytokine detection.

To obtain the splenic lymphocyte, mice were sacrificed 4 days after the last immunization (day 18). Individual spleens were aseptically removed, minced and then cell suspensions were prepared.

2.4.2. The production and expression of cytokines

For the evaluation of cytokine production, splenic lymphocyte suspensions were cultured 72 h in 24-well plates (1×10^6 cells/well) in the presence of PBS, TM (10 $\mu\text{g}/\text{mL}$), or TM (10 $\mu\text{g}/\text{mL}$) plus RPC (20, 40, 80 $\mu\text{g}/\text{mL}$). Levels of cytokines IL-4, interferon (IFN)- γ , and IL-13 were determined by sandwich-ELISA kit (Perprotech, Princeton, NJ, USA), according to the manufacturer's instructions.

Real-time reverse transcription quantitative PCR was performed by using a SYBR green protocol (Tiangen, Beijing, China) according to the manufacturer's instructions. The mRNA level of housekeeping gene β -actin was used to normalize the values obtained for all transcripts under examination. The program of the thermal cycler (Applied Biosystem 7300) was set up as described previously [3]. Real-time quantitative PCR was performed with the following primers: β -actin (5'-AGC TGC GTT TTA CAC CCT TT-3'; 5'-AAG CCA TGC CAA TGT TGT CT-3'), IL-4 (5'-ACA GGA GAA GGG ACG CCA T-3'; 5'-GAA GCC CTA CAG ACG AGC TCA-3'), IL-13 (5'-ACA GGA GAA GGG ACG CCA T-3'; 5'-GAA GCC CTA CAG ACG AGC TCA-3'), and IFN- γ (5'-TGG CAT AGA TGT GGA AGA AAA GAG-3'; 5'-TGC AGG ATT TTC ATG TCA CCA-3').

2.5. The signal pathways involved in RPC-induced IFN- γ production in the TM-treated mice

The lymphocytes from TM-treated mice were pretreated with 0.1% dimethyl sulfoxide (DMSO), 25 μ M SP600125 (inhibitor of JNK, CST, Danvers, MA, USA) in cultures containing 0.1% DMSO, 10 μ M SB203580 (inhibitor of p38 MAPK, CST, Danvers, MA, USA) in cultures containing 0.1% DMSO, or 10 μ M SD1008 (inhibitor of JAK2, Tocris, Bristol, United Kingdom) in cultures containing 0.1% DMSO for 1 h; and further incubated for 48 h with 80 μ g/mL RPC. The untreated cells were left in the culture medium alone for 48 h. The production of IFN- γ was quantified by ELISA.

2.6. The mast cell model assay in vitro

2.6.1. Cell culture

The rat mast cell line RBL-2H3 was obtained from the American Type Culture Collection (Bethesda, MD) and grown in minimum essential medium with Eagle's salts (EMEM) (Hyclone, Logan, USA), supplemented with 10% FBS (Hyclone, Logan, USA), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin (Gibco, New York, USA) at 37 °C in a humidified incubator with a 5% CO₂/95% air atmosphere (Forma 3111, Thermo, Waltham, MA, USA).

2.6.2. Assay for proliferative activity

RBL-2H3 cells in 10% FBS-EMEM were seeded in 96-well plates. Briefly, RPC (20–200 μ g/mL) and/or dinitrophenyl-bovine serum albumin (DNP-BSA, 500 ng/mL) (Biosearch, Petaluma, CA) were added to the wells, and incubated for 24 h, then the cells were treated with 20 μ L of MTS solution. Cell viability was examined using an MTS assay kit according to the manufacturer's instructions (Promega, Madison, USA). Relative cytotoxicity was quantified by measuring the absorbance at 570 nm.

2.6.3. Measurement of β -hexosaminidase

Degranulation was monitored by the release of β -hexosaminidase, as described by Han et al. [21]. RBL-2H3 cells were seeded at 1×10^5 cells per well in 48-well plates and incubated overnight with 200 ng/mL of anti-DNP-IgE (Sigma, St. Louis, MO, USA). IgE-sensitized RBL-2H3 cells were pre-treated with RPC (80–150 μ g/mL) for 1 h and stimulated with 500 ng/mL DNP-BSA for 6 h in Tyrode's buffer at 37 °C. The results were presented as the percentage of β -hexosaminidase activity measured in supernatant samples compared to the total levels in the corresponding cell lysates. The β -hexosaminidase activity of RBL-2H3 cells was measured with the 4-methylumbelliferyl-N-acetyl-D-glucosaminide substrate (Sigma, St. Louis, MO, USA). For measurement of total activity, the cells were lysed with 0.1% Triton-X 100 prior to removing the supernatant. The β -hexosaminidase activity was quantified by measuring the fluorescence intensity of the hydrolyzed substrate in a fluorometer (Infinite M200PRO, Tecan, Switzerland) using 360 nm excitation and 450 nm emission filters.

2.6.4. Histamine release assay

The IgE-sensitized RBL-2H3 cells were pre-incubated with RPC (80–150 μ g/mL) for 1 h and then incubated with DNP-BSA (500 ng/mL) for 15 min. Cell culture supernatant was collected for measurement of histamine (IBL, Hamburg, Germany).

2.6.5. Reactive oxygen species (ROS) measurement

The amount of intracellular ROS was measured using 2,7-dichlorodihydrofluorescein diacetate (H₂DCF-DA) (Sigma, St. Louis, MO, USA), which is deacetylated by intracellular esterases to non-fluorescent 2,7-dichlorodihydrofluorescein (DCFH), and the DCFH is in turn oxidized to the fluorescent compound 2,7-dichlorodihydrofluorescein (DCF) by ROS. IgE-sensitized RBL-2H3 cells (1×10^5 per well) were pre-incubated with 5 μ M of H₂DCF-DA for 30 min at 37 °C in 48-well plates, washed to remove excess H₂DCF-DA, and treated with RPC and/or DNP-BSA for 30 min. The fluorescence intensity was measured with

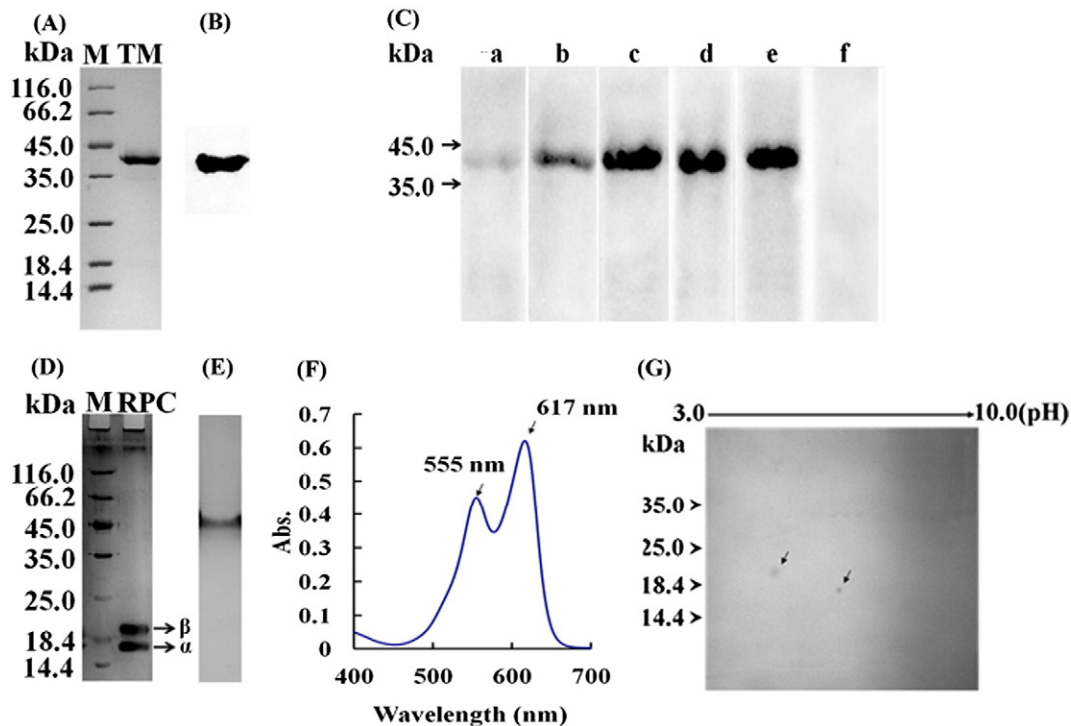


Fig. 2. Purification and identification of TM and RPC. (A) Tricine-SDS-PAGE of TM. (B) Western-blotting analysis of TM with the rabbit anti-TM polyclonal antibody (1:10,000 dilution). (C) Western-blotting analysis of TM with the sera of shellfish-allergic subjects (1:4 dilution). (D) Tricine-SDS-PAGE of RPC. (E) Native-PAGE of RPC. (F) Ultraviolet-visible absorption spectrum of RPC. (G) 2-DE map analysis of RPC. M, protein marker. TM, tropomyosin. a–e, Patients' sera. f, Normal sera. RPC, R-phycoerythrin.

excitation at 485 nm and emission at 530 nm using a fluorometer (Infinite M200PRO, Tecan, Switzerland).

2.6.6. Measurement of cytokine secretion

For cytokine immunoassays, IgE-sensitized RBL-2H3 cells were treated with RPC and/or DNP-BSA. Supernatant was harvested at the indicated time intervals, and the production of IL-4 (4 h) and TNF- α (6 h) were quantified by sandwich immunoassays using the protocol supplied by Perprotech (Princeton, NJ, USA).

2.7. Statistical analysis

All experiments were repeated at least three times *in vitro*, or six mice ($n = 6$) for each group of the *in vivo* studies. One-way analysis of variance (one-way ANOVA) comparison tests of SPSS statistics 17.0 software was used to evaluate the statistical significances of the differences between experimental groups. Differences were measured by Duncan's multiple range tests between groups and were considered significant at $P < 0.05$. Results are presented as means $\pm$ SD.

3. Results

3.1. Purification and identification of TM and RPC

The result of SDS-PAGE showed that purified TM from *S. paramamosain* revealed a band corresponding to a molecular mass of 38 kDa (Fig. 2A), which was recognized by the rabbit anti-TM polyclonal antibody (Fig. 2B) and the sera of shellfish-allergic subjects (Fig. 2C). After ammonium sulfate fractionation and Sephacryl S-200 and DEAE-Sephacryl column chromatography, a highly purified protein (A620/A280 > 7.0) which consisted of two subunits α and β (molecular weights were about 17.7 kDa and 20.5 kDa) in SDS-PAGE (Fig. 2D) was acquired, and the protein was shown to be a single band in the native-PAGE (Fig. 2E). The purified protein was identified as RPC with two special absorption peaks at 617 nm and 555 nm as monitoring by an ultraviolet spectrophotometer (Fig. 2F). On the 2-DE map of purified RPC, there were two spots with pI values of 4.9 and 6.5, respectively (Fig. 2G).

3.2. The inhibitory effects of RPC on systemic anaphylaxis in mice

The levels of the TM-specific serum IgE increased remarkably after the second intraperitoneal injection in the TM-treated mice, and were significantly suppressed ($P < 0.01$) by injections of RPC (Fig. 3A). Moreover, TM-specific serum IgE could be detected by Western-blotting analysis in the TM-treated mice, but no specific serologic reactions were observed in the mice of the negative group and TM + RPC-treated group (Fig. 3A).

Moreover, mice of the TM-treated group showed more serious allergic symptoms: half of the mice had symptoms of scratching around the nose and tail (symptom score 1) while the others had reduced activities along with diarrhea on day 32 (symptom score 2). In the TM + RPC-treated group, only one third of the mice had slight allergy symptoms (Fig. 3B).

As symptoms involving the gastrointestinal tract have been found in the murine model of food allergy, the histology of the jejunum tissues were further investigated. In the present histopathology analysis, lymphocytic infiltration, villi edema, and shrinkage were observed in the jejunum of the TM-treated group (Fig. 3C-a, b, c). RPC was found to significantly eliminate the symptoms excepting lymphocytic infiltration (Fig. 3C-a). The results indicated that mice treated with RPC had reduced allergic inflammation in the jejunum tissues.

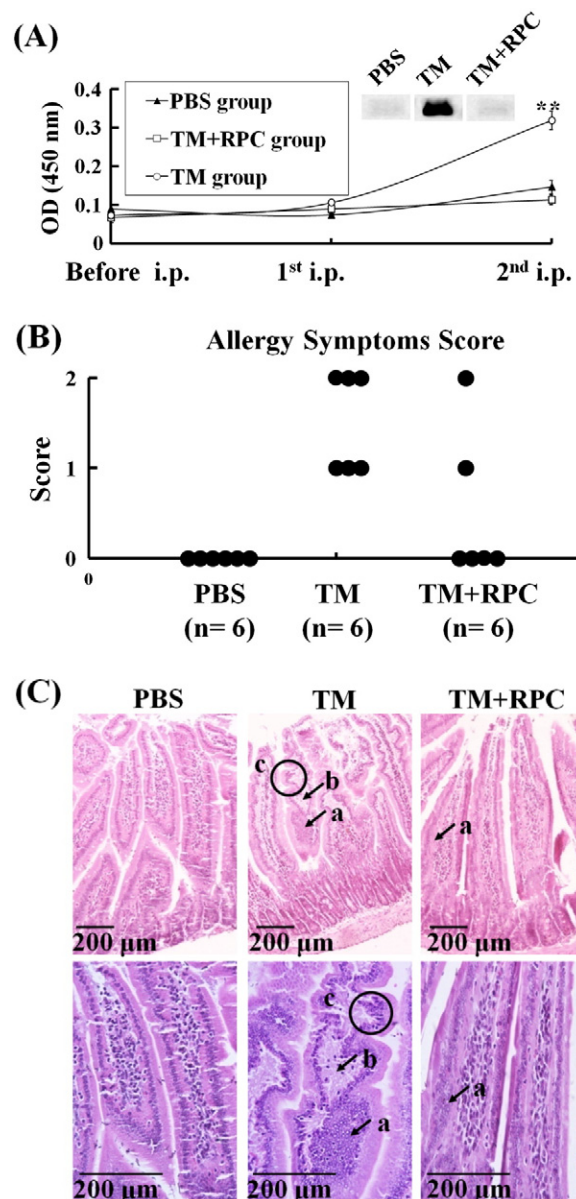


Fig. 3. The levels of TM-specific serum IgE, systemic anaphylaxis responses, and histological analysis of jejunum. (A) RPC reduced the levels of the TM-specific serum IgE. Sera were collected from mice before and 24 h after immunized by TM, TM + RPC, or PBS. The levels of the TM-specific serum IgE were determined by ELISA. The data represent the mean $\pm$ SD from three independent experiments. Statistical differences are indicated by P values (one-way ANOVA). * $P < 0.05$ and ** $P < 0.01$, compared with the TM-treated group. After second immunization, sera were detected in the IgE levels by Western-blotting (1:20 dilution). The result of Western-blotting was in the right upper corner of the diagram. Before i.p., before immunization. 1st i.p., the first immunization. 2nd i.p., the second immunization. (B) Systemic anaphylaxis responses after intragastric challenge. Responses of the 3 groups of mice challenged intragastrically with TM, TM + RPC, or PBS were evaluated for 30–40 min after challenge based on the scoring system described in methods. (C) RPC mitigated the inflammation of jejunum tissues in the mice of TM + RPC group. a, Lymphocytic infiltration. b, Villi edema. c, Villous atrophy.

3.3. Effects of RPC on cytokines in PLF and histamine in the feces and sera

RPC decreased effectively the levels of IL-4 (from 700 pg/mL to 200 pg/mL) and IL-13 (from 1700 pg/mL to 200 pg/mL), that induced by TM. Meanwhile, in the presence of RPC, IFN- γ level was also found to be promoted from 1000 pg/mL to approximate 1800 pg/mL in PLF (Fig. 4A).

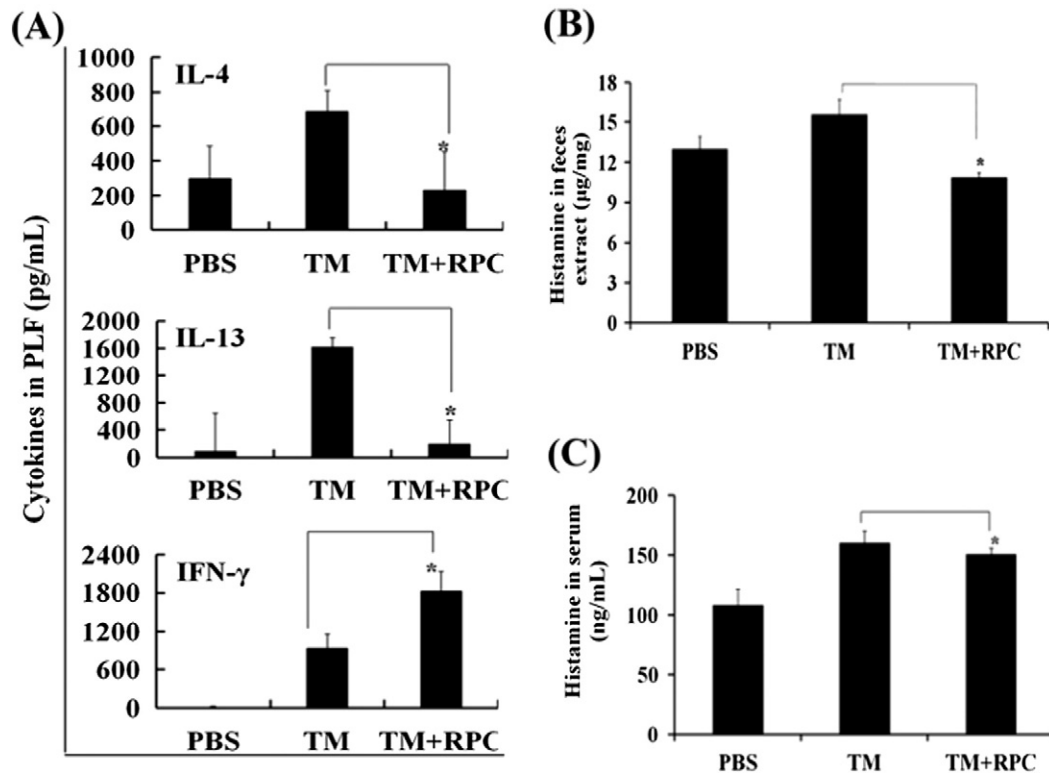


Fig. 4. Effect of RPC on cytokines in PLF and histamine in mice feces and sera. (A) RPC regulated Th1/Th2 cytokine production in PLF. Peritoneal lavage fluid was collected 24 h after the second immunization. Levels of IL-4, IL-13 and IFN- γ were measured by ELISA. (B) RPC reduced histamine levels in mice feces. Mice feces were collected 24 h after three times of intragastric challenges with TM and/or RPC. Histamine levels were determined by HPLC. (C) RPC reduced histamine levels in mice sera. Mice sera were collected 24 h after the second immunization for histamine detection. The data represent the mean $\pm$ SD from three independent experiments. Statistical differences are indicated by *P* values (one-way ANOVA). **P* < 0.05 and ***P* < 0.01, compared with the TM-treated group.

Mice feces were collected after three gavages. The content of histamine was measured by HPLC. The histamine characteristic peak of the standard sample arisen at a retention time of 5.6 min. After the challenge of TM, the feces from mice of the TM + RPC-treated group showed a reduction in histamine level (Fig. 4B), at the same time, there was also a reduction of histamine level in the sera (Fig. 4C).

3.4. Regulatory effects of RPC on Th1/Th2 cell responses in the TM-treated mice

The regulatory effects of RPC on Th1/Th2 cell responses in the TM-treated mice were examined. The production of Th1 cytokine (IFN- γ) and Th2 cytokines (IL-4 and IL-13) were assayed by stimulation of splenocytes with PBS, TM, or TM + RPC. As illustrated in Fig. 5A, the levels of IL-4 and IL-13 were promoted when spleen lymphocytes were stimulated by TM alone. Compared with TM-treated splenocytes, the levels of IL-4 and IL-13 were significantly decreased when the splenocytes were treated with TM in combination with RPC, whereas the production of IFN- γ was significantly increased when the splenocytes were treated with TM in combination with RPC. The data showed that there was a dose–response relationship between these effects and the concentration of RPC. The mRNA levels of cytokines were in accordance with their protein levels (Fig. 5B).

3.5. Synergistic effect between TM and RPC in the TM-treated mice

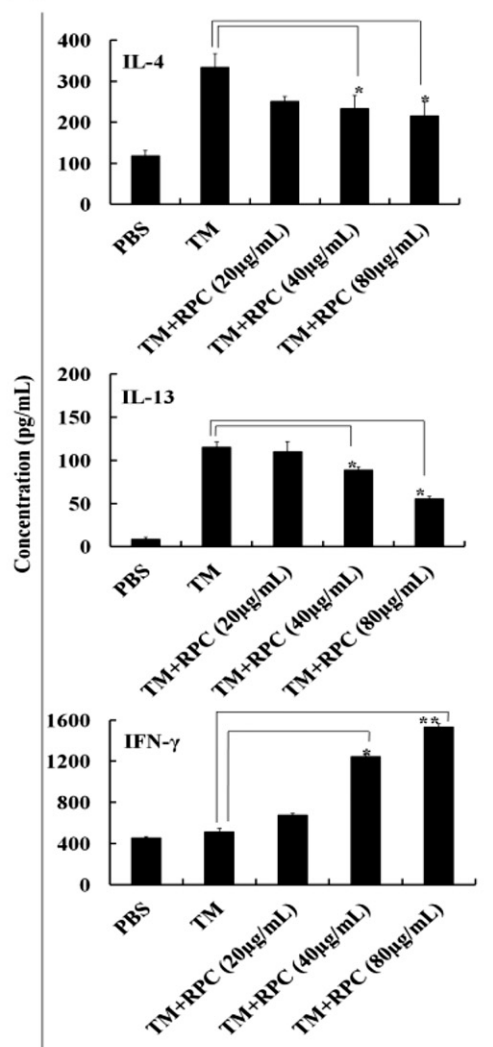
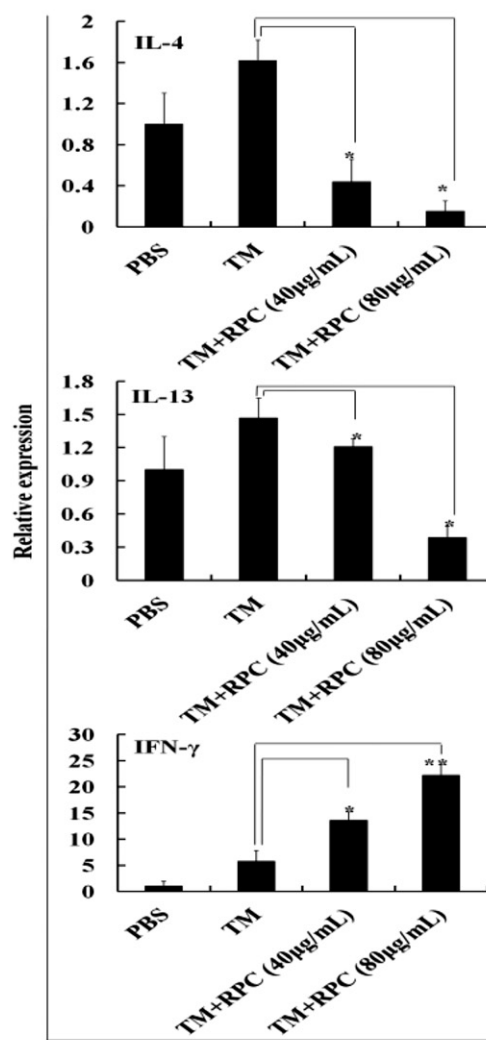
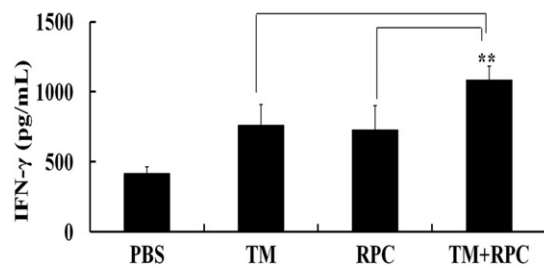
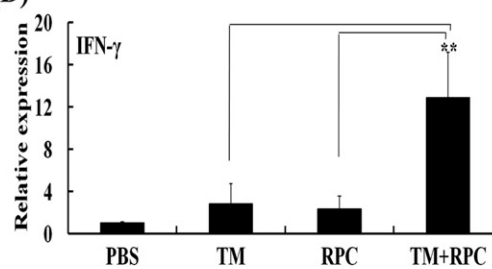
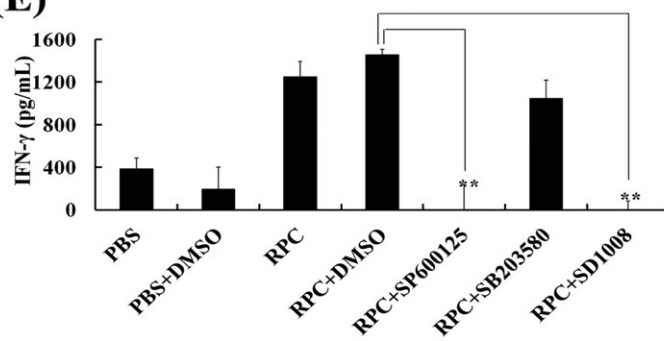
The splenic lymphocytes stimulated by TM in combination with RPC showed much higher secretion of IFN- γ , approximately 1100 pg/mL, than those treated by TM or RPC alone (Fig. 5C). Similar results were acquired at the mRNA level (Fig. 5D). Thus, whether the RPC-induced secretion of IFN- γ was affected by inhibitors of p38 MAPK, JNK, or JAK2 was further explored. Fig. 5E showed that in the splenic

lymphocytes from TM-treated mouse, the production of RPC-induced IFN- γ was significantly reduced by treatment of SP600125 and SD1008, compared with the levels of cytokines recovered from RPC-treated splenic lymphocytes in culture containing DMSO (0.1%). To the contrary, SB203580 did not affect the production of RPC-induced IFN- γ in splenic lymphocytes.

3.6. The inhibitory effect of RPC on IgE-mediated allergic response

To determine the potential cytotoxicity of RPC, MTT assay was operated. As shown in Fig. 6A, RPC did not affect cell viability, RPC and antigen (DNP-BSA) co-treatment also had no significant cytotoxic effect. To determine the anti-allergic activity of RPC, its effects on the degranulation of antigen-stimulated RBL-2H3 cells was determined. The β -hexosaminidase release was an index of degranulation, DNP-BSA induced degranulation in IgE-sensitized RBL-2H3 cells (Fig. 6B), and RPC significantly suppressed the antigen-induced degranulation (Fig. 6B). RPC also had the effect of inhibiting antigen-induced histamine release in IgE-sensitized RBL-2H3 cells and had the concentration dependence (Fig. 6C). Mast cells release ROS in response to various stimuli [22], and ROS from mast cells are involved in allergic inflammation [23]. The result showed that RPC reduced the production of ROS in these cells (Fig. 6D). These indexes had no significant difference between RBL-2H3 cells stimulated with RPC (100 μ g/mL) alone and PBS-stimulated cells (data not shown).

Mast cells play an important role in initiating and perpetuating the inflammatory response in allergic reactions by secreting large amounts of cytokines such as IL-4 and tumor necrosis factor (TNF)- α [24]. The results of ELISA demonstrated that RPC significantly inhibited the secretion of IL-4 and TNF- α in a dose-dependent manner in antigen-stimulated IgE-sensitized mast cells (Fig. 6E, F).

(A)**(B)****(C)****(D)****(E)**

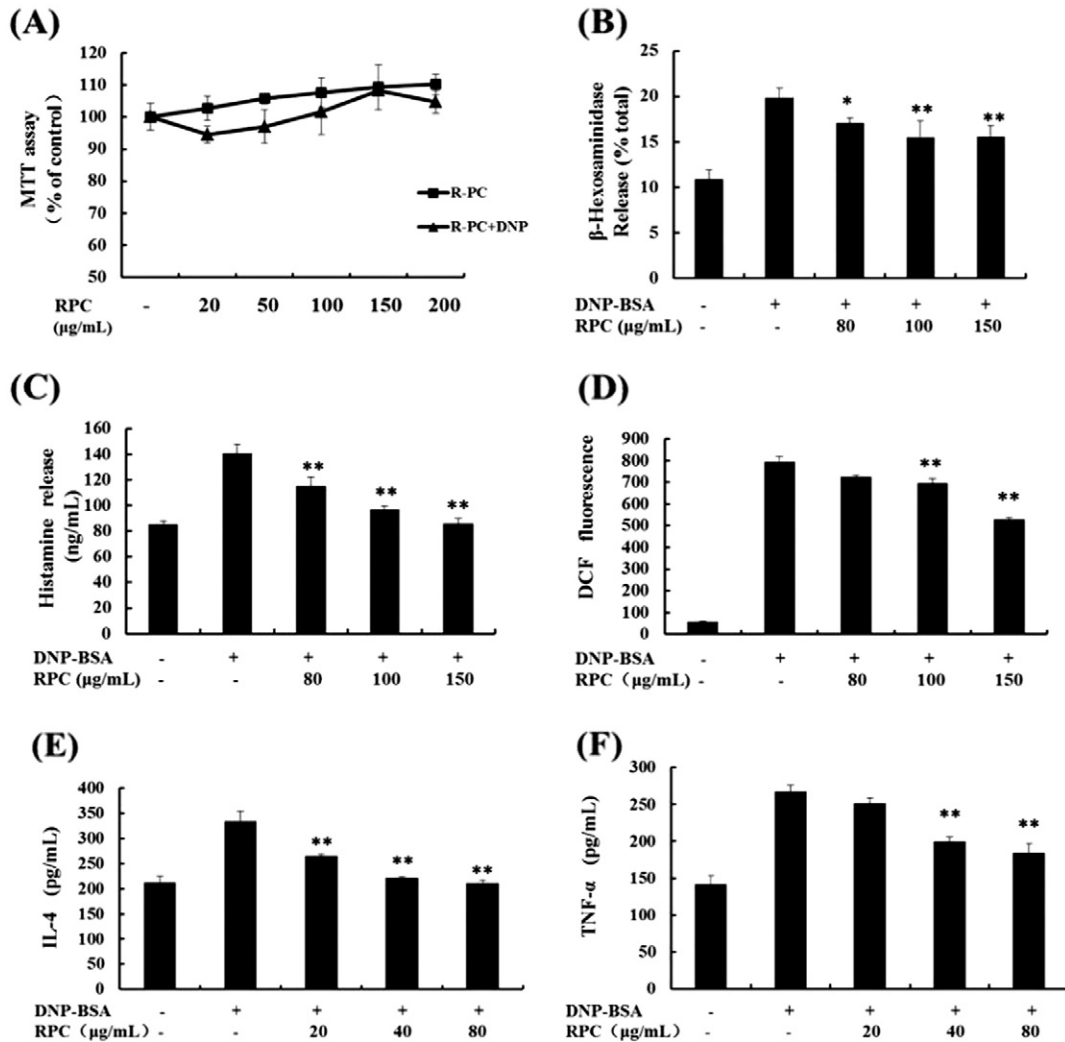


Fig. 6. The inhibitory effect of RPC on IgE-mediated allergic responses. (A) Effects of RPC on the cytotoxicity. The RBL-2H3 cells were seeded in 96-well plates and treated with the indicated concentrations of RPC and/or DNP-BSA (500 ng/mL) for 24 h. MTT assay was applied for detection. (B) Effects of RPC on the degranulation. The cells were incubated overnight in 48-well plates with 200 ng/mL of DNP-specific IgE in the medium. The medium was replaced by Tyrode's buffer containing the indicated concentrations of RPC (80–150 μg/mL) followed by stimulation with 500 ng/mL DNP-BSA for 6 h, and the release of β-hexosaminidase was measured. (C) Effects of RPC on the histamine release. Same as (B) except that stimulation with 500 ng/mL DNP-BSA for 15 min, and histamine was measured. (D) Effects of RPC on the antigen-induced ROS production. The fluorescence intensity was measured with excitation at 485 nm and emission at 530 nm for ROS production. (E, F) Effects of RPC on the amount of cytokines in antigen-stimulated mast cells. Cells were stimulated with DNP-BSA (500 ng/mL) for 4 h (IL-4) or 6 h (TNF-α) in medium alone or with RPC (80–150 μg/mL). The amount of IL-4 and TNF-α released into the culture supernatant were measured by ELISA. Each bar shows three independent experiments. * $P < 0.05$ and ** $P < 0.01$, significantly different from DNP-BSA alone (Duncan's multiple range tests).

These results suggested that RPC markedly inhibited the allergic reaction in IgE-mediated RBL-2H3 cells.

4. Discussion

Laver is cultivated over a much larger area and has a lower price than other edible economical red algae in China. RPC exists in red algae such as laver, porphyridium, and bangia. Our preliminary experiments suggested that the content of RPC in *P. haitanensis* was close to that of *B. atropurpurea* (approximately 2.0–3.0% dry weight). While, *P. haitanensis* is more popular in China for its larger cultivation area and lower price. It has been reported that RPC extracted from *Porphyridium cruentum* showed two

characteristic absorption peaks at 617 nm and 555 nm and included two different subunits, with molecular weights of about 18.2 kDa and 20.5 kDa, respectively [15]. In the present study, high purity of RPC (A620/A280 > 7.0) extracted from *P. haitanensis* showed similar characteristics except for α subunit, which had a molecular weight of 17.7 kDa. This difference might result from the different materials used in the experiment.

Allergen-specific serum IgE and histamine are essential in type I hypersensitivity reactions [25]. The results of the ELISA and Western-blotting in the present study indicated that after the second immunization, the injection of RPC together with TM completely inhibited the promotion of TM-specific IgE in sera. In previous studies, the serum

Fig. 5. Regulatory effect of RPC on the Th1/Th2 cell responses in TM-treated mice. (A) RPC reduced the production of Th2 cytokines but elevated the production of Th1 cytokines. Culture supernatant was collected and levels of IL-4, IL-13 and IFN-γ were measured by ELISA. TM 10 μg/mL, RPC 20, 40, 80 μg/mL. (B) RPC reduced the expression of Th2 cytokines but elevated the expression of Th1 cytokines. Lymphocyte precipitations were collected and mRNA expression levels of IL-4, IL-13 and IFN-γ were measured by QPCR. TM 10 μg/mL and RPC 40 and 80 μg/mL. (C) High levels of IFN-γ were induced by the synergistic effect between RPC and TM. Culture supernatant was collected and levels of IFN-γ were measured by ELISA. TM 10 μg/mL and RPC 80 μg/mL. (D) High levels of IFN-γ mRNA expressed in the RPC + TM group. Lymphocyte precipitations were collected and mRNA expression levels of IFN-γ were measured by QPCR. TM 10 μg/mL and RPC 80 μg/mL. (E) JNK and JAK2 pathways involved in RPC-induced IFN-γ production in the splenic lymphocytes of the TM-treated mice. The latter five groups add TM 10 μg/mL. RPC 80 μg/mL. The data represent the mean ± SD from three independent experiments. Statistical differences are indicated by P values (one-way ANOVA). * $P < 0.05$ and ** $P < 0.01$.

specific IgE of fucoidan-treated mice showed nearly 50% reduction compared with OVA-treated mice at the time of challenge [11]. Our results implied that RPC might be more effective in reducing IgE levels than fucoidan. However, the serum antigen-specific IgE levels were reduced after three gavages in the TM-treated mice (data not shown), this reduction of IgE was possibly due to the low dose of the gavages or food tolerance induced by high concentrations of antigen.

The levels of histamine in mice feces were higher than that in mice sera. Hence, HPLC and ELISA were used for detection in mice feces and mice sera, respectively. In this study, the histamine level of mice feces was significantly inhibited by RPC compared with the TM group ($P < 0.05$). Meanwhile, the concentration of histamine in the TM-stimulated mice was nearly 160 ng/mL. As previously reported, the serum histamine in antigen-stimulated mice could reach 1700 ng/mL [26]. The difference may be due to the time of blood collection.

A number of studies have reported that edema and lymphocytic infiltration were observed in the jejunum of mice sensitized with allergens [27,28]. In our histopathological examination, lymphocytic infiltration, villi edema, and villi shrinkage were observed in the jejunum of the TM-treated mice. In addition, the pathological symptoms of the TM + RPC-treated mice were mitigated for only lymphocytic infiltration.

As illustrated by Th1/Th2 cell polarization theory, the production of IgE antibody requires IL-4 and IL-13, but can be inhibited by IFN- γ . In particular, IL-4 is essential for IgE production [29] and promotes the switch from native T cells to Th2 cells [30]. So the effects of RPC on the characteristic cytokines were investigated in this study. The results showed that RPC could reduce the production and expression of IL-4 and IL-13 but promoted the production of IFN- γ in splenic lymphocytes and peritoneal cells. The results illustrated that the shift of the T-cell population from Th2 profile to Th1 profile induced by RPC was likely to protect against food allergy. However, splenic lymphocytes stimulated with TM or RPC alone could not produce such high levels of IFN- γ as the TM + RPC group. This result had been detected at both protein and mRNA levels. It indicated that higher levels of IFN- γ can be induced by the synergistic effect between TM and RPC.

The JAK-STAT4 pathway had been identified as the most important pathway for the production of IFN- γ [31]. Zhang et al. [32] found a p38 MAPK-dependent but STAT4-independent pathway of IFN- γ expression in activated T cells. Nakahira et al. [33] demonstrated that IL-18 could activate JNK, then induced the phosphorylation of c-Jun for IFN- γ expression. In this study, the signal pathways of IFN- γ production induced by RPC in TM-treated mice were investigated. The results showed that SP600125 (an inhibitor of JNK) and SD1008 (an inhibitor of JAK2) could significantly reduce IFN- γ levels in lymphocyte culture supernatants of the TM-treated mice, which implied that RPC in combination with TM induced IFN- γ synthesis through the pathways of JAK2 and JNK. The other signaling pathways of the production of IFN- γ and the other relevant allergic cytokines need to be further investigated.

Food-induced anaphylaxis is an IgE-mediated type I hypersensitivity reaction. In addition to the TM-sensitized murine model experiments, it has also been demonstrated that the anti-allergic effects of RPC extracted from *P. haitanensis* were discovered by the inhibition of IgE-mediated mast cell allergic reactions. Moreover, RPC inhibited the release and production of pro-inflammatory mediators (β -hexosaminidase and histamine), and pro-inflammatory cytokines (IL-4 and TNF- α) in activated mast cells. IL-4 is necessary for the development of Th2 cell response and promotes the production of IgE in B cells [34]. In the present study, RPC extracted from *P. haitanensis* was able to suppress the secretion of IL-4 in RBL-2H3 cells as well as mice splenic and peritoneal cells, indicating that RPC might mitigate the systemic anaphylaxis by inhibiting the switch from native T cells to Th2 cells.

In conclusion, the highly purified RPC extracted from *P. haitanensis* may be beneficial for allergic disorders, since it could potentially suppress Th2-type cell polarization but promote Th1-type cell polarization. Oral therapeutic administration of RPC could significantly reduce histamine release, relieve allergic symptoms and repair the pathology of the

jejunum in the TM-sensitized mice. In addition, RPC down-regulated the degranulation and histamine release of mast cells, and reduced the production of ROS and cytokines in IgE-mediated antigen-stimulated RBL-2H3 cells. Based on the results *in vivo* and *in vitro*, it can be speculated that RPC extracted from *P. haitanensis* had the ability of inhibiting not only TM-induced anaphylactic reaction, but also other allergens-induced IgE-mediated hypersensitivity reaction. Taken together, RPC extracted from *P. haitanensis* may provide insight into the prevention or treatment of food allergy anaphylaxis and may be used as function food such as RPC-infant formula or raw material of anti-allergy drugs.

Conflict of interest

The authors declare that there is no conflict of interests.

Acknowledgments

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