

Anti-Food Allergic Activity of Sulfated Polysaccharide from *Gracilaria lemaneiformis* is Dependent on Immunosuppression and Inhibition of p38 MAPK

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ABSTRACT: Polysaccharides from *Gracilaria lemaneiformis* in particular possess various bioactive functions, but their antiallergic activity remains incompletely defined. Sulfated polysaccharide from *Gracilaria lemaneiformis* (GLSP) was obtained by water extraction and ethanol precipitation followed by column chromatography. BALB/c mice, RBL-2H3, and KU812 cells were used for verifying the anti food allergic activity of GLSP. According to the results of mice experiment, GLSP was able to alleviate allergy symptoms, to reduce TM-specific IgE and IgG1, to suppress Th2 cell polarization, and to promote the function of regulatory T (Treg) cells. In addition, GLSP had the ability to inhibit the function of RBL-2H3 cells. Furthermore, GLSP inhibited the activation of KU812 via suppression of p38 mitogen-activated protein kinase (MAPK). In conclusion, immunosuppression as well as the reduction in the level of p38 MAPK may contribute to GLSP's putative activity against food allergy. GLSP may be used as a functional food component for allergic patients.

KEYWORDS: sulfated polysaccharide from *Gracilaria lemaneiformis*, food allergy, immunosuppression, p38 MAPK

INTRODUCTION

Food allergy is a rapidly growing public health concern because of its increasing prevalence and life-threatening potential. Food allergy is a kind of hypersensitivity caused by food containing some kind of allergen and manifests as a variety of symptoms that affects nearly 8% of children and 5% of adults worldwide, with growing evidence of an increase in prevalence.¹ Shellfish allergy in the Asia-Pacific region in particular ranks among the highest in the world and is the most common cause of food-induced anaphylaxis in the region.² In China, 16.7% of the rural population is sensitized to shellfish,³ in which tropomyosin (TM) is the major allergen.⁴ TM have been previously demonstrated to be a potent allergen in crab which could cause adverse hypersensitivity in a mouse model.⁵

After activation by allergens, the subset of CD4⁺ T-lymphocytes could produce a spectrum of cytokines such as interleukin (IL)-4, IL-5, and IL-13 that induce serum immunoglobulin E (IgE) production, which in turn mediate the clinical symptoms.⁶ The IgE immunoglobulins then attach specifically to Fc epsilon region Receptor 1 (FcεR1) membrane receptors of mast cells or basophils. Mast cells and basophils are key effector cells in allergy, once activated they can release cytokines, chemokines, proteases, leukotrienes, and bioactive polyamines.^{7,8} The activation of mast cells and basophils involves a complex network of signal transduction pathways and molecules, including various tyrosine kinases, Akt, and mitogen-activated protein kinases (MAPKs).⁹ MAPKs signaling cascades are important in the differentiation, activation, proliferation, degranulation, and migration

of various immune cells, including basophils and mast cells.¹⁰ In addition, regulatory T cells (Treg) are known to alleviate clinical symptoms of hypersensitivity to dietary food allergen by modulating the priming of allergen-specific T and B cell responses during oral sensitization and mast cell degranulation.¹¹

Indigenous people from southeast of China while eating shellfish simultaneously ingest marine algae products. In traditional Chinese medical literature, the marine algae are described as improver of digestion, enhancer of immunity among others. Recently, the role of marine algae as antiallergic agents has been demonstrated in vitro and in vivo studies.¹² It was shown that *Laminaria japonica* polysaccharides can significantly inhibit airway inflammation of asthmatic mice, adjust the balance of cytokines, and improve the pulmonary histopathological condition.¹³ Ishihara¹⁴ had reported that porphyran from red algae *Porphyra tenera* and *P. yezoensis* were capable of inhibiting the contact hypersensitivity reaction by decreasing the IgE serum level in BALB/c mice. Our previous studies also demonstrated that sulfated polysaccharides purified from *Porphyra haitanensis* (PHPS) which are composed mainly of galactose and sulfate have an antiallergic activity by suppressing the Th2 immune response in TM-sensitized mice.¹⁵ The Rhodophyta *Gracilaria lemaneiformis* has a similar polysaccharide content

Received: March 7, 2016

Revised: May 9, 2016

Accepted: May 17, 2016

and more affordable compare with *Porphyra haitanensis*. Several biological activities have been attributed to *Gracilaria lemaneiformis* among them antitumor, antiviral, hypoglycemic, and immunomodulatory effects.^{16–18} However, to the best of our knowledge, there are no studies describing the antiallergic activity of the sulfated polysaccharides from *G. lemaneiformis*.

In this study, the structural characteristics of sulfated polysaccharide isolated from *Gracilaria lemaneiformis* (GLSP) were described. The antiallergic activity of GLSP was evaluated employing the TM-sensitized mice and cellular models. Moreover, the key functional group of GLSP was shown to possess an antiallergic activity, and the effects of GLSP on signaling pathway molecules were further investigated in basophils.

MATERIALS AND METHODS

Reagents and materials. DEAE-cellulose 52 was obtained from Whatman (New York, U.S.A.). Imject Alum was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, U.S.A.). RPMI 1640, minimum essential medium with Eagle's salts (EMEM), penicillin, streptomycin solution, and fetal calf serum (FCS) were purchased from Invitrogen (New York, U.S.A.). Antidinitrophenyl (DNP)-IgE was obtained from Sigma (Louis, MO, U.S.A.), and DNP-BSA was purchased from Biosearch (Petaluma, CA). Human IgE, anti-human IgE, and goat anti mouse IgE, IgG1, IgG2a antibodies were purchased from Abcam (Cambridge, U.K.). Fluorescein isothiocyanate (FITC)-conjugated CD63 was obtained from BD pharmlingen (San Diego, CA). ELISA kit of histamine was purchased from IBL (Hamburg, Germany). Human phospho-MAPK array and ELISA kits of IL-4, IL-13, interferon (IFN)- γ , transforming growth factor (TGF)- β , and mouse mast cell proteinase (mMCP)-1 were purchased from R&D Systems (Minneapolis, MN, U.S.A.).

Gracilaria lemaneiformis was provided by the Third Institute of Oceanography, State Oceanic Administration, Xiamen, China.

Cells. The rat mast cell line (RBL-2H3) and human basophil cell line (KU812) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). The RBL-2H3 and KU812 cells were grown in EMEM and RPMI 1640, respectively, and supplemented with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin at 37 °C in a humidified incubator with a 5% CO₂/95% air atmosphere (Forma 3111, Thermo, Waltham, MA, U.S.A.).

Mice. Female BALB/c mice, 4–6 weeks of age, were obtained from the Animal Center of Xiamen University (China). Mice were housed in a Specific Pathogen Free (SPF) environment maintained at 22 $\pm$ 1 °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 (Xiamen, China), SCXK 2012–0005.

Purification of Polysaccharide from *Gracilaria lemaneiformis*. Polysaccharides from *Gracilaria lemaneiformis* were extracted and purified according to the procedures described by Shi.¹⁵ Briefly the algae was extracted in 95 °C water, ethanol precipitated, and fractionated in an anion exchange column (DEAE-cellulose 52). The carbohydrate content of the polysaccharide was monitored by the anthrone-sulfuric acid method.¹⁹ The endotoxin was removed from the purified polysaccharide fraction using Detoxi Endotoxin Removing Gel (Pierce, Rockford, USA). The endotoxin content of the polysaccharide fraction was determined to be <0.03 EU/mg as measured by the Limulus amoebocyte lysate assay (Associates of Cape Cod, New York, USA).

Characterization of Polysaccharide from *Gracilaria lemaneiformis*. Carbohydrate was quantified by the anthrone-sulfuric acid method using galactose as the standard.¹⁹ The 3,6-anhydrogalactose, sulfate, and protein contents were respectively determined as described by Shi.¹⁵ The molecular weight distribution of the polysaccharide was determined by high performance gel-permeation chromatography (HPGPC) (Agilent-1100, Santa Clara, U.S.A.) equipped with a ZORBAX Eclipse XDB-C18 column (250 $\times$ 4.6 mm², column temperature 30 °C). The monosaccharide composition of GLSP was

determined after acid hydrolyses of the polysaccharide by ion chromatography (IC) analysis (ICS-3000, Dionex, Sunnyvale, CA, U.S.A.) in a CarboPac PA20 column (3 $\times$ 150 mm²), using 0.25 M NaOH at a flow rate of 0.5 mL/min. The infrared spectra of the polysaccharide fraction was collected in a Fourier transformed infrared spectrometer (FTIR) (VECTOR-22, BRUKER) in the wavenumber range of 4000–500 cm^{–1} using the KBr-disk method.

Food Allergy Model. The purification of TM from mud crab (*Scylla paramamosain*) was carried out as previously described.²⁰ Mice were sensitized and monitored as described by Yamaki.²¹ Briefly, four groups of mice experiment were established to investigate the anti food allergic activity of GLSP, and the schemes were PBS group, TM group, GLSP-preventive group, and GLSP-treatment group, respectively. Twenty-four mice were randomized to four groups. For the sensitization, mice were immunized with 50 μ g of TM in 150 μ L of phosphate-buffered saline (PBS) emulsified with Imject Alum by intraperitoneal injection on days 0 and 14. Then mice were challenged 6 times from days 28 to 38 by gavage with 10 mg TM in 200 μ L of PBS every other day for establishing the food allergy model. In the preventive protocol, GLSP (5 mg/mouse in 200 μ L of PBS) was orally administered daily from the day before the first challenge to the day before sacrifice (from day 27 to day 40) (regarded as GLSP-preventive group). And in the treatment protocol, GLSP (5 mg/mouse in 200 μ L of PBS) intake daily from mice already challenged with TM 3 times (from day 33 to day 40) to evaluate the therapeutic effect of GLSP on food allergy (regarded as GLSP-treatment group). Meanwhile, the negative control mice (PBS group) were immunized with 150 μ L PBS at days 0 and 14, and given intragastrically of 200 μ L PBS at days 28, 30, 32, 34, 36, and 38.

Anaphylactic Symptoms. Anaphylactic symptoms and diarrhea rates (the proportion of diarrheal mice in each group) were scored for 1 h after each challenge.²² Rectal temperatures were measured for 0–1 h after the sixth challenge using a rectal temperature electronic thermometer (Prosper, Shanghai, China). And the concentrations of

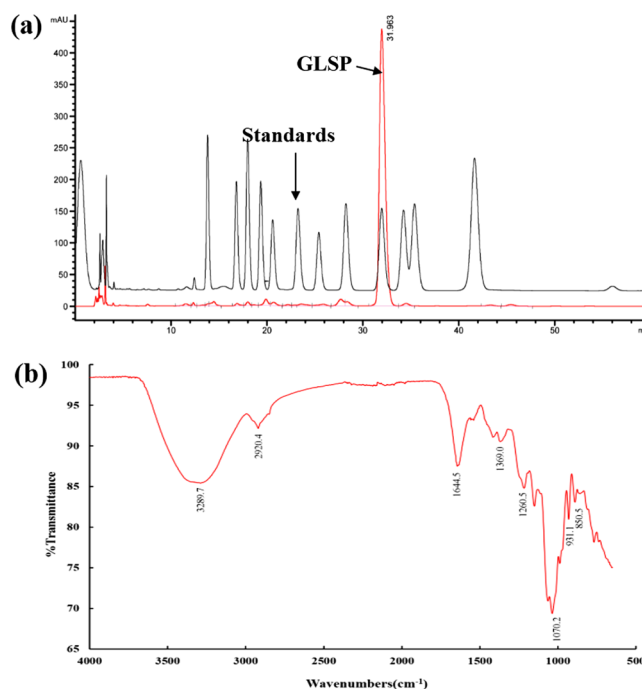


Figure 1. The structural analysis of GLSP. (a) IC analysis of the monosaccharide from GLSP, the gray line was standard substances and the red line was GLSP sample. The standards were mannose, glucosamine, ribose, rhamnose, glucuronic acid, galacturonic acid, galactosamine, glucose, galactose, xylose, arabinose, and fucoidan. (b) FTIR spectrum of GLSP in the wavenumber region between 4000 and 500 cm^{–1}.

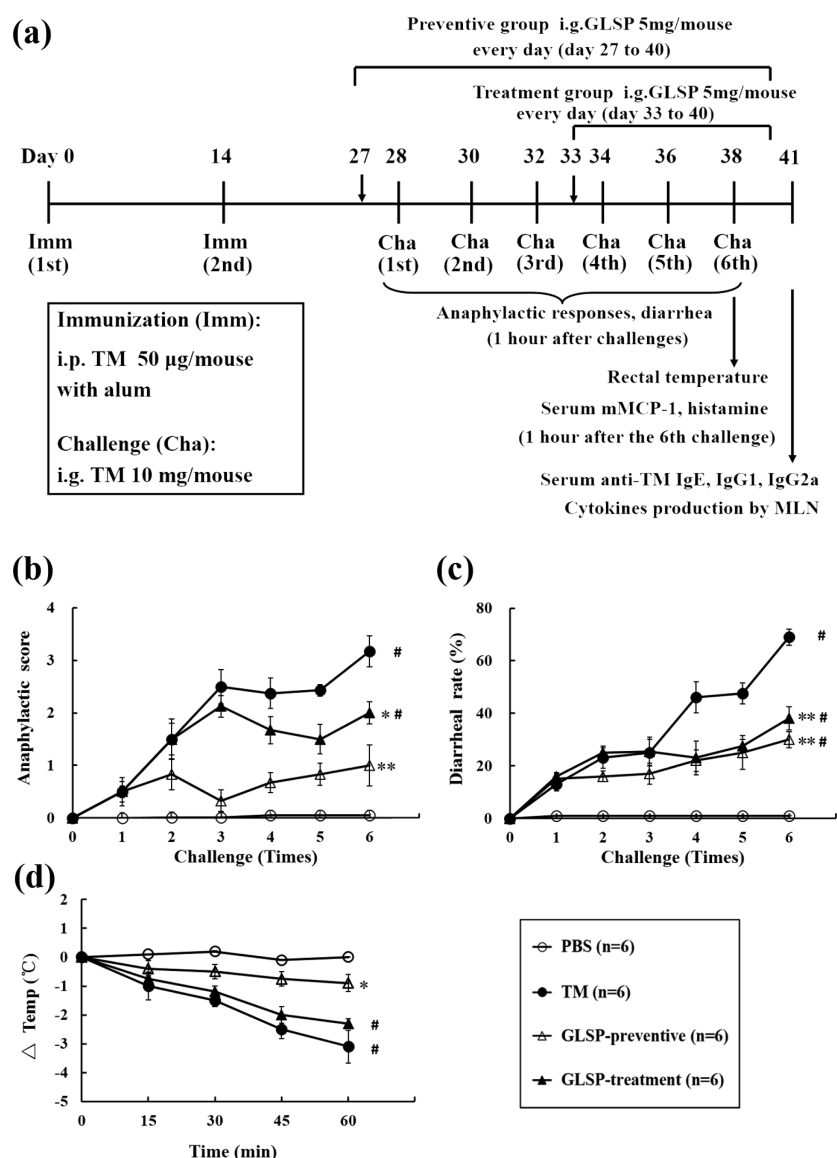


Figure 2. Animal experimental design and effects of GLSP on the allergy symptoms. (a) Protocol for the food allergy model. Groups of mice ($n = 6$ mice per group) were sensitized two times by intraperitoneal injection of TM (50 μ g/mouse), orally challenged with 10 mg TM for six times every other day. Mice were orally challenged with 5 mg GLSP every day from days 27 to 40 (regarded as GLSP-preventive group) or from days 33 to 40 (regarded as GLSP-treatment group). (b) The symptom scores of the anaphylactic symptom for 1 h after every challenge. (c) The rates of diarrhea for 1 h after every challenge. (d) The rectal temperature of mice for 0 to 1 h after the sixth challenge. i.p., intraperitoneal injection i.g., intragastrically. All experiments were repeated 3 times. Statistical differences are indicated by P values (one-way ANOVA). * $P < 0.05$, ** $P < 0.01$, compared with the TM-treated group, and # $P < 0.01$, compared with the PBS group.

histamine and mMCP-1 in serum obtained for 1 h after the sixth challenge were determined using commercially available ELISA kits following the manufacturer's instructions. All mice were sacrificed on day 41, the levels of serum TM-specific IgE, IgG1, and IgG2a were measured using ELISA as previously described.²³ Furthermore, Western blot analysis of pooled serum TM-specific was performed as described by Yamaki.²²

Mesenteric Lymph Node (MLN) Cells Restimulation for Cytokines Measurement. MLNs were isolated from mice and minced in RPMI 1640 medium with 10% fetal calf serum and 1% penicillin-streptomycin solution at day 41. Cells were cultured in 24-well plates and restimulated with the antigen TM (10 μ g/mL) for 3 days. Then the cell supernatants were collected for measuring the concentrations of IL-4, IL-13, IFN- γ , and TGF- β using ELISA kits following the manufacturer's instructions. And the cell precipitates were collected for mRNA preparation and real-time quantitative PCR (QPCR) for transcription factors according to the previous description.²⁴

RBL-2H3 Assay. The release of β -hexosaminidase and histamine by RBL-2H3 cell was measured as a model of IgE-mediated mast cell allergic reaction, using the sensitization of anti-DNP-IgE and the stimulation of DNP-BSA.^{24,25} The cytokines (IL-4 and TNF- α) were evaluated as previously described.²⁴ To evaluate the role of sulfate in cell degranulation, the sulfate of GLSP was removed by heating GLSP as a pyridinium salt in DMSO.²⁶ The sulfate content of GLSP was measured by barium chloride-gelatin method.¹⁵

Flow Cytometry Assay. To examine the effects of GLSP on the expression of activation-linked antigens of basophils, flow cytometry experiments were performed. Human basophil KU812 cells (1×10^6 cells) pretreated with or without GLSP (100–200 μ g/mL) for 24 h were incubated with 10 μ g/mL of human IgE for 3 h and stimulated with 20 μ g/mL of anti-IgE for 30 min in tyrode's buffer. Then, cells were washed and stained with FITC-conjugated CD63 for 20 min at 4 $^{\circ}$ C. Guava easyCyte 6–2L system and GuavaSoft 3.1.1 software (Millipore, Massachusetts, U.S.A.) were used for the

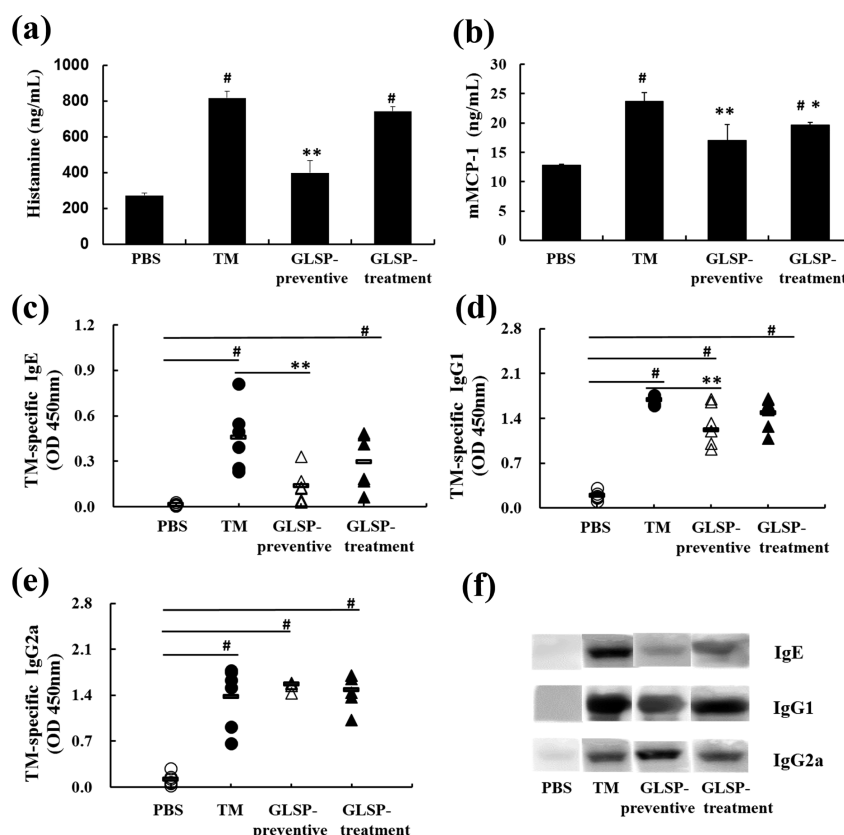


Figure 3. The inhibition of GLSP on serum histamine, mMCP-1, TM-specific antibodies in mice. Sera from the tail vein of mice were obtained for 1h after the last challenge, and the histamine levels (a) and mMCP-1 levels (b) were measured; Sera from eyeball blood at days 41 were collected to measure the TM-specific IgE (c), IgG1 (d) and IgG2a (e) levels through ELISA and Western blot analysis from pooled serum (f). All experiments were repeated 3 times. Statistical differences are indicated by *P* values (one-way ANOVA). **P* < 0.05, ***P* < 0.01, compared with the TM-treated group, # *P* < 0.01, compared with the PBS group.

signal-color flow cytometry analysis. IgE-induced upregulation of CD63 was calculated from mean fluorescence intensities (MFIs) obtained with stimulated, unstimulated cells, and GLSP pretreated cells.

Proteome Profiler array. KU812 cells after the same treatment with flow cytometry assay were lysed and relative phosphorylation levels of all three major families of MAPK (ERK1/2, p38 α / β / δ / γ , JNK1/2/3/pan) and Akt 1/2/3/pan, RSK1/2, GSK-3 α / β , MSK2, MEK3/6, and HSP27 as well as p70 S6 kinase were determined by human phospho-MAPK array according to the manufactures' instructions.

Statistical Analysis. Data were presented as means $\pm$ SD. Differences between means were analyzed by ANOVA of Duncan test. A *p* value of less than 0.05 was considered statistically significant. In the animal model study samples from individual mice were processed and analyzed separately. Each experiment was repeated at least 3 times.

RESULTS

Composition of Polysaccharide from *Gracilaria lemaneiformis*. A polysaccharide fraction from *Gracilaria lemaneiformis* was purified as single chromatography peak and called hereafter as GLSP. The fraction contained 3,6-anhydrogalactose, sulfate, and protein at levels of 8.23%, 11.26%, and 0.98%, respectively. The average molecular weight of GLSP was 152 481 Da as determined by HPLC analysis (data not shown). According to the description by Sun,²⁷ as shown in IC analysis results, the peak at 31.963 min was the galactose in standard substances (gray line in Figure 1a), and the strong peak at 31.963 min content of 87.49% peak area was shown in GLSP sample (red line in Figure 1a). The FTIR spectrum of the sample was shown in Figure 1b. GLSP exhibited absorption

peaks at 3289, 2920, and 1070 cm^{-1} , which were characteristic absorptions of —OH, C—H, and C—O, respectively.²⁸ The IR absorption at 931 cm^{-1} had the characteristic absorption of 3,6-anhydrogalactose. Infrared spectroscopy provided useful information for the position of sulfate groups of polysaccharide. The IR spectra show an absorption band at 1260 and 850 cm^{-1} , indicating the presence of the total sulfate ester.²⁹

Attenuation of Anaphylaxis by GLSP in TM-Sensitized Mice. To test the effect of GLSP on food allergy, the animal experiment was implemented according to the protocol shown in Figure 2a. Daily dose of GLSP moderately decreased the scores of the anaphylactic response (Figure 2b) and diarrhea rates (Figure 2c) for 1 h after the third-sixth challenge in both GLSP-preventive and GLSP-treatment groups. The average rectal temperature of TM group mice was 3 °C less than PBS group mice for 1 h after the sixth challenge. Hypothermia induced was remitted by the administration of GLSP in GLSP-preventive mice (*p* < 0.05) (Figure 2d). Meanwhile, GLSP has also shown to diminish the increase of the concentrations of serum histamine and mMCP-1 after the sixth challenge in the GLSP-preventive group (Figure 3a, 3b). In addition, the daily administration of GLSP in GLSP-preventive group significantly reduced TM-specific IgE (Figure 3c) and IgG1 (Figure 3d) serum levels, but did not anti-TM IgG2a (Figure 3e). The Western blot result from pooled sera was similar to the ELISA data (Figure 3f). These results suggest that the anti food allergic symptoms activity of GLSP in GLSP-preventive group was more pronounced than that in the GLSP-treatment group.

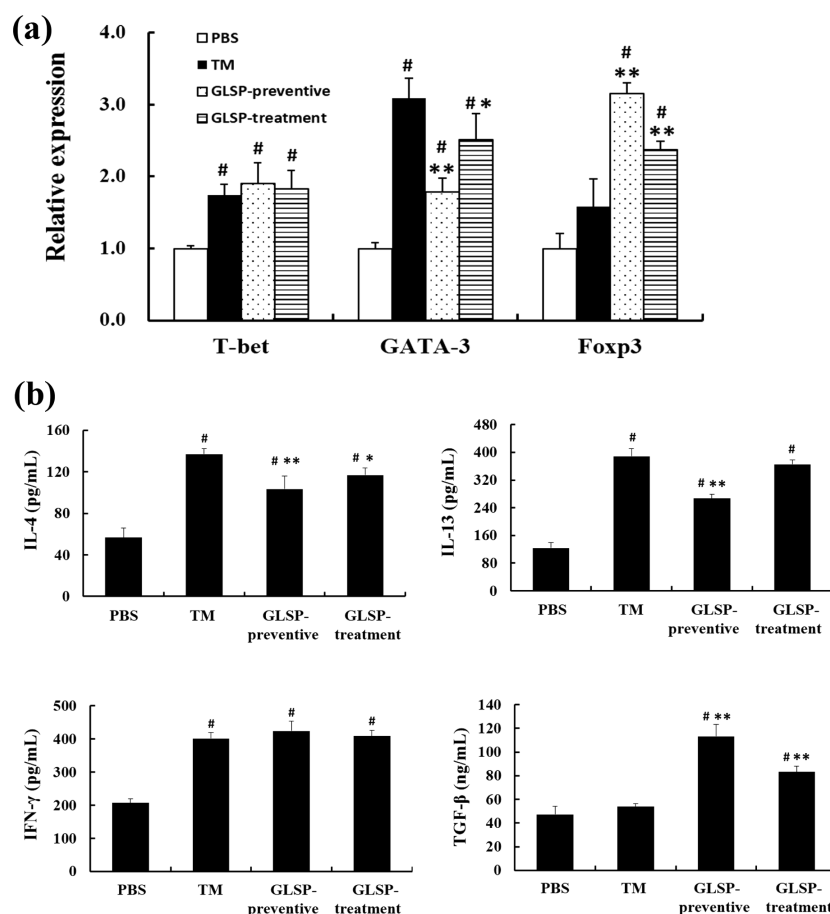


Figure 4. The regulatory effects of GLSP on T cell polarization in mice. (a) The MLN cells obtained from mice at day 41 were cultured with TM (10 $\mu\text{g/mL}$) for 3 days (1×10^6 cells/well). The transcription factors (T-bet, GATA-3 and Foxp3) were detected by QPCR. QPCR was performed with the following primers: β -actin (5'-AGC TGC GTT TTA CAC CCT TT-3'; 5'-AAG CCA TGC CAA TGT TGT CT-3'), T-bet (5'-CCC CTG TCC AGT CAG TAA CTT-3'; 5'-CTT CTC TGT TTG GCT GGC T-3'), GATA-3 (5'-AGG AGT CTC CAA GTG TGC GAA-3'; 5'-TTG GAA TGC AGA CAC CAC CT-3'), Foxp3 (5'-CTC ATG ATA GTG CCT GTG TCC TCA A-3'; 5'-AGG GCC AGC ATA GGT GCA AG-3'). (b) The production of cytokines (IL-4, IL-13, IFN- γ , and TGF- β) from cell culture supernatants were measured by ELISA. All experiments were repeated 3 times. Statistical differences are indicated by *P* values (one-way ANOVA). **P* < 0.05, ***P* < 0.01, compared with the TM-treated group, and # *P* < 0.01, compared with the PBS group.

Immunosuppression of GLSP in TM-Sensitized Mice.

Thus far, GLSP was known to inhibit the levels of Th2-dependent IgE and IgG1, but did not affect the Th1-dependent antibody. In order to examine whether T-cell polarization in the TM-sensitized mice was modulated by GLSP, the expression of the transcription factors T-bet, GATA-3, and Foxp3 as the reflection of, respectively, Th1, Th2, and Treg cells were evaluated. The QPCR results of both the GLSP-preventive and the GLSP-treatment group were shown in Figure 4a, GATA-3 expression tended to decrease and Foxp3 expression tended to increase in MLN cells, while T-bet expression was not modulated by GLSP. The results of cytokines secreted from TM-stimulated cultured MLN cells corroborated these results. Compared with the TM mice, the GLSP-preventive mice exhibited the inhibition of the production of Th2 cytokines (IL-4 and IL-13), the increase of the secretion of TGF- β , and little influence on Th1 cytokine (IFN- γ) (Figure 4b). The level of IL-4 in the cell culture supernatants from GLSP-treatment mice was lower than that from TM mice (Figure 4b). Therefore, GLSP was able to suppress Th2 cell polarization, and promote Treg cell polarization. Due to the immune suppressor capacity of Treg cell, it was boldly speculated that the antiallergic activity of GLSP in TM-sensitized mice was through immunosuppression

by up-regulating Treg, however a complete set of cytokine and more in depth analysis is still necessary to reach this conclusion.

Suppression Effect of GLSP on IgE-Mediated RBL-2H3 Cells.

The RBL-2H3 cell line has been widely used as a mast cell model for IgE-mediated cell study.³⁰ GLSP (20–200 $\mu\text{g/mL}$) alone or GLSP+DNP-BSA had no measurable cytotoxic effect on RBL-2H3 cells (data not shown). GLSP (100–200 $\mu\text{g/mL}$) significantly diminished the antigen-induced degranulation as measured by the reduction in the release of β -hexosaminidase (*p* < 0.01) (Figure 5a). And the levels of histamine were also significantly reduced by GLSP (100–200 $\mu\text{g/mL}$) (Figure 5b) in a concentration dependent manner. IL-4 and TNF- α are the important cytokines in allergic inflammation.³¹ Figure 5c, d demonstrates that GLSP significantly inhibited the secretion of IL-4 and TNF- α in a dose-dependent manner in IgE-mediated RBL-2H3 cells. None of the indexes above showed significant difference between RBL-2H3 cells treated with GLSP (200 $\mu\text{g/mL}$) alone (in the absence of DNP-BSA) and PBS-treated cells (data not shown).

The sulfate content of GLSP after desulfurization was dropped to 2.64%. The data in Figure 5e indicated that GLSP after desulfurization (the last three columns) could not inhibit the degranulation of RBL-2H3. Thus, these results indicated that

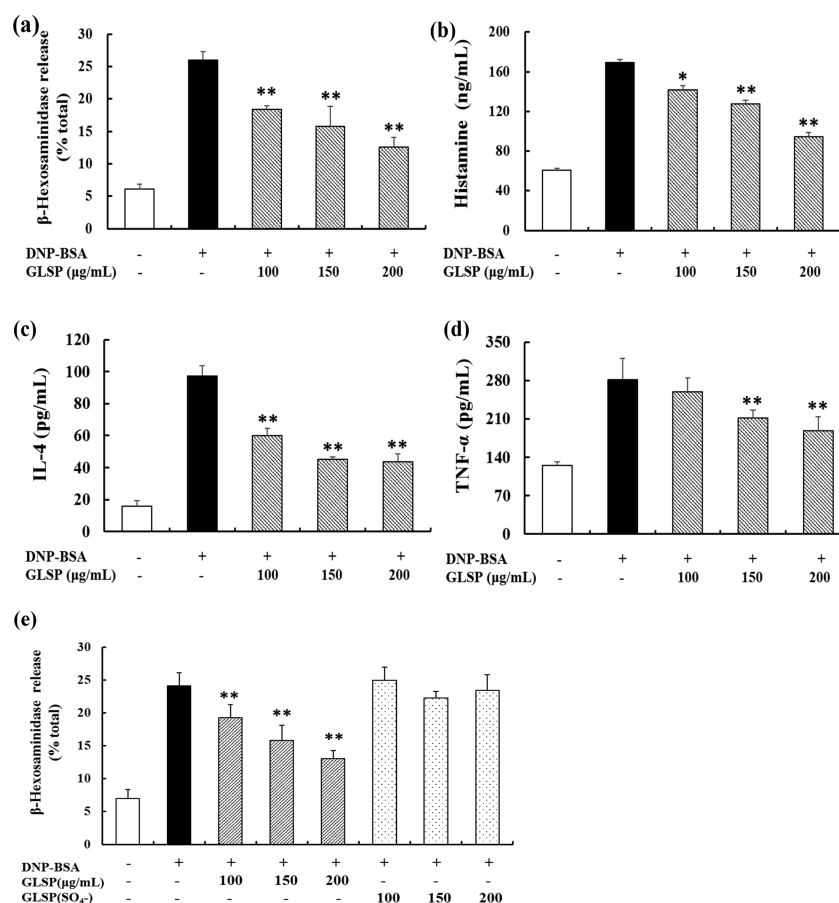


Figure 5. The inhibitory effect of GLSP in IgE-mediated allergic responses in RBL-2H3. (a) Effects of GLSP on the degranulation. The cells were incubated overnight in 48-well plates with 200 ng/mL of DNP-specific IgE in the medium. The medium was replaced by Tyrode's buffer containing the indicated concentrations of GLSP (100–200 μg/mL) followed by stimulation with 500 ng/mL DNP-BSA for 6 h, and the release of β-hexosaminidase was measured. (b) Effects of GLSP on the histamine release. Same as (a) except that stimulation with 500 ng/mL DNP-BSA for 15 min, and histamine was measured. (c) (d) Effects of GLSP 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 GLSP (100–200 μg/mL). The amount of IL-4 and TNF-α released into the culture supernatant were measured by ELISA. (e) Cells culture were same as (a), and the β-hexosaminidase release rates of RBL-2H3 cells pretreatment with GLSP (3–5 columns) or GLSP after desulfurization (the last three columns) were measured. GLSP(SO₄⁻): GLSP after desulfurization. Data represent the mean ± SD from three independent experiments. Statistical differences are indicated by *P* values (one-way ANOVA). **P* < 0.05, ***P* < 0.01, and significantly different from DNP-BSA alone.

the activation and function of RBL-2H3 cells were inhibited by GLSP, and the sulfate of GLSP play a crucial role.

Inhibitory Effect of GLSP on IgE-Mediated KU812 Cells. The human basophilic cell line KU812 was commonly used for the cell model of IgE-mediated allergic reaction.³² Flow cytometry was used to assess the activation of KU812 cells. In the CD63-FITC histogram plot (Figure 6a), the leftmost peak was the result of PBS stimulated KU812 cells (in absence of anti-IgE), the rightmost peak was reflected the anti-IgE stimulated cells result, and the result of cells pretreatment with 200 μg/mL of GLSP was the middle peak. The MFI of CD63 of KU812 cells stimulated by anti-IgE was 225, significantly higher than that of unstimulated cells, while GLSP was found to decrease the MFI of stimulated cells in a dose-dependent fashion. In addition, GLSP after desulfurization could not inhibit the expression of CD63 in KU812 cells (the MFI of CD63 was 226, Figure 6b).

Hindering Effect of GLSP on the Phosphorylation Levels of Distinct Signaling Molecules in KU812 Cells. To gain insight into how GLSP suppressed the activation of basophils, the phospho-activation of MAPKs was examined. As shown in Figure 7a, 26 kinases of signaling molecules were monitored using Proteome Profiler Array. As shown in

Figure 7b–d, the spot intensity of p38δ, Akt1, MEK3/6, and RSK2 in the GLSP pretreatment group were significantly lower than that of the IgE stimulated control. Although measuring the total proteins in the cytoplasm of the KU812 cells pretreatment with or without GLSP (200 μg/mL), the levels of the phosphorylated kinases MEK3 and MEK6 upstream of p38 MAPK were increased in anti-IgE stimulated KU812 cells (the second photograph), and they were impeded by GLSP (the bottom photograph). Meanwhile, the phosphorylation level of p38δ MAPK was suppressed by GLSP. GLSP also caused a large reduction of the activation of Akt1, compared to cells stimulated with anti-IgE. Furthermore, the phosphorylation level of RSK2 downstream of ERK was counteracted by GLSP, although GLSP enhanced mildly the phosphorylation of ERK1 and ERK2. Throughout the experiment, no effect of GLSP on JNK was observed.

DISCUSSION

During the past decade, active ingredients from marine algae have been shown to play an important role in the pharmaceutical industry in the development of novel drugs against

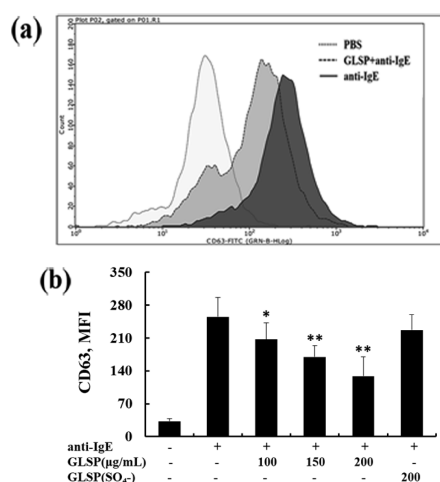


Figure 6. The inhibitory effect of GLSP on the expression of CD63 in KU812 cells. (a) KU812 cells (1×10^6) pretreated with or without GLSP (100–200 $\mu\text{g/mL}$) for 24 h were incubated with 10 $\mu\text{g/mL}$ of human IgE for 3 h and stimulated with 20 $\mu\text{g/mL}$ of anti-IgE for 30 min. The CD63-FITC histogram plot of flow cytometry was shown; (b) The MFIs of CD63-FITC were demonstrated in the column diagram. The experiment was repeated three times. Statistical differences are indicated by P values (one-way ANOVA). * $P < 0.05$ and ** $P < 0.01$, significantly different from anti-IgE stimulated cells. GLSP(SO₄⁻): GLSP after desulfurization.

allergic disorders, for example polysaccharides, phlorotannins, phycocyanins, among others.¹² Various studies had reported that marine polysaccharides were immunomodulators of allergic responses.^{13,14} *Gracilaria lemaneiformis* is an important marine

algae that is mainly cultured near the southeast coast of China and is known to contain bioactive material. In the present study, using our patented methodology (Patent No.: 201310424569.6), the purified sulfated polysaccharide isolated from *Gracilaria lemaneiformis* showed a similar monosaccharide composition and sulfate content as the PHPS reported by our group.¹⁵ Fan¹⁶ reported that the acidic polysaccharide isolated from *Gracilaria lemaneiformis* was exhibited the antitumor activity, however its molecular weight was 1.37×10^6 Da, which is more than that of GLSP (1.52×10^5 Da) reported here; the sulfate content was 6.13%, which is less than that of GLSP (11.26%); and the main monosaccharide is galactose, which is the same as that of GLSP. These differences might due to the different extraction methods and different geographical origins of the materials used in the experiment.

Food allergy is an immunologically adverse reaction caused by food, which encompasses a range of disorders including IgE-mediated anaphylaxis, decrease of body temperature, and gastrointestinal adverse reaction.^{1,33} A food allergic murine model sensitized by intraperitoneally followed by the orally challenge of TM was established in the present study. The allergic animal were exhibited the increase in Th2 cytokine levels, TM-specific IgE levels, serum histamine, and mMCP-1 levels. The present data suggested that GLSP had potent effects on the attenuation of allergic diseases. GLSP was responsible for the reduction in the production of serum mMCP-1 and histamine in GLSP-preventive mice.

Allergen-specific serum IgE is essential in type I hypersensitivity reactions. The Th2-dependent TM-specific IgE and IgG1 serum levels were significantly reduced by GLSP as well as the production of IL-4 and IL-13. This influence was similar

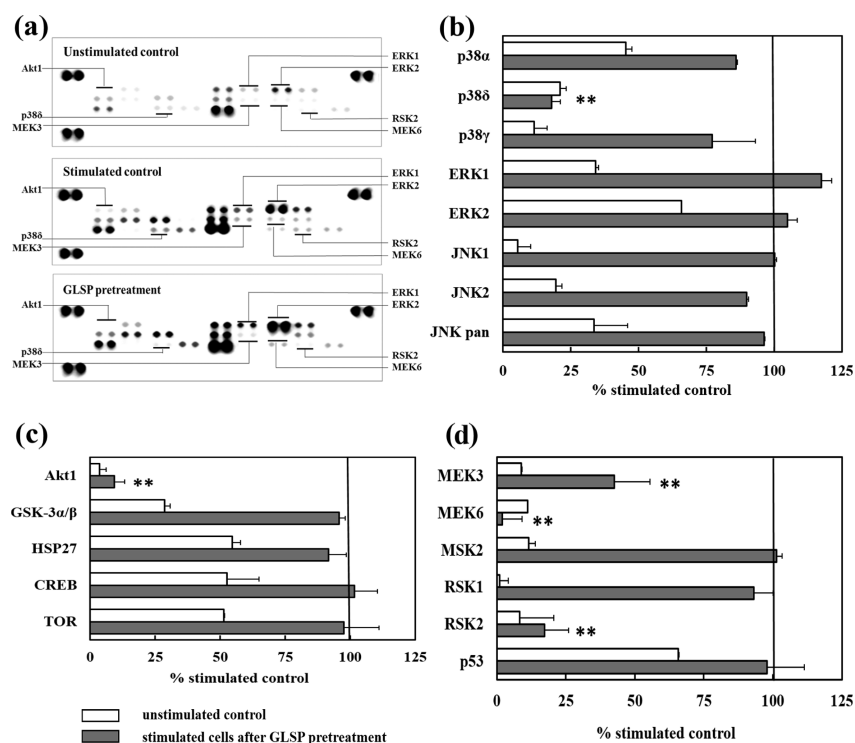


Figure 7. Effect of GLSP on phosphorylation levels of multiple signaling molecules in KU812 cells. (a) Representative pictures of Proteome Profiler Human Phospho-MAPK array of unstimulated control cells, stimulated control cells and stimulated cells after pretreatment with GLSP (200 $\mu\text{g/mL}$). (b–d) Densitometric analysis of selected spots as % of stimulated cells treated with anti-IgE is shown. Stimulated cells were incubated with IgE for 3 h and subsequently stimulated with anti-IgE for 30 min. The results of KU812 cells pretreated with GLSP were shown in the dark gray column, and cells incubated with PBS (light gray) could be the unstimulated control.

to the effect of PHPS, but the difference is that GLSP had no obvious effect on the level of Th1-dependent IgG2a and the production of IFN- γ . The expression of Foxp3 in mouse MLN cells was increased in mice of two GLSP treated groups, suggesting that Treg cell polarization was promoted by GLSP. TGF- β is an important factor in the Treg cell development and suppressor function.³⁴ The increased production of TGF- β further corroborated these. The GLSP was thus speculated to have immunosuppression by promoting Treg which can suppress T cells. Whether GLSP had some influence on Th9, Th17, and other immune cells need to follow research.

Food allergy reaction is induced by the rapid local and systemic release of inflammatory mediators such as histamine, serotonin, and various pro-inflammatory cytokines from mast cells and basophils.³⁵ The concentrations of serum histamine and mMCP-1, which are known inflammatory mediators⁷ increased in TM-sensitization of mice were inhibited by GLSP. So the effect of GLSP on the activation and function of mast cells and basophils were further confirmed using RBL-2H3 and KU812 cell lines. The activation of human basophils is associated with up-regulation of various surface CD-antigens, for instance, CD63.³⁶ The present results showed that GLSP had the ability to suppress the degranulation and the production of histamine, IL-4, and TNF- α in IgE-mediated RBL-2H3. Mouse mast cells have been shown to inhibit Treg suppressor function via histamine H1 receptor interaction and thus promote optimal activation of effector T cells.³⁷ The immunologic balance in TM-sensitized mice was regulated by GLSP, it was speculated that the preventive effect of GLSP on food allergic response were due to the inhibition of the release of mediators of mast cells. The poor therapeutic effect of GLSP on food allergic response may be to the result of short treatment term (day 33 to 40). Meanwhile, the MFI of CD63 was also inhibited by GLSP in KU812 cells. The little effect of GLSP after desulfurization on mast cells and basophils was in general agreement with the results reported by Ngo³⁸ in which it is stated that sulfated polysaccharides are the main bioactive agents from marine algae.

The activation of tyrosine kinases initiates an intracellular signaling cascade involving activation of MAPKs, Akt, and other molecules, which have been implicated in the gene expression of relevant cytokines.³⁹ The p38 MAPK play an important role in anaphylactic reaction in humans, and all four isoforms of p38 (α , β , γ , and δ) can be activated by MEK6; p38 α and p38 β are also activated by MEK.⁴⁰ RSK is a known downstream target of ERK1/ERK2.⁴⁰ It has been shown that sequential allergen desensitization of basophils may involved the phosphorylation of p38 MAPK and was correlated with the decrease in CD63 upregulation.⁴¹ CTB glycoprotein was exhibited to reduce allergic responses through p38 MAPK phosphorylation,⁴² and Yang⁴³ had reported that oleamide from *arctium lappa* L. undried roots had the antiallergic activity via regulating p38 MAPK signaling in RBL-2H3 Cells. In the present study, GLSP was able to inhibit the phosphorylation of Akt, and MEK3/6, P38 δ , as well as RSK2. Due to the inhibition of these signaling pathway molecules, GLSP was shown to be able to counteract the activation of basophils.

In conclusion, the present study showed that purified sulfate polysaccharide from *Gracilaria lemaneiformis* (GLSP) had an average mass of 152 481 Da. Dietary intervention with GLSP could alleviate the allergic symptoms of TM-sensitized mice by immunosuppression and the inhibition of mast cells. The activation of basophil was inhibited by GLSP via p38 MAPK signaling pathway. On the basis of the in vivo and in vitro

results, we suggested that GLSP possess an antifeed allergic activity. Finally, GLSP may provide insight into the preventive of food allergy induced-anaphylaxis and may be used as a functional food component to prevent food allergic diseases.

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Funding

This work was supported by grants from the National Natural Scientific Foundation of China (31171660 and U1405214), the Scientific Foundation of Fujian Province (2014N0014), the Marine Scientific Research Special Foundation for Public Sector Program (201505026-03), the Xiamen South Ocean Research Center Project (201405220001).

Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

DCF, 2,2'-Dichlorodihydrofluorescein; DNP, Dinitrophenyl; ERK, extracellular signal-regulated kinase; Foxp3, Forkhead box protein 3; GLSP, Sulfated polysaccharide from *Gracilaria lemaneiformis*; JNK, c-Jun NH2-terminal kinase; LPS, Lipopolysaccharide; MAPK, mitogen-activated protein kinases; mMCP, Mouse mast cell protease; TM, Tropomyosin; Treg, Regulatory T

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