Sulfated polysaccharide from *Gracilaria lemaneiformis* aleviates food allergy symptoms by immunosuppression and inhibition of Syk and p38 MAPK

Qing-mei Liu, MS,[a] Yang Yang, MS,[a] Soheila J. Maleki, PhD,[b] Marcos Alcocer, PhD,[c] Sha-sha Xu, MS,[a] Chao-lan Shi, MS,[a] Min-jie Cao, PhD,[a] Guang-ming Liu, PhD,[a]*

[a] *College of Food and Biological Engineering, Fujian Collaborative Innovation Center for Exploitation and Utilization of Marine Biological Resources, Jimei University, 43 Yindou Road, Xiamen, 361021, Fujian, P.R. China*

[b] *U.S. Department of Agriculture, Agriculture Research Service, Southern Regional Research Center, 1100 Robert E. Lee Boulevard, New Orleans, LA 70124, USA*

[c] *School of Biosciences, Sutton Bonington Campus, Loughborough, LE12 5RD, UK*

Running title: Anti-allergic activity of sulfated polysaccharide from *Gracilaria lemaneiformis*

*__Corresponding author:__

Guang-ming Liu, PhD

College of Food and Biological Engineering, Jimei University

Xiamen, 361021, P.R. China

Tel: +86-592-6180378

Fax: +86-592-6180470

Email: gmliu@jmu.edu.cn

ABSTRACT

Background: Polysaccharides from marine sources offer diverse therapeutic functions due to their multifarious biological nature. Polysaccharide from *Gracilaria lemaneiformis* possesses various bioactive functions, but its anti-allergic activity remains incompletely defined.

Objective: This study aimed to extract and analyze sulfated polysaccharide from *Gracilaria lemaneiformis* (GLSP), investigate the effects of GLSP on the anti-allergic activity in both animal and cell models, and explore its anti-allergic mechanisms.

Methods: GLSP was obtained by water extraction, ethyl alcohol deposition, and column chromatography. Tropomyosin (TM)-sensitized mice were treated with GLSP intragastrically for the prevention group and the treatment group. The effects of GLSP on the function of IgE-mediated RBL-2H3 and the activation of KU812 cell lines were also investigated. Finally, the inhibition of signaling molecules by GLSP was detected through Western blot and Proteome assays.

Results: GLSP was a 152,481 Da sulfated polysaccharose composed of galactose. GLSP could alleviate allergy symptoms, effectively reduce TM-specific IgE and IgG1, decrease the levels of histamine and mMCP-1, suppress Th2 cell polarization, and promote the function of regulatory T cells in mice. In addition, GLSP had the ability to inhibit the function of RBL-2H3 cells. And the sulfate of GLSP inhibited degranulation. Meanwhile, the expression of CD63 and CD203c were reduced by GLSP in activated KU812 cells. GLSP inhibited the activation of KU812 via suppressing Syk and p38 mitogen-activated protein kinases (MAPK).

Conclusion: Immunosuppression as well as the reduction in the levels of Syk and p38 MAPK may contribute to the ability of GLSP to suppress the symptoms of food allergy. The sulfate of GLSP may play an important role in the anti-allergic effect.

Key words: sulfated polysaccharide from *Gracilaria lemaneiformis*, food allergy, immunosuppression, Syk tyrosine kinase, p38 MAPK

Abbreviations used

DCF: 2,7-Dichlorodihydrofluorescein

DNP: Dinitrophenyl

Foxp3: Forkhead box protein 3

GLSP: Sulfated polysaccharide from *Gracilaria lemaneiformis*

LPS: Lipopolysaccharide

MAPK: mitogen-activated protein kinases

mMCP: Mouse mast cell protease

~~MTT: Methyl thiazolyl tetrazolium~~

~~SPF: Specefic pathogen free~~

Treg: Regulatory T

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INTRODUCTION

Food allergy is a rapidly growing public health concern because of its increasing prevalence and life-threatening potential. On the basis of numerous studies, food allergy is an immune provocation by food in susceptible individuals that affects nearly 8% of children and 5% of adults, with growing evidence of an increase in prevalence.[1] As a kind of food allergy, shellfish allergy in the Asia-Pacific region ranks among the highest in the world and is the most common cause of food-induced anaphylaxis.[2] In china, 16.7% of the rural population is sensitized to shellfish.[3] Tropomyosin (TM) has been identified as the major allergen of shellfish.[4] We have previously demonstrated that TM is an allergen in crab, which can cause adverse hypersensitivity in a mouse model.[5]

Food-induced anaphylaxis is an IgE-mediated Type I hypersensitivity reaction and is characterized by the development and activation of T helper 2 (Th2) cells.[1] After activation by allergens, the subset of CD4 (+) T-lymphocytes produce a spectrum of cytokines like IL-4, IL-5, IL-6, IL-9, and IL-13 that cause induction of serum immunoglobulin E (IgE) levels, which, in turn, mediate the clinical symptoms.[6] The IgE immunoglobulin attaches with Fragment crystallizable epsilon region Receptor 1 (FcεR1) of mast cells or basophils. Mast cells and basophils are key effector cells, which can release cytokines, chemokines, proteases, leukotrienes and bioactive polyamines after activation.[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] MAPKs signaling modules are divided into at least three groups: extracelluar signal-regulated kinase (ERK), p38 MAPK, and c-Jun NH_2-terminal kinase (JNK). In addition, Regulatory (Treg) cells which known as suppressor T cells can alleviate clinical signs 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]

The indigenous people often have a diet of marine algae and shellfish in the southeast coast of China. Marine algae are known to be one of the most important producers of biomass in the marine environment. Traditional Chinese medicine books have documented that marine algae could improve digestion, enhance immunity and other effects. Recently, the role of marine algae as anti-allergic agents has been determined in *vitro* and in *vivo*. [12] In a recent study, Lin et al. [13] demonstrated that Laminaria japonica polysaccharides can significantly inhibit airway inflammation of asthmatic mice, adjust the balance of cytokines, and improve the pulmonary histopathological condition. Ishihara et al. [14] reported that porphyran of red algae *Porphyra tenera* and *P. yezoensis* were capable of inhibiting the contact hypersensitivity reaction via decreasing the serum level of IgE in Balb/c mice. Our previous studies also have demonstrated that sulfated polysaccharide purified from *Porphyra haitanensis* (PHPS) which is composed of galactose and sulfate has an anti-allergic activity by suppressing Th2 immune response in the TM-sensitized mice.

[15] *Gracilaria lemaneiformis* has similar polysaccharide content and is as extensively distributed as *Porphyra haitanensis*. It is distributed widely in a marine environment and belongs to the family Gracilariaceae (Rhodophyta). *Gracilaria lemaneiformis* possesses various bioactive functions such as antitumor, antiviral, hypoglycemic, and immunomodulatory effects.[16,17,18] However, to the best of our knowledge, there are no studies reported on the anti-allergic activity of the sulfated polysaccharides from *Gracilaria lemaneiformis*, .

In this study, the structural characteristics of sulfated polysaccharide isolated from *Gracilaria lemaneiformis* (GLSP) were resolved. The anti-allergic activity of GLSP was evaluated by TM-sensitized mice and cellular models. Moreover, the key functional group of GLSP was shown for anti-allergic effect, and the inhibition of GLSP on signaling pathway was further investigated in basophils.

METHODS

Purification of polysaccharide from *Gracilaria lemaneiformis*

Gracilaria lemaneiformis was provided by the Third Institute of Oceanography, State Oceanic Administration. Polysaccharide from *Gracilaria lemaneiformis* was extracted and purified according to the procedures of Shi et al.[15] by 95℃ water extraction, ethyl alcohol deposition, and DEAE-cellulose 52 (Whatman, New York, USA) column chromatography. The carbohydrate content of the polysaccharide was detected by the anthrone-sulfuric acid method. [19] The lipopolysaccharide (LPS) was removed from the polysaccharide with Detoxi-Gel Endotoxin Removing Gel (Pierce,

Rockford, USA). The LPS content of the polysaccharide was found to be < 0.03 EU/mg as measured by the Limulus amebocyte 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. The 3,6-anhydrogalactose (3,6-AG), sulfate, uronic acid and protein contents were respectively measured according the description by Shi et al.[15] The molecular weight distribution of the polysaccharide was determined by high performance gel-permeation chromatography (HPGPC) (Agilent-1100, Santa Clara, USA) equipped with a ZORBAX Eclipse XDB-C18 column (250 mm ×4.6 mm, column temperature 30°C). To determine the monosaccharide composition of GLSP, the acid hydrolysate of the polysaccharide was applied to ion chromatography (IC) (ICS-3000, Dionex, Sunnyvale, CA, USA) analysis with CarboPac PA20 (3×150 mm), and separated using 0.25 M NaOH at a flow rate of 0.5 mL/min. Fourier transform infrared spectroscopy (FTIR) spectrum of the polysaccharide was collected with a Fourier transformed infrared spectrometer (VECTOR-22, BRUKER) in the wave number range of 4000-500 cm^{-1} using the KBr-disk method.

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Mouse food allergy model

Female BALB/c mice, 4-6 weeks of age, were obtained from the Animal Center of Xiamen University. Mice were housed in ~~an~~a Specefic Pathogen Free (SPF) ~~(define SPF)~~ environment maintained at 22±1°C with a relative humidity of 55±10%, and experiments were performed in conformity with the laws and regulations for

155 treatment of live animals in Jimei University, SCXK 2012-0005.

156 The purification of TM from mud crab (*Scylla paramamosain*) was carried out as
157 described previously.[20] Food allergy was induced in mice and monitored as described
158 by Yamaki et al.[21] Briefly, mice were immunized with TM (50μg/mouse) in
159 phosphate-buffered saline (PBS) emulsified with Imject Alum (Thermo Fisher
160 Scientific Inc. Waltham, MA, USA) at day 0 and 14. Mice were challenged from day
161 28 by oral administration TM (10 mg/mouse) every other day. The negative control
162 mice were given 0.2 mL of intragastric PBS (unimmunized PBS group). GLSP (5
163 mg/mouse) was orally administered to immunized mice from the day before the 1st
164 challenge to the day before sacrifice (from day 27 to day 40) in the prevention group.
165 And in the treatment group, GLSP (5 mg/mouse) was orally administered to mice
166 already challenged with oral TM 3 times (from day 33 to day 40) to evaluate the
167 therapeutic effect of GLSP on food allergy. Anaphylactic symptoms and diarrhea rates
168 (the proportion of diarrheal mice in each group) were scored for 1 h after each
169 challenges.[22] Rectal temperatures were measured 1 h after the 6th challenge and sera
170 was collected.

171 **Measure the production of histamine, mMCP-1, TM-specific antibodies, and**
172 **cytokines**

173 The concentrations of histamine and mMCP-1 in serum obtained 1 h after the 6th
174 challenge was determined using commercially available ELISA kits following the
175 manufacturer's instructions (IBL, Hamburg, Germany; TSZ, Boston, USA). Serum
176 levels of anti-TM IgG2a, IgE, and IgG1 were measured using ELISA as described

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previously.[15] Splenocyte suspensions were obtained on day 41, and were cultured with TM (10μg/mL) for 3 days to measure the release of cytokines IL 4, IL-13, interferon (IFN)-γ, transforming growth factor (TGF)-β) using ELISA kits following the manufacturer's instructions (R&D Systems, Minneapoils, MN, USA).

RNA preparation and real-time RT-PCR analysis

RNA preparation and real-time RT-PCR were performed according to the previous description.[23] Real-time quantitative PCR (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').

Western blot analysis

Western blot analysis of serum anti-TM IgG2a, IgE, and IgG1 was performed as described previously.[22] The detection of Syk (Cell Signaling Technology, Danvers, MA, USA), phosphor-Syk (Cell Signaling Technology, Danvers, MA, USA), and β-actin (Cell Signaling Technology, Danvers, MA, USA) of activated KU812 cells with or without GLSP pretreatment were performed according the description by Lee.[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. Meanwhile, the cell toxicity,

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reactive oxygen species (ROS) ~~(define ROS)~~ and cytokines (IL-4 and TNF-α) were described previously.[23] A solvolytic desulfation procedure was used wherein the GLSP as a pyridinium salt was heated in dimethyl sulfoxide.[25] The effect of GLSP after desulfation on degranulation were measured to verify the role of sulfate.

Flow cytometry assay

To examine the effects of GLSP on the expression of activation-linked antigens, flow cytometry experiments were performed. KU812 cells were incubated with GLSP (10-200μg/mL) for 1 h and then ~~(or?)~~ stimulated with 0.5μM Calcium Ionophore A23187 (Sigma, St. Louis, MO, USA) plus 1μM Phorbol Myristate Acetate (PMA) (Sigma, St. Louis, MO, USA) for 30 min in tyrode's buffer. Then, cells were stained with fluorescein isothiocyanate (FITC)-conjugated CD63 (BD pharmingen, San diego, CA) and phycoerythrin (PE)-labelled CD203c (BD pharmingen, San diego, CA) by signal-colour flow cytometry. Guava easyCyte 6HT system and GuavaSoft software (Millipore, Massachusetts, USA) were used for the analysis.

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Proteome Profiler™ array

KU812 cells were incubated with GLSP (200μg/mL) for 1 h and then stimulated with 0.5μM A23187 plus 1μM PMA for 30 min in tyrode's buffer. Then cells were lysed and ~~R~~relative phosphorylation levels of all three major families of MAPK (ERK1/2, p38α/β/δ/γ, JNK1/2/3/pan) and Akt 1/2/3/pan, RSK1/2, GSK-3α/β, MSK2, MEK3/6, HSP27 as well as p70 S6 kinase were determined by human phospho-MAPK array (R&D Systems, Minneapoils, MN, USA) according to the manufactures' instructions ~~(need a description of what you incubated with the array~~

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~~and how it was prepared~~).

Statistical analysis

Data were presented as means ± SDs. Differences between means were analyzed by using ANOVA. A *p* value of less than 0.05 was set as a significant criterion. Each experiment was repeated at least 3 times. In the animal model study samples from individual mice were processed and analyzed separately.

RESULTS

Composition of polysaccharide from *Gracilaria lemaneiformis*

The 3, 6-AG, sulfate, uronic acid and protein contents of GLSP were 8.23%, 11.3%, 8.59%, and 0.98%, respectively. The molecular weight of GLSP was 152,481 Da by HPLC analysis (data not shown)~~(data not shown?)~~. According to the description by Sun et al.,[26] the peak at 31.963 min was the galactose standard, and the monosaccharide in GLSP was galactose determined through IC analysis as shown in Fig. 1a~~ (the Fig1a description is too brief)~~. The FTIR spectrum of the sample was shown in Fig. 1b. GLSP exhibited absorption peaks at 3289 cm^{-1}, 2920 $cm^{-1}$and 1070 cm^{-1}, which were characteristic absorptions of -OH, C-H and C-O, respectively.[27] The IR absorption at 931cm^{-1} was the characteristic absorption of 3, 6-AG. Infrared spectroscopy provided useful information for the position of sulfate groups of polysaccharide. The IR spectra show an absorption band at 1260 cm^{-1} and 850 cm^{-1}, indicating the presence of the total sulfate ester. [28] So this polysaccharide was defined as a sulfated polysaccharide from *Gracilaria lemaneiformis* (GLSP).

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The anesis of anaphylaxis by GLSP in TM-sensitized mice

To test the effect of GLSP on food allergy, the animal experiment was implemented according the protocol shown in Fig. 2a. Daily dose of GLSP moderately decreased the scores of the anaphylactic response (Fig. 2b) and diarrhea rates (Fig. 2c) in a dose-dependent manner for 1 h after the ~~every~~ 3rd-6th challenge ~~(this is first mention on Diarrhea rates, need to add to methods)~~. The average rectal temperature of TM group mice was 35.5°C that was 2°C less than PBS group mice. Hypothermia induced at the 6th challenge was also remitted by the administration of GLSP in GLSP-preventive (p<0.01) and GLSP-treatment (p<0.05) group mice (Fig. 2d) ~~(this figure needs better description of the data. For example, what is on the Y axis vs the x axis? Are there any error bars? Does each line involve one mouse? How was diarrheal~~ treatment effect on food allergy. Meanwhile, GLSP diminished the increase in histamine and mMCP-1 serum concentrations, ~~(which indicates basophil degranulation?)~~ after the 6th challenge in the GLSP-prevention group (Fig. 3a, 3b). In addition, the daily administration of GLSP significantly reduced anti-TM IgE (Fig. 3c) and IgG1 (Fig. 3d) serum levels, but did not anti-TM IgG2a (Fig. ~~3c,3d,~~3e). The western blot result was similar to the ELISA data (Fig. 3f).

The immunosuppression of GLSP in TM-sensitized mice

GLSP ~~is~~ was known to inhibit the Th2-dependent anti-TM IgE and IgG1 levels, but it ~~is~~ was not known if it influence~~s~~d T~~Th1~~ cells. Therefore, to examine whether T-cell polarization in the TM-sensitized mice was modulated by GLSP, we evaluated the expression of the transcription factors T-bet, GATA-3 and Foxp3 as the reflection of

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Th1, Th2 and Treg cell activity, respectively. As shown in Fig. 4a, GATA-3 expression tended to decrease and Foxp3 expression tended to increase in splenic lymphocytes, while T-bet expression was not modulated by GLSP. The results of cytokines secreted from TM-stimulated cultured splenic lymphocytes confirmed these observations. GLSP reduced the production of Th2 cytokines (IL-4 and IL-13), increased the secretion of TGF-β, and had no effect on Th1 cytokine (IFN-γ) (Fig. 4b). ~~(belong in discussion)~~.

The suppression impact of GLSP in IgE-mediated RBL-2H3 cell

In Fig. 5a, methyl thiazolyl tetrazolium (MTT) assay was measured to determine the potential cytotoxicity of GLSP alone or conjugated to dinitrophenyl (DNP)-BSA. As shown in Fig. 5a, GLSP (20-200 μg/mL) alone or GLSP+DNP-BSA did not affect the cell viability (p>0.05). ~~As shown in Fig. 5a, GLSP (20-200 μg/mL) had no cytotoxicity (in the results section you have to describe the figure. For example: In Figure 5a, we measure cell lysis??….by the release of MTT as a measure of toxicity of GSLP alone or conjugated to DNP and the results are reported as the % of the….etc). (Also, what MTT is (?!) And there is no mention of toxicity measurements or MTT in methods section. What is the significance of using GSLP plus and minus DNP conjugation (or is BSA-DNP? In this case also fix the figure). This is important information that should also be included in Materials and methods as well as briefly described here).~~ GLSP (100-200 μg/mL) significantly diminished the antigen-induced degranulation as measured by the reduction in the release of ~~B~~β-hexasominodase (p<0.01) (Fig. 5b). ~~and~~ And the levels of histamine ~~(Fig. 5c)~~

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and ROS (as measured by the fluorescence of 2,7-dichlorodihydrofluorescein (DCF)) were significantly reduced by GLSP (100-200μg/mL) ~~(Define ROS and detail more in methods, and here what are you measuring and what does the figure show?)~~ (Fig. 5c, 5d). The inhibition of mediator release was also shown to be concentration dependent. IL-4 and TNF-α are important cytokines in allergic inflammation.[29] The IL-4 and TNF-α levels were increased by treatment of RB~~H~~L-2H3 ~~–~~cells with 500 ng/ml ~~dinitrophenyl (~~DNP~~)~~ ~~-~~BSA. Fig. 5e and Fig. 5f demonstrated that GLSP significantly inhibited the secretion of IL-4 and TNF-α in a dose-dependent manner in ~~DNP-BSA treated stimulated~~IgE-mediated RBL-2H3 cells. These indexes had no significant difference between cells stimulated with GLSP (200 μg/mL) alone and PBS-stimulated cells (data not shown).~~,~~ The data in Table 1 indicated that GLSP after desulfurization could not inhibit the degranulation of RBL-2H3. Thus, these results indicate that the activation and function of RBL-2~~3~~H3 cells were inhibited by GLSP, and the sulfate of GLSP plays a crucial role.

The inhibitory effect of GLSP on the activation of KU812 cells

Flow cytometry was used to assess the activation of KU812 cells (Fig 6). GLSP was found to counteract the expression of CD63 in KU812 cells that were activated by A23187~~+~~ plus PMA (A+P) treatment ~~(??what is A+P?)~~ at 20-200 μg/mL in a concentration dependent manner (Fig. 6a). Significantly less effect was seen by GLSP on A23187 plus PMA ~~–~~stimulated up-regulation of CD203c (Fig. 6b). As shown in Fig. 6c, in flow cytometric measurements GLSP was demonstrated to block the formation of the CD63+ (top panel) and reduce the expression of CD203c+ (bottom

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panel) subpopulations of KU812 cells. GLSP after desulfation could not inhibit the expression of CD63 and CD203c in KU812 cells (data not shown).

The 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, we examined its effects on signaling events, specifically the phospho-activation of tyrosine kinase like Syk, MAPKs and serine/threonine kinases like Akt. ~~Antigen (what is the antigen?, TM) induced tyrosine phosphorylation of Syk was inhibited by GLSP in KU812 cells (Fig. 7a).~~ As shown in Fig. 7a,~~This figure is not very clear. It needs to be described better, for example,~~ the p~~-SYK~~-Syk levels ~~is~~ were increased cells ~~are~~ were pre treated with 0.2 mg/mL~~l~~ of GLSP, the ~~pSYK~~ p-Syk levels ~~are~~ were decreased ~~to no A+P activation~~. No significan~~gt~~ change ~~is~~ was seen in the levels of and unphosphorylated Syk with the various treatments. We analyzed other signaling molecules using a Proteome Profiler™ Array is detecting 26 kinases simultaneously (Fig. 7b). Though measuring the total proteins in the cytoplasm of the KU812 cells with or without GLSP (0.2 mg/mL) pretreatment, the ~~increase~~levels of ~~This assay~~ kinases MEK3 and MEK6 upstream of MAPK p38 ~~(same as below?)~~ w~~as~~ere KU812 cells. And they were reduced by GLSP. Meanwhile, the phosphorylation level of MAPK p38δ~~–~~ ~~(?? Is this the same as MAPK p38? If it is, then it is a repeat of~~ the elimination of the ~~activation?~~increase of ~~A~~Akt1 phosphorylation ~~(? Or levels?~~ stimulated with A~~23187 plus~~ +P~~MA (A+P, defined way too late)~~. Furthermore, the molecule downstream of ERK, was reduced by GLSP, while the phosphorylation

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levels of ERK1 and ERK2 ~~was~~ were enhanced. ~~and~~ And JNK was not affected by

DISCUSSION

During the last decade, the active ingredients of marine algae, such as olysaccharides, phlorotannins, phycocyanins, carotenoids etc, play an important role in the pharmaceutical industry and the development of novel drugs against allergic disorders.[12] Polysaccharides from marine sources offer ~~diverse therapeutic~~ regulated function~~s~~ of allergic responses due to their biocompatible, biodegradablenontoxic, and physiologically inert properties.[30] ~~(should include at least one review article as a reference or multiple references).~~ It has been reported that several marine polysaccharides have been found to modulate allergic responses.[13,14] It was demonstrated that the structural characteristics and anti-allergic activity of GLSP are similar to PHPS[15], a polysaccharide from *Porphyra haitanensisin*. *Gracilaria lemaneiformis*, is an important murine 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 with PHPS. Fan et al.[18] reported that the acidic polysaccharide isolated from *Gracilaria lemaneiformis* showed anti-tumor activity, and its molecular weight is 1.37 $\times 10^6$ Da, which is more than GLSP (1.52×10^5 Da); the sulfate content is 6.13%, which is less than GLSP (11.3%); and the main monosaccharide is galactose, which is same as GLSP. These differences might

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result from the different extraction methods and or the distinctlyl different material 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] In this study, a previously established food allergic model in mice that were orally sensitized by TM. The allergic animal exhibited increase in Th2 cytokine levels, TM-specific IgE concentration, serum histamine, and mMCP-1 levels. The present data suggested that GLSP had potent effects on the prevention and treatment of allergic diseases, and the preventive effect was better than the treatment effect. GSLP was directly responsible for the reduction in mMCP-1 release and the serum histamine levels.

Allergen-specific serum IgE is essential in type I hypersensitivity reactions. The Th2-dependent anti-TM IgE and IgG1 serum levels were reduced observably by GLSP. In addition, the production of IL-4 and IL-13 was decreased by GLSP. This effect was similar to the effect of PHPS, but the difference is that GLSP had no obvious effect on Th1 cytokine IFN-γ and Th1-dependent anti-TM IgG2a. The expression of Foxp3 in mouse splenic lymphocytes was increased, indicating that Treg cell polarization was promoted by GLSP. TGF-β is an important factor in the Treg cell development and suppressor function.[301] The production of TGF-β was also increased in mice challenged with GLSP. So, we belive that GLSP diminishes the severity of food anaphylaxis in the TM-sensitized mice through immunosuppression by up-regulating Treg cells. Whether GLSP had the influence of Th9, Th17, and other

immune cells need to be further investigated.

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.[3~~1~~2] The concentrations of serum histamine and mMCP-1, which is a known inflammatory mediator[7] ~~(?)~~ increased by TM-sensitization of mice were inhibited by GLSP. So the effect of GLSP on the activation and function of basophils were further investigated by RBL-2H3 and KU812 cell lines. IL-4 which is thought to contribute to immunoglobulin class switching in B cells to produce IgE[3~~2~~3] is also reported to induce Th2 differentiation[3~~3~~4] and affect the survival/proliferation of ~~MCs~~ mast cells~~(define MCs)~~ leading to allergic diathesis.[3~~4~~5] The activation of human basophils is associated with up-regulation of various surface CD-antigens, including CD63 and CD203c.[~~35~~,36,37] The present results showed that GLSP had the ability to suppress the degranulation and the production of histamine, ROS, IL-4, and TNF-α in IgE-mediated RBL-2H3. The expression of CD63 and CD203c were also inhibited by GLSP in KU812 cells. Furthermore, just as the reported by Ngo et al.[3~~7~~8] sulfated polysaccharides, from marine algae, could act as bioactive agents, and the sulfate of GLSP was shown to be important in the inhibition of mediator release and cytokine secretion by of RBL-2H3 and KU812 cells.

Syk deficient mast cells do not degranulate after FcεRI aggregation, Syk is essential for FcεRI induced signal transduction of mast cells.[3~~8~~9] The activation of Syk and other tyrosine kinases initiates an intracellular signaling cascade involving activation of MAPKs, Akt, and other molecules, which have been implicated in gene expression of

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relevant cytokines.[3409] 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; p38α and p38β are also activated by MEK3.[401] RSK is a known downstream target of ERK1/ERK2.[401] Research has shown that camellia japonica suppressed allergic response by the inhibition of Syk kinase activation in mast cells,[23] homoisoflavanone prevented mast cell activation and allergic responses by inhibition of Syk signaling pathway,[412] and CTB glycoprotein reduced arachidonic acid release, COX-2 expression, and activator protein-1 transcriptional activation through p38 MAPK phosphorylation in RBL-2H3 cells.[423] In the present study, GLSP inhibited the phosphorylation of Syk tyrosine kinase, Akt, and MEK3/6, as well as P38δ. Due to the inhibition of these signaling pathway molecules, GLSP may be able to counteract the activation and function of basophils. It could be speculated that the mechanism of the inhibitory effect on mast cells is similar to basophils, and should be studied further.

In conclusion, the present study show that purified GLSP was a 152,481 Da sulfated polysaccharide from *Gracilaria lemaneiformis*. GLSP could alleviate the allergic symptoms of TM-sensitized mice by immunosuppression and the inhibition of basophils. The activation of basophil was inhibited by GLSP via Syk and p38 MAPK signaling pathways. Based on the results *in vivo* and *vitro*, we believe that GLSP could not only inhibit TM- mediated food allergy, but also reduce the severity of other allergic symptoms. Finally, GLSP may provide insight into the prevention or treatment of food allergy induced-anaphylaxis and may be used as a functional food

component or active pharmaceutical ingredient to treat allergic patients.

Conflict of interest

The authors declare that there is no conflict of interests.

Acknowledgements

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Figure Legends

Table 1. The β-hexosaminidase release rates of RBL-2H3 cells deal with GLSP or GLSP after desulfation

The 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, significantly different from

551 DNP-BSA alone.

552

553 **Fig. 1. The structural analysis of GLSP**

554 (a) IC analysis of the monosaccharide from GLSP;

555 (b) FTIR spectrum of GLSP in the wavenumber region between 4000 and 500 cm^{-1}.

556

557 **Fig. 2. Animal experimental design and effects of GLSP on the allergy symptoms**

558 (a) Immunization and challenge protocol;

559 (b) The symptom scores of the anaphylactic symptom 0 to 1 hour after every challenge;

560 (c) The rates of diarrhea of mice 0 to 1 hour after every challenge;

561 (d) The rectal temperature of mice 1h after the 6th challenge.

562 i.p., intraperitoneal injection i.g., intragastrically

563 Statistical differences are indicated by *P* values (one-way ANOVA). $*P < 0.05$, $**P < 0.01$,

564 compared with the TM-treated group.

565

566 **Fig. 3. The inhibition of GLSP on serum histamine, mMCP-1, TM-specific antibodies in mice**

567 (a) GLSP reduced the histamine levels in the prevention group;

568 (b) GLSP reduced the mMCP-1 levels in the prevention and the treatment group;

569 (c) GLSP inhibited serum TM-specific IgE in the prevention group after the last challenge;

570 (d) GLSP inhibited serum TM-specific IgG1 in the prevention group after the last challenge;

571 (e) GLSP had no effect on serum TM-specific IgG2a;

572 (f) Western blot analysis of the TM-specific antibodies in mice serum after the last challenge.

573 Statistical differences are indicated by *P* values (one-way ANOVA). **P* < 0.05, ***P* < 0.01,
574 compared with the TM-treated group.
575
576 **Fig. 4. The regulatory effects of GLSP on T cell polarization in mice**
577 (a) GLSP suppressed the expression of Th2 transcription factor (GATA-3), promoted the
578 expression of Treg transcription factor (Foxp3), and had no effect on Th1 transcription factor
579 (T-bet);
580 (b) The effects of GLSP on cytokines secreted from Th1, Th2, and Treg cells. GLSP could reduce
581 the production of Th2 cytokines (IL-4 and IL-13), increase the production of Treg cytokines
582 (TGF-β), and have no effect on Th1 cytokines (IFN-γ).
583 Statistical differences are indicated by *P* values (one-way ANOVA). **P* < 0.05, ***P* < 0.01,
584 compared with the TM-treated group.
585
586 **Fig. 5. The inhibitory effect of GLSP in IgE-mediated allergic responses in RBL-2H3**
587 (a) Effects of GLSP on cytotoxicity;
588 (b) GLSP (100-200μg/mL) reduced the release rate of β-hexosaminidase;
589 (c) GLSP (100-200μg/mL) inhibited the histamine levels;
590 (d) GLSP (100-200μg/mL) decreased the production of ROS;
591 (e) GLSP (100-200μg/mL) suppressed the secretion of IL-4;
592 (f) GLSP (150-200μg/mL) impeded the secretion of TNF-α.
593 DNP: DNP-BSA
594 The data represent the mean ± SD from three independent experiments. Statistical differences are

595 indicated by *P* values (one-way ANOVA). *$P < 0.05$, **$P < 0.01$, significantly different from
596 DNP-BSA alone.
597
598 **Fig. 6. The inhibitory action of GLSP on the expression of CD63 and CD203c on KU812 cell**
599 (a) GLSP inhibited the percentage of CD63+ cells, and had slight inhibition of CD203c+ cells;
600 (b) The result pictures of the expression of CD63 and CD203c by flow cytometry as an example.
601
602 **Fig. 7. The adjusted effects of GLSP on the signaling pathway molecules**
603 (a) GLSP (200μg/mL) reduced the phosphorylation of Syk tyrosine kinase in stimulated KU812
604 cells;
605 (b) GLSP (200μg/mL) reduced the phosphorylation of Akt, MEK3/6, MAPK p38δ, and RSK2,
606 and mildly enhanced the phosphorylation of ERK1 and ERK2.
607 A+P: 0.5μM A23187+1μM PMA