



Cite this: DOI: 10.1039/d1fo00335f

Degraded *Porphyra haitanensis* sulfated polysaccharide relieves ovalbumin-induced food allergic response by restoring the balance of T helper cell differentiation†

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We previously described that *Porphyra haitanensis* sulfated polysaccharide (PHSP) maintains the balance of pro-inflammation and immunosuppression. However, it is unclear whether degraded PHSP (DPHSP) still shows the immunomodulatory activity. Here, we degraded PHSP by four different methods alone or combined in pairs, and the results showed that the molecular weight and viscosity of DPHSP were significantly decreased, while the main chemical bonds and functional structure were consistent with those of PHSP. We then investigated the immunomodulatory function of DPHSP *in vitro* and *in vivo*. Actually, DPHSP enhances the inhibitory effects on mast cell activation and improves the suppression activity of PHSP on the food anaphylactic response. In an ovalbumin-induced food allergy mouse model, the production of allergic mediators and cytokines (interleukin-4 and 13, and interferon- γ) was inhibited by DPHSP. Meanwhile, DPHSP had a stronger ability to up-regulate the differentiation of regulatory T (Treg) cells and its related cytokines. These results suggested that DPHSP showed a better anti-food allergic ability than PHSP by regulating T helper cell balance and promoting Treg cell differentiation, which indicates that DPHSP is a novel potential nutrient component against food allergy.

Received 2nd February 2021,
Accepted 12th April 2021

DOI: 10.1039/d1fo00335f

rsc.li/food-function

1. Introduction

Food allergy is currently a major public health issue that has received increased concern because of the increasing prevalence. Estimates of food allergy indicate that the prevalence of food allergies in children (3–4%) is higher than that in adults (1–2%).¹ Allergic reactions can range from mild local symptoms, for example in atopic dermatitis, urticaria, oedema, and rhinitis, to severe life-threatening anaphylaxis, such as laryngeal oedema, acute asthma, and anaphylactic shock.² However, the current treatment options are limited due to some undesirable adverse effects, and a strict targeted eviction diet remains the standard of care, which seriously affects the

life quality of allergic people and their families.³ At the same time, natural anti-food allergic active substances have the characteristics of mild effect, less side effects and safety.⁴ Thus, it is necessary to develop and use safe and natural anti-allergic active substances.

Marine algae, the most abundant resources in the ocean, were considered as a key resource containing a rich source of functional metabolites such as polysaccharides, proteins, peptides, lipids, and polyphenols, which are used as therapeutic and health beneficial foods in various diseases.⁵ Polysaccharides are the main components in marine algae, hence a great deal of attention has been directed towards marine algal polysaccharides because of their numerous health benefits including immunomodulatory, antitumor, antioxidant, and hypolipidemic activities and so on.^{6,7} In our present study, *Porphyra haitanensis* sulfated polysaccharide (PHSP) was confirmed to have the ability to relieve food allergy.⁸ Nevertheless, more and more studies have shown that due to their lower molecular weights, higher solubilities, and easier absorption in the body, degraded polysaccharides tend to show higher biological activity than non-degraded polysaccharides.⁹ There exist a lot of methods to degrade polysaccharides, such as the ultrasonic method (US), high temperature

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†Electronic supplementary information (ESI) available. See DOI: 10.1039/d1fo00335f

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and pressure method (HP), H_2O_2 method, vitamin C method (VC), Fe^{2+} method and so on.^{10–13} Based on this, several methods were developed, for example H_2O_2 -VC method and ultrasound hydrogen peroxide method ($\text{US-H}_2\text{O}_2$).^{14,15} Usually, the degradation products of polysaccharides can retain or even enhance the biological activity after physicochemical treatment.¹⁶ Wang *et al.* found that shorter alginate oligosaccharides impart better nonspecific immunostimulatory effects than alginate polysaccharides.¹⁷ Liao *et al.* compared the hypoglycemic effects of the chemically degraded products of *Gracilaria lemaneiformis* polysaccharide and *Gracilaria lemaneiformis* polysaccharide and the results indicated that the oligosaccharides exhibited higher inhibitory effects than the polysaccharides.¹⁸ Therefore, we want to obtain the degraded *Porphyra haitanensis* sulfated polysaccharide (DPHSP) through the degradation of PHSP by physical or chemical methods, compare the biological activities between PHSP and DPHSP, and then analyze whether low molecular weight polysaccharides show a higher anti-food allergic activity.

In the process of immunoglobulin (Ig)E mediated food anaphylaxis, T cells, activated by the antigen-presenting cells, can stimulate B cells to differentiate into antibody-producing cells.¹⁹ Lymphokines such as interleukin (IL)-4, IL-5 and IL-13 released from T helper (Th) 2 cells have been postulated to be functional molecules regulating immune balance.²⁰ Priming mast cells (MCs) or basophils with IgE causes signaling events followed by the secondary exposure of allergens, degranulation and the release of various chemical mediators such as histamines and interleukin. These released mediators induce several kinds of allergy symptoms.²¹ In addition, regulatory T (Treg) cells, a small yet critical subset of CD4^+ T cells, have a role in relieving and preventing allergy because of their ability to maintain self-tolerance.²² Treg cells exert immunosuppressive effects by secreting IL-10 and transforming growth factor (TGF)- β which plays an important role in the generation of antigen-specific forkhead box protein 3 (Foxp3) Treg cells, and their abrogation *in vivo* leads to Th2 allergic asthma out of control.²³ Thus, alleviating allergy and restoring immune homeostasis through the promotion, activation or delivery of Treg cells has emerged as a focus for therapies aimed at curing or controlling autoimmune diseases. In previous studies, the sulfated polysaccharide from *Gracilaria lemaneiformis* (GLSP) was found to suppress the production of IL-4 and IL-13, and the degradation product sulfated oligosaccharide from *Gracilaria lemaneiformis* (GLSO) was found to significantly up-regulate Treg cell differentiation.^{24,25} However, the effects of PHSP and DPHSP on Treg cell differentiation and the balance of Th1/Th2 cells on a food allergy system remain unknown.

In the present study, the most suitable degradation method for PHSP among the different polysaccharide degradation methods was ensured. The anti-allergic activities of PHSP and DPHSP were explored to confirm whether PHSP and DPHSP were of value to apply to develop new health foods to prevent and alleviate food allergy using the IgE-allergic activity MC model and OVA-induced food allergy murine model.

2. Materials and methods

2.1. Materials and reagents

Porphyra haitanensis grown in Dadeng Island was purchased from a local market in Xiamen City, Fujian Province, China. Fetal bovine serum (FBS), RPMI 1640 medium, Eagle's Minimum Essential Medium (MEM), and trypsin solution were supplied by Hyclone (Logan, UT, USA). Penicillin-streptomycin solution and Inject $\text{Al}(\text{OH})_3$ adjuvant were obtained from Gibco (Grand Island, NY, USA) and Thermo Fisher Scientific Inc. (Waltham, MA, USA), respectively. Anti-dinitrophenyl (DNP)-IgE, 4-methylumbelliferyl-*N*-acetyl- β -D-glucosaminide, OVA, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-*H*-tetrazolium bromide (MTT) and bovine serum albumin (BSA) were purchased from Sigma (St Louis, MO, USA). DNP-BSA was obtained from Biosearch (Petaluma, CA, USA). Goat anti-mouse IgG1 and IgG2a antibodies were bought from Abcam (Cambridge, UK). Enzyme-linked immunosorbent assay (ELISA) kits for anti-OVA-IgE, histamine and mouse mast cell proteinase (mMCP)-1 were provided by Cayman (Ann Arbor, MI, USA), IBL (Hamburg, GER) and R&D Systems (Minneapolis, MN, USA), respectively. ELISA kits for mouse IL-4, IL-13, Interferon (IFN)- γ and recombinant mouse IL-3 protein and mouse stem cell growth factor were supplied by Peprotech (Rocky Hill, NJ, USA). ELISA kits of IL-10 and TGF- α were obtained from R&D Systems (Minneapolis, MN, USA). APC conjugated CD3, PerCP-Cy5.5-conjugated CD4, and Foxp3-PerCP-Cy5.5, and a flow cytometry analysis kit of Treg cells were supplied by BD Pharmingen (San Diego, CA, USA). All other chemicals and solvents were of analytical grade.

2.2. Preparation of DPHSP by different degradation methods

PHSP was prepared from *Porphyra haitanensis* through hot water extraction, ethanol precipitation and impurity removal by column according to our earlier research.^{8,26} The prepared PHSP powder was dissolved into 5 mg mL^{-1} solution with ultrapure water firstly, and then PHSP was degraded into DPHSP by different ways including ultrasonic treatment, high temperature and pressure treatment, hydrogen peroxide treatment and VC treatment. The specific degradation methods of PHSP are provided in the ESI.[†]

2.3. Analysis of the related physicochemical properties of DPHSP

The total carbohydrate and reducing sugar contents of PHSP and DPHSP were determined using the anthrone-sulfuric acid and dinitrosalicylic acid (DNS) solution.²⁷ Taking galactose as the standard, the concentration gradient was set in the range of $0\text{--}4 \text{ mg mL}^{-1}$, and the standard curve was drawn by reading the absorbance value at 630 nm and 520 nm, respectively. Using the formula for the standard curve, the total sugar and reducing sugar contents of PHSP and DPHSP were calculated.

The viscosities of PHSP and DPHSP were measured using an Ubbelohde viscometer and the specific method reported by Liu.²⁸ At the same time, the sulfate content was determined by the BaCl_2 turbidimetric method,²⁹ and the molecular weight

distribution of PHSP and DPHSP was determined by high performance volume exclusion chromatography. The molecular weight analysis and detection of the experiment was completed by the analysis and test center of Jiangnan University. The FT-IR spectra of freeze-dried PHSP and DPHSP were measured using a Thermo Fisher spectrometer.⁸ The FT-IR spectra of PHSP and DPHSP were measured using a Thermo Fisher spectrometer. And then the surface micrographs of the extracted polysaccharides were obtained through Scanning electron microscopy (SEM).³⁰ The prepared and numbered freeze-dried PHSP is attached to a sample holder for getting high resolution images by SEM.

2.4. Secretion of histamine and β -hexosaminidase assay of a mast cell model

The rat basophil line (RBL)-2H3 cell model and mouse bone marrow-derived mast cell (BMMC) assay were obtained as described previously.³¹ RBL-2H3 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Bone marrow cells from the femur of BALB/c mice were cultured in the presence of IL-3 (10 ng mL^{-1}) and SCF (50 ng mL^{-1}) for 4 weeks. The expression of the mast cell specific marker (c-kit⁺ Fc ϵ RI α ⁺) was detected by flow cytometry. The effects of PHSP and DPHSP on RBL-2H3 cell and BMMC viability were determined *via* MTT assays. Cells (5×10^5 cells per mL) were seeded into 96-well plates, and then added 95 μL of 10% FBS-MEM (RBL-2H3 cell) and 10% FBS-RPMI (BMMCs) and 5 μL of PHSP and DPHSP with different concentrations (50, 100, 200, 300, and 400 $\mu\text{g mL}^{-1}$). The plates were maintained under 5% (v/v) CO_2 at 37 $^\circ\text{C}$. After 48 h, these cells were washed with the phosphate buffer solution (PBS), dimethyl sulfoxide was added to stop the reaction, and the absorbance value was measured at 570 nm wavelength. The toxic effect was calculated and normalized to the PBS group.

The levels of histamine and β -hexosaminidase in RBL-2H3 cells or BMMCs treated with PHSP and DPHSP were measured as previously described, respectively.³² Cells were seeded into 96-well plates (5×10^5 cells per mL), anti-DNP-IgE (0.5 $\mu\text{g mL}^{-1}$) was added to stimulate the cells for 16 h, and then PHSP and DPHSP were added to cells with 200 $\mu\text{g mL}^{-1}$ final concentration. After the samples were treated for 1 h, DNP-BSA (2.5 $\mu\text{g mL}^{-1}$) was added for 1 h to stimulate the cells to release β -hexosaminidase. The cell supernatant and pyrolysis product were collected and incubated with 4-methylumbelliferyl-*n*-acetyl-*b*-D-glucosaminide for 30 min, and the absorbance value was read at 360 nm excitation wavelength and 450 nm emission wavelength. After DNP-BSA stimulated the cells for 15 min, the cell culture supernatant was collected, and the histamine content in the cell supernatant was determined using the ELISA Kit from IBL company.

2.5. Food allergy model protocol and assessment of allergic symptoms

Female specific pathogen free (SPF) BALB/c mice (6–8 weeks) were obtained from Weitong Lihua Inc. (Beijing, China). The housing environment for mice was maintained at 22 ± 2 $^\circ\text{C}$

and 50–70% relative humidity under a 12 hour light/dark cycle. The animal experiment was performed in strict accordance with the Guidelines for the Care and Use of Laboratory Animals of Jimei University. All protocols were approved by the Animal Research Ethics Board of Jimei University (Xiamen, China, no. SCXK 2016-0006).

After one-week of acclimation, mice were randomly divided into control group, OVA group, PHSP group, and DPHSP-1, DPHSP-2, and DPHSP-3 groups (six mice in each group). Mice in OVA, PHSP and DPHSP groups were sensitized with 100 μg of OVA and 2 mg of $\text{Al}(\text{OH})_3$ adjuvant in 200 μL of PBS by intraperitoneal (i.p.) injection on days 0 and 14, while mice in the PBS group were injected with 2 mg of $\text{Al}(\text{OH})_3$ adjuvant in 200 μL of PBS. Mice in the OVA, PHSP, and DPHSP groups received 50 mg of OVA in 200 μL of PBS on days 28, 31, 34, 37, and 40 by gavage, and mice in the PBS group were injected with 200 μL of PBS without OVA. From day 27 to day 40, mice in the PHSP, DPHSP-1, DPHSP-2 and DPHSP-3 groups received PHSP (250 mg PHSP per kg mouse body weight, hereafter referred to as 250 mg kg^{-1}), 50 mg kg^{-1} DPHSP, 150 mg kg^{-1} DPHSP, and 250 mg kg^{-1} DPHSP by gavage every day, respectively. On day 41, all mice were killed after removing their eyeballs and collecting their blood.

The state of mice in each group was observed for 30 min and allergy symptoms were scored after OVA challenge each time. Symptoms were evaluated by using a scoring system modified slightly from previous reports,³³ and scored as follows: 0 = no symptoms; 1 = scratching and rubbing around the nose and head; 2 = edema around the eyes and mouth, piloerection, decreased activity, and/or decreased activity with increased respiratory rate; 3 = wheezing, dyspnea, and cyanosis around the mouth and the tail; 4 = no activity after stimulation or tremor and convulsion; and 5 = death. At the same time, the rectal temperature and diarrhea rate were measured as indicators of food allergy according to a previous study.³²

2.6. Determination of the levels of specific antibodies, histamine, and mMCP-1

Tail vein blood samples were collected from mice after 1 h of OVA challenging on day 40 for histamine and mMCP-1 measurement using the corresponding commercial ELISA kits according to the manufacturers' instructions. On day 41, the orbital venous hemorrhage blood samples were collected for testing other indexes. Mouse blood was centrifuged at 3000g and 4 $^\circ\text{C}$ for 10 min and stored in a refrigerator at -80 $^\circ\text{C}$. The antigen-specific IgE was detected using the corresponding ELISA kit, and IgG1 and IgG2a were determined by indirect ELISA methods.

2.7. Determination of cytokine levels and the flow cytometry analysis of Treg cells

Blood samples were collected from orbital venous hemorrhage on day 41 for the determination of IL-4, IL-13, IFN- γ , IL-10, and TGF- β levels. They were measured using the corresponding commercial ELISA kits according to the manufacturers' instructions as previously reported.³²

Spleen and mesenteric lymph nodes (MLN) were separated from the body immediately after the mice were killed on day 41. The single cell suspension was obtained by grinding and centrifugation. The population of Treg cells was indicated by staining with anti-CD4 and anti-Foxp3 antibodies and analyzing by flow cytometry. The signal-color flow cytometry analysis was performed using the Guava easyCyte 6-2L system and GuavaSoft 3.1.1 software (Millipore, Massachusetts, USA).

2.8. Statistical data

All experiments were repeated at least three times. Statistical analysis was performed using the SPSS 17.0 program. The experimental data were expressed as mean $\pm$ standard deviation (mean $\pm$ SD). One-way analysis of variance was used to determine significant differences between the groups. The differences were measured by Duncan's multiple range test between the PHSP and DPHSP groups in comparison with the OVA group. Significance was defined as $P < 0.05$ or $P < 0.01$.

3. Results

3.1. Determination of PHSP degradation conditions

PHSP, which is composed of galactose, breaks the glycosidic bond upon treatment using physical and chemical methods, releases more reducing sugar ends, and results in DPHSP with a low molecular weight. Therefore, the reducing sugar content of degraded PHSP was determined to evaluate the degradation degree of PHSP. The results are shown in Table 1; the contents of reducing sugar in all the degraded PHSP groups were higher than that of PHSP without degradation (0.41%). However, the degradation effects of ultrasound treatment combined with H_2O_2 treatment (US- H_2O_2 group) and ultrasound treatment combined with VC treatment (US-VC group) were not satisfactory. The degradation effects of high temperature and pressure treatment combined with H_2O_2 treatment (HP- H_2O_2 group) and high temperature and pressure treatment with VC treatment (HP-VC group) were better, and the contents of reducing sugar in the HP- H_2O_2 and HP-VC groups were 23.23% and 24.00%, respectively. Considering that VC, as a food additive, is safer than H_2O_2 , the composite method of the HP-VC group was selected to degrade PHSP in the follow-up experiments.

Table 1 The different ways to degrade the PHSP

Degradation methods	Reducing sugar (%)
PHSP ^a	0.41 $\pm$ 0.07
DPHSP ^b	
US	1.81 $\pm$ 0.22
HP	11.65 $\pm$ 0.54
VC	5.21 $\pm$ 0.62
H_2O_2	4.11 $\pm$ 0.47
US-HP	13.26 $\pm$ 1.88
US- H_2O_2	2.60 $\pm$ 1.55
US-VC	7.20 $\pm$ 0.82
HP- H_2O_2	23.24 $\pm$ 7.41
HP-VC	24.00 $\pm$ 5.22
VC- H_2O_2	5.83 $\pm$ 1.97

^a PHSP: *Porphyra haitanensis* sulfated polysaccharide. ^b DPHSP: degraded *Porphyra haitanensis* sulfated polysaccharide.

3.2. Comparison of the related physicochemical properties of PHSP and DPHSP

The physicochemical properties of DPHSP obtained by combining high temperature and pressure treatment with VC treatment and PHSP without degradation are shown in Table 2. PHSP was shown to have an average molecular weight of 2960 kDa, 95.86% total sugar content, 0.41% reducing sugar, 10.94% sulfate content, and a viscosity of 1.76 dL g⁻¹. Compared with PHSP, the molecular weight of DPHSP was 132 kDa, the total sugar content was 96.22%, the reducing sugar content was 24.00%, and the viscosity was 0.14 dL g⁻¹. Thus, PHSP was successfully degraded using high temperature and pressure treatment combined with VC treatment.

The FT-IR spectra of PHSP and DPHSP are shown in Fig. 1A. The PHSP and DPHSP shared the similar spectral features. An absorption peak at 3350 cm⁻¹ appeared in PHSP and DPHSP, which indicates that there is a hydrogen and oxygen bonds between molecules. The absorption bands around 2935⁻¹ and 1015 cm⁻¹ are due to the antisymmetric stretching vibration of methylene and the strong characteristic absorption of C-O, respectively. These peaks indicated that PHSP and DPHSP had the structural characteristics of a polysaccharide backbone. At the same time, there were characteristic absorption peaks of sulfate at 1216 cm⁻¹ and 767 cm⁻¹ before and after degradation.

In order to observe the changes in PHSP and DPHSP, the microstructures of PHSP and DPHSP solid particles were observed by SEM at 200 $\times$, 500 $\times$ and 5000 $\times$ magnification, as shown in Fig. 1B. The surface of PHSP was smooth and dense, while the surface of DPHSP sample was no longer smooth, showing a broken and loose state. These results suggested that the macromolecular structure of PHSP was obviously destroyed and DPHSP with a smaller molecular weight was obtained after degradation upon high temperature and pressure treatment combined with VC treatment. Through FT-IR and SEM detection, it was found that the chemical bond and active group of PHSP and DPHSP did not change significantly. However, DPHSP had smaller molecular weight, higher reducing sugar content and larger changes in the microstructure than PHSP. Therefore, we studied their antiallergic activities and compared the different results.

3.3. Evaluation of anti-allergic activities of PHSP and DPHSP *in vitro*

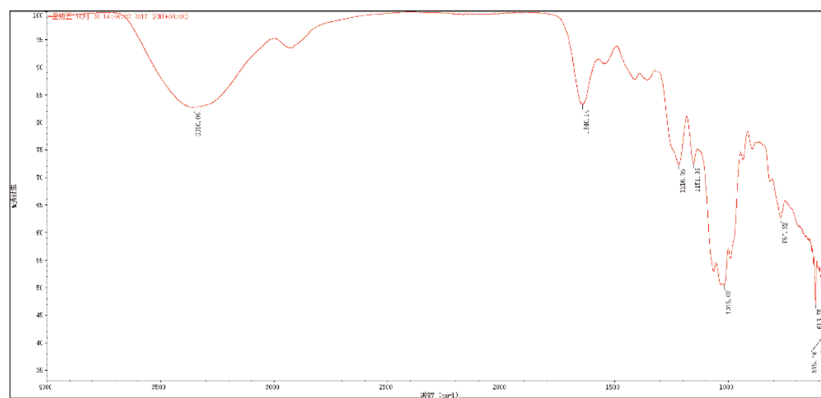
The cytotoxicity of PHSP and DPHSP was investigated before the cell experiment *in vitro*. Compared with the blank group (regarded as 100% non-toxic), the cell viability of the

Table 2 Physicochemical properties of PHSP and DPHSP

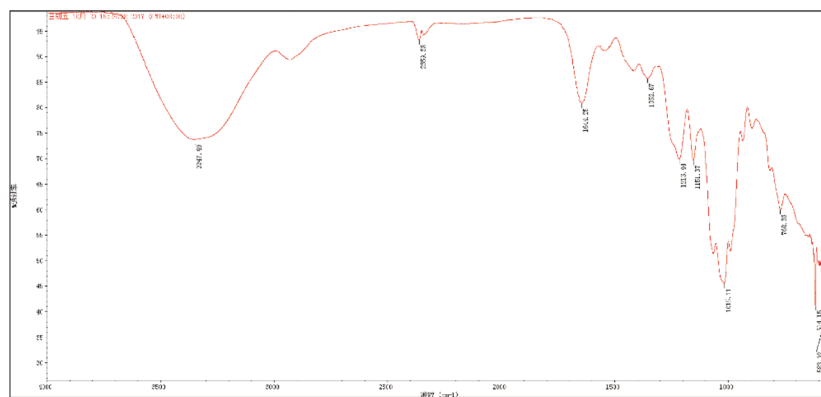
Physicochemical properties	PHSP	DPHSP
Total sugar (%)	95.86 $\pm$ 2.07	96.22 $\pm$ 1.68
Reducing sugar (%)	0.41 $\pm$ 0.07	24.00 $\pm$ 5.22
Viscosity (dL g ⁻¹)	1.76 $\pm$ 0.10	0.14 $\pm$ 0.03
Sulfate radical (%)	10.94 $\pm$ 1.77	11.41 $\pm$ 2.01
Molecular weight (kDa)	2960	132

A

PHSP

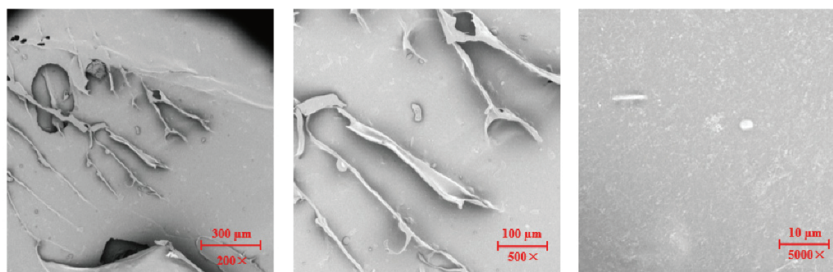


DPHSP



B

PHSP



DPHSP

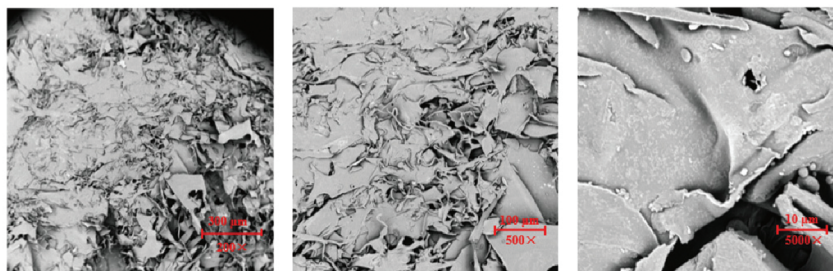


Fig. 1 FT-IR spectrum and electron microscope analysis diagram of PHSP and DPHSP. (A) FT-IR spectrum diagram of PHSP and DPHSP. The wave-number region of the FT-IR of PHSP and DPHSP is 4000–400 cm^{-1} . (B) SEM analysis diagram of PHSP and DPHSP.

RBL-2H3 group treated with PHSP and DPHSP ($200 \mu\text{g mL}^{-1}$) were 99.01% and 96.22%, respectively (Fig. 2A). The cell viability of BMMCs treated with PHSP and DPHSP were 98.23% and 97.56%, respectively (Fig. 2B). Therefore, PHSP and DPHSP had no cytotoxic effects on RBL-2H3 cells and BMMCs.

Many polysaccharides had been shown to inhibit degranulation of MCs.³⁴ In this study, the effects of RBL-2H3 cells and BMMCs treated with PHSP and DPHSP on degranulation of MCs were examined, and the release rates of β -hexosaminidase and histamine were measured and used as indicators of mast cell activation. As shown

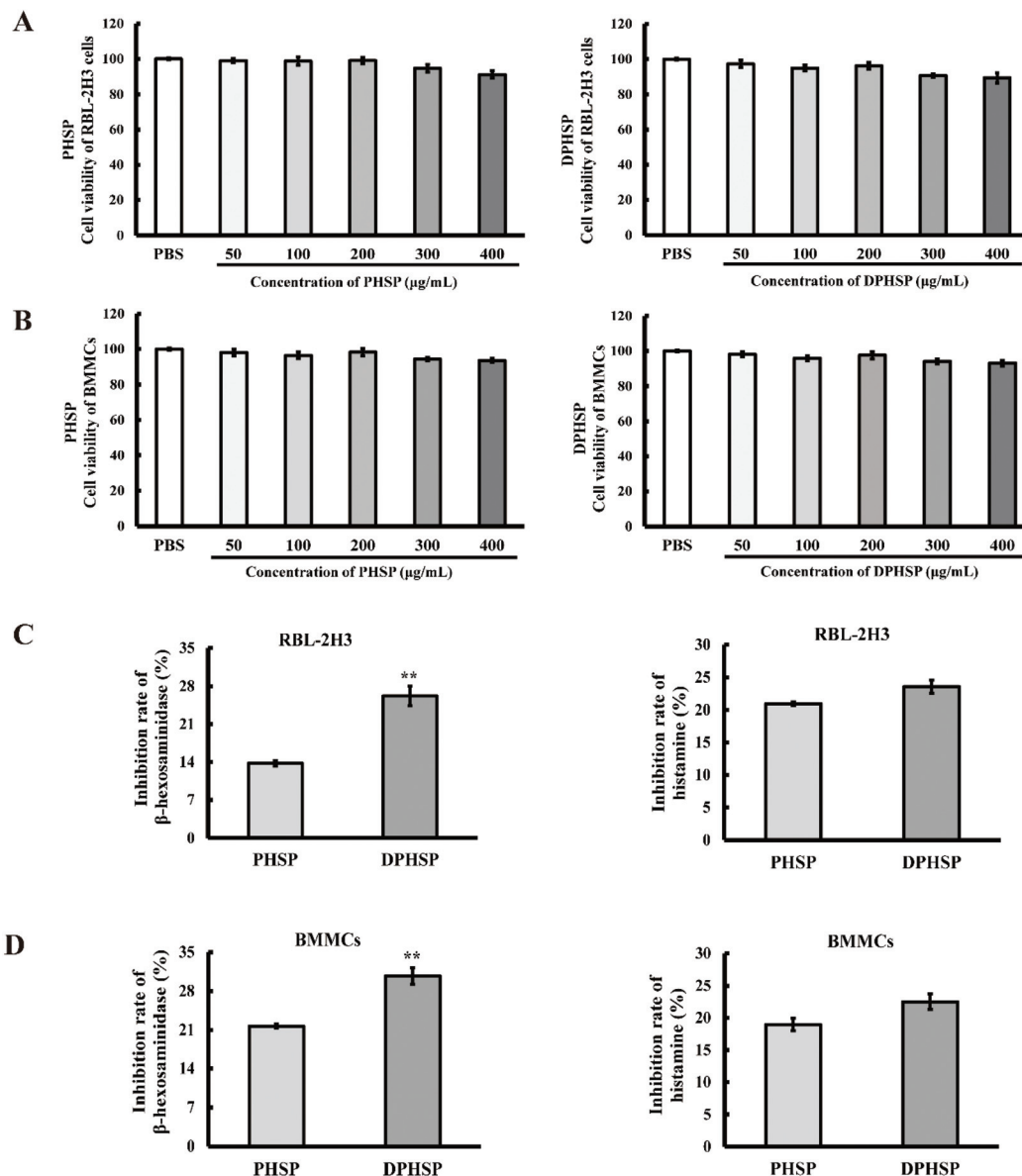


Fig. 2 Evaluation of antiallergic activities of PHSP and DPHSP *in vitro*. (A) Cytotoxicity of PHSP and DPHSP toward RBL-2H3 cells were measured with MTT assays. (B) Cytotoxicity of PHSP and DPHSP toward BMMCs were measured with MTT assays. RBL-2H3 cells and BMMCs were incubated with PHSP at concentrations of 50–400 $\mu\text{g mL}^{-1}$. (C) Effects of PHSP and DPHSP on the levels of β -hexosaminidase in RBL-2H3 cells and BMMCs to evaluate the antiallergic activities of PHSP and DPHSP *in vitro*. (D) Effects of PHSP and DPHSP on the levels of histamine in RBL-2H3 cells and BMMCs to evaluate the antiallergic activities of PHSP and DPHSP *in vitro*. Values are expressed as mean $\pm$ SD ($n = 5$), $^{**}P < 0.01$ compared with the PHSP group.

in Fig. 2C and D, when the concentration of DPHSP was 100 $\mu\text{g mL}^{-1}$, the inhibition rates of β -hexosaminidase release of RBL-2H3 cells and BMMCs were 27.48% and 31.65%, respectively, which were higher than that of PHSP ($P < 0.01$). Meanwhile, both PHSP and DPHSP could inhibit the histamine released from RBL-2H3 cells and BMMCs with no significant difference between them.

3.4. Effects of PHSP and DPHSP on allergic symptoms in OVA-induced mice

The mouse model of food allergy was established to investigate the anti-allergic activities of PHSP and DPHSP

in vivo (Fig. 3A). Daily doses of PHSP (250 mg kg^{-1}), DPHSP-1 (50 mg kg^{-1}), DPHSP-2 (150 mg kg^{-1}), and DPHSP-3 (250 mg kg^{-1}) more or less decreased the occurrence rate and severity score of the anaphylactic symptom (Fig. 3B), rectal temperature (Fig. 3C), and diarrhea rate (Fig. 3D). Mice in the DPHSP-3 group showed a lower allergy score and better preventive effect than the PHSP group (Fig. 3B). It could be seen from Fig. 3D that the DPHSP-3 and PHSP groups were able to significantly alleviate allergic diarrhea in OVA-induced food allergic mice ($P < 0.01$).

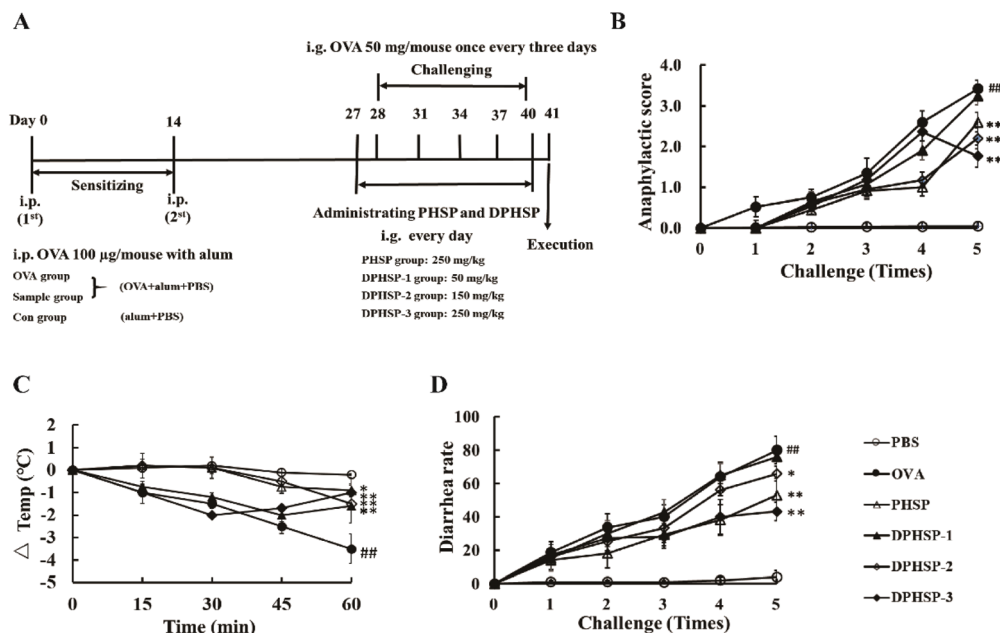


Fig. 3 Alleviating effects of PHSP and DPHSP on allergic symptoms in OVA-induced mice. (A) The protocol of the food allergy model of OVA-induced mice. (B) The anaphylactic score after each OVA challenge. (C) The rectal temperature after the fifth OVA challenge. (D) The situation of diarrhea in mice after each OVA challenge. Calculate the proportion of loose and total stools in the stool. * $P < 0.05$, ** $P < 0.01$ for all of the PHSP and DPHSP groups compared to the OVA group. # $P < 0.05$; ## $P < 0.01$, for the PBS group compared to the OVA group. The data represent mean $\pm$ SD ($n = 5$).

3.5. Effects of PHSP and DPHSP on antibody, histamine and mMCP-1 in OVA-induced food allergic mice

The levels of IgE, IgG1, and IgG2a in each group of mice were measured in the present study. As shown in Fig. 4A–C, the production of IgE, IgG1, and IgG2a in the serum of the OVA group was significantly different from that of the control group ($P < 0.01$). The allergic symptoms in the mice of the DPHSP-3 group were significantly reduced, and the content of OVA specific IgE in serum of mice was also significantly decreased ($P < 0.05$) (Fig. 4A). Therefore, histamine and mMCP-1 in serum of mice were detected subsequently. The content of histamine in the OVA group increased from 35.91 ng mL^{-1} (PBS group) to 300.49 ng mL^{-1} ($P < 0.01$), while the levels of histamine in the PHSP and DPHSP-3 groups decreased to 172 ng mL^{-1} and 101.11 ng mL^{-1} , respectively ($P < 0.01$) (Fig. 4D). Thus, both PHSP and DPHSP-3 groups could effectively reduce the level of histamine in serum of allergic mice, and the decrease in the level of histamine in the DPHSP-3 group is more obvious. The levels of mMCP-1 in each group are shown in Fig. 4E. The levels of mMCP-1 in the OVA group were significantly different from those in the PBS group ($P < 0.01$), while those in the PHSP and DPHSP-3 groups were significantly lower than those in the OVA group ($P < 0.01$). These data suggested that both PHSP and DPHSP had the inhibitory effects on the release of the anaphylactic mediator, and were consistent with the suppression of MC activation *in vitro*.

3.6. Effects of PHSP and DPHSP on cytokines in OVA-induced food allergic mice

Food allergy is caused by the imbalance between Th1 type cells and Th2 type cells. Generally, Th2 type cytokines can lead to an allergic reaction, while Th1 type cytokines may rise and reverse the balance to alleviate the allergic situation. What is more, Treg cells can alleviate food allergy symptoms by releasing the cytokines IL-10 and TGF- β to inhibit the proliferation and activation of Th1, Th2, Th17 and basophil cells.³⁵ In order to explore the effects of PHSP and DPHSP-3 on cytokines related to T cells in serum, the levels of IL-4 and IL-13 (Th2 cytokines), IFN- γ (Th1 cytokines) and IL-10 and TGF- β were detected by ELISA in our study. As shown in Fig. 5, compared with the PBS group, the expression of IL-4, IL-13, IFN- γ and TGF- β in the OVA group changed significantly ($P < 0.01$). The expression of IL-4 in the DPHSP-3 group decreased to 187.81 pg mL^{-1} ($P < 0.05$) while in the PHSP group it was not significantly different from the OVA group (Fig. 5A). In Fig. 5B, the production of IL-13 in the PHSP and DPHSP-3 groups was down-regulated to 98.67 pg mL^{-1} and 149.33 pg mL^{-1} ($P < 0.01$). As shown in Fig. 5C, the level of IFN- γ in the OVA group was 466.60 pg mL^{-1} , while in the PHSP and DPHSP-3 groups the levels were 226.80 pg mL^{-1} ($P < 0.01$) and 328.80 pg mL^{-1} ($P < 0.05$), respectively. The IL-10 levels in the OVA and PHSP groups did not change significantly when compared with the PBS group, while the expression of IL-10 in the DPHSP-3 group was up-regulated to 4135.75 pg mL^{-1} ($P < 0.01$) (Fig. 5D). Compared with the PBS group, the levels of TGF- β in the OVA

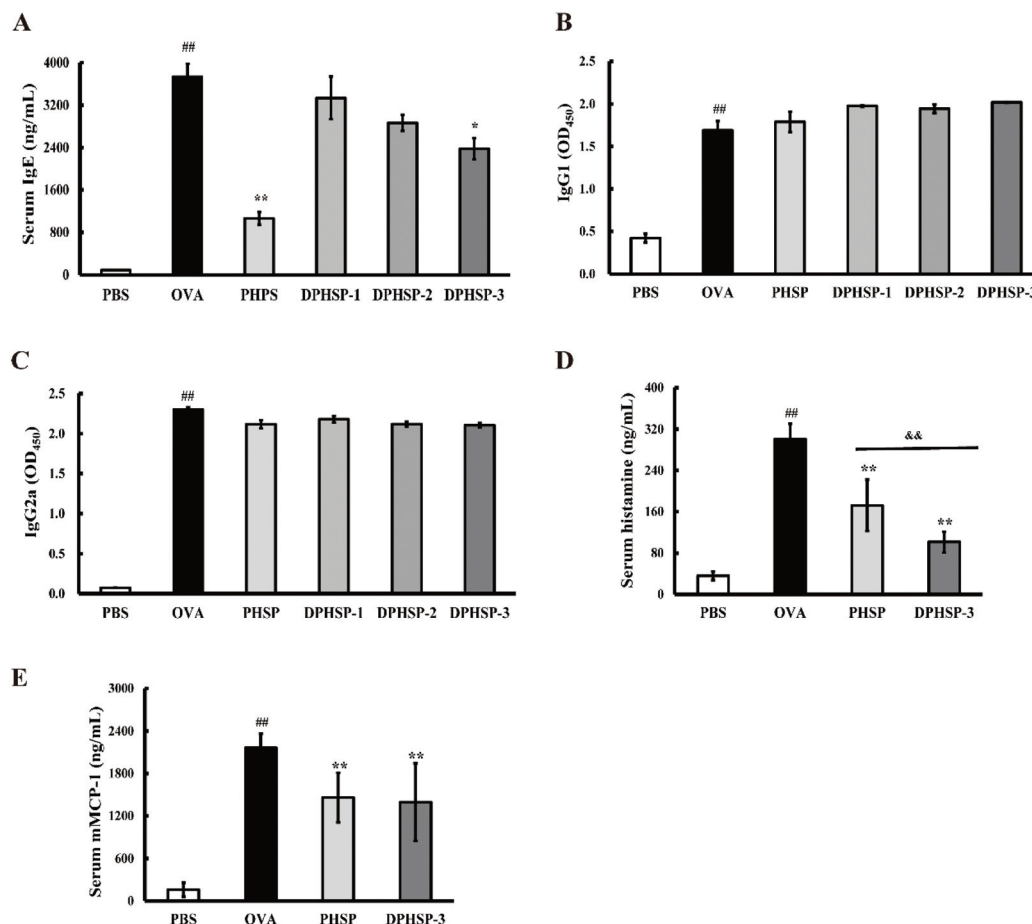


Fig. 4 Effects of PHSP and DPHSP on serum antibody, histamine and mMCP-1 in OVA-induced food allergic mice. (A) The effect of daily treatment with PHSP and DPHSP on serum OVA-specific IgE. The serum from the eyeball was collected on day 41. (B) The effect of daily treatment with PHSP and DPHSP on serum OVA-specific IgG1. (C) The effect of daily treatment with PHSP and DPHSP on serum OVA-specific IgG2a. (D) The effect of daily treatment with PHSP and DPHSP on serum histamine. The serum from the eyeball of mice was collected within 1 h after the last challenge. (E) The effect of daily treatment with PHSP and DPHSP on serum mMCP-1. * $P < 0.05$, ** $P < 0.01$ for all of the PHSP and DPHSP groups compared to the OVA group. # $P < 0.05$; ## $P < 0.01$, for the PBS group compared to the OVA group; ^a $P < 0.05$; ^a $P < 0.01$, for the DPHSP-3 group compared to the PHSP group. The data represent the mean $\pm$ SD of triplicate determinations at least.

group was down-regulated to 4051.25 pg mL⁻¹ ($P < 0.01$). In addition, the production of TGF- β in the DPHSP-3 group was significantly increased to 5152.50 pg mL⁻¹ (Fig. 5E).

3.7. Effects of PHSP and DPHSP on the population of Treg cells

From the previous experiments, it was found that DPHSP could promote the secretion of Treg cell related cytokines (IL-10 and TGF- β). In order to further determine the effect of PHSP and DPHSP on Treg cells, the subpopulation of CD4⁺Foxp3⁺ Treg cells was tested by flow cytometry in the present study. The cluster data of Treg cells in the spleen are shown in Fig. 6A, the proportion of Treg cells in the PBS group was 16.60% and that in the OVA group was 12.70% ($P < 0.01$). Compared with the OVA group, the subpopulation of Treg cells in the PHSP and DPHSP-3 groups increased to 13.38% and 16.85%, respectively. This indicated that both PHSP and DPHSP could promote the proliferation of Treg cells, and the

percentage of Treg cells in DPHSP-3 group mice was increased more significantly ($P < 0.01$). In Fig. 6B, the proportion of Treg cells from the MLN in the OVA group was significantly decreased from 8.50% (PBS group) to 6.64% ($P < 0.01$), while the proportions of Treg cells in the PHSP and DPHSP-3 groups were not significantly different from that of the OVA group.

4. Discussion

Polysaccharides are abundant in marine algae and have shown numerous bioactivities, such as antioxidant, anti-inflammatory anticoagulant, antiviral, and immunomodulatory activities, and gastroprotective and antidiarrheal effects.^{36,37} However, their activities are not always satisfactory mostly due to their structure and large molecular size, so that molecular modifications and degradations of polysaccharides are good ways to overcome this problem.^{38,39} Hence, a lot of methods were used

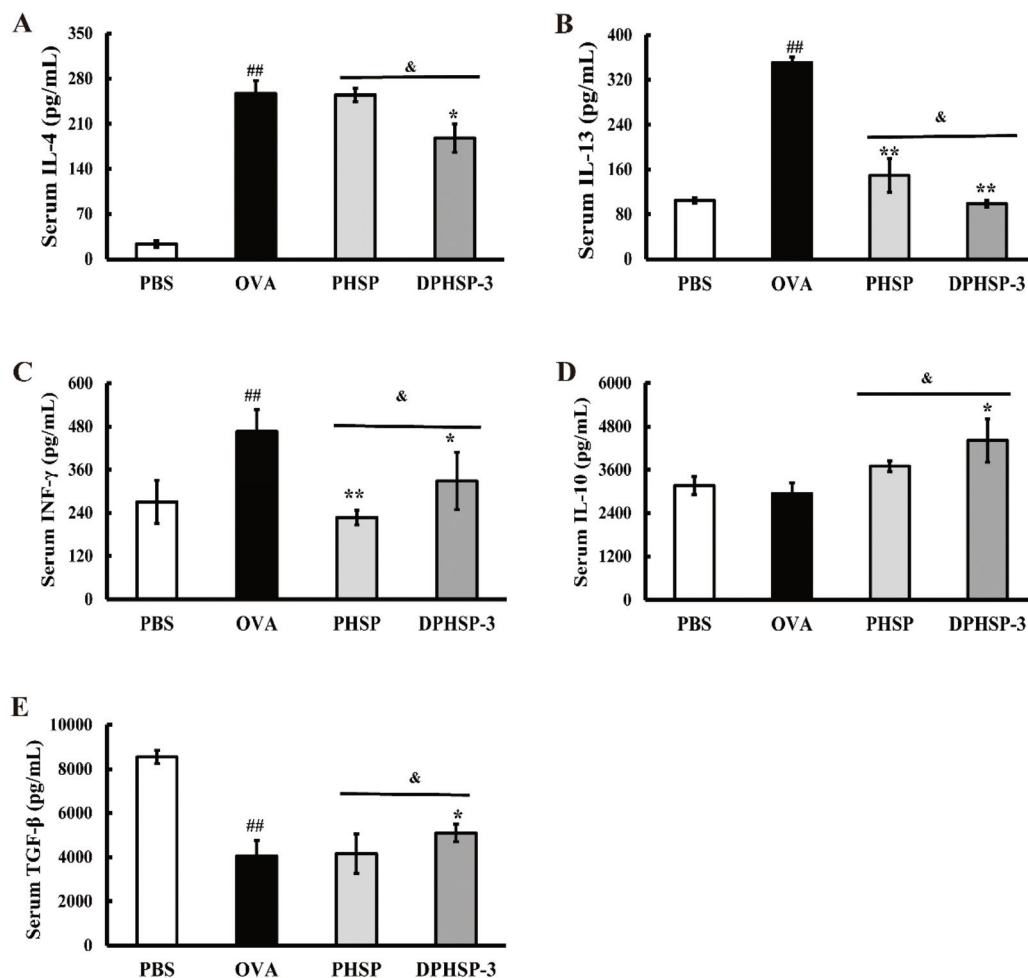


Fig. 5 The regulation of cytokines from serum T cells by PHSP and DPHSP in OVA-induced food allergic mice. (A) The effect of daily treatment with PHSP and DPHSP on serum IL-4. The serum from the eyeball was collected on day 41. (B) The effect of daily treatment with PHSP and DPHSP on serum IL-13. (C) The effect of daily treatment with PHSP and DPHSP on serum INF- γ . (D) The effect of daily treatment with PHSP and DPHSP on serum IL-10. (E) The effect of daily treatment with PHSP and DPHSP on serum TGF- β . * $P < 0.05$; ** $P < 0.01$ for all of the PHSP and DPHSP groups compared to the OVA group; # $P < 0.05$; ## $P < 0.01$, for the PBS group compared to the OVA group; & $P < 0.05$; ™ $P < 0.01$, for the DPHSP-3 group compared to the PHSP group. The data represent the mean $\pm$ SD of triplicate determinations.

by researchers to degrade polysaccharides. Studies have shown that the apparent viscosity of polysaccharides could be changed by heating and adding VC.⁴⁰ In our study, according to a proprietary polysaccharide degradation technology in our lab (original patent no. 201811073476.2), the results of viscosity and physicochemical properties of PHSP and DPHSP showed that there was obvious change in the reducing sugar content, the viscosity, microstructure, and molecular weight while total sugar, sulfate radical content and FT-IR had no apparent changes. The properties and the immunomodulatory activity of polysaccharides and oligosaccharides depend mainly on their structure and molecular weight, which are related to the viscosity and solubility.⁴¹ These results indicated that using the HP-VC method did not destroy the main structure of polysaccharides, which indicates that the inherent immunomodulatory activity of DPHSP is not reduced.

Due to their large molecular weights, complex structures and poor solubilities, polysaccharides have certain limitations in their immunomodulatory activities when they cross the membrane barrier. At the same time, it has been reported that the immunomodulatory activity of macromolecular polysaccharides is significantly improved after degradation in recent years.⁴² For example, the antioxidant activities of polysaccharides from *Porphyra haitanensis* and their degradation products were evaluated by Gong *et al.* and they found that the immunomodulatory activities of the degradation products were stronger.⁴³ Liao *et al.* reported that the chemically degraded products of *Gracilaria lemaneiformis* polysaccharides had a stronger hypoglycemic effect than *Gracilaria lemaneiformis* polysaccharides at the same dose.¹⁸ These findings were consistent with the results described in the present study. In the present animal experiments, the allergic symptom score and the diarrhea rate of the DPHSP-3 group were lower than those of the

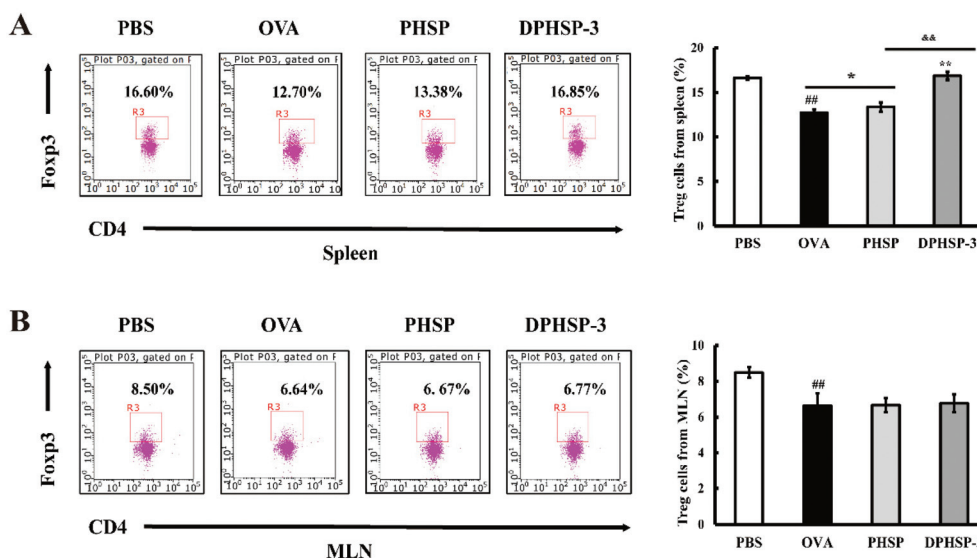


Fig. 6 Effects of PHSP and DPHSP on the population of Treg cells in the mouse spleen (A) and MLN (B). * $P < 0.05$, ** $P < 0.01$ for all of the PHSP and DPHSP groups compared to the OVA group. # $P < 0.05$; ## $P < 0.01$, for the PBS group compared to the OVA group. The data represent the mean $\pm$ SD of triplicate determinations.

PHSP group at the same dose, which means DPHSP had a stronger anti-allergic immunomodulatory activity than PHSP. However, the bioactivity of degraded GLSO was significantly improved compared with GLSP, while the activity of DPHSP was not much higher than that of PHSP. The main reason may be that GLSO has a higher degree of degradation and much lower molecular weight than DPHSP.^{24,25} The molecular weight will affect the absorption and transportation of polysaccharides. It was not easy for PHSP to enter cells through the cell membrane barrier after reaching the intestines because of its very large macromolecular weight. DPHSP has a lower molecular weight and higher immunomodulatory activity than PHSP. However, because the molecular weight of DPHSP is still relatively large, its immunomodulatory activity has not been enhanced as much as GLSO.

IgE-dependent mast cell activation plays an important role in food allergy; after the cross-linking of IgE and MCs, MCs are stimulated to degranulate and release β -hexosaminidase.⁴⁴ The production of immune cells and related cytokines is the important basis of promoting or alleviating the development of food allergies. Several marine polysaccharides have been found to be modulators of the allergic response by improving the integrity of the intestinal epithelial villi, inhibiting IgE production, restoring the balance of Th1/Th2 cells, or suppressing the degranulation of MCs.^{34,45} In our work, PHSP and DPHSP inhibited mast cell activation *in vitro* and *in vivo*. Interestingly, the release rate of β -hexosaminidase in the PHSP group was higher than that in the DPHSP group *in vitro*, which showed that DPHSP inhibited the degranulation of MCs. However, the level of IgE in PHSP group mice was significantly lower than that in the DPHSP group *in vivo*, and low IgE levels in serum are beneficial to the alleviation of mast cell degranulation by reducing serum IgE and mast cells in the surface of the Fc ϵ RI

binding. The reason for the decrease of IgE levels in the PHSP group was surmised that PHSP with a huge molecular weight may affect the phagocytosis of allergens or B cell function and need further research, while DPHSP with a low molecular weight could influence the T lymphocyte differentiation directly.

In addition to IgE-dependent activation of MCs, T lymphocyte has been identified to play a central role in food allergy. It could be an effective strategy for the treatment of food allergy as it affects the secretion of key cytokines during the allergy response and reverses the imbalanced Th1/Th2 cytokine pattern.⁴⁶ The current study showed that DPHSP exhibited obvious suppression activities of the levels of IFN- γ produced by Th1 cells, and also reduced the production of IL-4 and IL-13 related with Th2 cells. It was speculated that DPHSP was able to restore the immune balance of Th1/Th2 cells. Several sophisticated regulatory mechanisms are used to maintain immune homeostasis and prevent autoimmunity. Being the principal pathway among the inhibitory pathways of CD4⁺Foxp3⁺ Treg cells, the TGF- β and IL-10 production from Treg cells, which is a non-cell-cell interaction inhibitory effect, maintains the balance of Th1/Th2 differentiation and the peripheral immune tolerance.^{47,48} Thus, based on the inhibitory effects of DPHSP on the Th cells, here we further verified the up-regulation of IL-10 and TGF- β levels by DPHSP. In addition, flow cytometry showed that DPHSP could enhance the proportion of CD4⁺Foxp3⁺ Treg cells in the spleen of allergic mice, inhibit the polarization of Th2 cells and alleviate the symptoms of food allergy. Therefore, DPHSP could play an anti-food allergy role via regulating the function of Treg cell and T helper cells. DPHSP-3 administration restored the number of Treg cells and significantly increased the level of IL-10, while the level of TGF- β did not show a downward or an upward trend compared

with the control group, which was similar to the results published by Lin, that is, IL-10 did not increase synchronously when TGF- β increased with the upregulation of Treg cells.⁴⁹ In our results, we found that IL-10 in the DPHSP-3 group increased significantly with the upregulation of Treg cells, indicating that DPHSP affected the secretion of IL-10 by Treg cells to affect the immune balance.

Meanwhile, MLN also participates in immune regulation as part of the immune system. Although the spleen and MLN are parts of the lymphatic system, the populations of T cells in the spleen were higher than those in the MLN, the results obtained for the spleen and MLN are not always consistent.⁵⁰ Zhang *et al.* found that bacterial translocation increased in the spleen and other immune organs of rats with severe shock, but did not change in the MLN due to the immune barrier function.⁵¹ Therefore, the MLN has the immune barrier function and is located near the gastrointestinal tract, which may also be regulated by intestinal flora and its metabolites. In our experiment, PHSP and DPHSP did not promote the upregulation of Treg cells in the MLN, which was inconsistent with the results for the spleen. The reason may be that as high molecular weight compounds, PHSP and DPHSP may interact with intestinal microflora and affect the differentiation of immature T cells in the MLN.

In a mouse model, PHSP and DPHSP have more significant inhibitory effects on mast cell degranulation *in vivo*, compared with the release rates of β -hexosaminidase and histamine in animal and cell models. Combined with the allergic process, PHSP and DPHSP promoted the differentiation of Treg cells by regulating the Th1/Th2 cell balance, and then affected mast cell activation so as to alleviate the allergic reaction. Therefore, the inhibitory effects of PHSP and DPHSP on allergic mediators in mast cells without T cells *in vitro* are less obvious than that *in vivo*.

4. Conclusion

In summary, DPHSP with low molecular weight and low viscosity was obtained by the HP-VC method. The antiallergic activity of DPHSP *in vitro* was stronger than that before degradation through stable mast cell activation. The animal models confirmed that both PHSP and DPHSP could alleviate food allergy symptoms and inhibit allergic mediators, but DPHSP with the same concentration showed a higher activity. Moreover, DPHSP could promote the differentiation of Treg cells and regulate the Th1/Th2 cell balance to relieve allergic reactions. Therefore, DPHSP was able to inhibit food allergy more effectively when compared with PHSP, and it is expected to be used as a raw material for the production of new anti-allergic special foods.

Abbreviations

BMMCs	Bone marrow-derived mast cells
BSA	Bovine serum albumin
DNP	Dinitrophenyl

DNS	Dinitrosalicylic acid
DPHSP	Degraded <i>Porphyra haitanensis</i> sulfated polysaccharide
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FT-IR	Fourier transform infrared
GLSO	<i>Gracilaria lemaneiformis</i> sulfated oligosaccharide
GLSP	<i>Gracilaria lemaneiformis</i> sulfated polysaccharide
HP	High temperature and pressure method
IFN- γ	Interferon γ
Ig	Immunoglobulin
IL	Interleukin
MCs	Mast cells
mMCP-1	Mouse mast cell proteinase 1
MLN	Mesenteric lymph node
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2- <i>H</i> -tetrazolium bromide
OVA	Ovalbumin
PBS	Phosphate buffer solution
PHSP	<i>Porphyra haitanensis</i> sulfated polysaccharide
RBL	Rat basophil line
SPF	Specific pathogen free
TGF- β	Transforming growth factor β
Th	T helper
Treg	Regulatory T
US	Ultrasonic
VC	Vitamin C

Author contributions

Qingmei Liu conducted anti-food allergic experiments and wrote the initial draft. Yu Zhou conducted anti-food allergic experiments and wrote the initial draft. Jinlv Sun and Guangming Liu designed and coordinated the project. Yuanyuan Gao created the food allergy mouse model and assisted this experiment. Zhendan Shu and Jun Zhang reviewed or revised the draft. Minjie Cao contributed to the coordination of the project. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declared that they have no conflicts of interest to this work.

Acknowledgements

This work was supported by the grants from the National Natural Science Foundation of China (32001695, 32072336, and 31871720), the Science and Technology Program of Fujian Province (2018R0071), the Educational Research Project for Young and Middle-aged Teachers of Fujian Education Department (JAT190344), and the Special Fund for Marine and Fishery Development of Xiamen City (19CZP011HJ08).

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Online Supplementary Material

Supplementary Methods

The different degradation methods of *Porphyra haitanensis* sulfated polysaccharide (PHSP)

Ultrasonic method (US): A major factor ultrasonic treatment is ultrasonic power in fragmentation, whereas temperature and ultrasonic time are minor factors. PHSP solution was treated with different ultrasonic power (240, 320, 400, 480, 600, 700, 800, 900 and 1000 W) for 1 h, every ultrasonic for 2 seconds, interval pause for 2 seconds. And then different degradation time was set as 0, 10, 30, 45, 60, 120, 180 min under 600 W ultrasonic power. The DPHSP precipitates were obtained by adding 5 times volume of ethanol to the treated solution after different ultrasonic power and treatment time, respectively. The DPHSP products were obtained after centrifugation and redissolution.

High temperature and pressure method (HP): PHSP solution was placed in a pressure cooker and treated with different pressure parameters (0 MPa, 0.05 MPa (110 °C), 0.15 MPa (121 °C), 0.16 MPa (127 °C)) for 2 h. Then the pressure parameters of 0.16 MPa (127 °C) were selected to degrade PHSP with different degradation time (0, 0.5, 1.0, 2.0, 2.5, 3.0, 4.0, 5.0 h). The DPHSP precipitates were obtained by adding 5 times volume of ethanol to the treated solution after different pressure and treatment time, respectively.

H₂O₂: PHSP solution was treated with different concentrations of H₂O₂ (5, 10, 20 mM) and different time (0, 30, 60, 90, 120 min) by orthogonal design. The DPHSP

precipitates were obtained by adding 5 times volume of ethanol to the treated solution, respectively.

Vitamin C method (VC): PHSP solution was treated with different concentrations of VC (10, 20, 30, 40, 50 mM) and different time (0.1, 0.5, 1.0, 2.0, 3.0 h) by orthogonal design. VC was removed by ethanol precipitation with 5 times of volume. The DPHSP product was obtained after redissolution and freeze-drying.

In this study, the optimal power of ultrasonic degradation of PHSP was 600 W and the optimal time was 60 min. The optimal temperature and time of HP concentration was 127 °C and 5 h. The degradation conditions of H₂O₂ were 20 mM and 120 min. The optimal concentration of PHSP degradation by VC was 30 mM, and the optimal time was 30 min. These four methods were combined in pairs to treatment PHSP under the optimum conditions:

US-HP: PHSP solution was degraded by 600 W ultrasound for 60 min and then treated at 0.16 MPa (127 °C) for 5 h.

US-H₂O₂: PHSP solution was treated with H₂O₂ (final concentration: 20 mM) for 2 h after being degraded by 600 W ultrasound for 60 min.

US-VC: PHSP solution was treated with VC (final concentration: 30 mM) for 30 min after being degraded by 600 W ultrasound for 60 min.

HP-H₂O₂: PHSP solution was degraded at 0.16 MPa (127 °C) for 5 h, and then treated with H₂O₂ (final concentration: 20 mM) for 2 h.

HP-VC: PHSP solution was degraded at 0.16 MPa (127 °C) for 5 h, and then treated with VC (final concentration: 30 mM) for 30 min

VC-H₂O₂: PHSP solution was treated with VC (final concentration: 30 mM) for 30 min and H₂O₂ (final concentration: 20 mM) for 2 h, respectively.