

Chitosan Oligosaccharide Exerts Anti-Allergic Effect against Shrimp Tropomyosin-Induced Food Allergy by Affecting Th1 and Th2 Cytokines

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Keywords

Shrimp tropomyosin · Degree of polymerization · Chitosan · Anti-allergy · Mouse model

Abstract

Background: Shrimp-derived allergen has a serious impact on people's health. Chitosan oligosaccharide (COS) has anti-allergic action but its function on shrimp allergen-induced allergy and related molecular mechanisms remain unclear. **Methods:** COS and its degrees of polymerization (DP) were selected to interact with shrimp tropomyosin (TM) and IgE was measured. A mouse model of food allergy was established by receiving shrimp TM intraperitoneally. The models were treated with different concentrations of COS. Fecal and serum histamine, serum IgE, IgG1 and IgG2a, and inflammatory cytokines were measured. **Results:** The main products for COS were DP2–6 with the contents of 6, 40, 26, 16, and 4%, respectively, and reacted with shrimp TM increasingly when COS DP was increased. Severe symptoms of food al-

lergy were observed in the TM group (diarrhea, anaphylactic response, and rectal temperature). In contrast, COS treatment improved these symptoms significantly ($p < 0.05$). The sensitized mice were desensitized after they were treated with 1 mg/kg COS. COS treatment significantly reduced serum IgE and IgG1 levels, and increased IgG2a levels ($p < 0.05$). COS consumption decreased fecal and serum histamine. COS treatment reduced Th2 cytokine (IL-4, IL-5, and IL-13) levels and increased the Th1 cytokine (IFN- γ) level ($p < 0.05$). **Conclusions:** COS showed anti-allergy properties by regulating the levels of Th1 and Th2 cytokines.

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Introduction

Food allergy is an adverse reaction caused by the immune response, often accompanied by a series of inflammatory symptoms, including gastrointestinal, skin [1], respiratory, and even systemic allergic reaction, and is

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sometimes fatal [2]. Epidemiological surveys show that about 1% to 2% of adults, and [3] 5% to 7% of children have allergic reaction to food worldwide [3]. The main allergic food includes milk, peanuts, and crustaceans. Shrimp is an important category among the eight categories of allergic food proposed by the Food and Agriculture Organization of the United Nations. Shrimp food has always attracted the attention of consumers. There are serious safety hazards to potential allergic people, some of which are even life-threatening. There is still no effective treatment for food allergy, although the allergy still occurs frequently.

Allergens play an important role in the induction of food allergic responses. There are many allergens in shrimp, and tropomyosin (TM) is recognized as the main shrimp-derived allergen [4]. Most food allergies are IgE-mediated type I hypersensitivity reactions. When allergens enter the body, they activate Th2 cells, which secrete cytokines such as IL-4, IL-5, and IL-13, which induce B cell production, antibody production, and isotype switching to IgE [5]. IgE is bound by Fc epsilon RI (the high affinity receptor for IgE), and that degranulation only occurs upon binding of the allergen, resulting in Fc epsilon RI crosslinking. Inflammatory mediators such as histamine and interleukin, etc. are released, thereby causing allergic reaction [6]. IgE can affect the expression of miRNA, which also causes histamine release [7].

It is highly necessary to control shrimp-derived allergy by using natural products with few side effects. Chitosan oligosaccharide (COS) is a low molecular weight oligosaccharide with high biological activity. It is water-soluble and easily absorbed and composed of 2–10 acetylglucosamines linked by β -1,4 glycosidic bonds. Studies have shown that COS has anti-allergy effects [8]. However, the effects of COS on shrimp-derived food allergy have not been reported and the related molecular mechanism remains unclear. Th1 and Th2 cells are important T cell subsets and involved in the pathogenesis of food allergy [9]. Oligosaccharides have been reported to be a potential food agent in the prevention of food allergy by modulating oral sensitization to food antigens and attenuating the induction of intestinal Th2 cytokine responses [10]. COS may also ameliorate shrimp-caused allergy by affecting Th1 and Th2 cells. In this paper, a mouse allergy model was established using shrimp TM. The anti-food allergy effect of COS was explored by using animal allergic models, and different inflammatory cytokines were measured.

Materials and Methods

Reagents

COS mixture, chitobiose (DP2), chitotriose (DP3), chitotetraose (DP4), chitopentaose (DP5), and chitohexaose (DP6) were purchased from Qingdao Bozhi Huili Biotechnology Co., Ltd. (Qingdao, China). SPF female BALB/c mice were purchased from Shanghai Lake Laboratory Animal Co., Ltd. (Shanghai, China). Shrimp TM was purchased from Indoor Biotechnologies (Charlottesville, VA, USA). The protein assay kit was purchased from Bio-Rad (Hercules, CA, USA). Alum adjuvant was purchased from Thermo Company (Rockford, IL, USA). HRP-labeled goat anti-mouse IgE antibody was purchased from Southern Biotech (Birmingham, AL, USA). HRP-labeled goat anti-mouse IgG1 and IgG2a antibodies were purchased from Abcam (Chicago, IL, USA). Mouse lymphocyte separation fluid was purchased from Shenzhen Dake Bio. (Shenzhen, China).

Preparation of Standard Solution

The standards DP2–6 were separately prepared into a standard solution with a mass fraction of 1.5, 2.5, 5.0, 7.5, and 10%, filtered through a 0.22- μ m filter, and stored at 4 °C for use. The COS mixture was also prepared with same concentration, filtered through a 0.22- μ m filter, and stored at 4 °C.

Chromatographic Analysis

LC-1200 high-performance liquid chromatograph (HPLC) was purchased from Agilent (Palo Alto, CA, USA) equipped with a Shodex Asahipak NH2P50 column (4.6 $\times$ 250 mm, 5 μ m). The following HPLC condition was used: column, Shodex Asahipak NH2P-50 4E (4.6 mm $\times$ 250 mm, 5 μ m); mobile phase, acetonitrile:water = 75:25 (v/v); mobile phase flow rate 0.6–1.2 mL/min; column temperature, 26–34 °C, and injection volume, 20 μ L. The prepared standard and COS solution were separately injected into HPLC, and the retention time and peak area of the standards and COS mixture were recorded.

Acetonitrile-water was selected as the mobile phase and the amino column was used for COS separation. After comparing the results of COS with different column temperatures, the final determination of the column temperature was 30 °C. At this temperature, the peaking time of COS was suitable, and 2–6 DPs of COS were effectively separated. Therefore, the determined analytical conditions were acetonitrile:water = 75:25 (v/v), column temperature 30 °C, and mobile phase flow rate 1.2 mL/min.

Precision Test

Qualitative and quantitative analysis of the COS sample solution. The COS sample solution was repeatedly measured 7 times. Online supplementary Table S1 (for all online suppl. material, see www.karger.com/doi/10.1159/000500720) shows that the relative standard deviation was 0.1–1.0%, both less than 2%, and the precision was high, indicating that this analytical method was reliable.

Interaction between TM and COS

The shrimp TM was prepared as 5 mg/mL and a certain amount of COS and COS DP2–6 was dissolved in the TM protein solution, respectively. The mixture was incubated at 37 °C and reacted for 60 min. Subsequently, the mixture was centrifuged at 12,000 g for 30 min, and the supernatant and precipitate were separated respectively. The protein content in the supernatant and the precipitate was analyzed by using SDS-PAGE.

Establishment of a Food Allergy Model in Mice

All protocols were approved by Animal Research Ethics Committee of China-Japan Union Hospital of Jilin University (Changchun, China, approval No. 20171215CJD). The female BALB/c mice of 6–8 weeks were divided into PBS, TM, and COS groups with 6 mice in each group. Online supplementary Figure S1 shows the treatment processes among different groups. Mice in the TM and COS groups were intraperitoneally injected with TM and alum adjuvant (TM 100 µg/mouse and 2 mg alum adjuvant in PBS) at 0 and 14 days, and only PBS (8.00 g NaCl, 0.20 g KCl, 2.9 g Na₂HPO₄·12 H₂O) was injected into the mice in the PBS group at the same time. In the COS group, COS (100 µg/mouse in PBS) was intraperitoneally injected three times a week. From the 28th day, the mice in the TM group and the COS group were intragastrically administered with TM (10 mg/mouse) every 3 days. The mice in the COS group were also intragastrically administered with COS (5 mg/mouse). The mice in the PBS group were intragastrically administered with 200 µL PBS as a negative control. Feces were collected from all mice at 49 days. Tail vein blood were collected from all mice and centrifuged (5,000 g, 10 min, 4 °C) to collect the serum.

Measurement of the Symptoms of TM-Induced Allergy

The common symptoms of food allergy were measured, including diarrhea, anaphylactic response, and rectal temperature. Diarrhea and anaphylaxis were observed by visually monitoring mice for 60 min after intragastric administration. Diarrhea and anaphylactic responses were scored according to a previous report [11]. Rectal temperature was measured by using a Diatek digital thermometer (Welch Allyn, Skaneateles Falls, NY, USA).

Measurement of TM-Specific IgE, IgG1, and IgG2a Levels in the Serum of the Mice

The content of TM-specific immunoglobulin (IgE, IgG1, and IgG2a) in mouse serum was determined by using corresponding ELISA kits from Dingguo Biotech (Beijing, China). The absorbance was measured at 450 nm with a microplate reader.

Measurement of Histamine

Histamine concentration should be measured in both feces and serum [12]. 0.8 g of feces with 2 mL of 5% trichloroacetic acid were mashed with a tissue masher, centrifuged (8,000 g, 20 min), and filtered to obtain the supernatant. The pH of the supernatant was adjusted to 9.5, and 300 µL of the supernatant was mixed with 600 µL of dansyl chloride solution and incubated at 40 °C water bath for 20 min. 100 µL of ammonia water was added to the mixture to remove excess dansyl chloride, left to stand for 30 min in the dark, diluted to 1.5 mL with acetonitrile, and loaded on HPLC. The HPLC analysis parameters were as follows: the column was an Agilent ZORBAX Extend-C18 column (50 mm × 4.6 mm, 5 µm); the mobile phase was acetonitrile:water = 70:30 (v/v); the flow rate was 1 mL/min; the column temperature was 35 °C; the injection volume was 20 µL, and the UV detection wavelength was 254 nm.

Serum histamine content in the mice was measured by using the kit from Shanghai Blue-based Biotechnol. Ltd. (Shanghai, China) according to the manufacturer's instruction.

Measurement of Inflammatory Cytokines

All mice were sacrificed at 49 days to isolate spleen, and splenocytes were isolated by homogenizing the tissues with a

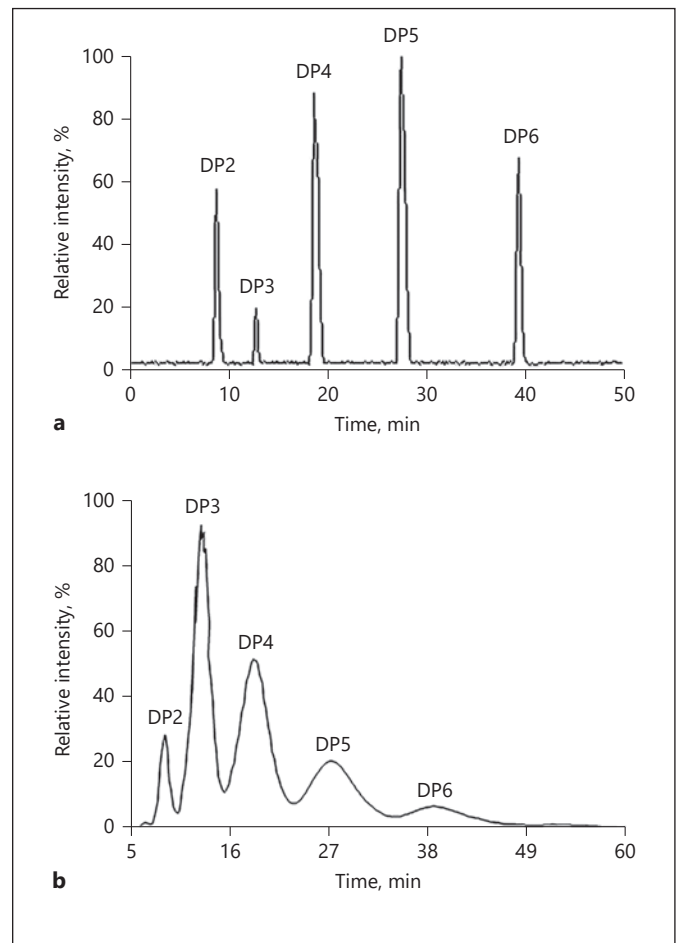


Fig. 1. HPLC analysis of COS. **a** The standards of COS DP2–6. **b** COS mixture with DP2–6.

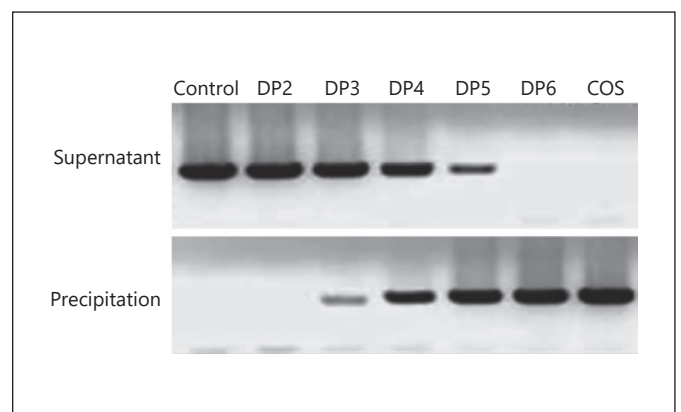


Fig. 2. SDS-PAGE analysis of the interaction of COS and TM. TM concentration in the supernatants was reduced, while the concentration was increased in the precipitation with the increase in COS DPs.

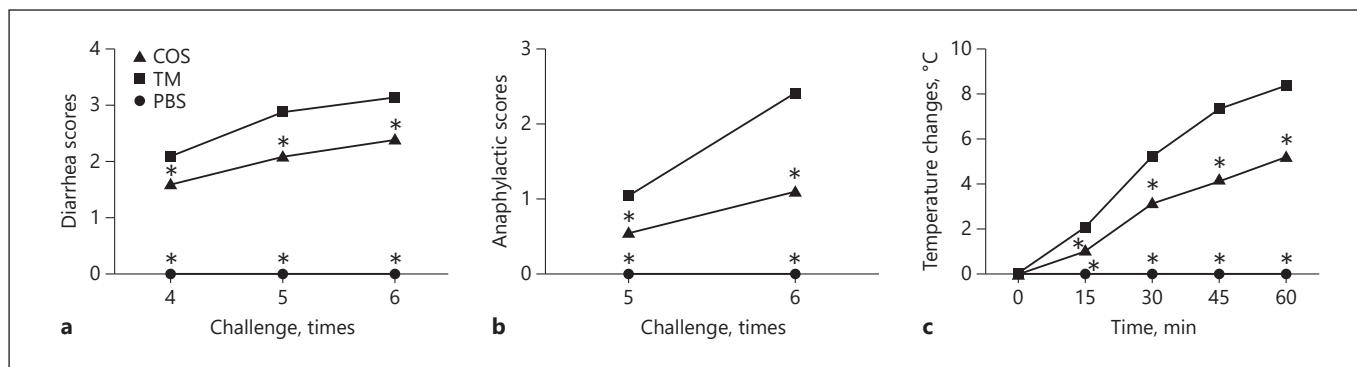


Fig. 3. The effects of COS on the symptoms of TM-included food allergy. **a** Diarrhea scores. **b** Anaphylactic scores. **c** Temperature changes. The mice were intragastrically administered with TM, TM and COS, and PBS in the TM, COS, and PBS groups, respectively. $n = 6$ for each group. $*p < 0.05$ versus the TM group.

tissue grinder. The cells were centrifuged (5,000 g, 10 min, 4 °C), and the inflammatory cytokine in the supernatants were analyzed by using ELISA kits. RNA was isolated from cell pellets by using Trizol reagent, and the RNA was reverse transcribed into cDNA. The cDNA was used as a template, and the gene expression of Th1 cytokine (IFN- γ) and Th2 cytokines (IL-4, IL-5, IL-13) was detected by real-time PCR. β -Actin was used as an internal reference. The sequence of the primers used (online suppl. Table S2) is referred to the method of Capobianco et al. [13].

Statistical Analysis

All data in the experiment were analyzed by SPSS 16.0 software. ANOVA was used for variance analysis. The significance test method was Duncan's multiple test method. The test limit was 0.05, and the experimental data was measured in triplicates.

Results

Recovery Rate of COS

Four aliquots of COS solution were taken, one of which was used as the background, and the other aliquots with different COS DPs were added to determine the peak area of each COS DP. The samples were tested in triplicates, and the COS recovery was between 96.9 and 98.2%, indicating that the method has a higher accuracy and high recovery rate of COS (online suppl. Table S3).

Measurement of COS DPs

Figure 1a shows the HPLC analysis of reference standards from chitobiose to chitohexaose, in which the peak retention time was 8.78, 12.77, 18.72, 27.52, and 39.39 min, respectively. The COS DP2–6 had a fraction of 6, 40, 26, 16, and 4%, respectively. The total mass fraction of the six degree-of-polymer COS was 91% (Fig. 1b).

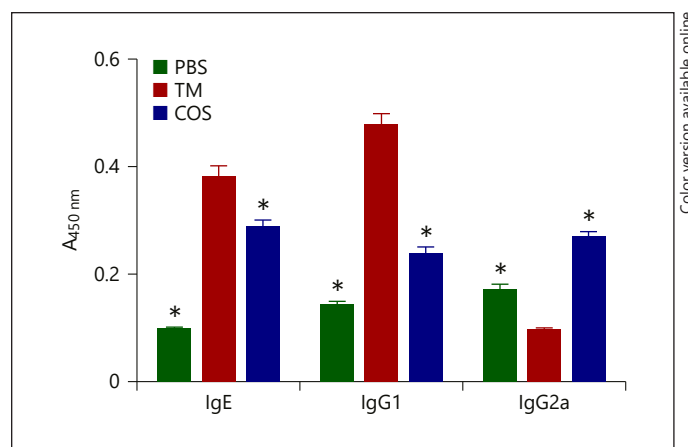


Fig. 4. The effects of COS mixture on IgE, IgG1, and IgG2a. The mice were intragastrically administered with TM, TM and COS, and PBS in the TM, COS, and PBS groups, respectively. $n = 6$ for each group. $*p < 0.05$ versus the TM group.

The Binding Power of COS and TM Was Dependent on COS DPs

SDS-PAGE analysis showed the concentration of TM in the supernatants was reduced and the TM concentration was increased in the precipitation with the increase in the DPs of COS (Fig. 2). The results suggested that COS DP2 did not interact with TM, COS DP3 interacted with TM weakly, COS DP4 interacted with TM normally, COS DP5 interacted with TM strongly, and COS DP6 and COS mixture sample interacted with TM completely. The binding power of COS and TM was dependent on COS DPs.

Fig. 5. The effects of COS mixture on the levels of histamine. **a** Feces. **b** Serum. The mice were intragastrically administered with TM, TM and COS, and PBS in the TM, COS, and PBS groups, respectively. $n = 6$ for each group. * $p < 0.05$ versus the TM group.

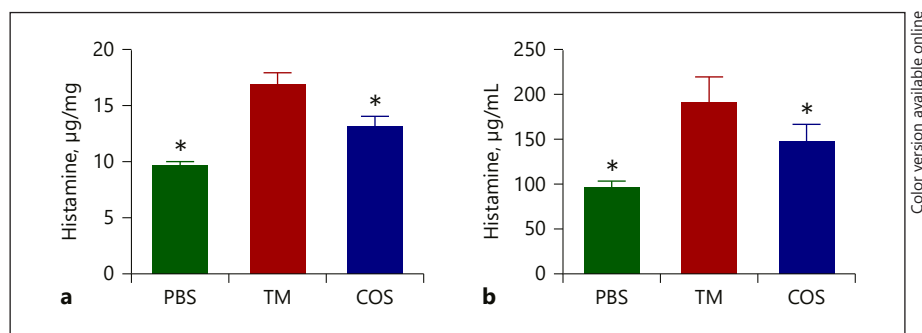
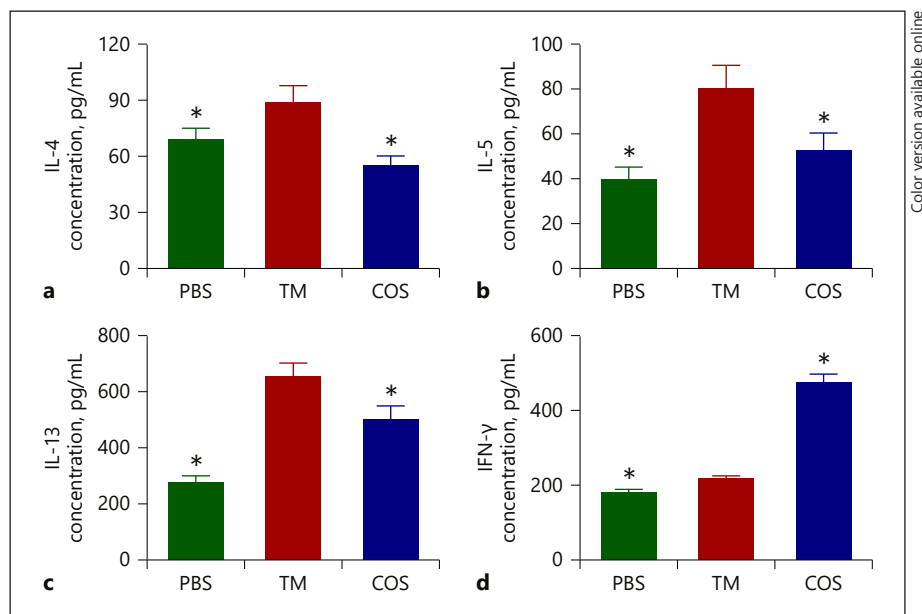


Fig. 6. The effects of COS mixture on the concentration of inflammatory cytokines. **a** IL-4. **b** IL-5. **c** IL-13. **d** IFN- γ . The mice were intragastrically administered with TM, TM and COS, and PBS in the TM, COS, and PBS groups, respectively. $n = 6$ for each group. * $p < 0.05$ versus the TM group.



COS Treatment Improved the Symptoms of TM-Induced Allergy

The severe symptoms of TM-induced food allergy were observed in the TM group, including diarrhea scores (Fig. 3a), anaphylactic response scores (Fig. 3b), and rectal temperature (Fig. 3c) ($p < 0.05$). In contrast, COS treatment improved these symptoms significantly (Fig. 3) ($p < 0.05$).

Effects of COS on Serum IgE, IgG1, and IgG2a in Mice

Blood was collected 24 h after the second injection of TM, and the levels of TM-specific IgE, IgG1, and IgG2a in the serum were examined by ELISA. The levels of serum IgE and IgG1 in the mice injected with COS was significantly lower than in the TM group, while the level of IgG2a in the COS group was significantly higher than in the TM group (Fig. 4) ($p < 0.05$).

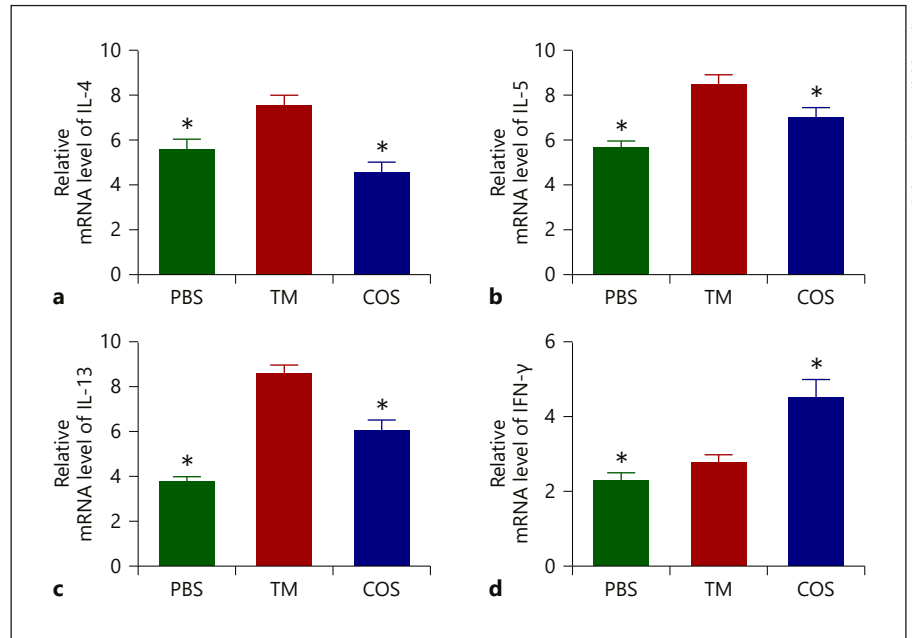
Histamine Content

The content of histamine in the feces of the PBS group was 9.07 $\mu\text{g}/\text{mg}$, and the content of histamine in the TM group increased to 18.75 $\mu\text{g}/\text{mg}$, while the content of histamine in the feces of mice fed with COS decreased to 13.35 $\mu\text{g}/\text{mg}$ in feces (Fig. 5a). The content of histamine in the serum of the PBS group was 95.32 $\mu\text{g}/\text{mL}$, and the content of histamine in the TM group increased to 190.41 $\mu\text{g}/\text{mL}$, while the content of histamine in the serum of mice fed with COS decreased to 147.29 $\mu\text{g}/\text{mL}$ in serum (Fig. 5b).

COS Regulated the Levels of IL-4, IL-5, IL-13, and IFN- γ

ELISA analysis showed that the concentrations of IL-4 (Fig. 6a), IL-5 (Fig. 6b), and IL-13 (Fig. 6c) in the PBS and COS groups were lower than in the TM group ($p < 0.05$),

Fig. 7. The effects of COS on relative mRNA levels of inflammatory cytokines. **a** IL-4. **b** IL-5. **c** IL-13. **d** IFN- γ . The mice were intragastrically administered with TM, TM and COS, and PBS in the TM, COS, and PBS groups, respectively. $n = 6$ for each group. * $p < 0.05$ versus the TM group.



whereas the concentration of IFN- γ (Fig. 6d) in the COS group was higher than in the PBS and TM groups ($p < 0.05$). In the similar results, real-time qPCR analysis showed that relative mRNA levels of IL-4 (Fig. 7a), IL-5 (Fig. 7b), and IL-13 (Fig. 7c) in the PBS and COS groups were lower than in the TM group ($p < 0.05$), whereas the level of IFN- γ (Fig. 7d) in the COS group was higher than in the PBS and TM groups ($p < 0.05$).

Discussion

The main products of COS were 2–6 DPs. The interaction between COS and shrimp TM increased with the increase in COS DPs. The results suggest that high DP of COS may have better therapeutic effects on food allergy. COS treatment improved the severe symptoms of TM-induced food allergy, including diarrhea scores, anaphylactic response scores, and rectal temperature. The results were consistent with previous reports: COS intervention reduced diarrhea incidence [14] and anaphylaxis [15] in animal models. The effects of COS on the changes of body temperature were seldom reported.

IgE is the decisive indicator for evaluating allergic condition. The determination of Th1 type specific index IgG2a and Th2 type specific index IgG1 can also well indicate the polarization of Th1/Th2, indicating the allergic responses [16]. In this experiment, COS was injected

from the 7th day before sensitization, and the level of TM-specific antibody in the serum of mice was detected after the second immunization. The results showed that COS significantly inhibited the levels of IgE and IgG1 in serum and significantly increased serum IgG2a level in the mice. These results suggested that COS was an anti-food allergy preventive.

Elevated histamine is associated with the increased risk of food allergy [17]. The present results showed that the histamine content in the feces and serum of the COS group was significantly lower than that in the TM group. Allergen immunization can cause the changes in microbiota composition in the food allergy model [18]. Other results showed that COS could increase the probiotic mixture [19], which may be beneficial to prevent allergy too.

When humans are suffering from allergy, Th2 cells will produce IL-4, IL-15, and IL-13, which induces B cells to produce antibodies such as IgE, so the content of these cytokines is an important indicator for evaluating food allergy [20]. Th1 type cells are characterized by secreting IFN- γ , which can alleviate the inflammatory response and resist the invasion of pathogens [21]. COS significantly increased the content of Th1 cytokine IFN- γ and significantly inhibited the Th2 cytokines IL-4, IL-5, and IL-13. Similar results were obtained by detecting the levels of IL-4, IL-13, and IFN- γ in the supernatant of mouse splenocytes by ELISA.

Conclusions

This study used the animal model of food allergy to determine allergic related indicators, such as IgE, IgG1, IgG2a, and histamine, which further confirmed that COS was a potential food allergy preventive agent with anti-allergy properties.

Disclosure Statement

All authors state no conflict of interests.

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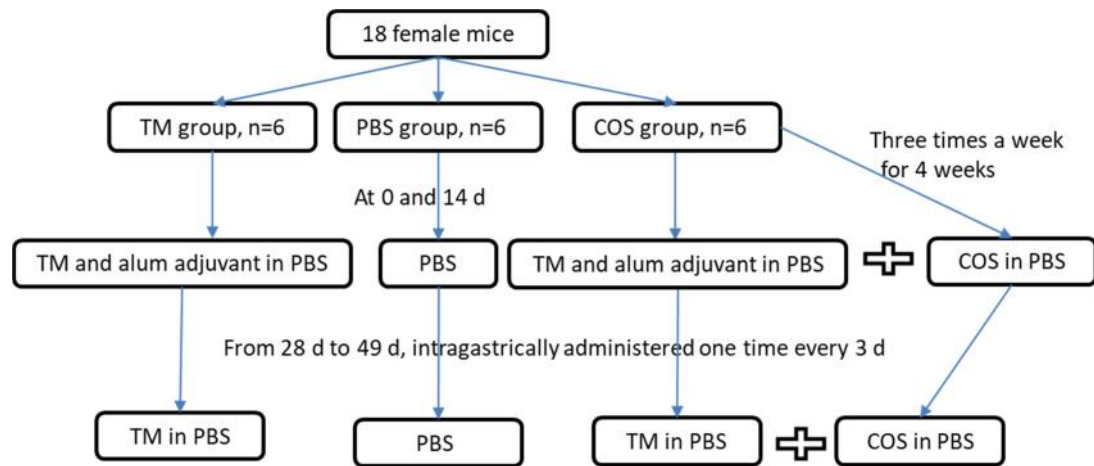


Figure S1. Schematic diagram illustrating the treatment processes among different groups.

Table S1. Precision test of COS by HPLC

	Chitobios	Chitotrios	Chitotetraos	Chitopentaos	Chitohexaos
	e	e	e	e	e
1	1953803	12269881	12540317	7140803	1763421
2	2011153	12355292	12546748	7169688	1763647
3	1974267	12259044	12512356	7174966	1764035
4	2003048	12326894	12594204	7139806	1759823
5	1966482	12279467	12558540	7150455	1760324
6	2000895	12287664	12537133	7151403	1762865
7	1942995	12303068	12555033	7176342	1757310
RS	1.0	0.3	0.2	0.2	0.1
D					
(%)					

Table S2. Pairs of primers used in the real-time qPCR analysis of cytokine gene expression in splenocytes

	Forward	Reverse
IFN- γ	5'-TGGCATAGATGTGGAAGAAAAGAG-3'	5'-TGCAGGATTTTCATGTCACCA-3'
IL-4	5'-ACAGGAGAAGGGACGCCAT-3'	5'-GAAGCCCTACAGACGAGCTCA-3'
IL-5	5'-CAGCACTCTGTCTTCTAACAAGAA-3'	5'-TCTGTGAAGGTTTCTCCTCC-3'
IL-13	5'-ACAGGAGAAGGGACGCCAT-3'	5'-GAAGCCCTACAGACGAGCTCA-3'
β -actin	5'-CGCCACCAGTTCGCCATGGA-3'	5'-TACAGCCCGGGGAGCATCGT-3'

Table S3. The recovery rate of COS

Polymer	Peak area of	Peak area of	Addition	of	Recovery
degrees	sample	standard	standard		rate (%)
Chitobiose	19538035	5618366	7405581		97.8
Chitotriose	12269881	1774508	13609013		96.9
Chitotetraose	125403178	8856095	21011276		98.2
Chitopentaose	7140803	10096350	16771750		97.3
Chitohexaose	1763421	6698981	8233917		97.3