Immunomodulatory effects of flazin from *Crassostrea sikamea* on splenic lymphocytes of Sprague-Dawley rats

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[ABSTRACT] *Crassostrea sikamea* (*C.sikamea*) is an important edible and medicinal seafood in China. In the present study, a compound named flazin was separated and identified from the ethyl acetate extract of *C.sikamea* (EAECs) for the first time. In addition, the 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay revealed that EAECs and flazin inhibited the transformation of splenic lymphocytes in vitro. Moreover, flazin (20 μg·mL⁻¹) altered the populations of splenic lymphocyte subtypes. Real-time quantitative PCR (RT-qPCR) analysis and enzyme-linked immunosorbent assay (ELISA) showed that flazin suppressed the mRNA expression and secretion of TNF-α and IL-2, and reversed Concanavalin A (ConA)-induced mRNA up-regulation and protein secretion of TNF-α and IL-2. Western blot results showed that flazin reversed ConA-induced increases in p-ERK1/2 and p-p38 in splenocytes. In conclusion, flazin exhibits effective immunomodulatory function and may be useful for treating immune-related disorders, which indicates the application potential of *C.sikamea* as a functional food or immunomodulator.

[KEY WORDS] Flazin; *Crassostrea sikamea*; Cytokine secretion; Immunomodulatory effect; Splenic lymphocyte


Introduction

Currently, bioactive compounds derived from marine origins have attracted substantial interest because of their rich nutritional value, diverse biofunctions and potent bioactivities [1]. Oysters are referred to as “the milk of the sea” as they provide essential amino acids, lipids, especially omega-3 fatty acids, vitamin B12 and essential elements, including iron, zinc and copper [2, 3], and exert many biological effects [4-6]. Considerable evidence indicated that oysters may be used as potential functional foods that exhibit beneficial health effects.

*C.sikamea* oyster, also known as Kumamoto oyster, belongs to the *Ostreidae* family. It is known for its smooth texture and sweet fruity flavor in spite of its small size and slow growth, and it has been cultivated and become a locally important fishery resource in China, Japan, Korea, the United States and some European countries [7, 8]. Previous studies on *C.sikamea* oysters mainly focused on the fertilization, survival and growth of hybrids, while the chemical constituents and biological activity of *C.sikamea* are less clarified. Thus, to further utilize *C.sikamea*, it is vital to screen out more novel bioactive compounds from *C.sikamea* and explore their potential biological activities.

Our current study was aimed to investigate the immunomodulatory effect of flazin, a compound that was separated and identified from *C.sikamea* for the first time. Our results revealed that flazin inhibited the transformation of splenic lymphocytes and altered the proportion of T lymphocyte subtypes in vitro. Furthermore, we found that flazin regulated the levels of inflammatory cytokines and related with the signaling pathways in splenic lymphocytes. Our present work...
provides novel evidence suggesting that *C. sikamea* has considerable potential for immunoregulation, which lays foundation for further investigating the possible role of *C. sikamea* in the immune response.

**Materials and Methods**

**Extraction and isolation**

*C. sikamea* samples were obtained from Zhanjiang (Guangdong Province, China) and transported to our laboratory. The edible parts were collected and finely cut with scissors, and then immersed in acetone (5000 mL × 2) for 7 days before sonicating for 30 min. The extracts were concentrated under reduced pressure. After concentration, the residue was dissolved in distilled water and then extracted with petroleum ether, chloroform, ethyl acetate and butyl alcohol (in that order). The ethyl acetate extract was separated into several fractions by medium- and low-pressure column chromatography [SiO2; elution with petroleum ether/ethyl acetate] with the help of UV absorbance. Fraction 3 (with a total amount of 24.4 g) was purified over a silica gel column using CHCl3-MeOH (15 : 1) as solvent system to give five subfractions. The third subfraction was then purified over a Sephadex LH-20 column by elution with CHCl3-MeOH (1 : 1), yielding flazin (5.2 mg) with a purity of 85.16%. Flazin used in the subsequent experiments was a standard compound purchased from Biobiopharma Co., Ltd. (Kunning, China), with a purity of 98% by HPLC, 1H-NMR and 13C-NMR.

**Animals**

Male Sprague-Dawley rats (8–10 weeks) were purchased from the Experimental Animal Center, Jilin University (Changchun, Jilin, China). The rats were housed in a temperature- and humidity-controlled environment with a 12 h/12 h light/dark cycle with standard pellet food *ad libitum*. All animal experiments were approved by the Institutional Animal Care and Use Committee of Northeast Normal University.

**Preparation of splenic lymphocyte suspension**

The spleens of the rats were minced in D-Hanks medium (Solarbio, Beijing, China). Then, cell suspension was prepared by teasing the spleen apart with the plunger of a syringe in a petri dish with D-Hanks medium (Solarbio, Beijing, China). The cell suspension was passed through a fine wire mesh screen (100 μm) to remove debris. Total spleen cells were collected by centrifugation, and erythrocytes were lysed using lysis buffer (Solarbio, Beijing, China). Then, the cells were centrifuged and washed three times with cold D-Hanks medium. Subsequently, the cells were adjusted to a concentration of 1 × 10^6 cells/mL in RPMI-1640 medium (Gibco, CA, USA).

**Splenetic lymphocyte culture**

The splenetic lymphocytes were cultivated in RPMI-1640 medium supplemented with 10% (V/V) fetal bovine serum (Gibco, CA, USA), 100 U·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin. All cells were incubated in a standard incubator or at 37 °C with 5% CO2.

**Cytotoxicity assay**

To assess the induced cytotoxicity by flazin treatment, the splenic lymphocytes were exposed to acridine orange/propidium iodide according to the Calcein/PI Live/Dead Viability/Cytotoxicity Assay Kit (Beyotime Biotechnology, Shanghai, China). Briefly, the cells at a density of 2 × 10^3 per well were seeded in a 96-well plate with 100 μL of cell culture medium containing 10% FBS and incubated 24 h. The cells were treated with a concentration of 1, 5, 20 and 40 μg·mL^{-1} of flazin for 24 h. After treatment, the cells were washed twice with cold phosphate buffered saline (PBS) and detached from their culture vessel with 100 μL of acridine orange/propidium iodide. Then, the cell suspension was incubated in the darkness at 4 °C for 30 min. Then, the mixture was added to a 96 well assay plate (corning, costar, USA), and the number of live and dead cells was counted using a fluorescence microplate reader (BMG labtech GmbH, FLUOstar OPTIMA, Germany).

**Lymphocyte transformation assay**

MTS assay (Promega, Wisconsin, USA) was used to evaluate the transformation of splenic lymphocytes according to the manufacturer’s instructions. The splenic lymphocytes were separately incubated with flazin at different concentrations (0, 1, 5 and 20 μg·mL^{-1}) or ConA (5 μg·mL^{-1}) in a 96-well plate for 48 h. ConA was used as the positive control. After incubation for 48 h, MTS was added to each well and the cells were incubated for another 3 h. The optical density (OD) values were measured at 490 nm by an automated microplate reader (Molecular Devices, CA, USA).

**Flow cytometry**

The splenic lymphocytes were collected in a 1.5 mL centrifugal tube and then stained with primary antibodies (eBiosciences, California, USA) for cell surface phenotyping. The cells were stained in the darkness at 4 °C for 30 min and washed with PBS. Then, the cells were analyzed on a FACSCalibur (BD Biosciences, San Jose, CA, USA) with CellQuest Pro software (BD Biosciences) and further analyzed using FlowJo Analysis Software (Tree Star Inc., Ashland, OR, USA). In each sample, a total of 10,000 events were collected. In this study, we defined the CD3^1^CD4^1^ population as helper T (Th) cells, the CD3^1^CD8^1^ population as cytotoxic T (Tc) cells, and the CD3^1^CD45RA^1^ population as B lymphocytes [9, 10].

**RNA isolation and real-time PCR**

Total RNA was extracted from the splenic lymphocytes using TRIzol reagent (Invitrogen, CA, USA) and then reversely transcribed using TransScript SuperMix (TransGen Biotech, Beijing, China) according to the manufacturer’s protocol. The mRNA levels of TNF-α, IL-2, IL-12 and IFN-γ were measured by real-time PCR using FastStart Universal SYBR Green Master Mix (Roche, Mannheim, Germany) and a Bio-Rad real-time PCR detection system. All primers from the 5’ to 3’ end were listed as follows: TNF-α, F: GCCTCCTCTCCTCCATCAAG and R: CTCCAAAGTAGACC-
TGCCCG; IL-2, F: CCTGCAAAGAAGACACGC and R: CAAATCCACAGCGTCCA; IL-12, F: AGTTCTTC- GTCCGATCAG and R: CTGGACGCAGAT ATTC-GCC; IFN-γ, F: CAACCCAGAATTCGGACCA and R: TCGACGGACCTCTCTTC; GAPDH, F: GACATGC-GCGCTGGAGAAAAC and R: AGCCCGGATGCCCTTT-AGT.

Cytokine secretion assay

The splenic cells were seeded in 6-well plates and incubated with flazin at 20 μg·mL⁻¹ for 48 h. The levels of TNF-α, IL-2, IL-12 and IFN-γ in the supernatant were measured by ELISA kits (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China) by quantitative enzyme immunoassay according to the manufacturer's instructions.

Western blot

Western blot was performed according to our previous method[11]. The lysates of splenic lymphocytes was separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to 0.2 μm microporous polyvinylidene fluoride membrane (Millipore). After blocking with 5% skimmed milk powder, the membrane was incubated with different antibodies at 4 °C overnight and then with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. Blots were detected with high-sig ECL (Tanon, Shanghai, China) using a specific imaging system. Antibodies against p38, p-p38, ERK1/2 and p-ERK1/2 were purchased from Cell Signaling Technology (Cell Signaling Technology, Beverly, MA, USA).

Statistical analysis

All experiments were performed in triplicate with three independent experiments. The data are expressed as the mean ± SD. The Student’s t test and one-way ANOVA were performed in SPSS 20.0 software. P < 0.05 was considered significant.

Results

Crude extracts of C.sikamea decrease the transformation of splenic lymphocytes

First, to evaluate the immunomodulatory effect of C.sikamea on splenocytes, the cells were exposed to crude extracts from petroleum ether, chloroform, ethyl acetate and butyl alcohol layers for 48 h. Then, the splenic lymphocyte transformation was determined by MTS assay. As shown in Fig. 1, the extracts of petroleum ether, chloroform and ethyl acetate layers suppressed the transformation rate of splenic lymphocytes in a concentration-dependent manner. Notably, the extract of ethyl acetate layers (100 μg·mL⁻¹) presented the strongest inhibitory effect. These results suggested that C.sikamea had immunomodulatory activity in vitro.

Cytotoxicity of flazin against splenic lymphocytes in vitro

The cytotoxicity of flazin against splenic lymphocytes at the concentrations of 1, 5, 20 and 40 μg·mL⁻¹ for 48 h was detected by the Calcein/PI Cytotoxicity Assay Kit. Compared with the control group, flazin at the concentration of 40 μg·mL⁻¹ was cytotoxic. Meanwhile, flazin at the concentrations of 1, 5 and 20 μg·mL⁻¹ did not show obvious cytotoxicity on splenic lymphocytes (Fig. 2). Thus, we chose flazin at the concentrations of 1, 5 and 20 μg·mL⁻¹ to evaluate the transformation of splenic lymphocytes.

Flazin treatment suppresses the transformation of splenic lymphocytes

Flazin was isolated and identified from the extract of the ethyl acetate layer (Fig. 3A), and its effect on the transformation of splenic lymphocytes was further analyzed. As shown in Fig. 3B, treatment with flazin at 1, 5 and 20 μg·mL⁻¹ for 48 h significantly suppressed the viability of splenocytes in a dose-dependent manner compared to that of untreated spleno-
Cytokines are the critical regulators of the immune system by activating and modulating the function of immune cells. Activated immune cells generate and sequentially secrete a variety of inflammatory cytokines, such as TNF-α, IL-2, IL-12 and IFN-γ, which have been shown to play important roles in the body’s immune response. In the current study, the mRNA levels of inflammatory cytokines, including TNF-α, IL-2, IL-12 and IFN-γ were measured in splenic cells by RT-PCR to determine the potential effects of flazin. As shown in Figs. 5A–D, treatment with flazin alone suppressed the mRNA expression of TNF-α and IL-2 in splenic cells, without effects on the expression of IL-12 and IFN-γ. In addition, flazin reversed ConA-induced up-regulation of the mRNA expression of TNF-α, IL-2 and IFN-γ.

Thus, we further detected the protein levels of TNF-α, IL-2 and IFN-γ in the supernatant by ELISA. As shown in Figs. 5F–G, the concentrations of TNF-α, IL-2 and IFN-γ significantly increased with ConA stimulation. Flazin treatment reduced the secretion of TNF-α (Fig. 5E) and IL-2 (Fig. 5F) compared with the negative control. Additionally, in accordance with the changes in mRNA expression, flazin decreased ConA-induced up-regulation of TNF-α, IL-2 and IFN-γ secretion (Fig. 5E–G). These results indicated that flazin suppressed the expression and secretion of inflammatory cytokines in splenic cells at the transcriptional and protein levels.

Flazin treatment inhibits the p38 and ERK1/2 MAPK signaling pathways

Many previous studies have shown that the mitogen-activated protein kinase (MAPK) signaling pathway plays a vital role in the regulation of the inflammatory response by activating its downstream cytosolic proteins and nuclear transcription factors. Thus, we examined the phosphorylation of p38 and ERK1/2 in splenocytes by Western blot. As shown in Figs. 6A–C, flazin significantly inhibited the phosphorylation of ERK and reversed ConA-induced increases in p-ERK1/2 and p-p38 in splenocytes. These findings indicated the involvement of the ERK and p38 MAPK pathways in the regulation of flazin-mediated immunosuppression activity.

Discussion

The immune system is involved in regulating various...
proportions of T and B lymphocyte subtypes were detected by specific antibodies and evaluated by flow cytometry. (A, B) Proportions of T and B lymphocyte subtypes. (C−F) Quantification of lymphocyte subtypes. Data are expressed as the mean ± SD (n = 3). *P < 0.05 vs control

biological processes, and discovering effective immunomodulatory compounds is important in the treatment of immune disorders [19]. According to recent studies, different marine species and their constituents exert various immunomodulatory effects on the immune system [17].

Oysters represent a high-quality nutritional source and contain polysaccharides, proteins, lipids, peptides, phenolic compounds and minerals [1, 4]. Previous reports [18, 19] have indicated that the constituents of oysters have potential immunomodulatory activity in vitro and in vivo. Thus, further investigating the constituents and bioactivity of C. sikamea is of great importance for the development of new immunomodulatory lead compounds.

In our pre-experiment, several crude extracts were collected from C. sikamea to determine their effects on splenic lymphocyte transformation (Fig. 1). The extracts of petroleum ether, chloroform and ethyl acetate layers suppressed the transformation of splenic lymphocytes, indicating that C. sikamea exhibited an immunomodulatory effect.

To explore the main active components, the ethyl acetate layer extract was further separated and purified. Flazin was identified from the ethyl acetate layer and significantly inhibited the transformation of splenic lymphocytes. Flazin is a β-carboline-derived alkaloid found in Japanese fermented foods, such as Japanese rice wine lees, rice vinegar, soy sauce and soy paste; it has also been found in a marine bacterium and a fungus from China [20]. In the current study, flazin was separated and identified from C. sikamea for the first time.

β-Carboline alkaloids are a class of pharmacologically active compounds and important in clinical treatment. Published studies [21] reported that β-carboline alkaloids exhibited many pharmacological activities, including antitumor, antioxidant, anti-inflammatory, antimalarial and antimicrobial effects. As reported, flazin served as a potent xanthine oxidase inhibitor [22] and a promising Nrf2 pathway activator [20]. However, limited data are available on the immunoregulatory activity and the underlying mechanism of flazin. Our study provides novel evidence suggesting that flazin may contribute to immunoregulation in splenic lymphocytes.

Splenocytes are composed of various immune cells, such as T cells, B cells, macrophages and dendritic cells, which have different immune functions [23, 24]. These kinds of immune cells participate in the complex immune response of the body. CD4 T cells and CD8 T cells are both important sub-
sets of T cells in the immune system. CD4+ T cells can enhance cellular immune function, while CD8+ T cells can kill target cells labeled with specific antigens [29]. Therefore, the change in the number of CD4+ T cells and CD8+ T cells is an important indicator of cellular immune function [29]. We analyzed the effects of flazin on the populations of the subtype of T lymphocytes and found that the percentages of CD3+CD4+ T lymphocytes and CD3+CD8+ T lymphocytes decreased after flazin treatment at the concentration of 20 μg·mL⁻¹, which indicated that the cellular immune function was weakened. There were no significant changes in the population of B lymphocytes after flazin treatment compared with the control group. Interestingly, Cheng et al. [27] investigated the effects of an oyster (Crassostrea gigas) polysaccharide (OPS) extract on antigen-specific T-cell immunity, and their results showed that OPS polarized the Th1/Th2 immunobalance toward the Th1-dominant direction, which may be attributed to up-regulation of Th1 cell development. These results suggested that the extracts of oysters exert specific immune functions by influencing the balance of T lymphocyte

Fig. 5 The mRNA expression and secretion of TNF-α, IL-2, IL-12 and IFN-γ in splenic cells. Splenic cells were cultured with RPMI-1640 medium, ConA (5 μg·mL⁻¹), and ConA co-incubated with flazin (20 μg·mL⁻¹) for 48 h. (A–D) The mRNA levels of TNF-α, IL-2, IL-12 and IFN-γ. (E–G) The concentrations of TNF-α, IL-2 and IFN-γ. Data are presented as the mean ± SD (n = 3). *P < 0.05, **P < 0.01 and ***P < 0.001 vs control.

Fig. 6 Effect of flazin on the phosphorylation of p38 and ERK1/2 proteins of the MAPK pathway (A–C). (A) Western blot bands. (B, C) Quantification of p-p38/p38 and p-ERK/ERK. Data are presented as the mean ± SD (n = 3).

subsets. However, in our study, the molecular mechanisms underlying the changes in the proportion of T lymphocyte subsets need further investigations.

Activated splenic lymphocytes generate and sequentially secrete a variety of inflammatory cytokines to regulate the immune response. The expression of cytokines, such as TNF-α, IL-12 and IFN-γ, represent the immune response. In the current study, the levels of cytokines determined by ELISA showed that flazin decreased the secretion of TNF-α and IL-2 in splenic cells and rescued ConA-induced up-regulation of the secretion of TNF-α, IL-2 and IFN-γ. This finding confirmed that flazin was more effective in regulating cell-mediated immune response. TNF-α is a key mediator of T lymphocyte and macrophage activation. IL-2 is considered to enhance the vitality of NK cells, cytotoxic T cells, monocytes, and macrophages and improve proliferation. Our results showed that the decreased secretion of TNF-α and IL-2 was positively correlated with inhibited splenocyte viability.

To further investigate the potential mechanism of the effect of flazin on the expression of inflammatory cytokines, we explored the involvement of the MAPK pathways in flazin-mediated immunosuppressive effects on splenocytes. Previous studies indicated that MAPK family plays a vital role in regulating cell growth, apoptosis and response to inflammation or stress and activated MAPKs can regulate the expression of inflammatory cytokines. In mammalian cells, there are three major MAPK pathways including the p38 MAPK pathway, the extracellular signal-regulated kinase pathway (ERK) and c-Jun N-terminal kinase/stress-activated protein kinase pathway (JNK). Thus, in our study, we first detected the activation of three major MAPK pathways. The results showed no obvious change in JNK pathway under flazin treatment (data not shown).

Treatment with flazin alone significantly reduced the levels of p-ERK1/2. Surprisingly, the inhibitory effect of flazin on the phosphorylation levels of p38 was not statistically significant. However, flazin reversed the activation of the p38 and ERK pathways induced by ConA, indicating that the effects of flazin were more obvious under ConA stimulation. Collectively, our data suggested that flazin exerted immunomodulatory function in part by inhibiting the ERK and p38 MAPK pathways. Furthermore, we speculated that flazin-induced decrease in the production of TNF-α and IL-2 might be mediated by the MAPK signaling pathways. However, the underlying mechanism need to be confirmed by the blockage of the MAPK signaling pathways. Further research is still needed to investigate the possible involvement of other pathways such as the NF-κB signaling cascade in the regulation of flazin.

Conclusions

The current study demonstrates that the ethyl acetate extract of *C. sikamea* (EAECs) and flazin suppress the transformation of splenic lymphocytes and alters the proportion of T lymphocyte subtypes and B lymphocytes in vitro. Furthermore, flazin regulates the mRNA expression and secretion of inflammatory cytokines in splenic cells. Treatment with flazin inhibits the MAPK pathway by reducing the phosphorylation of p38 and ERK1/2. These results suggested that flazin is a promising immunomodulatory component and deserves further investigation in future studies.

References


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