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Biological Immune Suppression Activity of Protein Peptide in Oyster Survived in Guangxi, China

YANHUA CHEN[1], CHAOZHU LI[2], YANHUI CHEN[2], SHUYU ZHU[3], DANRONG LI[3,*] and SHUFEI JIAO[2]

[1]Tumor Hospital Affiliated to Guangxi Medical University, Nanning 530021, P.R. China
[2]Qinzhou University, Qinzhou 535000, P.R. China
[3]Guangxi Medical University, Nanning 530021, P.R. China

*Corresponding author: Tel: +86 13197778557; E-mail: chen_yanhui@163.com

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In this paper, the biological immune suppression activity of protein peptide in oyster survived in Guangxi, P.R. China was investigated. The spleen lymphocyte proliferation of mouse influenced by oyster bioactive peptides was observed from MTT assay, lymphocyte proliferation test *in vitro* inhibition, lymphocyte proliferation of mice *in vitro* activity, respectively. After the rat spleen lymphocytes was influenced by oyster bioactive peptides, the changes of cytokines IL-2, IFN-γ secreted the supernatant of which was detected by ELISA. It was shown that oyster bioactive peptides played an important role in immunosuppressive effects of lymphocyte proliferation. The immunosuppressive effects could be influenced by the concentration of oyster active peptide. Furthermore, the decrease of the secretion of cytokines IL-2 and IFN-γ was observed as the presence of oyster active peptide. It is suggested that the oyster bioactive peptides exhibit an efficient immunosuppressive biological activity. Significantly, the efficient oyster bioactive peptides can be developed to an immunosuppressive drug with perfect prospect.

Key Words: Oyster bioactive peptides, Immune regulation, Lymphocyte proliferation.

INTRODUCTION

Bioactive peptides are peptide compounds with efficient physiological function, which play an important role in living organisms with useful life activity and are also known as functional peptide. Each peptide has a unique composition and different peptide structure is endowed with different functions as their different composition. Especially, the active substances in the sea exhibit many unique properties as the special circumstances, such as various structural specificity, efficient activity and low toxicity and so on. Significantly, it is shown that bioactive peptides in halobios exhibit significant physiological activity, such as immunomodulatory, antithrombotic, antihypertensive, antibacteria, antivirus, anticancer effects, free radical scavenging effects and so on[1,2]. Thus ocean is considered to be the treasure house of natural medicine.

Oyster is commonly known as Da Hao, yellow oyster, *etc*., which is a tender and delicious high nutritional value of seafood with a great medicinal value. In recent years, pharmaceutical research based on oyster has been deeply investigated by domestic and foreign researchers. It is shown that extract of oyster exhibits some unique properties, such as anti-tumor, cancer cell radiosensitization, scavenge oxygen free radicals, oyster animal immune regulation, *etc*.[3-8]. However, the research of the immune regulation of oyster, especially its immunosuppression is still rarely reported. In this study, the extracts of Guangxi oysters that was treated by enzymatic, chromatographic separation, ultrafiltration. The oyster bioactive peptides with a low molecular weight about 3000 Da were obtained. Especially, the immunosuppressive activity of oyster was investigated by lymphocyte proliferation assay and mixed lymphocyte experiments using mouse spleen lymphocytes as experimental models.

EXPERIMENTAL

Fresh oysters was collected from Guangxi Beibu Gulf, peeled, washed and homogenized, stocked under -20 ºC. Animal protease were purchased from Guang Xi Pangbo Bio Co. Ltd.

SPF grade BALB/c mice and Kunming mice (Experimental Animal Center of Guangxi Medical University), male, weighing 22-24 g, weeks 6 to 8 weeks.

RPMI 1640 medium (Thermo Fisher Biochemical Products (Beijing) Co. Ltd.). Fetal calf serum (Hangzhou Sijiqing Institute of Biological Materials). Phosphate buffered saline (PBS). Lymphocyte separation medium. PHA (Huizhou Hongyu Technology Co. Ltd.). MTT (Sigma Company). Concanavalin a (ConA), sigma product, 2 mg/mL of double-distilled water solution, filter sterilization.

Low-speed centrifuge (Beijing Medical Centrifuge Factory). Low-temperature plate centrifuge (sigma company). CO_2 incubator (Thermo Corporation). Enzyme-linked immunosorbent assay instrument (Thermo (Shanghai) Instrument Co. Ltd.). Inverted optical microscope (Nikon China Co. Ltd.). Clean Bench (Suzhou Antai Air Technology Co. Ltd.).

Preparation of oyster protein peptides: Adjust oyster homogenate to pH 5.5 and add animal proteolytic enzymes in accordance with oyster tissue weight of 2 ‰. Enzymatic hydrolysis was performed by heating in water bath at 50 ºC for 5 h. Discard precipitate by centrifuge. Filter the supernatant using MW < 3000 membrane filtration. The filtrate was freeze-dried and the oyster bioactive peptides were obtained. Configured 250, 125, 62.5, 31.6, 15.6, 7.8 μg mL^{-1} concentration gradient according to the experimental requirements.

Lymphocyte proliferation test

Preparation of lymphocyte suspension and primary culture method: Killed the BALB/c mice by cervical dislocation, disinfection of the skin of mice using 75 % alcohol, disinfection of the abdomen repeatedly, remove the spleen, tear the spleen in a dish with PBS buffer, filter using 200 mesh filter, preparation of spleen lymphocyte suspension (on ice). Then, centrifuge under 2000 rmp for 5 min, discard the supernatant. The spleen cell suspension was added to centrifuge tube with equal amount of pre-installed with lymphocyte separation by volume ratio of 1:1. Centrifuge 30 min (2000 rmp). Draw interface layer of mononuclear cells (white blood cell layer) after centrifuge, washed with PBS buffer again and centrifuged 5 min (500 rmp). Discard the supernatant, re-suspended cells in PRMI-1640 medium containing 10 % FCS. And the cell concentration was adjusted to 1×10^6 mL^{-1}.

Add to 96-well plates after the cell concentration was adjusted, each well 100 μL, adjust the ConA concentration to the 20 μg mL^{-1} (final concentration of 10 μg mL^{-1}) using PRMI-1640 medium containing 10 % fetal bovine serum. The final peptide concentrations were 250, 125, 62.5, 31.6, 15.6, 7.8 μg mL^{-1}, each well 100 μL; parallel to each do three holes and use the controlled 3 holes with lymphocyte and ConA.

Place the 96-well plates in CO_2 incubator and cultured for 48 h, adding 5 mg mL^{-1} MTT, each well 20 μL. 4 h later, centrifuge (3000 rmp, 4 ºC) for 10 min, discard supernatant, add 0.4 mol L^{-1} hydrochloric acid of isopropyl alcohol, each well 100 μL. Then, the absorbance was measured using a microplate reader at 492 nm until the crystals dissolved.

Mixed lymphocyte experiments: Take the BALB/c mouse spleen lymphocytes suspension as reactive cell (A). Take appropriate 50 μg mL^{-1} mitomycin and add to a Kunming mouse spleen lymphocyte suspension with the final concentration of 25 μg mL^{-1}. Insulate 30 min under 37 ºC, wash twice using PBS. Adjusted a certain concentration of cells using the 10 % fetal calf serum containing RPMI-1640 suspension cells, which was denoted as mitomycin treated stimulator cells (B). Good preparation of the above two kinds of lymphocytes Add the above lymphocytes (A and B) at a 1:1 volume mixture to a 96-well plates. And 100 μL culture medium and 100 μL mixed spleen lymphocytes were added in the control group, the dosing group was added with 100 μL of different concentrations of bioactive peptide and 100 μL mixed lymphocyte. Place the 96-well plates in 5 % CO_2 incubator and cultured for 48 h at 37 ºC, add 5 mg mL^{-1} MTT at 4 h before the end of culture, each well 20 μL, observe the MTT of crystallization after the culture. Then use the low-temperature plate centrifuge (3000 rmp, 4 ºC) centrifuge, 10 min; discard supernatant, add 0.4 mol L^{-1} hydrochloric acid of isopropyl alcohol, each well 100 μL. Then, the absorbance was measured using a microplate reader at 492 nm until the crystals dissolved.

Detection of the ability of production of cytokine *in vitro* under ELISA method: The mouse spleen lymphocytes were activated by concanavalin. Then, detect the changes of cytokines IL-2 in cell culture supernatant. On the one hand, primary cultures of mouse spleen lymphocytes, regulate of cell suspension (2×106 mL^{-1}). Add 24-well culture plate, each well 1 mL, culture for 4 h in 5 % CO_2 incubator. Add 0.5 mL ConA (final concentration of 5 μg mL^{-1}) and different concentrations of oysters active peptides, including of low-dose 15.6 μg mL^{-1}, the dose of 62.5 μg mL^{-1}, high-dose 125 μg mL^{-1} each 0.5 mL. Place the 96-well plates in 5 % CO_2 incubator and cultured for 48 h at 37 ºC. Then the supernatant was collected and stock under -20 ºC. Subsequently, the IL-2 and IFN-γ were detected by ELISA.

Statistical analysis: The statistical analysis was assayed by compare methods using of multiple samples of SPSS17.0. The measurement data was illustrated as the mean ± standard deviation (x ± s) with considered statistically significant of $P < 0.05$.

RESULTS AND DISCUSSION

Mouse spleen lymphocyte proliferation and mixed lymphocyte growth influenced by oyster bioactive peptides: As shown in Table-1, the growth inhibition rate of mouse spleen lymphocytes can be influenced by oyster bioactive peptides with the dose-effect relationship. Typically, the inhibition rate increases or decreases with higher or lower concentrations in certain concentration range. IC_{50} = 125 μg mL^{-1}.

TABLE-1
INHIBITION RATE OF LYMPHOCYTE PROLIFERATION BY OYSTER PEPTIDES (n = 3, x ± s)

Concentration (μg mL^{-1})	Inhibition rate of lymphocyte proliferation (%)	Inhibition rate of mixed lymphocyte proliferation (%)
7.81	7.53 ± 0.33	16.51 ± 5.19
15.62	14.88 ± 2.35	20.91 ± 2.23
31.25	26.74 ± 1.31	21.38 ± 1.18
62.5	25.98 ± 0.56	23.74 ± 1.44
125	52.18 ± 0.65	45.38 ± 5.34
250	62.39 ± 1.25	60.12 ± 1.35

The standard curves of cytokines IFN-r and IL-2 are given in Fig. 1.

The secretion of mouse spleen lymphocytes factor IFN-r and IL-2 influenced by oyster bioactive peptides.

The cytokines in the culture supernatant of mouse spleen lymphocytes were detected using double sandwich ELISA method. It is suggested that the oyster bioactive peptides can efficiently inhibit the cytokines IL-2 and IFN-γ in the culture supernatant of mouse spleen lymphocytes activated by PHA with a dose-dependent manner, as shown in Figs. 2 and 3.

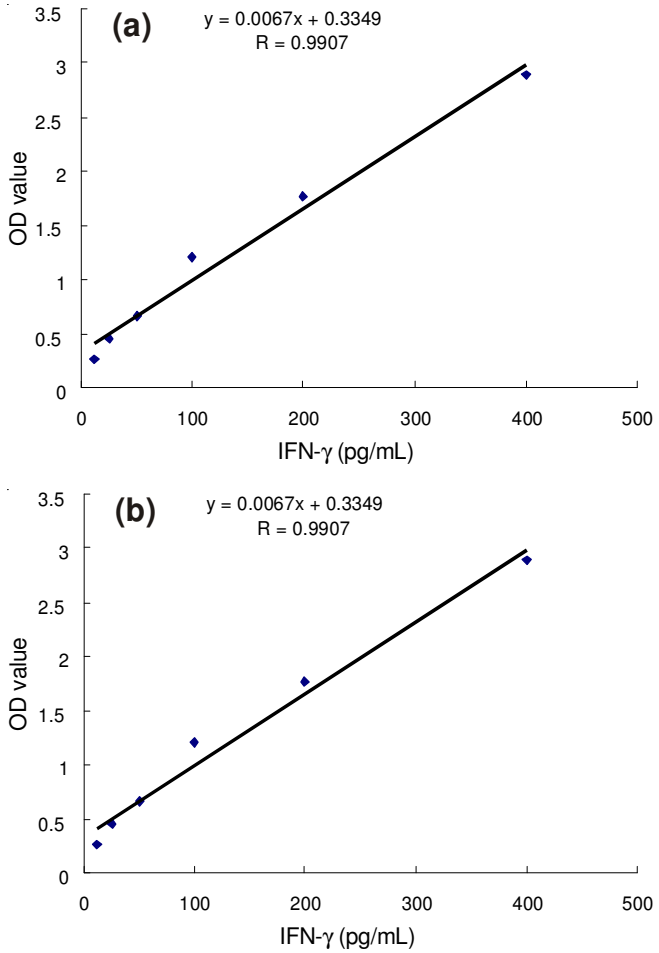

Fig. 1. Standard curve of cytokines IFN-r (a) and IL-2 (b)

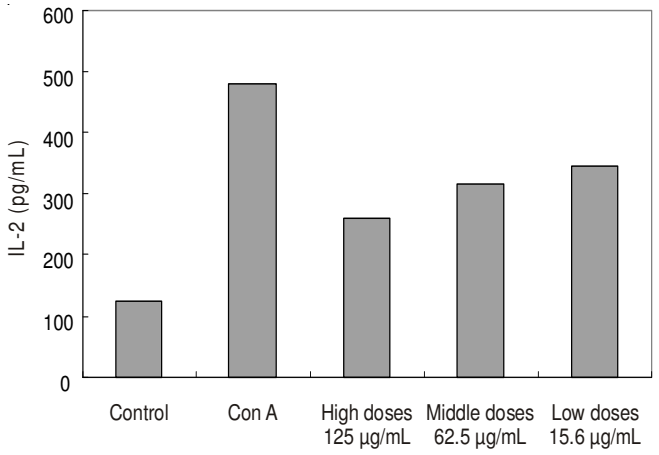

Fig. 2. Detection of the IL-2 in supernatant influenced by oyster bioactive peptides using ELISA method

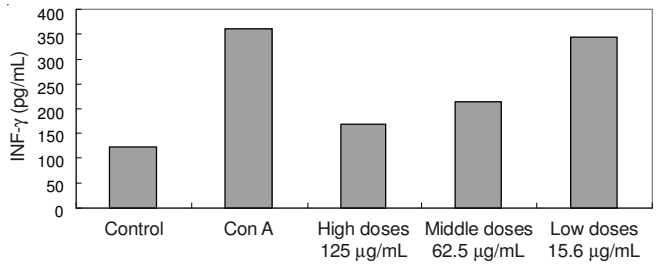

Fig. 3. Detection of the IFN-r in supernatant influenced by oyster bioactive peptides using ELISA method

Generally, lymphocyte proliferation is thought to be one of the effective indicators reflecting the immune function. The lymphocyte proliferation is related to the response to excessive activation of *T. lymphocytes*, proliferation and infiltration. Consist of *T. lymphocytes* and *B. lymphocytes*, spleen lymphocytes play an important role in cellular and humoral immune process *in vivo*. Considering that the *T. lymphocytes* and *B. lymphocytes* endowed with antigen receptors and mitogen receptors on their surface, they can result in the corresponding proliferation of lymphocytes as the antigen-specific stimulation. For example, concanavalin ConA is a *T. lymphocyte* mitogen, which can activate the proliferation of T cell *in vitro* selectively and promote the production of mitogen of cytokine based on T-cell and monocyte[9]. And the lipopolysaccharide (LPS) then stimulate the proliferation of *B. lymphocytes*. Therefore, the spleen T, *B. lymphocytes* function as the important target for the exploration of drug immune function. It was suggested that the construction of the T and *B. lymphocyte* proliferation model was the significant basement for the investigation of the immune activity of oyster bioactive peptides.

Cytokines is one kind of biologically active peptides or glycoproteins secreted by immune cells and non-immune cells, which consists of lymphocytes, monocytes and cytokines in other cells[10]. Typically, the IL-2 mainly secreted by mature *T. lymphocytes*, which can promote lymphocytes proliferation, activation secretion of lymphokines, enhancement of NK cell activity. Extensive investigations have shown that the inflammatory cytokines IL-2 levels in serum or lymphocytes supernatant were significantly increased in systemic lupus erythematosus, rheumatoid arthritis, autoimmune hepatitis, polymyositis, ulcerative colitis, endometriosis, autoimmune diseases[11]. Considering that IFN-γ can enhance the abnormal expression of the antigen-presenting cells or antigen-presenting cells in target organ, promote or increase the recognition of their antigen and the presenting function, activate the autoreactive T cells, IFN-γ may induce some autoimmune diseases, such as autoimmune hepatitis, autoimmune thyroiditis and so on. It is shown that oyster bioactive peptides can inhibit lymphocyte proliferation and inhibit the secretion of IL-2, IFN-γ with a significant immunosuppressive activity.

Conclusion

Based on the research of spleen lymphocyte proliferation of mouse influenced by oyster bioactive peptides, lymphocyte proliferation test *in vitro* inhibition, lymphocyte proliferation of mice *in vitro* activity, the changes of cytokines IL-2, IFN-γ secreted by the supernatant, it was shown that oyster bioactive peptides played an important role in immunosuppressive effects of lymphocyte proliferation. And the immunosuppressive effects could be influenced by the concentration of oyster active peptide. Furthermore, the decrease of the secretion of cytokines IL-2, IFN-γ was observed as the presence of oyster active peptide. So the bioactive peptides can be developed to an immunosuppressive drug with perfect prospect.

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