Activation of Macrophages in vitro by Phospholipids from Brain of Katsuwonus pelamis (Skipjack Tuna)

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Abstract: The biological activities of phospholipids (PLs) have attracted people’s attention, especially marine phospholipids with omega-3 polyunsaturated fatty acids DHA and EPA. In this study, we investigated the immune activation of macrophages in vitro by phospholipids from skipjack brain. The phospholipids were extracted with hexane and ethanol ultrasonication instead of the traditional method of methanol and chloroform. The content of phospholipids from Skipjack brain was 19.59 g/kg by the method (the ratio of hexane and ethanol 2:1, 40 min, 35°C, 1:9 of the ratio of material to solvent, ultrasonic power 300W, ultrasonic extraction 2 times). The RAW264.7 macrophages were stimulated by the phospholipids from the Skipjack, by which the volume, viability and phagocytosis of macrophages were increased. The concentration of NO and the activity of SOD of the cells were also enhanced. The gene expressions of IL-1β, IL-6, iNOS and TNF-α mRNA assayed by RT-PCR were up-regulated. Phospholipids from brain of Skipjack Tuna could activate macrophages immunity which displayed to induce pro-inflammatory cytokines mRNA expression.

Key words: brain of Skipjack Tuna, phospholipids, immunity activation, macrophages

1 Introduction

Fisheries processing produce a large number of by-products (about 50% of the whole fish), including fish heads, skin, roe, bone and so on¹. These by-products are mainly used for fertilizer, animal feed, nowadays, some of which are used as fish oil materials. However, nutritional value of fish by-products is not fully utilized, leading to negligible market value. Biochemical compositions of fish by-products have been widely studied, including collagen from fish skin and bone, fish oil from fish eggs and fish guts², ³. Few articles pertaining to comprehensive utilization of fish heads have been reported, except lipids and PLs were extracted with Protamex from sardine heads by Dumay et al., which might increase the added value of fish heads⁴.

Marine fish oils, such as DHA and EPA potentiate health benefits of people, especially in the phospholipids form⁵-⁷, called marine phospholipids or omega-3 PLs. At present, one of omega-3 PLs resource from fish by-product is fish roe, which contains between 38%-75% of their lipids in the form of PLs with phosphatidylcholine⁸ (PC). Compared to PLs without DHA or EPA, many studies have demonstrated that omega-3 PLs exert superior biological and nutritional functions, such as antioxidant activity, improved memory, reduced blood and tissue lipids⁹, reducing obesity¹⁰-¹². In addition, immunity effect of omega-3 PLs has been discussed and exhibited antitumor activity¹³. But the function of omega-3 PLs on immunity system has been studied barely, besides some anti-inflammatory actions have been reported. For example, Liu et al. reported EPA-enriched PL reduced serum TNF-α and IL-6, and omega-3 fatty acids decreased lipopolysaccharide (LPS)-induced TNF-α secretion¹⁴.

The purpose of this paper is to study a new extraction condition for omega-3 PLs from brain of Skipjack Tuna with hexane and ethanol, and to evaluate the effects of omega-3 PLs on macrophages immunity activation. In addition, the molecular mechanism by which omega-3 PLs acts on macrophages was also investigated.

2 Experimental

2.1 Materials

Brain of Skipjack Tuna was collected from Shandong Jinghai industrial Co., Ltd. Standards of cholesterol, PC, phosphatidyl ethanolamine (PE), lysophosphatidylcholine...
LPC, sphingomyelin (SM) were purchased from Sigma. ToxinSensor™ Single Test Kit was purchased from Nanjing Genscript Biotechnology Company.

2.2 Main nutrient content
Dry matter content was estimated gravimetrically after freeze-drying. Ash content was determined by heating samples at 600°C overnight. Protein was measured by Kjeldahl determination. Total lipid was estimated by Soxhlet extraction. Cholesterol was determined according to the GB/T 5009.128-2003. Phospholipid content was measured according to the GB/T 5537-2008. The materials in the experiments above were all dried samples.

2.3 Lipids extraction
Frozen fish brain was eluted by hexane and ethanol with ultrasonic after broken, and then the mixture was filtrated with vacuum. The filtered liquor was blended and separated. The upper phase was concentrated and purified by cold acetone remaining the precipitant as crude PLs.

2.4 Phospholipids class distribution, fatty acid analysis and endotoxin detection
The fish brain PLs sample were recovered by chloroform, and analyzed by thin-layer chromatography with chloroform-methanol-water (65:25:10, V/V). The spots were colored by Iodine steam, and the rate of flow (Rf) was measured. The fish brain PLs were methylated by the boron trifluoride method, and then were analyzed by gas chromatography according to GB/T 17377-2008. Endotoxin was checked with ToxinSensor™ Single Test Kit.

2.5 Experimental design and Statistical analysis
Orthogonal test was used to investigate the influence of PLs extraction variables: ratio of hexane and ethanol, ratio of fish brain and solvent, ultrasonic time, ultrasonic temperature. Range analysis was conducted by the software SPSS 10.0 package (SPSS Inc., Chicago, IL, USA). One-way ANOVA and Student’s T-test were used to analyze statistical significance. Value of $p < 0.05$ was considered statistically significant.

2.6 Macrophage culture
Macrophage RAW264.7 was cultured in the medium of RPMI 1640, including 10% heat-inactivated fetal calf serum, penicillin (100 U/mL) and streptomycin (100 U/mL). The cells were incubated at 37°C and 5% CO$_2$ atm.

2.7 Macrophage morphologic observation and cells activity
The macrophage morphologic changes were observed by light microscopy. The cells activity were measured by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT).

2.8 Nitric oxide (NO) content and Superoxide dismutase (SOD) activity assay
NO of macrophage cells was evaluated by the method of Griess$^{14}$. SOD activity was measured by SOD Assay WST.

2.9 Analysis of gene expression
Macrophage mRNA levels were measured by RT-PCR. Total RNA was extracted from macrophage by a Trizol Reagent (Invitrogen, Japan). 1 µg total RNA was reverse transcribed into cDNA using random primer, at 42°C for 60 minutes. The resulting cDNA was amplified using specific primers for each gene. The primers used were as follows:

| Genes | Size (bp) | Primer sequences (5'→3') |
|-------|-----------|-------------------------|
| Actin | 246       | Sense: GTCGGCGCTTCTAGGCCACCA  
            Anti-sense: CGGTTGGCCTTAAGGGTCAGGGGG |
| TNF-α | 363       | Sense: GCGGTGCTATGCTCA  
            Anti-sense: GGCAAGCTTGTACCCTGA |
| IL-1β | 350       | Sense: ATGGCAACTTCTGAACTC  
            Anti-sense: TTAGGAAGACACAGATTCATGG |
| IL-6  | 383       | Sense: CTTCCTGGGACTGATGCTTG | G |
| IL-8  | 433       | Anti-sense: CGCTTGGCTTTGCTCCTGTA |
| IL-12 | 618       | Sense: ATCGACCTTCCAGCTGCGCCGCTG |
| iNOS  | 650       | Anti-sense: TGGAGCGAGTTGTGGATTTGTC |

Table 1 Primers for PCR.
min, 95°C for 5 min. The primers of selected genes were listed in Table 1\(^{15,16}\). PCR conditions were as follows: 1 cycle of 94°C for 2 min, 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, 1 cycle of 72°C for 5 min. The PCR products were analyzed by agarose gel electrophoresis.

### 3 Results

#### 3.1 Phospholipids extraction from skipjack brain

The approximate chemical composition of skipjack brain was 82.3% moisture, 1.2% ash, 9.9% protein, 5.9% lipids, including 2.2% phospholipids and 0.11% cholesterol (Table 2).

According to the experiment results of single factor, the orthogonal experiment was designed to analyze the influence of extraction variables: ratio of hexane and ethanol, ratio of fish brain and solvent, ultrasonic time, ultrasonic temperature (Table 3), and to optimize the influence of these four variables. The results of range analysis showed that the most significant effect was the ratio of hexane and ethanol. The order of influence effect was the ratio of extraction solution, ultrasonic time, the ration of fish brain and solvent, ultrasonic temperature, and the best extraction process was A3B2C2D3, which was shown in the Table 4. According to the extraction method, 19.59 g phospholipids were obtained from 1 kg fish brain (wet weight), the extraction rate of which reached 87.1%, compared with 22.49 g extracted by the traditional method of chloroform and methanol. But the method of chloroform and methanol was restricted, because of toxicity to damage retinas and lead to cancer. The method of phospholipids extraction in this paper was relatively safe and effective, which could replace chloroform and methanol.

#### 3.2 Phospholipids composition and fatty acids analysis

The phospholipids composition was analysed by thin layer chromatography (TLC). According to the rate of flow (Rf) of standards, phospholipids components of fish brain were confirmed as showed in Fig. 1. The contents of samples were measured, and the results showed PC, PE and SM were 33.3%, 32.6%, 8.2%, respectively.

The fatty acid composition of phospholipids of fish brain was determined by gas chromatography, the results were showed in Table 5. Fatty acid of the phospholipids mainly consisted of C16:0, C18:0, C18:1n-9c, C22:6n-3, and DHA and EPA content were 21.1% and 2.4%, which explained the phospholipids were rich in DHA and EPA.

#### 3.3 Activation of macrophages by phospholipids of fish brain

The morphology of macrophages could be affected by exogenous factors, sometimes would related to function of macrophages. In this paper, we studied the influence of the phospholipids of fish brain on morphology of macrophages. It was shown that macrophages were activated by the 40 μg/mL phospholipids, by which the cells became larger and irregular in shape, such as diamond, even with long pseudopodia. Meanwhile, the cells without the phospholipids treatment were usually round or oval in shape (shown in Fig. 2).

Then, the macrophages viability with different treatment was detected. The results showed that viability of the macrophages treated by phospholipids from fish brain were higher than the PBS control group (Fig. 3-1), which were

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**Table 2** Main nutrient content of the Skipjack brain.

| Main nutrient content | Moisture (%) | Lipid (%) | Protein (%) | Ash (%) | Cholesterol (%) | Phospholipid (%) |
|-----------------------|--------------|-----------|-------------|---------|-----------------|------------------|
| Wet weight (W/V)      | 82.3 ± 0.76  | 5.9 ± 0.42| 9.9 ± 0.93  | 1.2 ± 0.89| 0.11 ± 0.02     | 2.2 ± 0.76       |

**Table 3** Factors and levels of orthogonal test.

| level | factor | A ultrasonic time (min) | B solvent ratio (V/V) | C solid-liquid ratio (W/V) | D Temperature (°C) |
|-------|--------|-------------------------|-----------------------|---------------------------|-------------------|
| 1     | 1      | 1                       | 1                     | 1                         | 1                 |
| 2     | 2      | 2                       | 2                     | 2                         | 2                 |
| 3     | 3      | 3                       | 3                     | 3                         | 3                 |
| 4     | 4      | 4                       | 4                     | 4                         | 4                 |
| 5     | 5      | 5                       | 5                     | 5                         | 5                 |
| 6     | 6      | 6                       | 6                     | 6                         | 6                 |
| 7     | 7      | 7                       | 7                     | 7                         | 7                 |
| 8     | 8      | 8                       | 8                     | 8                         | 8                 |
| 9     | 9      | 9                       | 9                     | 9                         | 9                 |

**Table 4** Result analysis of orthogonal experiment.

| NO. | A | B | C | D | Relative extraction rate (%) |
|-----|---|---|---|---|-----------------------------|
| 1   | 1 | 1 | 1 | 1 | 58.3                        |
| 2   | 1 | 2 | 2 | 2 | 75.5                        |
| 3   | 1 | 3 | 3 | 3 | 59.3                        |
| 4   | 2 | 1 | 2 | 3 | 80.5                        |
| 5   | 2 | 2 | 3 | 1 | 78.9                        |
| 6   | 2 | 3 | 1 | 2 | 63.5                        |
| 7   | 3 | 1 | 3 | 2 | 80.7                        |
| 8   | 3 | 2 | 1 | 3 | 82.9                        |
| 9   | 3 | 3 | 2 | 1 | 69.7                        |
| k1  | 64.4 | 73.2 | 68.2 | 69.0 |
| k2  | 74.3 | 79.1 | 75.2 | 73.2 |
| k3  | 77.8 | 64.2 | 73.0 | 74.2 |
| R   | 13.4 | 14.9 | 7.0  | 5.3  |

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The cells viability were rising gradually treated by the phospholipids with 5-20 μg/mL, and the cells viability were not significantly different by the phospholipids with 20-40 μg/mL. Furthermore, the cells viability was strengthened with longer treatment time.

As important active indices, SOD and NO of macrophages were measured. It was shown that SOD activity of the group treated with fish brain phospholipids obviously increased and the SOD activity of macrophages treated with 20 μg/mL fish brain phospholipids were higher than the positive control (Fig. 3-2). The NO production of macrophages enhanced after 24h phospholipids treatment (10, 20 μg/mL) and it showed a dose dependent manner (Fig. 3-3). The NO content of group of fish brain phospholipids were slightly higher than the group of soybean phospholipids, and lower than group of LPS.

Macrophages mRNA expression of genes that promote inflammation and activate immunity were examined. The mRNA expression of IL-8 and IL-12 were similar among the groups, but the expressions of other inflammation genes, such as IL-1, IL-6, TNF-α, iNOS were markedly increased with dose-depended by the fish brain phospholipids, since endotoxin contamination in the phospholipid samples have not been found. Furthermore, IL-1, IL-6, TNF-α, iNOS induced by fish brain phospholipids were not obviously different with LPS control group, and higher than soybean phospholipids group (Fig. 4).

### 4 Discussion

The extraction methods of omega-3 PLs were widely studied, because of its biological and nutritional functions. The majority of phospholipids were obtained by the traditional method of chloroform and methanol\(^9, 17\). Besides organic solvent extraction, enzymatic hydrolysis and supercritical fluid extraction were also used to produce phospholipids\(^18, 19\). But these methods are often combined with organic solvents, and the extraction efficiencies are not high. Because of the higher toxicity of chloroform and methanol, we tried to find a way to extract phospholipids from fish brain with the relatively lower toxicity and higher extraction efficiencies. Hexane and ethanol were used in this paper, whose toxicity was far lower than chloroform and methanol, and extraction rate was 87.1% of traditional method, which was conducive to industrial production.

The phospholipids composition of skipjack brain was preliminary analyzed by TLC, which showed it mainly included PC, PE, SM, and SM content was higher than mussel, soybean and yolk\(^20\). Some evidences have proved SM is employed by TNF-α and IL-1β to affect signal transduction\(^21, 22\), which may be the reason of TNF-α and IL-1β...
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Macrophages occupy a unique niche in the immune system. Macrophages make major contributions to respond to infection and enhance immune responses by secretion of myriad inflammatory cytokines and mediators, such as TNF-α, IL-1, IL-6, ROS and so on\(^24\). During inflammation, TNF-α exerts protective effects in early infection via inhibiting bacterial growth and macrophage death. IL-1 as an early response cytokine secreted by the activated macrophages is a central mediator of lymphocyte activation that augments thymocyte proliferation. NO generated by inducible nitric oxides synthase endows macrophages with cytostatic or cytotoxic activity against bacteria, viruses and tumor cells\(^25\). The cytokines IL-6 is produced by cells of adaptive and innate arms of the immune system and appears to play a key role in genetically diverse autoimmune diseases and respond to pathogens\(^26\). There is mounting evidence that the cytokines are related to Toll-like receptors (TLRs)/down-stream NF-κB pathway, which suggested M1 macrophage are activated\(^27, 28\).

Phospholipids play critical signaling roles as second messengers in a number of cellular signaling cascades via their derivatives and cleavage products\(^29\). It has been reported that phospholipids have anti-inflammatory action on acute exudation of inflammatory modes induced by LPS\(^30\), but the direct immune of PLs has barely reported. In more recent years, PL derivatives have been implicated in the regulation of immune system\(^31\), such as innate immune response to products of phospholipid peroxidation\(^32\). It has been reported mmLDL (minimally oxidized low-density li-

Fig. 3 Effects of phospholipids on macrophages activation. (1) Effect of phospholipids on cell viability. A Effect of phospholipids from soybean or fish brain on macrophages for 24h. B Effect of phospholipids on macrophages for 48h. (2) Effect of phospholipids on SOD activity of macrophages. (3) Effect of phospholipids on the production of NO in macrophage cells. Compared with the CK group, * means \(p<0.05\).

Fig. 4 Effect of phospholipids from soybean or fish brain on genes expression.

expression induced by the PLs (Fig. 4). It is as expected that the phospholipids are rich in polyunsaturated fatty acids, and the content of EPA and DHA is 23.5% of fatty acids, which has presented many beneficial effects in human health and disease. For example, DHA and EPA affect immune cells including phagocytosis, T cell signaling and antigen presentation capability by changing the fatty acid composition of immune cells\(^33\).

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poprotein) induced modest levels of expression of pro-inflammatory cytokines, IL-1β and IL-6 in macrophages, and ROS generation, which had been suggested to play a major role in the NF-κB signaling pathway [30]. In this paper, phospholipids from fish brain, which was not peroxidative, also induced pro-inflammatory cytokines in macrophages, such as IL-1β, IL-6, TNF-α and iNOS. Besides oxidized phospholipids, SM was reported to mediate TNF-α signal transduction in vivo [31], so it was possible that PLs from fish brain induced pro-inflammatory cytokines expression, which may be related to M1 macrophages activation and TLRs pathways. But the inducing mechanism was not clear. Some evidence has suggested that mmlDL-induced activation of TLR4 was different from LPS-induced signaling, which showed costimulation of macrophages by mmlDL and by low levels of bacterial LPS. On the other hand, anti-inflammatory properties of OxPAPC (oxidized 1-palmitoyl-2-arachidonoyl-phosphatidylcholine) have been reported in LPS-induced sepsis or acute lung injury, which may be explained by direct antagonism of LPS recognition [32]. Thus the macrophage activation mechanism induced by phospholipids from fish brain will be further studied in the future, such as receptor recognition, signal transduction and regulation mechanisms.

5 Conclusions

In summary, PLs from brain of Katsuwonus pelamis contain a large number of DHA and EPA, which could be extracted by hexane and ethanol instead of the traditional method of methanol and chloroform. The PLs showed new bioactivity, which could activate macrophages by inducing pro-inflammatory cytokines, such as NO, IL-1, IL-6, TNF-α. The findings would be useful to develop healthy foods containing the PLs contributing to boost immunity of humans.

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