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Jellyfish skin polysaccharides: Extraction and inhibitory activity on macrophage-derived foam cell formation

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ABSTRACT

In this work, response surface methodology was used to determine optimum conditions for extraction of polysaccharides from jellyfish skin (JSP). The optimum parameters were found to be raw material to water ratio 1:7.5 (w/v), extraction temperature 100 °C and extraction time 4 h. Under these conditions, the JSP yield reached 1.007 mg/g. Papain (15 U/mL) in combination with Sevag reagent was beneficial in removing proteins from JSP. After precipitation with ethanol at final concentration of 40%, 60% and 80% in turn, three polysaccharide fractions of JSP1, JSP2 and JSP3 were obtained from JSP, respectively. The three fractions exhibited different physicochemical properties with respect to molecular weight distribution, monosaccharide composition, infrared absorption spectra, and glycosyl bond composition. In addition, JSP3 showed strong inhibitory effects on oxidized low-density lipoprotein (oxLDL) induced conversion of macrophages into foam cells, which possibly attributed to the down-regulation of some atherogenesis-related gene expressions.

1. Introduction

The jellyfish *Rhopilema esculetum* Kishinouye belongs to the class Scyphozoa, the order *Rhizostomeae* and the family Rhopilema (Liu, Guo, Yu, & Li, 2012). It has been used in Chinese cooking for more than one thousand years (Dong et al., 2009). Among the edible species, jellyfish *R. esculetum* is the most abundant species distributed in the areas of South China Sea, Yellow Sea and Bohai Sea (Yu et al., 2005). Since jellyfish is rich in proteins and low in fat/cholesterol, it is a typical nutritious food (Ding et al., 2011). In recent years, jellyfish has been considered to possess good potential for treating obesity, hypertension, gastric ulcers, asthma, constipation and arthritis as well as relieving fatigue (Liu et al., 2012). Previous studies revealed that proteins, amino acids, vitamins and inorganic elements are present in jellyfish (Ding et al., 2011; Liu et al., 2012). Among these nutrients, the active proteins have been reported to have antioxidant, antifatigue and immune-modulatory activities (Ding et al., 2011). In addition to the above nutrients, we found that jellyfish contained some active polysaccharides. To the best of our knowledge, little information has been published on polysaccharides of jellyfish.

Atherosclerosis, the complex interaction of serum cholesterol with the cellular components of the arterial wall, is the leading cause of cardiovascular disease (Pan et al., 2010). In the past, due to unknown atherosclerotic pathogenesis, effective pharmaceuticals have not been developed for the treatment of this disease. Even up to now, restrictions of streptokinase use within certain time period of atherosclerotic plague formation, drives the scientists' attention to seek for natural products. Therefore, seeking natural products has become of great interest for the prevention and treatment of atherosclerotic lesions in recent years (Chanet et al., 2012; Koh, Han, Oh, Shin, & Quon, 2010). It is well known that high serum concentration of total cholesterol (TC), triglycerides (TG) and low density lipoproteins (LDL) are the major factors contributing to atherogenesis as well as the overproduction of reactive oxygen species (ROS) (Armstrong, Voyle, Armstrong, Fuller, & Rutledge, 2011; Diepen, Berbee, Havekes, & Rensen, 2013). ROS modify LDL to produce oxidized LDL (oxLDL). Macrophages in arterial walls take up oxLDL via scavenger receptors, accumulating lipids in intracellular space and then become foam cells (Min, Um, Cho, & Kwon, 2013). Accordingly, the conversion of macrophages into foam cells is considered to be an initial and critical process in the development

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of atherosclerosis (Mclaren, Michael, Ashlin, & Ramji, 2011), suggesting modulation of macrophages function might be an effective strategy for prevention of atherosclerosis.

In this study, we demonstrated an effective method for extraction and purification of jellyfish skin polysaccharides (JSP), followed by the analysis of physicochemical properties including molecular weight, monosaccharide composition and structural features. In addition, the effect of JSP on conversion of macrophages into foam cells was examined and the possible molecular mechanisms underlying JSP action were discussed using RAW264.7 macrophage cell lines. This work presents the first study of extraction and physicochemical properties of polysaccharides from jellyfish as well as the activity of polysaccharides against foam cell formation.

2. Materials and methods

2.1. Materials and reagents

Jellyfish skin (containing about 95% water) was obtained from Dalian Shuiyuan Seafood Co. Ltd. (Liaoning province, China). L-glucose (Glu), D-rhamnose (Rha), L-xylose (Xyl), D-galactose (Gal), L-mannose (Man) and L-arabinose (Ara) were purchased from Sigma–Aldrich (MO, USA). Dextran standards were purchased from Fluka Co. (Gallen, Switzerland). The Oil Red O and Hematoxylin were obtained from Solarbio Technology Co. Ltd. (Beijing, China). Both total cholesterol measured kit and free cholesterol measured kit were purchased from PPLYGEN Co. Ltd. (Beijing, China). The oxidized low-density lipoprotein (oxLDL) was purchased from Gaochuang Chemical Technology (Shanghai, China). TRIzol® reagent was obtained from Invitrogen (Darmstadt, Germany). Both the iScript™ cDNA Synthesis kit and iTaq™ Universal SYBR® Green supermix kit were purchased from Bio-Rad laboratories (Hercules, CA). All primers were obtained from Sangon Biotech Co. Ltd. (Shanghai, China). All other reagents are analytical grade.

2.2. Optimization of parameters for polysaccharide extraction

2.2.1. Single-factor test

To investigate the effects of ratio of raw material to water (RMW) on polysaccharide extraction, 50 g jellyfish skin were ground in a blender, followed by mixing with 100, 200, 300, 400 and 500 mL deionized water. Subsequently, all mixtures were heated in a water bath at 50 °C for 1 h. After this incubation, centrifugation was performed to remove the residues at 10,000 rpm for 15 min. The supernatant was collected and concentrated to a certain volume on a rotary evaporator. Then, ethanol was added to the concentrates at a final concentration of 80% (v/v). After 24 h precipitation at room temperature, the precipitate was collected by centrifugation (10,000 rpm, 20 min), dissolved in distilled water, deproteinized by Sevag method (Staub, 1965), and lyophilized to give crude jellyfish skin polysaccharides (JSP).

To investigate the influence of temperature on polysaccharide extraction, based on the above results, the extraction temperature was changed from 20 to 100 °C at the fixed RMW ratio of 1:8 and extraction time at 1 h. To study the effects of extraction time on polysaccharide production, extraction time was set at 0.5, 1, 1.5, 2, 2.5 and 3 h while the RMW and extraction temperature were fixed at 1:8 and 100 °C, respectively. The other experimental procedures were carried out as described above. The polysaccharide contents were measured by phenol-sulfuric acid method, using D-glucose as a standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

2.2.2. RSM optimization

The extraction parameters including RMW (X_1), extraction temperature (X_2) and extraction time (X_3) were optimized by RSM (Zhou et al., 2013). According to the results of single-factor tests, the range and center point values of three independent variables were selected as shown in Table 1. Each variable was coded at three levels (−1, 0, +1). The Box–Behnken design (BBD) was employed to determine the best combination of different variables for polysaccharide extraction, containing 5 replicates of the central point and 12 factorial points (Table 1). The BBD data were further analyzed by second-order regression to fit an empirical model. Accordingly, the optimal conditions could be predicted for JSP extraction using this model.

2.3. Removal of proteins

2.3.1. Sevag method

Fifteen milligrams crude JSP were dissolved in 50 mL distilled water, followed by addition of 20 mL Sevag reagent (1-butanol:chloroform = 1:4). After the mixture was stirred at 1000 rpm for 1 h using a magnetic stirrer, centrifugation was carried out at 12,000 rpm for 20 min. Then, the supernatant was collected and the protein was measured by Lowry method (Lowry, Rosenbrough, Farr, & Randall, 1951). This process was repeated until the protein content reached a constant value. The polysaccharide in the supernatant was determined by phenol-sulfuric acid method.

2.3.2. TCA method

TCA was added to crude JSP aqueous solution at a final concentration of 4%, 8%, 12%, 16% and 20%, respectively. The mixture was stirred at 1000 rpm for 12 h using a magnetic stirrer. After centrifugation at 12,000 rpm for 20 min, the supernatant was collected for protein and polysaccharide analysis.

2.3.3. Papain method

Crude JSP (15 mg) were dissolved in 50 mL phosphate buffer (0.2 M, pH 4.0) and transferred into a 100 mL flask. After heating at 50 °C in a water bath, papain was added to the solution at the final concentration of 5, 10 and 15 U/mL. All mixtures were maintained at 50 °C for 2 h, followed by boiling for 20 min to terminate the reaction. Then, centrifugation (12,000 rpm, 20 min) was performed to get the supernatant for protein and polysaccharide analysis.

2.3.4. Papain in combination with TCA or Sevag reagent method

Papain was employed to remove proteins at final concentrations of 5, 10 and 15 U/mL, respectively, followed by the addition of TCA (4%, w/v) or Sevag reagent (20 mL) to each solution. The other experimental procedures were the same as described above.

2.4. Polysaccharide precipitation with ethanol

Crude polysaccharides were fractionated using ethanol precipitation at final concentrations of 40%, 60% and 80% (v/v), respectively. To obtain JSP1, the deproteinized crude JSP solution (1.2 mg/mL) was poured into ethanol to a final concentration of 40% and centrifuged at 12,000 rpm for 20 min, leading to the precipitate JSP1. Ethanol was further added to the above supernatant to the final concentration of 60%, leading to the precipitate JSP2. Similarly, JSP3 was obtained from the supernatant of JSP2 precipitation by the addition of ethanol to the final concentration of 80%. To investigate the effects of precipitation conditions on polysaccharide yield, precipitation temperature and time were varied from 4 °C to 37 °C and 4 h to 24 h, respectively.

2.5. Analysis of physicochemical properties

As described in our previous report (Zha et al., 2013), the molecular weight (MW) and monosaccharide composition of

Table 1
Experimental data and predicted polysaccharide production extracted from jellyfish skin with different combinations of ratio of raw material to water, extraction temperature and extraction time designed by response surface methodology.

Run	Coded variables[a]			Uncoded variables			Polysaccharide content (mg/g)		
	X_1	X_2	X_3	X_1	X_2	X_3	Experimental (Y_0)	Predicted (Y_1)	$Y_0 - Y_1$
1	1	−1	0	1:10	90	2	0.258	0.220	0.038
2	−1	0	−1	1:6	80	3	0.596	0.612	−0.016
3	0	−1	1	1:8	100	2	0.480	0.535	−0.055
4	0	1	−1	1:8	80	4	0.999	0.944	−0.055
5	0	0	0	1:8	90	3	0.661	0.665	−0.004
6	−1	1	0	1:6	90	4	0.838	0.876	−0.038
7	0	0	0	1:8	90	3	0.685	0.665	0.020
8	0	0	0	1:8	90	3	0.692	0.665	0.027
9	1	0	−1	1:10	80	3	0.422	0.429	−0.007
10	0	0	0	1:8	90	3	0.656	0.665	−0.009
11	1	1	0	1:10	90	4	0.665	0.713	−0.048
12	0	0	0	1:8	90	3	0.631	0.665	−0.034
13	−1	0	1	1:6	100	3	0.682	0.675	0.005
14	0	−1	−1	1:8	80	2	0.439	0.470	−0.031
15	1	0	1	1:10	100	3	0.532	0.516	0.016
16	0	1	1	1:8	100	4	1.061	1.030	0.031
17	−1	−1	0	1:6	90	2	0.448	0.400	0.048

[a] X_1: ratio of raw material to water; X_2: extraction temperature (°C); X_3: extraction time (h).

polysaccharides were determined by high performance liquid chromatography (HPLC) and gas chromatography (GC), respectively. FT-IR spectra of polysaccharides were recorded on a Nicolet Nexus 470 spectrometer in a range of 400–4000 cm^{-1}. Periodate oxidation was employed to analyze the glycosyl bonds in polysaccharides by detecting the consumption of $NaIO_4$ and the formation of formic acid (Sun & Liu, 2009).

2.6. Effects of polysaccharides on macrophage-derived foam cell formation

2.6.1. Cell line and cell culture

The RAW 264.7 macrophage cell line was supplied by Prof. Jian Liu (Hefei University of Technology, Hefei, China). The cells were cultured at 37 °C in a humidified incubator with 5% CO_2.

2.6.2. Oil Red O staining

To investigate the effects of JSP3 on the conversion of macrophage into foam cell, cells were treated with 50 μg/mL oxLDL in the presence or absence of JSP3 at 100 μg/mL. The oxLDL and JSP3 were dissolved in the culture medium of DMEM medium supplemented with fetal bovine serum (10%, v/v), streptomycin (100 μg/mL) and penicillin (100 U/mL). After 48 h of culture, the cells were collected, washed with PBS, fixed with paraformaldehyde, and stained with Oil Red O (Min et al., 2013). The stained cells were photographed under a microscope at 400× magnification. The stained cells were destained with ethanol (99.99%) and the absorbance of destaining ethanol was measured at 500 nm. Control cells (normal group) were incubated over the same time period in cell culture medium without oxLDL and JSP3.

2.6.3. TC and CE determination

The total cholesterol (TC) and free cholesterol (FC) in the treated cells were determined using the commercial kits according to the manufacturer's instructions. The difference between TC and FC represents the production of cellular cholesteryl ester (CE).

2.6.4. Quantitative real-time PCR (qPCR)

As described above, macrophages were treated with 50 μg/mL oxLDL in the presence or absence of JSP3 at 100, 200 and 300 μg/mL for 48 h. Control cells (normal group) were incubated over the same time period in cell culture medium without oxLDL and JSP3. Total RNA was extracted with TRIzol reagent, and cDNA was prepared using iScript™ cDNA Synthesis kit according to the manufacturer's instructions. The qPCR was carried out in a reaction volume of 20 μL containing 10 μL 2 × iTaq™ Universal SYBR® Green supermix, 0.4 μL of each forward and reverse primer, 1 μL cDNA and 8.2 μL PCR grade sterile water. The following primers were used for the amplification of mouse CD36, PPAR-γ, ACAT-1, COX-2, IL-1β, TNF-α, MCP-1, ICAM-1 and GAPDH: CD36 (forward) 5′-GTCTTCCCAATAAGCATGTCTCC-3′ and (reverse) 5′-ATGGGCTGTGATCGGAACTG-3′, PPAR-γ (forward) 5′-TGGCATCTC-TGTGTCAACCATG-3′ and (reverse) 5′-GCATGGTGCCTTCGCTGA-3′, ACAT-1 (forward) 5′-CTTCCCATTCTGCCACTTTTAG-3′ and (reverse) 5′-CAAGACAGCCAGGACTACACAG-3′, COX-2 (forward) 5′-TGAGT-CTGCTGGTTTGGAATAG-3′ and (reverse) 5′-GTGCCTGGTCTGAT-GATGTATG-3′, IL-1β (forward) 5′-TCTTTTGGGGTCCGTCAACT-3′ and (reverse) 5′-GCAACTGTTCCTGAACTCAACT-3′, TNF-α (forward) 5′-AGATAGCAAATCGGCTGACG-3′ and (reverse) 5′-ACGGCATGGA-TCTCAAAGAC-3′, MCP-1 (forward) 5′-CCATTCCTTCTTGGGGTCAG-3′ and (reverse) 5′-TCACCTGCTGCTACTCATTCAC-3′, ICAM-1 (forward) 5′-TAAGACTCGGGGAGCAATAAGA-3′ and (reverse) 5′-ACACGGCTTGGGACTGTTT-3′, and GAPDH (forward) 5′-CTCG-CTCCTGGAAGATGGTG-3′ and (reverse) 5′-GGTGAAGGTC-GGTGTGAACG-3′. The thermal cycling conditions were used as follows: initial denaturation at 94 °C for 1 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 15 s, and extension at 72 °C for 6 s. The cycle threshold (C_t) values were normalized to the expression levels of GAPDH.

2.7. Statistical analysis

Three or more separate experiments were performed. The data were analyzed by the SPSS software. The values were expressed as mean ± standard deviation (SD). Comparisons between groups were made using one-way analysis of variance (ANOVA) and Dunnett's *t*-test for multiple comparisons. Values of $p < 0.05$ were considered to be statistically significant.

3. Results and discussion

3.1. Polysaccharide extraction from jellyfish skin

As shown in Fig. 1A, both polysaccharide and total sugar extracted from raw materials increased gradually when the RMW ratio changed from 1:2 to 1:8 g/mL with a maxima occurring at

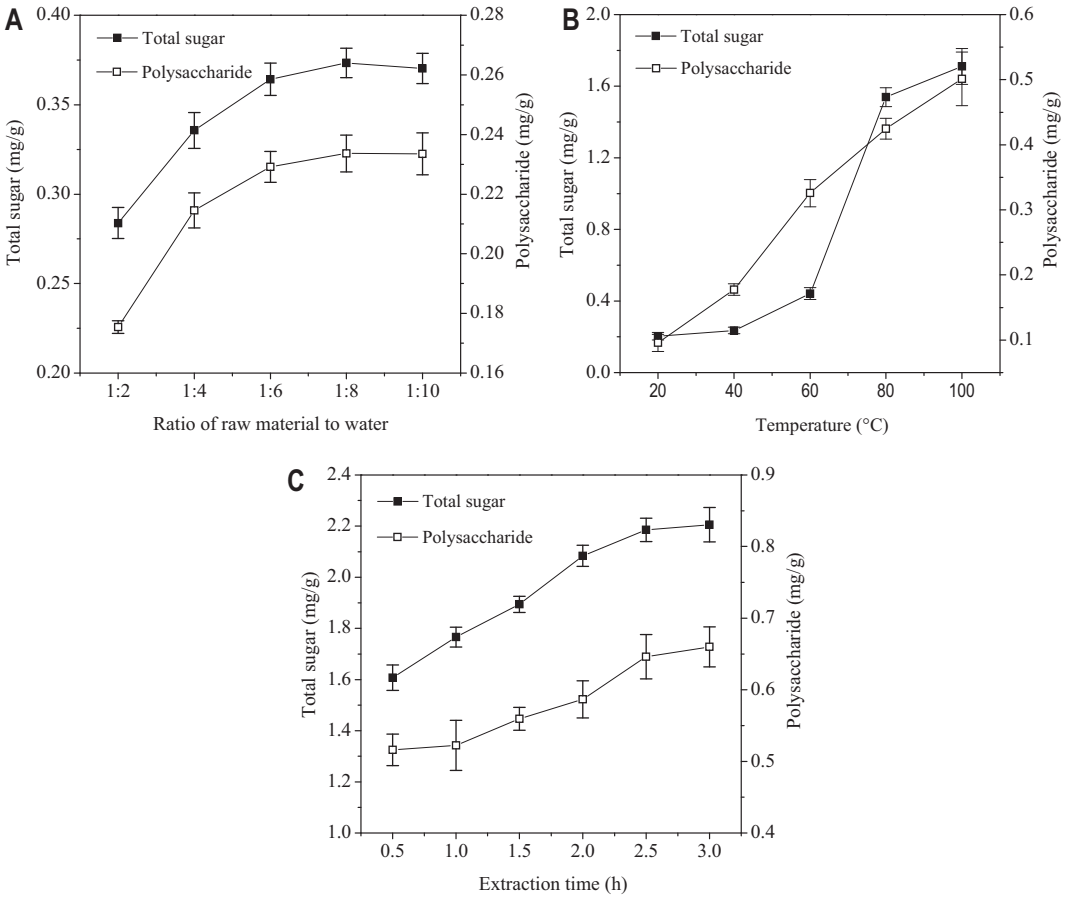

Fig. 1. Effects of independent variables on sugar extraction efficiency by single-factor test: (A) ratio of raw material to water; (B) extraction temperature; and (C) extraction time.

1:8. Addition of more water after this point did not lead to any further increase in the quantity of polysaccharide and total sugar that was extracted (Fig. 1A). Similar patterns of extraction were also reported in other polysaccharide extractions (Ye et al., 2013; Samavati, 2013). The possible explanation is that different amounts of extraction solvents could change the driving force for mass transfer of polysaccharides by altering the pressure difference between inside and outside of the cells (Samavati, 2013). Since the maximum production of polysaccharide (0.24 mg/g) and total sugar (0.37 mg/g) were obtained at 1:8 of RMW, the RMW was fixed at 1:8 in the following experiments for selection of other optimal extraction conditions.

Data presented in Fig. 1B suggests that temperature is also an important factor for sugar extraction from jellyfish skin. Both polysaccharide and total sugar yields were significantly enhanced when the extraction temperature was increased from 20 to 100 °C. Similar pattern effect of temperature on polysaccharide extraction has been reported by other researchers (Samavati, 2013; Hou & Chen, 2008). This effect of temperature might be caused by an increased solubility of the polysaccharide at higher temperatures. In this experiment, the peak value of 0.5 mg/g for polysaccharide and 1.7 mg/g for total sugar were observed when the extraction temperature was raised to 100 °C, which improved 7.5-fold and 4-fold than those obtained at 20 °C. Thus, extraction temperature of 100 °C was considered to be optimal for the following experiments.

As shown in Fig. 1C, the quantities of polysaccharide and total sugar displayed a significant increase from 0.52 mg/g to 0.65 mg/g and 1.6 mg/g to 2.18 mg/g as extraction time increased from 0.5 h to 2.5 h, respectively. If the extraction time was increased to over 2.5 h, extraction of both polysaccharide and total sugar increased only slightly, but there was no significant difference ($p < 0.05$). The maximum quantities of polysaccharide (0.66 mg/g) and total sugar (2.2 mg/g) were observed when extraction time was set at 3 h. Similar effect of extraction time on polysaccharide extractions has been reported before (Samavati, 2013; Hou & Chen, 2008).

Based on the above data of single-factor tests, Box–Behnken design was employed to optimize extraction conditions of jellyfish skin polysaccharide. The design matrix and corresponding results are shown in Table 1. Multiple regression analysis was performed on the experimental data to get an empirical model, revealing the relationships between predicted response variable and independent variable. The model can be expressed by the following second-order polynomial equation: $Y = 0.67 - 0.086X_1 + 0.24X_2 + 0.037X_3 + 4.25 \times 10^{-3} X_1X_2 + 6 \times 10^{-3}X_1X_3 + 5.25 \times 10^{-3}X_2X_3 - 0.15{X_1}^2 + 0.037{X_2}^2 + 0.043{X_3}^2$, where Y is the predicted polysaccharide production, X_1, X_2 and X_3 represent RMW, extraction temperature and extraction time, respectively.

As shown in Table 1, the predicted values of polysaccharide quantity were calculated using the regression model. The p-value of the model was 0.0001, indicating the model was very significant. The determination coefficient (R^2) with a high value of 0.9721 suggested that only 2.79% of total variations were not explained by this model. The coefficient of variation (CV) was calculated as 8.12%, implying a high degree of veracity and good reliability of experiment data. According to the above regression model, the maximum polysaccharide extraction was predicted to be 1.04 mg/g under optimal extraction conditions as follows: 1:7.5 of RMW, 100 °C of extraction temperature and 4 h of extraction

Table 2
Comparison of deproteinization efficiency and polysaccharide loss by different methods in removing proteins.

Method	Protein content (mg/mL)	Protein loss (%)	Polysaccharide content (mg/mL)	Polysaccharide loss (%)
Original sample	0.1103 ± 0.0056	–	0.08929 ± 0.0027	–
Sevag method to remove proteins				
Chloroform/1-butanol = 4/1	0.0817 ± 0.0028	25.97 ± 2.15	0.0838 ± 0.0018	6.11 ± 1.57
TCA method to remove proteins				
4% (w/v)	0.0714 ± 0.0037	35.31 ± 4.21	0.0646 ± 0.0035	27.63 ± 2.23
8% (w/v)	0.0517 ± 0.0023	53.12 ± 2.34	0.0606 ± 0.0023	32.16 ± 1.92
12% (w/v)	0.0500 ± 0.0045	54.67 ± 4.35	0.0590 ± 0.0066	33.97 ± 4.11
16% (w/v)	0.0491 ± 0.0018	55.52 ± 1.97	0.0587 ± 0.0038	34.31 ± 2.37
20% (w/v)	0.0486 ± 0.0027	55.93 ± 2.86	0.0582 ± 0.0042	34.86 ± 2.52
Papain method to remove proteins				
5 U/mL	0.0317 ± 0.0041	71.22 ± 5.68	0.0836 ± 0.0041	6.42 ± 1.73
10 U/mL	0.0224 ± 0.0072	79.72 ± 6.21	0.0847 ± 0.0026	5.14 ± 0.75
15 U/mL	0.0186 ± 0.0038	83.13 ± 4.98	0.0840 ± 0.0033	5.98 ± 0.97
Papain method in combination with TCA method to remove proteins				
5 U/mL trypsinase + TCA (4%)	0.0250 ± 0.0026	77.35 ± 2.69	0.0651 ± 0.0028	27.13 ± 1.62
10 U/mL trypsinase + TCA (4%)	0.0174 ± 0.0026	84.18 ± 3.52	0.0609 ± 0.0033	31.75 ± 2.13
15 U/mL trypsinase + TCA (4%)	0.0063 ± 0.0021	94.27 ± 1.99	0.0573 ± 0.0025	35.88 ± 1.46
Papain method in combination with Sevag method to remove proteins				
5 U/mL trypsinase + Sevag reagent	0.0293 ± 0.0027	73.43 ± 3.13	0.0830 ± 0.0031	6.95 ± 2.56
10 U/mL trypsinase + Sevag reagent	0.0180 ± 0.0022	83.65 ± 2.89	0.0817 ± 0.0033	8.45 ± 2.79
15 U/mL trypsinase + Sevag reagent	0.0088 ± 0.0031	92.03 ± 4.65	0.0733 ± 0.0042	11.53 ± 3.63

time. To verify this prediction, verification experiments were further carried out using this optimal condition for three times. Results displayed that there is no significant difference between experimental value (1.007 mg/g) and predicted value (1.04 mg/g), confirming that the response model is satisfactory and accurate for prediction of polysaccharide extraction from jellyfish skin.

3.2. Purification of polysaccharides by removing proteins

Proteins are the main impurities in crude polysaccharides extracted from organisms, which are free or conjugated with carbohydrates. In order to avoid their interference in determining molecular weight, characterizing structure and screening bioactivity, proteins must be removed from crude polysaccharides. In the present work, five methods were employed to remove proteins from crude JSP and we compared their deproteinization efficiencies as well as polysaccharide losses (Table 2).

Application of Sevag reagent has been considered to be an effective method to remove free proteins, but not proteins combined with polysaccharides (Ge & Yang, 2010). After repeating the deproteinization with Sevag reagent 13 times, both protein and polysaccharide content became stable in JSP preparation. As shown in Table 2, the protein loss and polysaccharide loss were 25.97% and 6.11%, respectively.

TCA at five concentrations of 4%, 8%, 12%, 16% and 20% (w/v) was employed to remove proteins from JSP. Results showed that both protein loss and polysaccharide loss increased with increasing TCA concentration. When TCA concentration was fixed at 20%, the protein loss and polysaccharide loss reached a maximum, corresponding to 55.93% and 34.86%, respectively. Although the deproteinization efficiency was higher with TCA method as compared to that with Sevag method, the polysaccharide remaining in solution was lower with TCA method. The possible reason is the reaction between TCA and proteins, which leads to the degradation of polysaccharides resulting in a significant loss of polysaccharides.

Proteinase is an effective reagent to hydrolyze proteins, which was often applied to remove proteins in polysaccharide preparation (Liu et al., 2007; Peng & Zhang, 2003). For this purpose, papain was selected as the candidate in this study. As shown in Table 2, deproteinization efficiency was enhanced with an increase in enzyme concentration. It was revealed that 83.13% proteins were sloughed by papain at 15 U/mL, which was much greater than those of Sevag method and TCA method, indicating that most polysaccharide-bound proteins were hydrolyzed. Moreover, compared to the above two methods, higher polysaccharide yields were also observed with this method. In the tested papain concentration range (5–15 U/mL), only 4.96–5.98% JSP were lost in comparison with the original sample.

Combining the advantages of different deproteinization methods, applications of papain in combination with Sevag reagent or TCA were further carried out to remove proteins. On applying papain in combination with TCA, although most proteins were removed from JSP solutions, a higher degree of polysaccharide

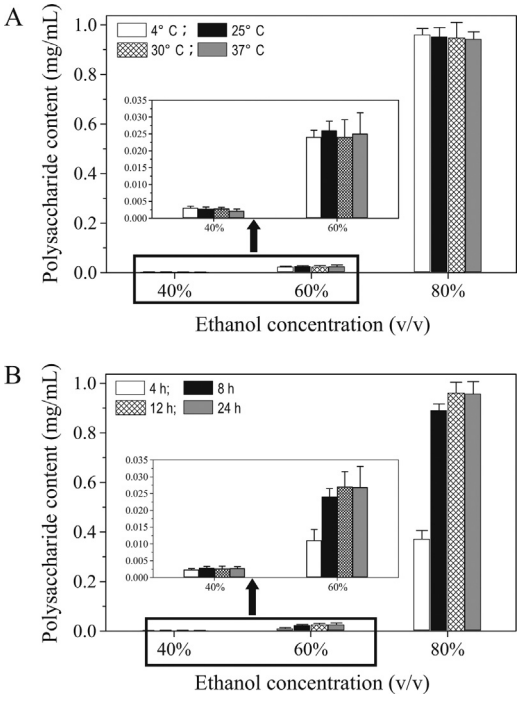

Fig. 2. Polysaccharide precipitation with ethanol: (A) precipitation temperature and (B) precipitation time.

loss (27.13–35.88%) was also seen (Table 2). When using papain in combination with Sevag reagent, both the loss of proteins and polysaccharides were enhanced with an increase in papain concentration. However, the increase in proteolysis was much stronger than the decrease in polysaccharides yield. When 15 U/mL papain was used in combination with Sevag reagent to remove proteins from JSP, the protein loss and polysaccharide preservation were observed to be 93.03% and 88.47%, respectively.

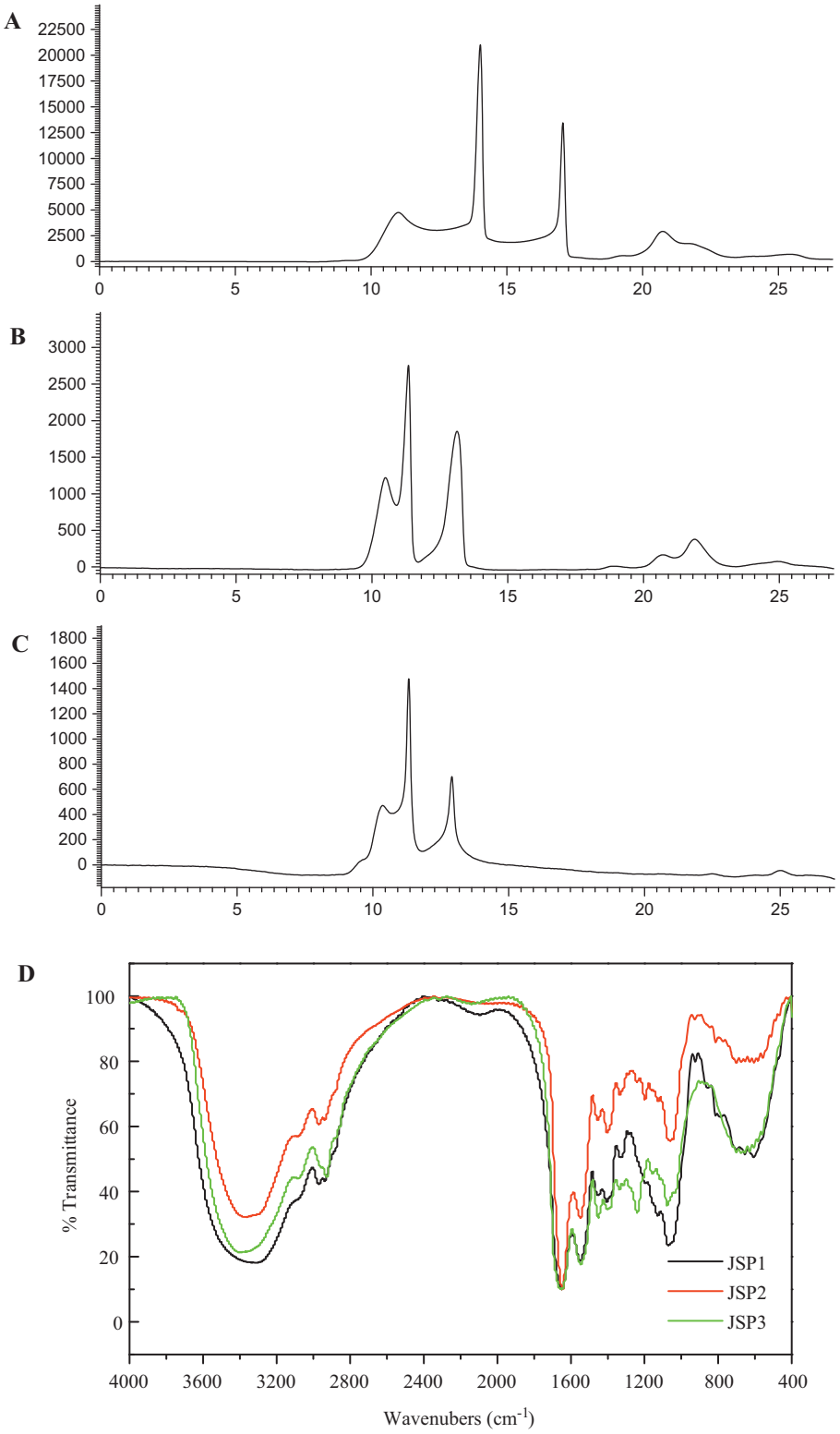

Fig. 3. Molecular weight distribution and FT-IR spectra of different polysaccharide fractions separated from the crude JSP: (A) JSP1, polysaccharide precipitated with 40% ethanol; (B) JSP2, polysaccharide precipitated with 60% ethanol; (C) JSP3, polysaccharide precipitated with 80% ethanol; (D) FT-IR spectra, where the black line, red line and green line represent the spectra of JSP1, JSP2 and JSP3, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.3. Precipitation of polysaccharides with ethanol

In order to obtain polysaccharides from crude extracts, ethanol is often employed to precipitate polysaccharide and remove water-soluble impurities. Nevertheless, the precipitation conditions including ethanol concentration, precipitation time and precipitation temperature, were often neglected. For this test, polysaccharide precipitation was carried out by the addition of ethanol at final concentration of 40%, 60% and 80% (v/v). Fig. 2A displays the effects of temperature on polysaccharide precipitation when precipitation time was fixed at 12 h. For each ethanol concentration, there was no notable difference in polysaccharide production at different temperatures, suggesting no effect of temperature on polysaccharide precipitation ($p < 0.05$). Fig. 2B shows the effects of precipitation time on polysaccharide production. In the group using 40% ethanol, no significant difference was observed in polysaccharide quantities obtained with different precipitation times (4–24 h). In the group using 60% and 80% ethanol, the precipitation time exhibited a pronounced influence on polysaccharide precipitation when varied from 4 to 12 h, and then no obvious changes in quantity of precipitates were observed, revealing 12 h as the optimal time for JSP precipitation by ethanol. In all cases, it is apparent that polysaccharide precipitation increased with an increase in final ethanol concentration. The fraction precipitated with 80% ethanol (JSP3) was the main component in crude JSP, corresponding to the yield of 97% which was about 323-fold and 36-fold of that precipitated with 40% (JSP1) and 60% ethanol (JSP2), respectively.

3.4. Molecular weight distribution of polysaccharides

Fig. 3 displays the HPLC chromatogram of JSP precipitated with ethanol at different final concentrations. JSP1 contained four fractions including JSP11, JSP12, JSP13 and JSP14, corresponding to the retention times of 11.005 min, 14.023 min, 17.063 min and 20.082 min, respectively (Fig. 3A). According to the standard curve between MW and retention time, the MW of JSP11, JSP12, JSP13 and JSP14 were calculated as about 4.86×10^6 Da, 5.18×10^5 Da, 5.44×10^4 Da and 5.8×10^3 Da, respectively. As shown in Fig. 3B, JSP2 consisted of four fractions of MW of 7.08×10^6, 3.78×10^6, 1.0×10^6 and 1.53×10^3 Da. JSP21, JSP22 and JSP23 were the main components in JSP2, accounting for about 87% of the total sample. The HPLC profile of JSP3 is depicted in Fig 3C, which was similar to that of JSP2 and comprised three polysaccharide fractions with MW of 7.84×10^6, 3.85×10^6 and 1.19×10^6 Da. These results revealed that JSP1 was very different from JPS2 and JSP3.

3.5. FT-IR analysis of polysaccharides

JSP1, JSP2 and JSP3 exhibited similar IR spectra (Fig. 3D). All samples possessed a broad characteristic stretching vibration peak at 3420 cm^{-1} for —OH and a weak C—H stretching vibration band ranging from 2930 to 2970 cm^{-1} for $—CH_2$ as well as $—CH_3$. A strong absorption peak at 1654 cm^{-1} in the three polysaccharide fractions implied the presence of carboxyl groups. The weak peak at near 1340 cm^{-1} was indicative of carbonyl groups that indicated the characteristic IR absorption of alduronic acids. The bands in the region of 1450 and 1243 cm^{-1} could be attributed to C—O stretch and O—H or C—H bending. In addition, all spectra displayed a relative strong stretching vibration from 1200 to 1000 cm^{-1}, which was induced by the overlapping of sugar rings through C—O—C and C—OH.

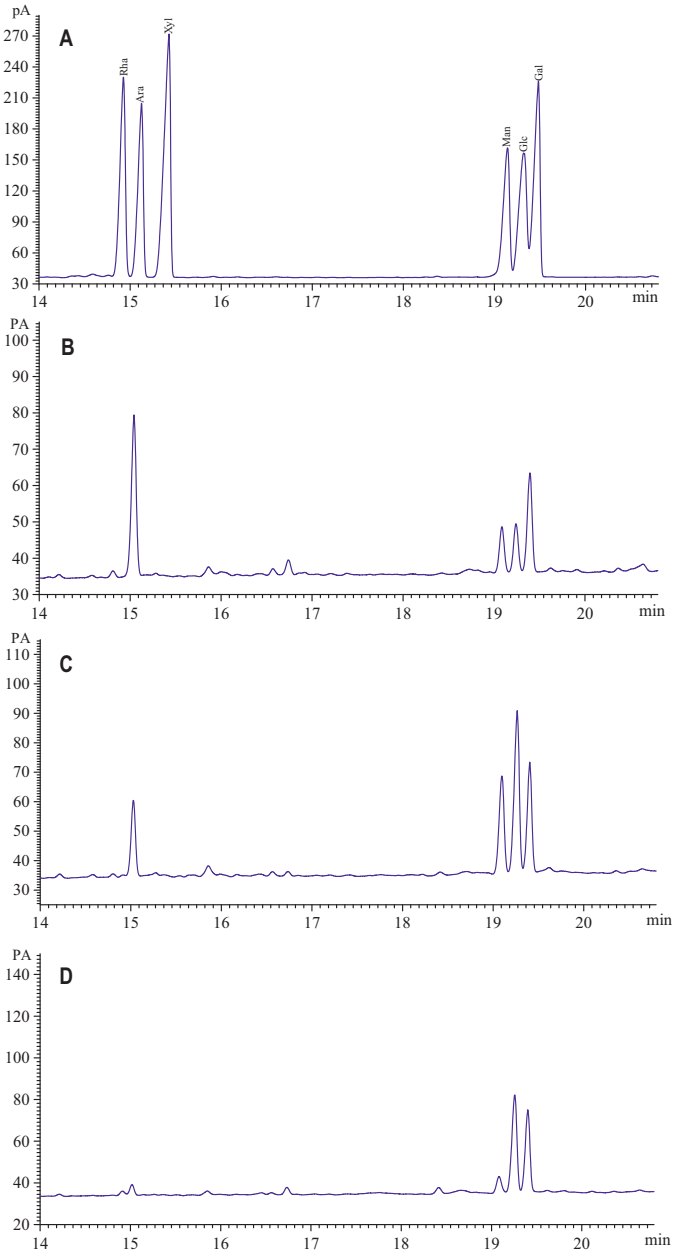

Fig. 4. GC chromatogram of monosaccharides in different polysaccharide fractions: (A) monosaccharide standards; (B) JSP1; (C) JSP2; and (D) JSP3.

3.6. Monosaccharide analysis of polysaccharides

As shown in Fig. 4A, the monosaccharide standards of Rha, Ara, Xyl, Man, Glc and Gal were well separated by HP-5 capillary column on GC system, corresponding to the retention time of 14.956, 15.149, 15.439, 19.170, 19.339 and 19.495 min. Results showed that JSP1, JSP2 and JSP3 exhibited obvious differences in monosaccharide composition. Both JSP1 and JSP2 were composed of Ara, Man, Glc and Gal in a molar ratio of 1.927:0.628:0.564:1 and 0.794:1.389:1.926:1, respectively. In JSP3, Glc and Gal were observed to be the dominant monosaccharides in a molar ratio of 1.51:1. Compared to the molecular weight distribution, although the HPLC chromatogram of JSP2 is very similar to that of JSP3, the difference existed in their monosaccharide compositions.

3.7. Glycosyl linkage analysis of polysaccharides

Periodate oxidation is considered as an effective method to study glycosyl bonds in polysaccharides, attributing to the special rupture in C—C bond on which two or three —OH are linked to different carbon atoms in series (Wang, Luo, & Liang, 2004). Based on the average molar mass of a glycosyl residue (160), a total consumption of 1.08 mol, 1.21 mol and 0.86 mol $NaIO_4$ was observed per mole of sugar residues for JSP1, JSP2 and JSP3, corresponding to 0.18 mol, 0.43 mol and 0.32 mol HCOOH formations, respectively. Thus, it was concluded that JSP1 contained about 18% non-reducing terminal residues and (1 → 6)-linked glycosyl bonds, 72% (1 → 2)-/(1 → 4)-linked and <10% (1 → 3)-linked glycosyl bonds. In JSP2, the non-reducing terminal residues and (1 → 6)-linked glycosyl bonds amounted to 43%, with (1 → 2)-/(1 → 4)-linked and (1 → 3)-linked glycosyl bonds amounting to 35% and <22%, respectively. JSP3 was revealed to comprise 32% non-reducing terminal residues and (1 → 6)-linked glycosyl bonds, 22% (1 → 2)-/(1 → 4)-linked and <6% (1 → 3)-linked glycosyl bonds. These results suggested that the glycosyl linkages were very different in JSP1, JSP2 and JSP3.

3.8. Effects of polysaccharides on macrophage-derived foam cell formation

As foam cell accumulation is an initial event in the development of atherosclerosis, the model of macrophage-derived foam cell formation induced by oxLDL is often used to screen anti-atherosclerotic agents. In this work, JSP3 was selected as the candidate to study the effects of jellyfish polysaccharide on macrophage-derived foam cell formation, because JSP3 was the main components in crude JSP. Fig. 5 depicts the conversion of macrophages into foam cells induced by oxLDL in the presence or absence of JSP3. Results showed that oxLDL caused intracellular lipid accumulation in macrophages as revealed by Oil Red O staining, but this lipid accumulation was drastically suppressed by 100 μg/mL JSP3 treatment (Fig. 5A and B). Compared to the normal cells, TC and CE were significantly higher in macrophages treated with oxLDL alone. However, macrophages treated with JSP3 exhibited a considerable decrease in TC and CE concentrations (Fig. 5C). It is generally accepted that macrophage has been converted into foam cell when CE percentage was higher than 50% in TC. As presented in Fig. 5D, the CE percentage is observed to be 38.1%, 69.7% and 39.8% in normal cells, cells treated with oxLDL alone and cells treated with oxLDL in addition to JSP3, respectively. These results suggested that jellyfish polysaccharide has good potential for inhibiting macrophage-derived foam cell formation induced by oxLDL.

The conversion of macrophages into foam cells is due to the lipid accumulation resulting from an imbalance between lipid uptake and export in macrophages (Mclaren et al., 2011). Macrophages take up oxLDL through scavenger receptors (SR). Among multiple SR, CD36 is the major one accounting for 40% and 60–80% of the uptake of oxLDL in human macrophages and peritoneal macrophages, respectively (Min et al., 2013). As shown in Fig. 6A, oxLDL drastically enhanced the expression of CD36, which was reduced markedly by JSP3 in a concentration range of 100–200 μg/mL. PPAR-γ is a major transcription factor that induces CD36 expression in oxLDL-treated cells, and thus modulates lipid metabolism (Chen, Tsai, Wang, & Lin, 2013; Min et al., 2013). In the present study, we found that oxLDL significantly stimulated PPAR-γ mRNA expression while JSP3 could block this promotion in the tested dosage range (Fig. 6B). These data imply that JSP3 modulates PPAR-γ signaling to reduce CD36 expression in oxLDL-treated macrophages. Acyl-coenzyme A: cholesterol acyltransferase-1 (ACAT-1) has been reported to be a key enzyme which promotes intracellular CE accumulation in macrophages (Yang et al., 2004). Previous reports have shown that ACAT-1 is highly expressed in macrophage-derived foam cells in human atherosclerotic lesions (Kusunoki et al., 2001), indicating a crucial role of ACAT-1 in the pathogenesis of atherosclerosis. As presented in Fig. 6C, macrophages treated with oxLDL alone exhibited a very high level of ACAT-1 mRNA expression, which was enhanced about 11-fold that of normal cells. However, this high expression was significantly weakened in the presence of 100–200 μg/mL JSP3, suggesting an important role for jellyfish polysaccharides in

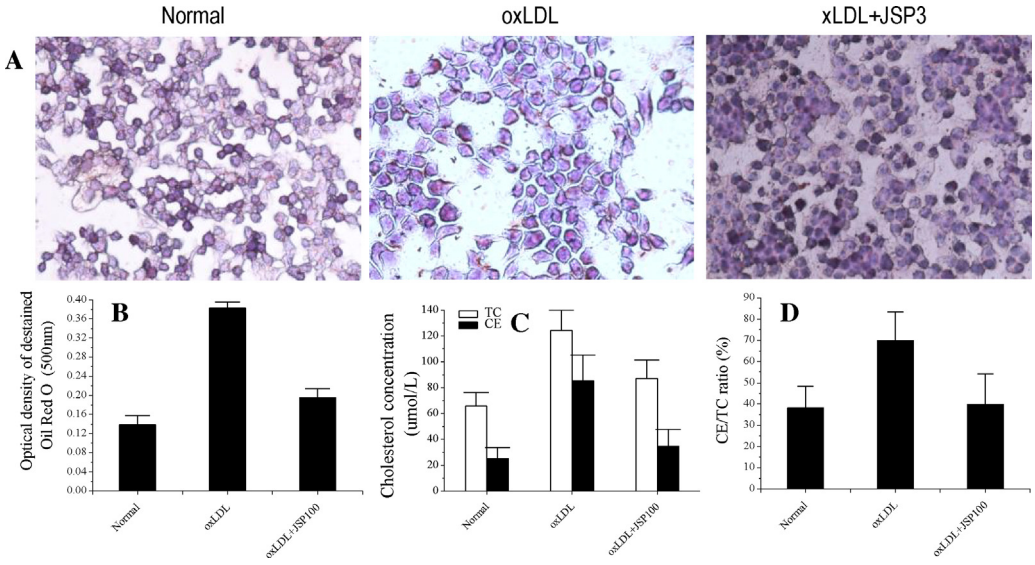

Fig. 5. Effects of JSP3 on the oxLDL-induced foam cell formation and intracellular lipid accumulation. The RAW 264.7 macrophage cells were treated with 50 μg/mL oxLDL in the presence or absence of 100 μg/mL JSP3 for 48 h. (A) After the incubation, the cells were stained with Oil Red O and then observed under the microscope (×400). The red droplets accumulated in the cells were indicated as the stained lipid. (B) Adding 1.0 mL ethanol to the stained culture dish, the extracted dye solution was diluted with double distilled water to five times, and then the absorbance was tested at 500 nm. (C) Intracellular total cholesterol (TC) and cholesteryl ester (CE) levels in cells with different treatment. (D) Quantification of percentage of cellular CE in TC. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

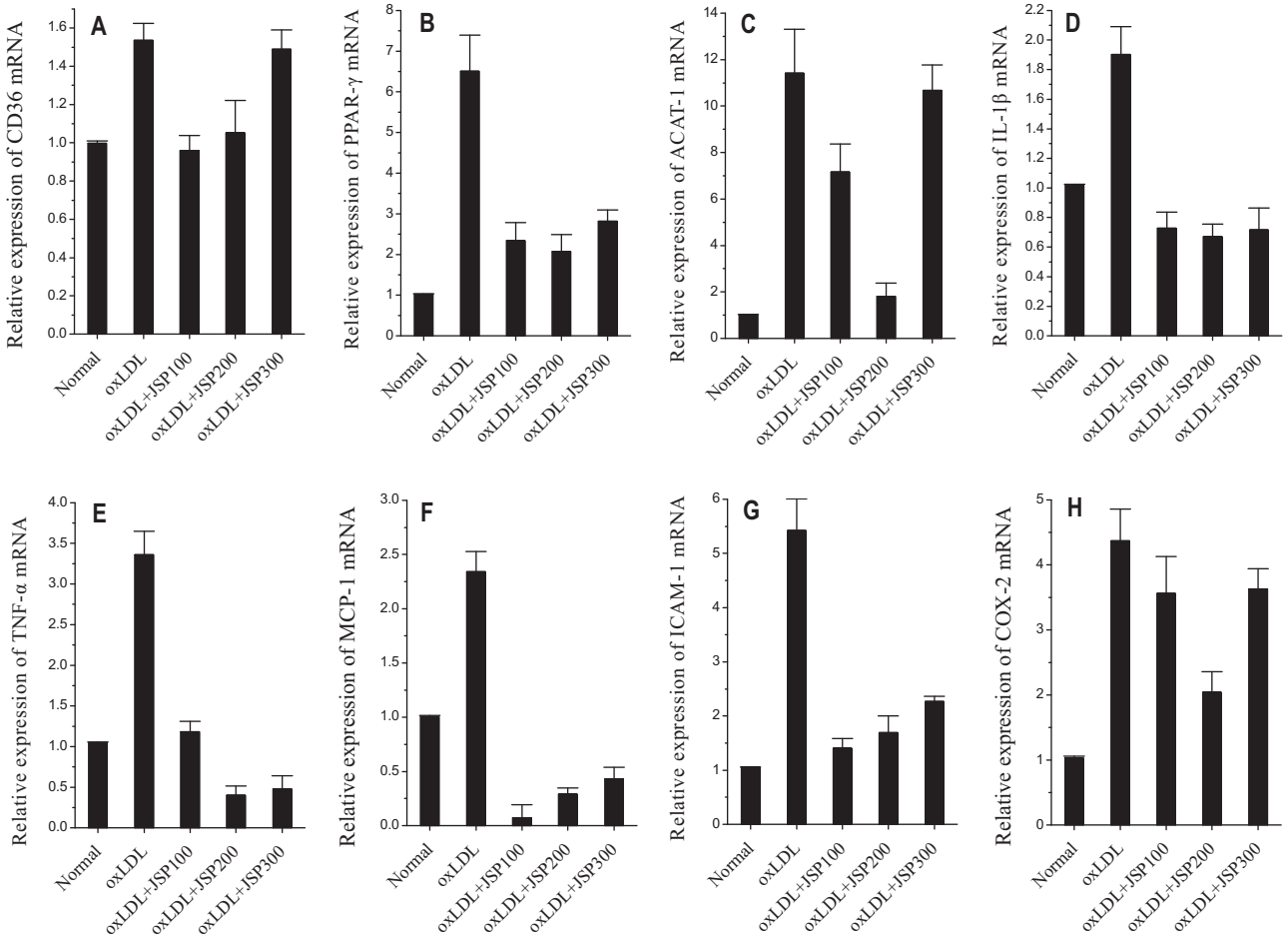

Fig. 6. Effects of JSP3 on the gene expression of CD36, PPAR-γ, ACAT-1, IL-1β, TNF-α, MCP-1, ICAM-1 and COX-2 in RAW 264.7 macrophage cells: (A) CD36; (B) PPAR-γ; (C) ACAT-1; (D) IL-1β; (E) TNF-α; (F) MCP-1; (G) ICAM-1; and (H) COX-2.

preventing foam cell formation. The variation of CE accumulation in macrophages also confirmed this conclusion (Fig. 5C and D).

In recent years, atherosclerosis is increasingly being considered as an inflammatory disease, since the macrophage-derived foam cells can amplify the inflammatory response through intracellular inflammatory pathways in the process of atherogenesis (Bobryshev, 2006). These include the expression of proinflammatory cytokines, chemokines and adhesion molecules in multiple type cells (Diepen et al., 2013). Tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) are the critical proinflammatory cytokines that mediate inflammation. Previous evidences have revealed that promotion of TNF-α and IL-1β expression could elevate the development of atherosclerosis (Diepen et al., 2013). Fig. 6D and E summarize the effects of oxLDL and JSP3 treatment on TNF-α and IL-1β expression in macrophages. Compared to the normal cells, oxLDL significantly promoted the expression of these cytokines. However, these promotions were attenuated to a lower level than that in normal cells by JSP3. Monocyte chemoattractant protein-1 (MCP-1), an important chemokine, has been considered to be critical for the initiation and development of atherosclerotic lesions by attracting inflammatory cells to the areas of developing inflammation (Diepen et al., 2013; Mclaren et al., 2011). Results showed that JSP3 could decrease the overexpression of MCP-1 in macrophages induced by oxLDL (Fig. 6F). Besides MCP-1, cellular adhesion molecules were also reported to participate in the recruitment of inflammatory cells to inflammation areas (Diepen et al., 2013; Mclaren et al., 2011). Among different adhesion molecules, intercellular adhesion molecule-1 (ICAM-1) has been suggested to play an important role in the development of atherosclerosis (Diepen et al., 2013; Mclaren et al., 2011). As shown in Fig. 6G, oxLDL induced significant increase in mRNA expression of ICAM-1, compared to that in normal cells. After treatment with JSP3 for 48 h, ICAM-1 expression decreased to about 26%, 30% and 43% with the escalating doses (100, 200 and 300 μg/mL), compared to that in the cells treated with oxLDL alone. There is evidence to confirm that cyclooxygenase-2 (COX-2) is expressed in atherosclerotic lesions of both humans and mice, contributing to lesion formation through modulation of inflammatory response (Burleigh et al., 2005). In the present study, the variation of COX-2 mRNA expression in cells treated under different conditions was similar to those of TNF-α and IL-1β (Fig. 6L). Thus, it is postulated that one possible mechanism of TNF-α and IL-1β expression alleviated by JSP3 is related to the down-regulation of COX-2 expression.

4. Conclusions

RSM was employed to optimize the extraction conditions of jellyfish skin polysaccharide. The optimal conditions were characterized as follows: 1:7.5 of raw material to water ratio, 100 °C of extraction temperature, and 4 h of extraction time. Papain in combination with Sevag reagent was beneficial in removing proteins from crude JSP. JSP3 precipitated with 80% ethanol was the main component in crude JSP. Notable differences in physicochemical properties were observed in fractions precipitated with different ethanol concentration. JSP3 exhibited good potential for inhibiting macrophage-derived foam cell formation, which possibly can be

attributed to the down-regulation of some atherosclerosis-related gene expression.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.01.041.

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