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Characteristics and immune-enhancing activity of pectic polysaccharides from sweet cherry (*Prunus avium*)

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ABSTRACT

Two water soluble polysaccharides components PAPS-1 and PAPS-2 with homogeneously distributed molecular weight were obtained from *Prunus avium*. PAPS-1 and PAPS-2 contained GalA: Ara: Gal: Rha: GluA: Glu in 49.38: 32.39: 10.68: 4.66: 1.94: 0.48 and 77.18: 14.91: 3.39: 3.46: 0.93: 0.19 M ratios respectively, as well as trace amount of mannose and fucose. Infrared spectroscopy (IR), nuclear magnetic resonance (NMR) and methylation analysis indicated that both fractions were type I rhamnogalacturonan (RG-I) pectic polysaccharides with glycan side chains constituted mainly of arabinose with minor amount of galactose. Galacturonic acid methylation and sugar acetylation was found in both PAPS-1 and PAPS-2. Both PAPS-1 and PAPS-2 significantly induced the NO release from RAW264.7 cells and the expression of several immune-related molecular (TNFα, IL6, IL10, GCSF, iNOS, COX-2) was induced in RAW264.7 cells.

1. Introduction

Polysaccharides had been found to possess a wide range of bioactivities, such as immune regulating activity (Sun, Gao, Xiong, Huang, & Xu, 2014), antioxidant activity (Song, & Tang, 2016), antitumor activity (Chihara, Maeda, Hamuro, Sasaki, & Fukuoka, 1969). Plants are a rich source of polysaccharides which function mainly as either structural or energy storage components. Several studies have indicated that polysaccharides from plants have immune-enhancing activity (Ferreira, Passos, Madureira, Vilanova, & Coimbra, 2015; Zhang, Qi, Guo, Zhou, & Zhang, 2016).

Sweet cherry (*Prunus avium*) is one of the most popular fruits containing various bioactive components, such as phenolics (Cao et al., 2015), dietary fiber (Nawirska & Kwasniewska, 2005), melatonin (González-Gómez et al., 2009). Among these, the phenolics have been the most studied compounds in sweet cherry and found to be beneficial for their antitumor (Olsson, Gustavsson, Andersson, Nilsson, & Duan, 2004) and antioxidant properties in humans (Kelebek & Selli, 2011: Liu et al., 2001). There have, however been few health-related studies on sweet cherry polysaccharides, which have been reported to be mainly composed of pectins, hemicelluloses and cellulose (Nawirska & Kwasniewska, 2005). Only a few studies on their bioactivity have been carried out and structural studies have been limited to the monosaccharides composition (Basanta, Pla, Stortz, & Rojas, 2013; Salato, Ponce, Raffo, Vicente, & Stortz, 2013). According to the pervious research, sweet cherry might have a beneficial effect on the immune system in animals (Kelley, Rasooly, Jacob, Kader, & Mackey, 2006). As polysaccharides are known to be good immunomodulators, and sweet cherry is a popular fruit, the effects on the immune system of their polysaccharides is worthy of further study.

Macrophage is one of the important components of non-specific immunity, which play an important role in regulating innate and adaptive immune responses via the production of cytokines, such as tumor necrosis factor α (TNFα), interleukin 6 (IL6), interleukin 10 (IL10), and interferon γ (IFNγ). Macrophages also regulate immune responses by releasing inflammatory molecules such as nitric oxide (NO) (Commins, Borish, & Steinke, 2010; Le Page, Génin, Baines, & Hiscott, 2000; Medzhitov, & Janeway, 2000). RAW264.7 cells are macrophage-like cells that can easily be activated by immune-enhancing reagents. The activated RAW264.7 cells are able to release NO, followed by the release of a series of immune-related cytokines, which can be easily detected. Thus, RAW264.7 cells are commonly used as model for the in vitro evaluation of immune regulatory activity (Simas-Tosin et al., 2012; Zhu, et al., 2012).

In the present study, the water-soluble polysaccharides were isolated and classificated from *Prunus avium* and their structural characteristics and the immune regulatory active in vitro using RAW264.7 cells were studied.

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2. Materials and methods

2.1. Materials and reagents

The fruit of *Prunus avium* cv. Black pearl was collected at commercial maturity from the breeding nursery of Yantai Agricultural Sci & Tech Institute, Shandong Province. Disease and mechanical damage-free fruits with uniform shape and color were pitted and frozen quickly in liquid nitrogen, then stored at −80 °C for later use.

Cell culture media were the products of Gibco BRL (Gaithersburg, MD, USA). Lipopolysaccharide (LPS) and polymyxin B was purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA). NO detection kit and Bradford protein quantization kit were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Elisa kits for mice TNFα, IL6 and IL10 quantization were purchased from R&D Systems China Co. Ltd. (Shanghai, China). The primers for qRT-PCR were synthesized by Invitrogen Co. Ltd. (Carlsbad, CA, USA). HCl-methanol solution was purchased from the Xiya Reagent Co. Ltd. (Sichuan, China). Chromatographically pure 1-Phenyl-3-methyl-5-pyrazolone (PMP), glucose, galactose, arabinose, rhamnose, fucose, xylose, galacturonic acid, and glucuronic acid were purchased from Jingchun Bio-Chem Technology Co. Ltd. (Shanghai, China). Acetonitrile and trifluoroacetic acid (TFA) were of chromatographic grade from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA). All other chemicals used were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Isolation and purification of polysaccharides

The pulp of cherry fruit (600 g) was mixed with 3.5 L absolute ethyl alcohol and homogenized. The homogenate was placed at −20 °C for 24 h and then filtered. The residue was extracted twice (2h each time) with 80% ethanol in a solid-liquid rate of 1:20 (m:v) and filtered. The residue was subsequently refluxed with 300 mL ethanol to further remove the ethanol-soluble impurities. The residue was dried and extracted three times with hot water at 90 °C (2h each time). The filtrates were combined and concentrated ten times in a rotary concentrator. Then, ethyl alcohol was added slowly to the concentrated filtrate to a final concentration of 80%, and maintained at 4 °C for 24 h to fully precipitate the crude polysaccharide.

The precipitate was collected by centrifugation, and washed subsequently with ethanol, acetone and absolute ether. After removing all the organic solvents by drying, the residue was dissolved in ddH_2O (20 mg/mL) and subjected to ion-exchange chromatography using DEAE-Sepharose Fast Flow (1.6 × 40 cm, GE Co., Pittsburgh, PA, USA), eluting subsequently with ddH_2O, 0.05, 0.1, 0.2, 0.3 and 0.4 mol/L NaCl (3 column bed volume for each gradient) at a flow rate of 1 mL/min. The eluent was collected in 8 mL fractions per tube. All the fractions were measured by phenol-sulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and results were expressed as a histogram. The peaks with high polysaccharide content was collected, concentrated at 45 °C and dialyzed for 48 h, following by lyophilization to obtain the polysaccharides.

The polysaccharides fractions were dissolved in 0.2 mol/L NaCl solution (10 mg/mL) for further purification using a HiPrep 26/60 Sephacryl S200 HR column (GE Co., Pittsburgh, PA, USA). NaCl solution (0.2 mol/L) was used as elution solvent, with flow rate of 1 mL/min. The eluent was collected in 4 mL aliquots per tube. All the fractions were measured by the phenol-sulphuric acid method (Dubois et al., 1956). The peaks with high polysaccharide content were collected, concentrated at 45 °C and dialyzed for 48 h, following by lyophilization to obtain the purified polysaccharides.

2.3. General analytical methods

2.3.1. Purity assessment

The polysaccharide content was determined by the phenol-sulphuric acid method (Dubois et al., 1956) and the total phenolics impurities in the polysaccharides were determined by the Folin-Ciocalteu method (Zhang et al., 2008). The total protein content in the polysaccharide was determined following the instruction for the Bradford protein quantization kit. The UV–Visible spectrum of the water solution of sample (1 mg/mL) was measured on a DU-8000 spectrophotometer (Beckman Coulter, Brea, CA, USA) in a wavelength range of 200–800 nm.

2.3.2. Homogeneity and molecular weight evaluation

The homogeneity and molecular weight of polysaccharides were determined by gel permeation chromatography (GPC) in a Waters 515 system coupled with 2410 refractive index detector (Waters, Milford, MA, USA), using a Biosep G4000SWXL column (Tosoh, Japan). The linear regression was calibrated with T-series dextran standards (76.9 kDa, 43.5 kDa, 21.4 kDa and 10.5 kDa) (GE Co., Pittsburgh, PA, USA) and the molecular weights of the polysaccharides were expressed as the dextran equivalent molecular weight.

2.3.3. Monosaccharides composition

The monosaccharides composition of polysaccharides was determined by high-performance liquid chromatography (HPLC) after pre-column derivatization according to the method described by Sun et al. (2009).

Briefly, 1 mg polysaccharide sample was hydrolyzed with 1 mL 30% HCl-methanol at 80 °C for 16 h. The solvent was removed by rotary evaporation at 30 °C. The residue was dissolved in 1 mL 2 mol/L trifluoroacetic acid, and hydrolyzed in a sealed condition at 120 °C for 2 h. Then the solvent was removed by rotary evaporation at 30 °C. The residue was washed with methanol several times to remove the excessive TFA. The residue was finally dissolved in 100 μL ddH_2O for further derivatization. Three repetitions were carried out for each sample.

For the pre-column derivatization, 100 μL of hydrolysate solution or 10 mg/mL monosaccharides standards solution were mixed with 120 μL 0.5 mol/L methanol solution of 1-phenyl-3-methyl-5-pyrazolone and 100 μL 0.3 mol/L NaOH solution, and incubated at 70 °C for 1 h. Then 100 μL 0.3 mol/L HCl solution was added to neutralize the NaOH. Finally, 1 mL dichloromethane was added, and the mixture was vigorously shaken and centrifuged for 5 min. The supernatant, containing the labeled carbohydrates, was filtered through a 0.22 μm membrane and 10 μL of the resulting solution was subjected to HPLC analysis.

The HPLC analysis of labeled monosaccharides was carried out in a Waters 2695-2996 HPLC system (Waters, Milford, MA, USA) coupled with a Sunfire C18 analytical column (4.6 × 250 mm, Waters, USA) operated at a column temperature of 25 °C. The mobile phase consisted of 0.1 mol/L KH_2PO_4 buffer (pH 6.7) (eluent A) and acetonitrile (eluent B). The gradient program was as follows: 0–30 min, 18% of B; 30–60 min, 18–25% of B; 60–65 min, 25–18% of B. The flow rate of the mobile phase was 1 mL/min and the compounds were detected at 245 nm. Sugar identification was by comparison with monosaccharide standards.

2.3.4. Methyl and acetyl esterification detection

The degree of esterification was determined according to the methods of Nergard et al. (2005). The polysaccharide samples (about 2 mg) were hydrolysed with 1 mol/L HCl (0.1 mL) at 100 °C for 2 h before being applied to the GC. The released methanol and acetic acid concentrations were calculated according to their standard curves respectively. 1 mol/L HCl was used as solvent control. Three repetitions were carried out for each sample. The gas chromatography was carried out on an Agilent 6890N (Agilent Technologies Inc., CA, USA) with a flame ionization detector. The column HP-INNOWAX

Table 1
Sequences of primers used for qRT-PCR detection.

Genes	Sequences	Genes	Sequences
Actin	F: CACTGTGCCCATCTACGAGG	COX-2	F: ATGACTGCCCAACTCCCATG
	R: ATGTCACGCACGATTTCCCT		R: ACTGATGGGTGAAGTGCTGG
TNFα	F: CGGGCAGGTCTACTTTGGAG	iNOS	F: CGGCAAACATGACTTCAGGC
	R: ACCCTGAGCCATAATCCCCT		R: GCACATCAAAGCGGCCATAG
IL6	F: GAGACTTCCATCCAGTTGCCT	IFNγ	F: AAGACTGTGATTGCGGGGTT
	R: CAGGTCTGTTGGGAGTGGTA		R: CATCTCCTCCCATCAGCAGC
IL10	F: CAGAGAAGCATGGCCCAGAA	IL12a	F: ATGATGACCCTGTGCCTTGG
	R: GCTCCACTGCCTTGCTCTTA		R: CACCAGCATGCCCTTGTCTA
GCSF	F: CAGCATCTGGTCTCCCTTCC	IL12b	F: ATCATCAAACCAGACCCGCC
	R: TAAACCTGGCTGCCACTGTT		R: CCCCTCCTCTGTCTCCTTCA

TNFα, tumor necrosis factor α; IL6, interleukin 6; IL10, interleukin 10; GCSF, granulocyte colony stimulating factor; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; IFNγ, interferon γ; IL12a, interleukin 12a; IL12b, interleukin 12b.

(30 m × 0.25 mm × 0.25 μm) was used at a temperature of 125 °C, and the injector and detector temperature of 150 °C and 160 °C, respectively.

2.3.5. Infrared spectroscopy (IR)

For IR analysis, 1 mg of the lyophilized polysaccharide was mixed with KBr powder, ground and then pressed into 1 mm pellets for Fourier transform infrared (FT-IR) measurement. FT-IR spectra of the polysaccharides were measured in the frequency range of 4000–400 cm^{-1}, a resolution of 4 cm^{-1} and scan number of 32.

2.3.6. Nuclear magnetic resonance (NMR) spectroscopy

For the NMR spectroscopy, the lyophilized polysaccharide was dissolved in D_2O to a final concentration of 40 mg/mL, and then its ^1H- and ^{13}C NMR spectra were recorded on a Bruker Ascend 600 MHz NMR magnet system (Bruker Daltonik GmbH, Bremen, Germany). The ^1H NMR spectra were obtained at 25 °C after 128 scans. The ^{13}C NMR spectra were obtained at 25 °C after 75,400 scans. The Chemical shift was expressed as δ ppm, using the resonances of acetone (δ_H 2.1 ppm, δ_c 32.8 ppm) as internal reference.

2.3.7. Methylation analysis

The uronic acids were reduced using the carboxyl reduction methods of Taylor and Conrad (1972) and the products were subjected to methylation analysis according to the methods developed by Anumula and Taylor (1992). Briefly, polysaccharides (2 mg) were diluted in 0.6 mL of DMSO for 30 min, then mixed with 0.6 mL of saturated NaOH-DMSO solution and 0.6 mL of methyl iodide, vortex thoroughly for 7 min, and the reaction terminated with 4 mL of ddH_2O. The methylated products were extracted five times with chloroform followed by vacuum concentration at 40 °C. The methylation procedure was repeated until exhaustive methylation was confirmed by IR detection. The materials were then submitted to reduction and acetylation as described above for monosaccharides composition. The products were examined by GC–MS using an Agilent 7890 GC coupled to a 5975 MSD scanning (Agilent Technologies Inc., CA, USA). A capillary column of HB-5MS (30 m × 0.25 mm × 0.25 μm) was used at a column temperature of 120 °C for 1 min, then programmed at 10 °C/min to 240 °C and held at this temperature for 7 min.

2.4. Immuno-enhancing activity analysis

2.4.1. Determination of NO produced in response to polysaccharide

RAW264.7 cells were cultured in DMEM complete medium (containing 10% fetal bovine serum) at 5% CO_2 and 37 °C in a humidified incubator. The cells were planted onto a 96 well plate at a density of 3×10^4 cells/well and cultured for 12 h for cell adhesion. Then, fresh medium containing different concentrations of polysaccharides was added, following by another 24 h culturing. The NO content in the medium in each well was detected by NO detection kit. LPS at a final concentration of 0.1 μg/mL was used as positive control and PBS as solvent control. Three independent experiments were performed with three parallel wells in each experiment.

To eliminate the possibility of LPS pollution during the polysaccharide isolation, the polysaccharides samples were mixed with polymyxin B of different concentrations for 30 min before adding to the medium.

2.4.2. qRT-PCR assay

RAW264.7 cells were planted onto the 24 well plates at a density of 1.2×10^5 cell per well, and cultured for 12 h for cell adhesion. Then, the cells were cultured with fresh medium containing different concentrations of polysaccharides. LPS at a final concentration of 0.1 μg/mL was used as positive control and PBS as solvent control. After 6 h treatment, the cells were collected for RNA extraction using Trizol reagent (Invitrogen, Carlsbad, CA, USA), following by cDNA synthesis using iScript cDNA synthesis kit (BioRad, Berkeley, CA, USA). SsoFast EvaGreen Supermix (BioRad, Berkeley, CA, USA) was added to the qRT-PCR reaction mixture. The sequences of the primers for qRT-PCR are listed in Table 1. qRT-PCR reactions were carried out in a BioRad CFX96 (BioRad, Berkeley, CA, USA) according to the following procedure: pre-denaturation at 95 °C for 3 min, 45 cycles of 95 °C for 10 s and 60 °C for 30 s. Three independent experiments were performed with three parallel wells in each experiment.

2.4.3. Elisa assay

RAW264.7 cells were planted onto 96 well plates (3×10^4 cells per well for IL6 and TNFα detection, 6×10^4 cell per well for IL10 detection), and cultured for 12 h for cell adhesion. Then, fresh medium containing different concentrations of polysaccharides was added, following by another 24 h culturing. The media were collected and diluted to an appropriate concentration for Elisa assay. LPS at a final concentration of 0.1 μg/mL was used as positive control and PBS as solvent control. Three independent experiments were performed with three parallel wells in each experiment.

2.5. Statistics

Statistical analyses were carried out using SPSS version 20.0 (IBM, Armonk, NY, USA). Data were analyzed by one-way ANOVA. Multiple comparisons between the groups were performed using the Tukey method. Values were expressed as the mean ± standard deviation.

3. Results and discussion

3.1. Isolation of polysaccharides PAPS-1 and PAPS-2 from sweet cherry pulp

After hot water extraction, ethanol precipitation and DEAE-Sepharose chromatography, 133 mg crude water soluble

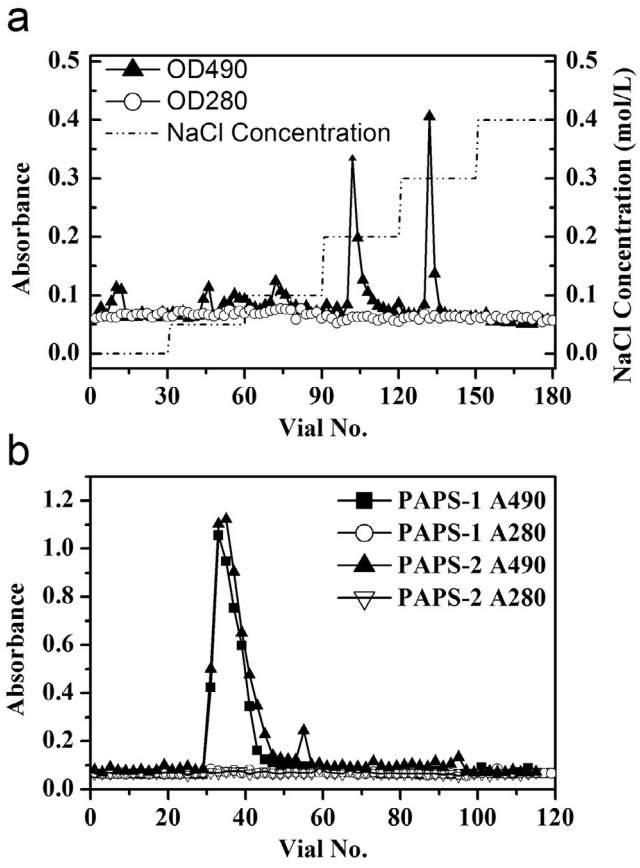

Fig. 1. Separation of PAPS-1 and PAPS-2. (a) DEAE-Sepharose elution curve; (b) Sephacryl S-200 elution curve.

polysaccharides were obtained from 100 g fresh sweet cherry pulp. According to the results of DEAE-Sepharose chromatography (Fig. 1a), the sweet cherry pulp water soluble polysaccharides were mainly composed of acidic polysaccharides which could be eluted by 0.05–0.3 mol/L NaCl solutions, with a small amount of neutral polysaccharide (eluted by water). Most of the acidic polysaccharides were concentrated in the 0.2 mol/L NaCl eluent and 0.3 mol/L eluent, thus, these two fractions were collected and desalted by dialysis for further purification by gel filtration chromatography using HiPrep 26/60 Sephacryl S200 HR column.

Fig. 1b shows the result of Sephacryl S200 chromatography. One peak was apparent from the 0.2 mol/L NaCl eluted fraction and one from the 0.3 mol/L eluted fraction. The GPC results suggested that these two fractions had a homogeneous molecular weight distribution of 144 kDa and 139 kDa according to their respective retention times compared with dexran (Supplement materials, Fig. S1). These two components were named PAPS-1 and PAPS-2 respectively.

After the purification treatments, most of the impurities such as the phenolics and proteins were removed. No phenolics components were detected by Folin-Ciocalteu method (Table 2). The UV–Visible spectroscopy of the PAPS-1 and PAPS-2 also confirmed this result (Supplement materials, Fig. S2). A protein content of 0.53% and 0.37% (w/w) were detected using the Bradford method. The total carbohydrate purity of PAPS-1 and PAPS-2 were 78.65% and 87.31%, respectively (Table 2).

3.2. Structural characteristics of PAPS-1 and PAPS-2

In the IR spectra, the signals of some typical groups of PAPS-1 and PAPS-2 were found (Supplement materials, Fig. S3). The strong peak at 3400 cm^{-1} was assigned to the hydroxyl stretching vibration. The absorption peak at 2920 cm^{-1} was assigned to the C–H stretching vibration, and that at 1411 cm^{-1} was assigned to the C–H Variable angle vibration. The ester carbonyl C=O asymmetric stretching vibration at 1740 cm^{-1} indicated the existence of acetylation, the non ester carbonyl C=O asymmetric stretching vibration at 1610 cm^{-1} indicated the existence of uronic acid. The absorption peak at 1238 cm^{-1} was assigned to the C–O stretching vibration. The peak at 832 cm^{-1} indicated the presence of an α-glycosidic bond.

Table 2
Characteristics of PAPS-1 and PAPS-2.

	PAPS-1	PAPS-2
Yield (mg/g FW)	0.51	0.43
Total carbohydrate (%, w/w)	78.65 ± 3.02	87.31 ± 0.48
Protein content (%, w/w)	0.53 ± 0.20	0.37 ± 0.13
Phenolics content (%, w/w)	ND	ND
Monosaccharide composition (mol percentage)		
Mannose	0.33	Trace
Rhamnose	4.66	3.46
Glucose	0.48	0.19
Galactose	10.68	3.39
Xylose	ND	ND
Arabinose	32.39	14.91
Fucose	0.13	Trace
Glucuronic acid	1.94	0.93
Galacturonic acid	49.38	77.18
Monosaccharide ratio		
(Ara + Gal)/Rha	9.26	5.29
Ara/Gal	6.96	4.31
GalA/Rha	10.61	22.32

ND, not detected.

The monosaccharides composition of PAPS-1 and PAPS-2 is shown in Table 2. In previous studies, the water-soluble polysaccharides from *Prunus avium*, *Prunus cerasus* and *Prunus tomentosa* were shown to be consist of 6–7 monosaccharide, including arabinose, galactose, glucose, rhamnose, mannose, fucose, xylose and uronic acid (Basanta et al., 2013; Kosmala, et al., 2009 Salato et al., 2013). However, the composition of uronic acids was not identified in these studies. In the present study, by using PMP derivation and HPLC, the glucuronic acid and galacturonic acid can be clearly separated (Supplement materials, Fig. S4). We found that the PAPS-1 and PAPS-2 were composed of two uronic acids (galacturonic acid and glucuronic acid), and six neutral monosaccharides (arabinose, galactose, rhamnose, glucose, mannose and fucose). The ratio of galacturonic acid was much higher than that of glucuronic acid. The xylose detected in a previous study on sweet cherry (Salato et al., 2013) were not detected in PAPS-1 and PAPS-2. The monosaccharides ratio varied between these two fractions. The major difference was that the PAPS-2 had higher ratio of galacturonic acid (77.185%) than PAPS-1 (49.38%), and far lower ratio of arabinose (14.91%) and galactose (3.39%) than PAPS-1 (32.39% and 10.68% respectively). The monosaccharides composition of PAPS-1 and PAPS-2 in the present study were also similar to that of the pectic polysaccharides of *Prunus domestica* and *Prunus persica* (Kosmala et al., 2013; Popov et al., 2014; Simas-Tosin et al., 2012). The gas chromatography results indicated the existence of methyl esterification of galacturonic acids and acetylation of sugars (Supplement materials, Fig. S5). The amount (mol%) of galacturonic acids methylesterified was relatively high in PAPS-1 (53.17%) compared to PAPS-2 (43.66%).

The ^{1}H NMR and ^{13}C NMR spectra of PAPS-1 and PAPS-2 are shown in Fig. 2. In the ^{1}H NMR spectra, the signals were mainly distributed in 3.0–5.5 ppm. The signals at 4.97 ppm and 4.94 ppm were assigned to the H-1 of 1,4-α-d-GalA, the signals at 5.02 ppm was assigned to the H-1 of 1,4-α-d-GalA-6-OMe, the signals at 5.18 was assigned to the H-1 of terminal α-l-Ara, and the signals at 5.05 was assigned to the H-1 of 1,5-α-l-Ara. The signal at 1.12 ppm was assigned to the H-6 of α-Rha. A typical acetyl group hydrogen signal (1.95 ppm) was found (Fig. 2a and

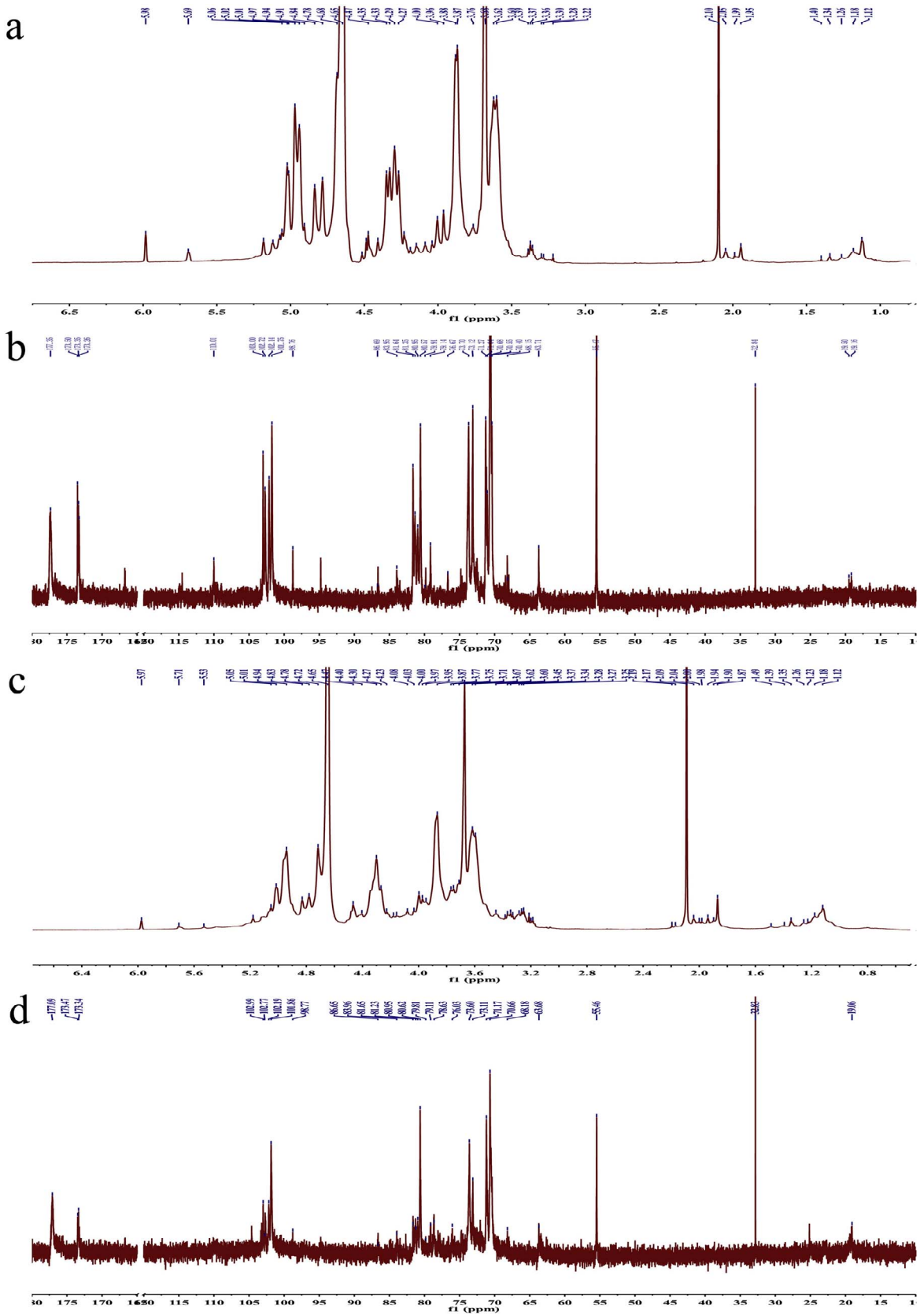

Fig. 2. NMR spectrum of PAPS-1 and PAPS-2. (a) ^{1}H NMR spectrum of PAPS-1; (b) ^{13}C NMR spectrum of PAPS-1; (c) ^{1}H NMR spectrum of PAPS-2; (d) ^{13}C NMR spectrum of PAPS-2.

Table 3
^{13}C NMR spectral assignments of PAPS-1 and PAPS-2 (600 MHz, D_2O).

	C-1	C-2	C-3	C-4	C-5	C-6
PAPS-1						
→4)-α-D-GalA-(1→	102.72	70.7	71.3	80.6	73.7	177.4
→4)-α-D-GalA-6-OMe (1→	101.75	70.7	71.1	80.6	73.1	173.5 (55.5 OMe)
→5)-α-L-Ara-(1→	110.0		76.7	84.0	68.2	
α-L-Ara-(1→	110.0		76.7	86.6	63.7	
1,4-α-D-Gal	98.76			79.79		
→2)-α-L-Rha-(1→		79.1		74.7		19.1
→2,4)-α-L-Rha-(1→		79.1		83.4		19.5
PAPS-2						
→4)-α-D-GalA-(1→	101.67	70.7	71.2	80.6	73.7	177.1
→4)-α-D-GalA-6-OMe (1→	100.48	70.7	71.0	80.6	73.1	173.4 (55.5 OMe)
→5)-α-L-Ara-(1→	110.1		76.6	84.0	68.1	
α-L-Ara-(1→	110.1		76.6	86.5	63.6	
1,4-α-D-Gal	98.76			79.79		
→2)-α-L-Rha-(1→		79.1		74.7		19.1
→2,4)-α-L-Rha-(1→		79.1		83.4	69.2	19.1

c).

In the ^{13}C NMR spectra, the carbon signals of 1,4-α-d-GalA, 1- or 1,5-α-l-Ara, 1,2- or 1,2,4-α-l-Rha were found in PAPS-1 and PAPS-2 (Fig. 2b and 2d). The signals of methoxyl group-linked C-6 of 1,4-α-d-GalA (173.35 ppm) and methyl group carbon (55.5 ppm) in the C-6 of 1,4-α-d-GalA indicated the methyl esterification in C6 of galacturonic acid in both PAPS-1 and PAPS-2 (Table 3) (Popov et al., 2011, 2014; Westereng, Michaelsen, Samuelsen, & Knutsen, 2008). These were also in agreement with the results of IR spectroscopy (Supplement materials, Fig. S3), as well as the methyl esterification determination (Supplement materials, Fig. S5). The signals identified at 110.0, 76.7, 84.0 and 68.2 ppm could be assigned to the C1, C3, C4 and C5 of 1,5-α-l-Ara, while the signal identified at 110.0, 76.7, 86.6 and 63.7 ppm could be assigned to the C1, C3, C4 and C5 of terminal-α-l-Ara (Popov et al., 2014; Wu, Ai, Wu, & Cui, 2013). The signals at 74.7 ppm and 83.4 ppm were assigned to the C4 of rhamnose with 1,2- or 1,2,4-linkage, respectively (Popov et al., 2014). However, due to the low content of rhamnose (lower than 5%), the NMR signals of other carbons of rhamnose were weak and overwhelmed or not detected (Table 3). For the same reason, the signals of the other five monosaccharides (glucuronic acid, mannose, fucose, and glucose) were not exactly identified. The galactose was linked mainly with a terminal-α or 1,4-α-glycosidic bond, with signals of C1 (98.76 ppm) and C4 (79.79 ppm) identified in the NMR spectrum (Popov et al., 2014). The signals of the other carbons of galactose were overwhelmed by other monosaccharides and not exactly identified.

Methylation analysis identified the signal of 2,3-di-O-methylarabinitolacetate (MW 306.131 amu, RT 9.294 min), indicating the existence of a 1,5-linkage of arabinose. Besides, the signals of 2,3,4-tri-O-methyl-Galactitolacetate (MW 350.158 amu, RT 12.302) and 2,3,4,6-tetra-O-methyl-Galactitolacetate (MW 322.163, RT 9.909 min) were also found, indicating the existence of a 1,6-linkage of galactose and terminal galactose, respectively. However, the signals of 1,6-α-galactose was not significant in the NMR results, indicating that the 1, 6-linkage was not the chief type of galactose linkage in PAPS-1 and PAPS-2. Further studies using additional methods of analysis were required to confirm the nature of the side chains.

Combining the results of monosaccharides composition, IR spectrum analysis, NMR analysis and methylation analysis, the PAPS-1 and PAPS-2 were the acidic polysaccharides consisted of two uronic acids and six monosaccharides, with 1,4-α-d-GalA as the chief uronic acid, and 1- or 1,5-α-l-Ara as the chief monosaccharide. The rhamnose was present in either 1,2- or 1,2,4- linkage. Thus, it can be deduced that both the PAPS-1 and PAPS-2 were type I rhamnogalacturonan (RG-I) pectic polysaccharides (Paulsen, & Barsett, 2005; Ridley, O'Neill, & Mohnen, 2001). The main chain was constituted mainly of RG-I and homogalacturonan (HG), and the side chains consisted mainly of the glycans of arabinose and galactose, attached to the C4 of rhamnose in the RG-I backbone. The PAPS-2 had higher HG proportion than PAPS-1, which can be deduced from the observation that the ratio of GalA/Ara was higher in PAPS-2 (22.32) than in PAPS-2 (10.61) (Table 2). Both PAPS-1 and PAPS-2 had a certain degree of acetylation and methylesterification, which is in agreement with the previously reported characteristics of RG-I pectic polysaccharides (Paulsen, & Barsett, 2005; Ridley et al., 2001). The structural characteristics of sweet cherry water-soluble polysaccharides obtained in present study were similar to that of *Prunus domestica* and *Prunus persica* (Cantu-Jungles et al., 2014;

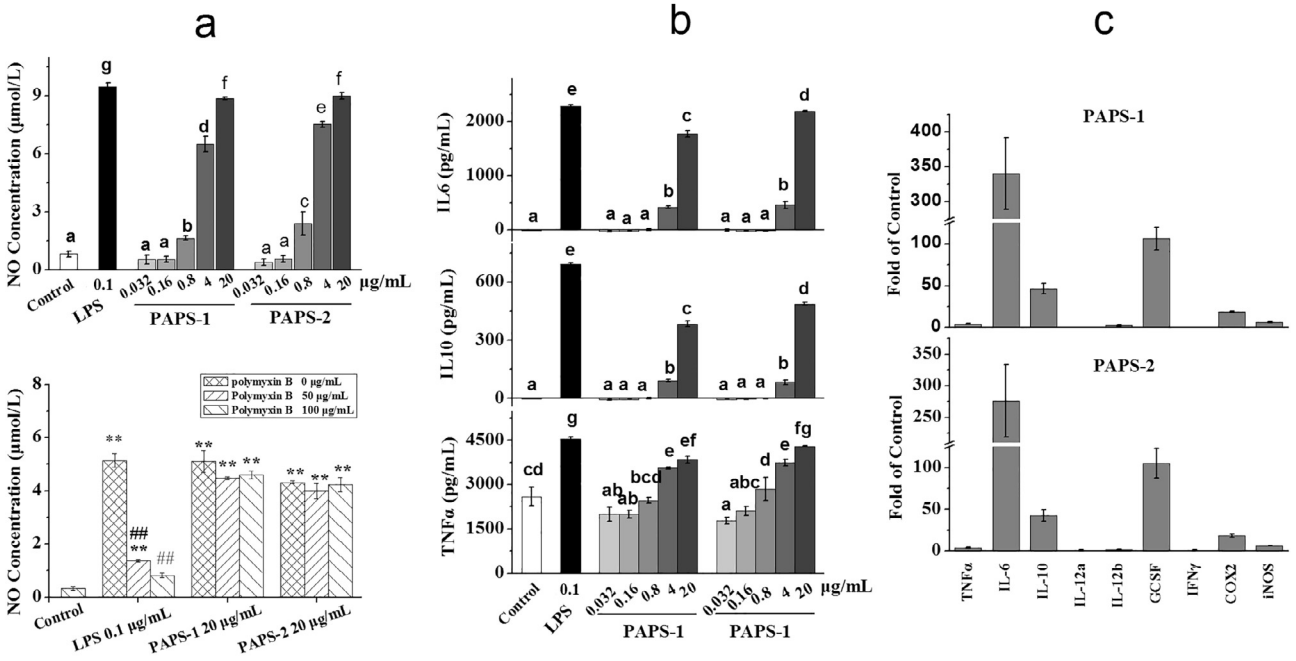

Fig. 3. RAW264.7 cell activation by PAPS-1 and PAPS-2. (a) NO release detection; (b) Elisa detection of IL-6, IL-10 and TNFα; (c) mRNA expression detection by qRT-PCR.

Kosmala et al., 2013; Popov et al., 2014; Simas-Tosin et al., 2012).

3.3. Immune-enhancing activity of PAPS-1 and PAPS-2

RAW264.7 cells, which release NO in an activated state, has been commonly used in evaluating the immune regulation activity of compounds. In the present study, both PAPS-1 and PAPS-2 significantly induced the NO release by RAW264.7 cells, with significant dosage effects (Fig. 3a). The mRNA level of the enzyme responsible for inducible NO synthesis, iNOS, was significantly elevated by PAPS-1 and PAPS-2 treatment (Fig. 3c). To eliminate the possibility that the induction was due to the pollution of LPS during the cherry polysaccharides extraction, a concentration gradient of polymyxin B (a specific LPS inhibitor) (Pugh et al., 2008) were applied to neutralize the effect of LPS. The results showed that, the effects of PAPS-1 and PAPS-2 could not be inhibited by the polymyxin B, while the effect of LPS treatment was significantly inhibited, indicating that the effect of PAPS-1 and PAPS-2 was not due to the contamination by LPS (Fig. 3a).

Various immune factors were induced by PAPS-1 and PAPS-2. By Elisa analysis, cellular release of TNFα, IL6 and IL10 were shown to be elevated by PAPS-1 and PAPS-2, with significant dosage effects (Fig. 3b). Several immune-related genes were further detected by qRT-PCR. The immune-associated cytokines (TNFα, IL6, IL10, GCSF), and a key enzyme required for the conversion of arachidonic acid into prostaglandins (COX-2) were significantly elevated at the mRNA level (Fig. 3c), while IL12 and IFNγ were not affected.

Immune regulation activity is one of the most important activities of polysaccharides. Several studies have indicated that pectic polysaccharides, which are important structural polysaccharides in plants, have good immunomodulatory effects. For example, the pectic polysaccharides from peach (*Prunus persica*) and mulberry (*Morus alba*), which are composed of uronic acids and seven neutral monosaccharides, were effective in activating mouse peritoneal macrophage (Simas-Tosin et al., 2012) and inducing NO release from RAW264.7 cells, and meanwhile inducing the expression of immune-related genes including TNFα, IL6 and COX-2 (Lee et al., 2013). The result of present study was in agreement with the studies on peach and mulberry.

However, not all pectic polysaccharides can induce the immunoreaction. For example, the pectic polysaccharides fraction from *Achillea millefolium* increased the cytokine secretion from LPS induced THP-1 monocytes, while not inducing cytokine secretion from THP-1 monocytes in the absence of LPS (Freysdottira, Logadottir, Omarsdottir, Vikingsson, & Hardardottir, 2016). Some pectic polysaccharides even inhibited the activation of immune cells. For example, the pectins with 30–90% esterification from citrus inhibited the LPS-induced increase of nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) of macrophages (Chen et al., 2006), and inhibited the LPS-induced increase of TNF and IL6 from human peripheral blood mononuclear cells (Salman, Bergman, Djaldetti, Orlin, & Bessler, 2008). So far, the structure-function relationship of pectic polysaccharides is still not clear. Ho et al. (2015) proved that the reticulate structure of the polysaccharides was necessary for the bioactivity of polysaccharides. The PAPS-1 and PAPS-2 obtained in the present study possessed branched structures formed from an RG-I type main chain and glycan side chains, which might be the reason for their immune regulatory activities.

4. Conclusions

Combining ethanol precipitation, DEAE-Sepharose chromatography and Sephacryl S200 chromatography, two water soluble polysaccharides components PAPS-1 and PAPS-2 were isolated from the flesh of *Prunus avium*. According to the results of monosaccharides analysis, IR analysis and NMR spectroscopy, the PAPS-1 and PAPS-2 were deduced to be RG-I pectic polysaccharides, with glycan side chains composed of arabinose and galactose. PAPS-2 possessed a higher proportion of galacturonic acid than PAPS-1. Both PAPS-1 and PAPS-2 showed immune-enhancing activity, and significantly induced the release of NO from macrophage-like RAW264.7 cells, while inducing the expression of several immune-related mRNAs (TNFα, IL6, IL10, GCSF, iNOS, COX-2) in RAW264.7 cells.

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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.foodchem.2018.01.145.

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