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Characterization of polysaccharides with marked inhibitory effect on lipid accumulation in *Pleurotus eryngii*

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ABSTRACT

Mushrooms have a great potential for the production of useful bioactive metabolites. To explore the bioactive compounds from edible mushrooms for interfering with the development of macrophage-derived foam cells, which is recognized as the hallmark of early atherosclerosis, eight types of mushrooms polysaccharides had been selected to be tested. Consequently, different mushrooms polysaccharides displayed diverse component profiles. Of polysaccharides that we tested, the *Pleurotus eryngii* polysaccharide had the strongest inhibitory effect on lipid accumulation. Furthermore, through fractionation of DEAE-52 and Sephadex G-100, the polysaccharide from *P. eryngii* had been successfully purified and identified. By the analysis of IR, GC, and HPLC, the purified polysaccharide was estimated to be 30–38 kDa for the average molecular weight with the monosaccharide composition mainly composed of D-types of mannose, glucose and galactose. Findings presented in this report firstly provide direct evidence, which links the purified polysaccharide moiety with the biological function in foam-cell model.

1. Introduction

Atherosclerosis is a common disorder and a leading cause of heart and vascular diseases throughout the world (Glass & Witztum, 2001). It occurs when fat, cholesterol and especially macrophage-derived foam cells build up in the walls of the arteries and form hard structures called plaque (Aviram & Fuhrman, 2002). It is generally accepted that the main reason for the formation of foam cell is macrophages' uncontrolled uptake of oxidized low-density lipoprotein (oxLDL), which leads to excessive lipid accumulation inside the cells (Berliner & Heinecke, 1996; Marsche, Zimmermann, Horiuchi, Tandon, Sattler & Malle, 2003; Steinberg, 1997). Several lines of evidence suggest that the macrophage-derived foam cells play integral roles in all stages of atherosclerosis (Li et al., 2004; Liu, Lee, Shih, Chyau & Wang, 2008). Thus, effectively disrupting lipid accumulation can hinder the formation of foam cells and prevent the development of atherosclerosis (Aviram & Fuhrman, 1998; Chen et al., 2011). However, the limited efficacy of current treatment strategies for stopping foam-cell formation requires new therapeutic options to be explored. Therefore, searching for new bioactive compounds to inhibit the development of macrophage-derived foam cells is particularly promising strategy.

Mushrooms have been treasured as a natural way to support health for thousands of years; they are a very popular food in Asia (Bisen, Baghel, Sanodiya, Thakur & Prasad, 2010; Lindequist, Niedermeyer & Julich, 2005). Moreover, it has been demonstrated that many kinds of mushrooms, have medicinal or functional properties in remedies for diseases, including *Lentinus edodes*, *Ganoderma lucidum*, *Trametes versicolor*, *Schizophyllum commune*, and *Flammulina velutipes*, etc. (Wasser & Weis, 1999; Kues & Liu, 2000). Over the past decade, the polysaccharides from edible mushrooms have received increasing attention and been extensively used for preventing or attenuating human diseases, such as cancers, inflammatory diseases, hyperlipidemia, diabetes, atherosclerosis, and liver damage, etc. (Zong, Cao & Wang, 2012). The potent ability of bioactive polysaccharides in medicinal mushrooms to modulate various important immune cells may be due to their structural diversity (Lindequist, Niedermeyer & Julich, 2005) (Sharon & Lis, 1993). Several reports have already confirmed that mushroom polysaccharides possess hyperlipidemic and anti-atherosclerotic properties, and these pharmaceutically active polysaccharides can be derived from fruit bodies (Chen et al., 2008; Chen, Mao, Yong, Li, Wei & Lu, 2012; Li, Zhang & Ma, 2010), fermented mycelia (Koh,

Abbreviations: oxLDL, oxidized low density lipoprotein; PEPE, polysaccharide from *P. eryngii* fruiting body; HPLC, high-performance liquid chromatography; IR, infrared; GC, gas chromatography; NMR, nuclear magnetic resonance.

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Kim, Chang & Suh, 2003) and cultural broth (Jeong et al., 2007; Lee et al., 2003; Yang, Jeong & Song, 2002; Yang, Park & Song, 2003). Notably, our previous study has verified that the crude polysaccharide from *Pleurotus eryngii*-PEPE was able to decrease the lipid level in high-fat loaded mouse model (Chen, Mao, Yong, Li, Wei & Lu, 2012). However, little is known for the real bioactive components in *P. eryngii* or whether polysaccharides from other mushrooms have the similar function to that of PEPE. In addition, it is hard and time-consuming to screen these bioactive components effectively using mouse model. Thus, in this study, through the oxLDL-inducing approach, we set up a macrophage-derived foam cell system to screen for and identify the biologically functional polysaccharides from the eight types of edible mushrooms. Our results indicated that different types of edible mushrooms have the diverse polysaccharide components. Among them in selected edible mushrooms, *P. eryngii* polysaccharide had the strongest ability to inhibit lipid accumulation. To our knowledge, this is the first report showing the evidence that linked the purified polysaccharide from mushroom with the function of inhibitory effect on lipid accumulation in foam cell model.

2. Materials and methods

2.1. Materials and reagents

The mushroom fruiting bodies of *Pleurotus eryngii*, *Lentinus edodes*, *Pleurotus ostreatus*, *Pleurotus nebrodensis*, *Hypsizygus marmoreus*, *Flammulina velutipes*, *Ganoderma lucidum* and *Hericium erinaceus* were purchased from Dashan group Ltd. Co. of China. The dried fruiting bodies were pulverized in an electric mill to become the powder at a size which was able to pass through a 0.425 mm sieve, and then the powder was packed in a glass jar and stored at room temperature until use. DEAE cellulose-52 and Sephadex G-100 were purchased from GE Healthcare (England, UK). Oxidative-LDL was obtained from Yiyuan biotechnology Ltd. Co. (Guangzhou, China). Standard monosaccharide, trichloroacetic acid and standard dextrans (molecular weight 500, 70, 40, 20 and 10 KD) were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other un-described reagents used in this study belonged to the analytical grade category.

2.2. Extraction and preparation of the crude polysaccharides

Extraction and preparation of the crude polysaccharides from mushroom fruiting bodies were carried out as previously described (Chen, Mao, Yong, Li, Wei & Lu, 2012). The crude polysaccharide content was measured by the phenol–sulfuric acid method using glucose as standard (Dubois, Gilles, Hamilton, Rebers & Smith, 1956).

2.3. Purification of the polysaccharide from P. eryngii

The crude polysaccharide from *P. eryngii* fruiting body (PEPE) was dissolved in the distilled water and passed through a DEAE-52 cellulose anion-exchange chromatography column (30 cm × 2.6 cm, GE Healthcare, UK), and then eluted with 0.05 mol/L PBS, followed with gradient solution of 0.05–1.0 mol/L NaCl at a flow rate of 1.0 ml/min. After that, all eluted fractions (each for 8 ml), were collected and quantified for the polysaccharide content by the phenol–sulfuric acid method using glucose as standard (Dubois, Gilles, Hamilton, Rebers & Smith, 1956). Based on the quantified curve, the major polysaccharide fraction-PEPE-A was obtained and further loaded on sephadex G-100 gel column (60 cm × 1.6 cm, GE Healthcare). After eluted with 0.1 mol/L PBS at a flow rate of 0.5 ml/min, two major fractions referred PEPE-A1 and PEPE-A2 were collected, dialyzed and then lyophilized to the pellets. For the evaluation for the polysaccharide purity, high-performance liquid chromatography (HPLC) with the analysis column (Bio-rad Aminex HPX-87H, USA) was performed according to the previously described (Chen, Mao, Yong, Li, Wei & Lu, 2012).

2.4. Structural characterization of purified polysaccharides

2.4.1. Infrared (IR) spectra of purified fractions

Above purified polysaccharides were ground with KBr powder and then pressed into pellets for IR measurement in the frequency range of 4000–500 cm^{-1}. IR spectra of polysaccharides were recorded on an infrared spectrometer (Nicolet, USA).

2.4.2. Molecular weight determination and monosaccharide composition analysis

The molecular weight of the purified polysaccharide was determined by HPLC instrument (Agilent 1100, USA) with a carbohydrate analysis column (Bio-Rad Aminex HPX-87H, USA), using a mobile phase condition (5 mmol/l sulphuric acid at a rate of 0.5 ml/min) and a refractive index detector. The calibration curve was made based on a series of different molecular sizes of dextrans (500, 70, 40, 20 and 10 kD) as standard.

Monosaccharide composition of polysaccharides was determined by gas chromatography (GC) (CP3800, Varian, USA) according to the previously described method (Luo, He, Zhou, Fan, Luo & Chun, 2010) with some modifications. Briefly, the samples were hydrolyzed with 4 ml of 2 M trifluoroacetic acid at 110 °C for 4 h, and the resulting solution was concentrated in vacuum and the excess of acid was removed by repeated co-distillations with methanol. Then, the hydrolyzed products were acetylated with 8 mg of hydroxylamine hydrochloride and 0.5 ml of pyridine for 30 min at 90 °C. After that, the mixture was cooled at room temperature, and then added by 0.3 ml of acetic anhydride and incubated for another 30 min at 90 °C. After cooling, the solution was added 1 ml of water and 0.5 ml of methylene chloride, and then the lower layer solution was collected to be dried in a vacuum. The pellet was dissolved by methylene chloride for further analysis. Alditol acetates for standards (glucose, mannose, rhamnose, galactose, xylose, fructose and arabinose) were also carried out using the same approach as above described for the sample. Lastly, all above prepared samples and standards were loaded onto an CP-8 column (Varian, USA) for the further analysis under the followed condition: N_2: 1.0 ml/min; injection temperature: 240 °C; detector temperature: 240 °C; column temperature programmed: 160 °C for 2 min, then increased to 240 °C at 5 °C/min and maintained for 15 min at 240 °C.

2.4.3. Nuclear magnetic resonance (NMR) spectrum analysis

The ^{1}H and ^{13}C NMR spectra of the polysaccharide were recorded using Bruker AV-500 NMR spectrometer (Bruker, Rheinstetten, Germany). The polysaccharide sample was dissolved in D_2O and then examined at 27 °C. Chemical shift was expressed in ppm.

2.5. Cell culture, oil red O staining, lipid content measure, and MTT assay

RAW264.7 cells (a murine macrophage/monocyte like cell line, ATCC, TIB-71) were cultured in DMEM (glutamine, high glucose) supplemented with 10% fetal bovine serum (Wisent Ltd. Co., Canada) at 37 °C for 1 h, and then incubated with 70 μg oxLDL per ml medium for 12 h. After that, polysaccharides or Simvastatin at the indicated concentration were added to media and incubated for 24 h. Then, the cells were fixed in 4% paraformaldehyde for 30 min and stained by oil red O for 1 h. After removed floating color by 60% isopropyl alcohol, the image for cells was taken by an inverted microscope (Ti-S, Nikon, Tokyo, Japan) equipped with a CCD camera

(DS-Qil, Nikon), and analyzed with a NIS-Elememts D 3.0 software (Nikon, Tokyo, Japan). Final images used in figures were assembled in Adobe Photoshop CS3 (Adobe, San Jose, CA).

The assay for the lipid content in RAW264.7 cells was performed according to the previously described (Lin et al., 2011). Briefly, cells were extracted with isopropanol for 24 h after oil red O staining, and then the crude extract was centrifuged at 5000 × *g* for 5 min. The absorbance of the supernatant was measured at 510 nm using a microplate reader (SpectraMax M2, Molecular Devices, USA). All treatments were carried out in triplicate. Normalized data for the lipid content were calculated according to the followed equation: Lipid content (%) = $[(A_S - A_N)/(A_C - A_N)] \times 100$, where A_S is the absorbance of sample group, A_N is the absorbance of the normal group, and A_C is the absorbance of the control group. The measurement for total or free cholesterol content was performed according to previously described (Gamble, Vaughan, Kruth & Avigan, 1978). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was carried out as described previously (Hamid, Rotshteyn, Rabadi, Parikh & Bullock, 2004).

3. Results

3.1. Screening of polysaccharides from mushrooms for the inhibiting lipid accumulation ability

To extract and obtain mushroom polysaccharides, eight different types of mushroom fruiting bodies were selected. As indicated in Section 2, hot-water extraction was used to obtain all crude extracts. To compare the polysaccharide yield among these selected mushrooms, the content and the extraction rate of polysaccharide were measured (Dubois, Gilles, Hamilton, Rebers & Smith, 1956). As shown in Table 1, the weight percentage of the polysaccharide to dried weight in *Pleurotus nebrodensis* reached to 19.33% in crude extracts so that it was the highest one in these selected mushrooms and almost 17 times of that in *Ganoderma lucidum*, which only had 1.13% content of polysaccharide. It is not excluded that hot water extraction possibly is not the suitable method to obtain polysaccharide effectively due to *G. lucidum* having a much harder wall structure than the others. In comparison, *Hericium erinaceus*, *Pleurotus ostreatus*, *Flammulina velutipes*, *Pleurotus eryngii, Lentinus edodes* and *Hypsizygus marmoreus* had relatively lower polysaccharide yields compared to the highest one as shown in Table 1. Most interestingly, the polysaccharide component profile analyzed by HPLC clearly indicated that the different types of edible mushrooms had the completely diverse patterns as shown in Fig. 1. These data suggest that each of selected polysaccharides may have its own specific polysaccharide components.

To further screen and identify these mushroom polysaccharides for the potential biological activities in inhibiting the lipid accumulation, we used macrophage-derived foam cells induced by oxLDL as a model. The murine RAW 264.7 macrophages were incubated with oxLDL, and then the eight treatment groups were amended with related polysaccharides in the media for 24 h. In comparison, the same volume of media was added to the control group. For normal group, no oxLDL was added in the media for all the cultured time. As expected, in the control group, oxLDL successfully induced the formation of foam cells, resulting in many accumulated lipid droplets stained by the oil red O in murine RAW 264.7 macrophages (Fig. 2A). Interestingly, the lipid accumulation showed statistically significant decreases in seven groups treated by polysaccharides from *P. eryngii, L. edodes, P. ostreatus, P. nebrodensis, H. marmoreus, H. erinaceus* ($p < 0.01$) and *F. velutipes* ($p < 0.05$) compared to the control group (Fig. 2A and B). However, there was no detectable difference in the *G. lucidum* polysaccharide-treated group compared to the control group. These data suggest that the polysaccharides extracted from different species of edible mushrooms have varying biological functions on lipid accumulation. Notably, the *P. eryngii* polysaccharide had the strongest inhibitory effect on lipid accumulation, resulting in only about 28.06% of lipid content left inside the cell compared to 100% in the control. In addition, in case of side effect of *P. eryngii* polysaccharide, MTT assay was carried out in the murine RAW 264.7 macrophages. As predicted, it had no detectable effect on the cell viability (Fig. S1). Thus, the polysaccharide from *P. eryngii* (hereafter referred to as PEPE) was selected to be studied further.

To test whether PEPE affects the lipid accumulation in murine RAW 264.7 macrophages in a dose-dependent way, different concentrations of PEPE (50, 100, and 200 μg/ml) were used in the cultured media. Because Simvastatin has been used as a clinical drug for preventing hyperlipidemia (Toolbar, 1994), we used this drug as a control. As predicted, PEPE significantly lowered the lipid content to about 62.42 ± 12.65%, 41.71 ± 9.92%, and 29.11 ± 7.63% of the control at the concentrations of 50, 100, and 200 μg/ml, respectively, in a dose-dependent manner ($p < 0.01$) (Fig. 3B). Surprisingly, the effect of PEPE at the concentration of 200 μg/ml was comparable with that of Simvastatin at the same concentration (Fig. 3A and B). These results clearly indicated that PEPE is a potentially effective reagent with its ability to decrease lipid accumulation in foam cells. As shown in Fig. S2, the data of lipid content analyzed by oil red O staining is comparable to the content of total and free cholesterol. Thus, we chose oil red O staining to quantify the lipid content in our followed experiments.

3.2. Purification of polysaccharides from the fruiting bodies of P. eryngii and identification of its functional components

To separate the bioactive component, dissolved PEPE was fractionated on a DEAE-52 cellulose anion exchange column. As shown in Fig. 4A, two main polysaccharide fractions were obtained: PEPE-A and PEPE-B. At the 200 μg/ml concentration, PEPE-A was able to significantly decrease the amount of lipid droplets, resulting in 18.82 ± 0.82% ($p < 0.01$) of lipid content in murine RAW 264.7 macrophages compared to the control group induced by oxLDL (Fig. 4B). In comparison, PEPE-B had no detectable effect on lipid accumulation under the same condition. These results suggest that PEPE-A (but not PEPE-B) may possibly be a main

Table 1
Polysaccharide yield measurement in eight common mushrooms.

Strain	Crude extracts[a] (g/100 g dried weight)	Polysaccharide content[a] (g/100 g dried weight)
Pleurotus eryngii	7.69 ± 0.10	7.46 ± 0.10
Pleurotus nebrodensis	20.41 ± 0.69	19.33 ± 0.65
Hericium erinaceus	12.18 ± 0.33	11.77 ± 0.32
Ganoderma lucidum	1.28 ± 0.19	1.13 ± 0.17
Lentinus edodes	3.51 ± 0.07	3.33 ± 0.07
Pleurotus ostreatus	9.79 ± 0.20	9.21 ± 0.19
Hypsizygus marmoreus	6.19 ± 0.34	5.65 ± 0.31
Flammulina velutipes	10.05 ± 0.37	9.03 ± 0.33

[a] Values are presented as mean ± standard deviations.

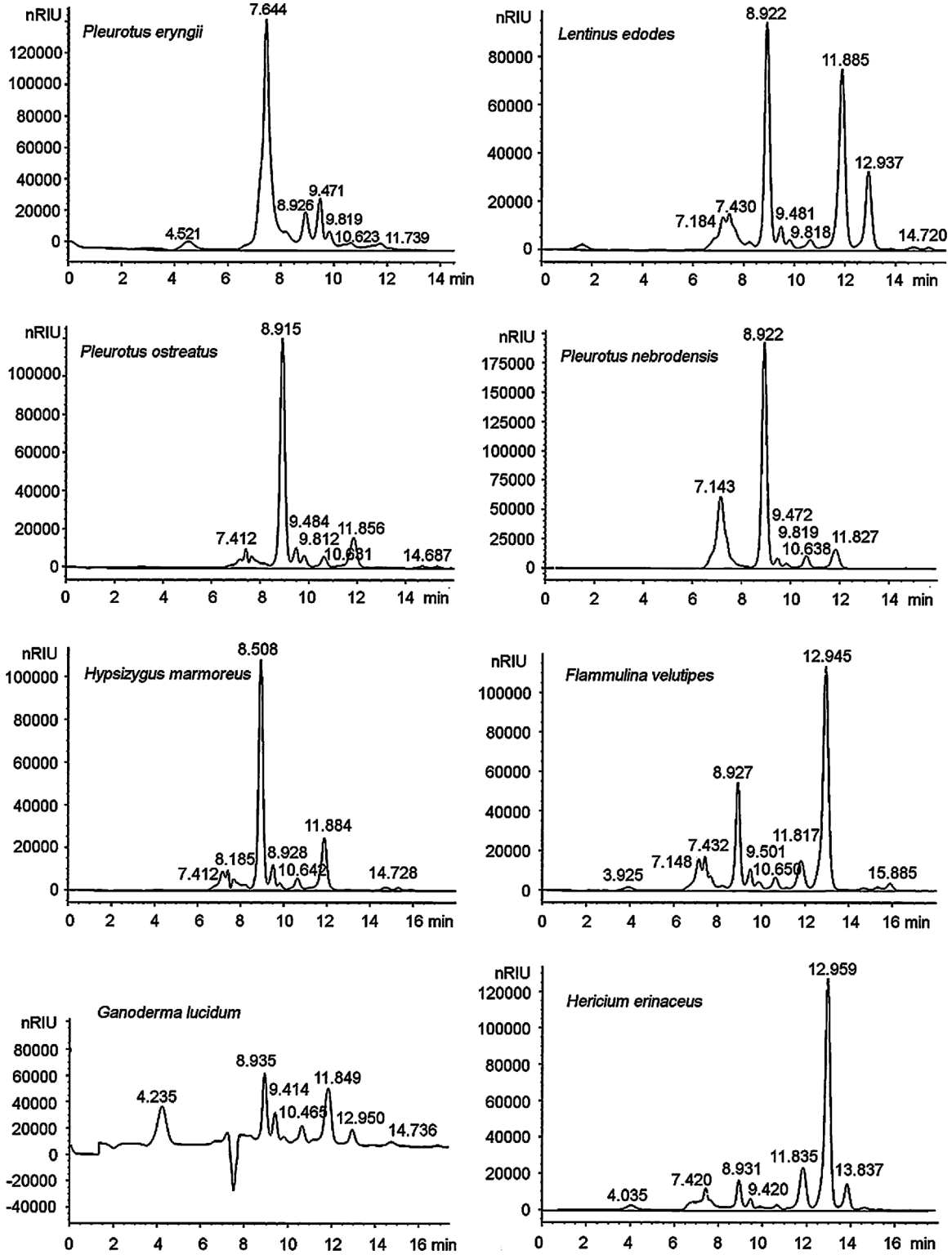

Fig. 1. The polysaccharide component profile from eight types of tested mushrooms by HPLC using RI (refractive index) detector with 5 mM H_2SO_4 at 0.5 ml/min (Bio-Rad, Aminex HPX-87H).

bioactive component in PEPE that attenuates the lipid accumulation of oxLDL-induced foam cells. Because PEPE-A produced two undivided peaks when observed with HPLC (Fig. S3), the Sephadex G-100 column (which separated substances according to their different molecular weights) was used to further purify PEPE-A. As we expected, two major polysaccharides from PEPE-A were isolated: PEPE-A1 and PEPE-A2 (Fig. 5A). Analysis with HPLC revealed that both PEPE-A1 and PEPE-A2 displayed a single peak; this may indicate that each is composed of only one component (Fig. 5B). To investigate the purified components' ability to decrease lipid content, PEPE-A1 and PEPE-A2 were modified with media as described above. As shown in Fig. 5C and D, both PEPE-A1 and PEPE-A2 significantly reduced the lipid content to $20.71 \pm 6.00\%$ and $18.49 \pm 1.59\%$ of the control group induced by oxLDL, respectively,

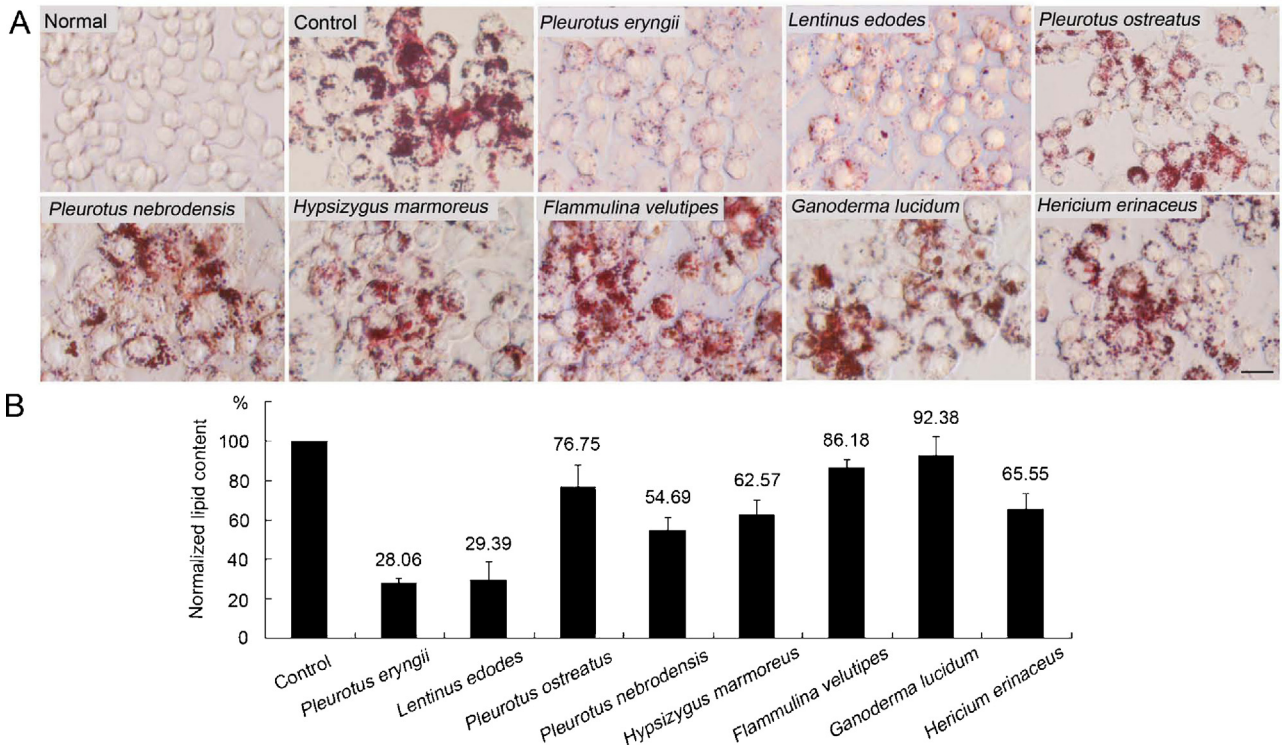

Fig. 2. Screening for the bioactive polysaccharides from edible mushrooms that inhibited lipid accumulation in macrophage-derived foam cells. (A) Murine RAW 264.7 macrophages were cultured in DEME containing with 10% fetal bovine serum at 37 °C for 1 h in the 12-well plate, and incubated with 70 μg oxLDL per ml medium for 12 h, then the cells were divided into nine groups, each group included three cultured cells in the 12-well plate for three independent experiments. Among them, the eight different groups were exposed to selected polysaccharides at the final concentration of 200 μg/ml in the medium for 24 h. The control group was added with the same amount volume of media as polysaccharides. For a normal group, no oxLDL was added in the media for all the cultured time. Cells were fixed, and stained with oil red O. Bar, 20 μm. (B) Quantification data of lipid content correlated to treatment in (A). Data represent mean ± SD from three independent experiments.

at the 200 μg/ml concentration. In terms of their ability to inhibit lipid accumulation in foam cells, no remarkable difference between PEPE-A1 and PEPE-A2 was observed.

3.3. Structural characterization of purified PEPE-A1 and -A2

To analyze the structural characteristics of the aforementioned functional components, the following experiments were conducted.

3.3.1. IR spectra of purified fractions

IR spectra of the purified PEPE-A1, PEPE-A2 were analyzed with a Nicolet 560 IR spectrometer that was equipped with a DTGS detector. As shown in Fig. 6A, both the two bioactive components showed similar absorption bands, which appeared at 3424 cm^{-1} (hydroxyl stretching vibration), 2926 cm^{-1} (C—H stretching vibration), 1600–1650 cm^{-1} (C═O vibration), 1430–1390 cm^{-1} (carboxyl groups), and 950–1200 cm^{-1} (pyranose ring). Based on published data, this analysis clarified that purified PEPE-A1 and PEPE-A2 had the typical absorption of polysaccharides (Ge, Duan, Fang, Zhang & Wang, 2009; Xie, Xie, Nie, Shen, Wang & Li, 2010).

3.3.2. Molecular weight determination and monosaccharide composition analysis

The molecular weights (Mw) of these purified polysaccharides were evaluated and determined using HPLC. A series of different molecular sizes of standard dextrans (T-500, T-70, T-40, T-20, and T-10) were used for the calibration curve based on the retention time of HPLC. As a result, the equation of the standard curve was as follows: $M_w = -0.0012\ t + 7.833$ (where t represents retention time) with a correlation co-efficient of 0.975. Consequently, the average molecular weights of PEPE-A1, PEPE-A2 were estimated to be 37.50 kDa and 30.00 kDa, respectively. Moreover, these polysaccharides were hydrolyzed and acetylated as described in Section 2, and GC was performed to further analyze the monosaccharide composition. Interestingly, as shown in Fig. 6B, the monosaccharide composition of the two fractions was similar. All were mainly composed of D-mannose, D-glucose, and D-galactose according to the retention time deduced from monosaccharide standards (L-rhamnose, D-arabinose, D-xylose, D-mannose, D-glucose, D-galactose, and D-fructose) (Fig. 6B). However, there were some small differences related to the ratio of the monosaccharide composition. PEPE-A1 included 96.26%, 2.32% and 1.42% of glucose, galactose and mannose, respectively, whereas PEPE-A2 comprised 95.54% glucose, 3.28% galactose and 1.18% mannose, instead.

3.3.3. NMR spectrum analysis

To analyze the glycosidic linkage of molecular structure for these polysaccharides, NMR spectrum analysis was performed. As shown in Fig. 7A, the proton NMR spectrum (500 MHz) of PEPE-A1 displayed the five different signals in the anomeric region at 4.63, 4.61, 5.36, 5.40, and 5.41 ppm in a molar ratio of (roughly) 3:2:1:1:1. These anomeric signals were labeled “a”, “b”, “c”, “d”, and “e”, respectively. Meanwhile, the carbon NMR spectrum (125 MHz) of PEPE-A1 in Fig. 7B also showed five different signals that appeared at 103.9, 103.8, 102.4, 102.1, and 101.7 ppm in a molar ratio of (roughly) 2:3:1:1:1, which corresponded to anomeric signals of “b”, “a”, “e”, “d”, and “c”, respectively. For residue “a”, the proton signals (4.63 ppm) and the coupling constants ($J_{H\text{-}1,H\text{-}2}$ (~8 Hz) and $J_{C\text{-}1,H\text{-}1}$ (~160 Hz)) verified its β-configuration. Large coupling constants $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ (~10 Hz) indicated there was a D-glucopyranosyl moiety. In addition, the downfield shift of C-3 (80.6 ppm) and C-6 (72.9 ppm) with respect to the standard values

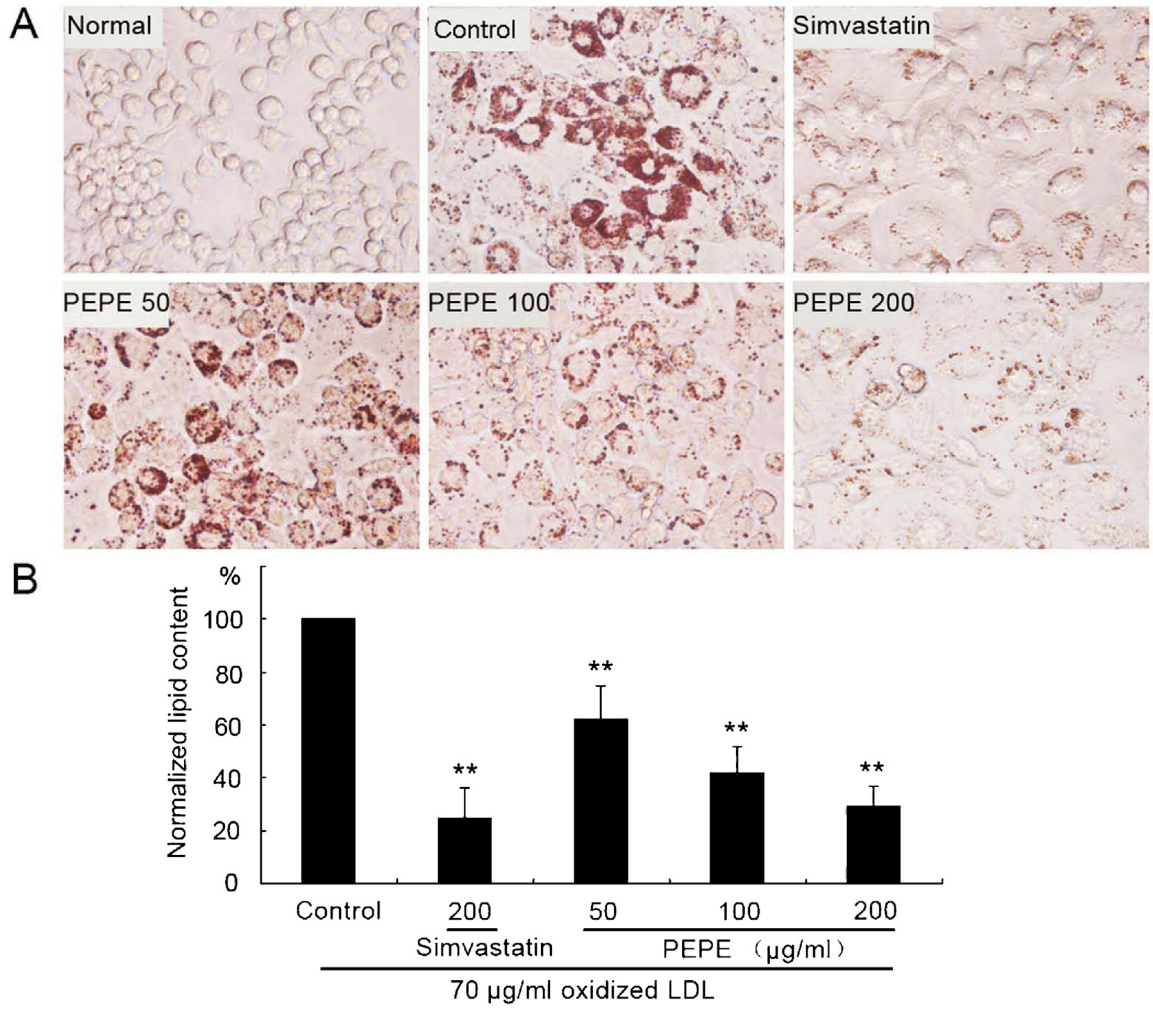

Fig. 3. PEPE lowered the lipid content in dose-dependant manner in oxLDL-induced foam cells. (A) Murine RAW 264.7 macrophages were pretreated with 70 μg oxLDL per ml medium for 12 h, and then added with the indicated concentrations of PEPE for additional 24 h. Cells were fixed, and stained with oil red O. Bar, 20 μm. (B) Quantification data of lipid content correlated to (A). Data represent mean ± SD from three independent experiments. The significance was set at the levels $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the control group.

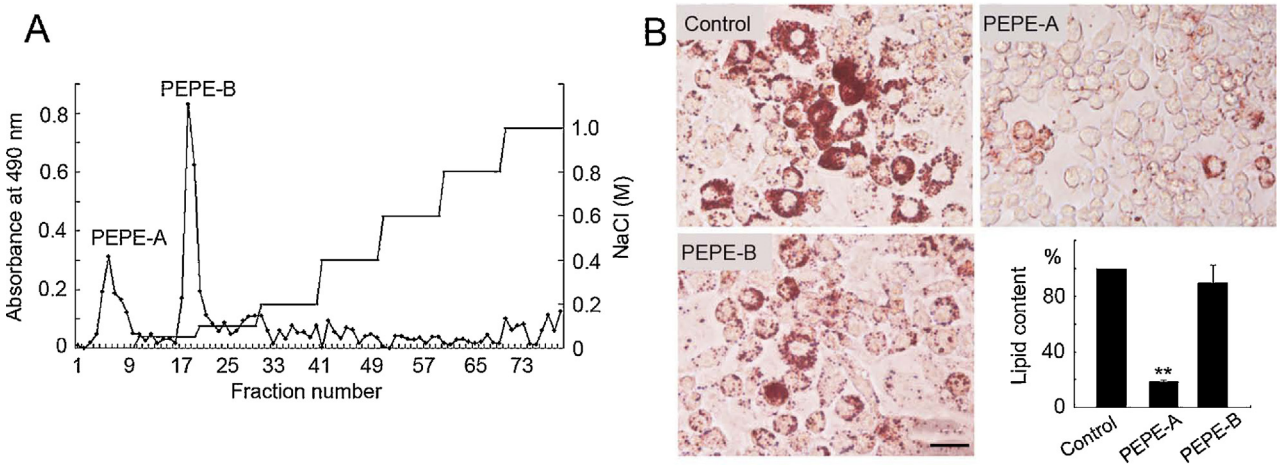

Fig. 4. Separation and identification of functional components in *P. eryngii* polysaccharides. (A) Elution profiles of PEPE on a DEAE-cellulose column (30 cm × 2.6 cm, GE Healthcare) eluted with 0.05 mol/L PBS and followed with a gradient of 0.05, 0.1, 0.2, 0.4, 0.8, and 1.0 mol/L NaCl at 1 ml/min. (B) Comparison of PEPE-A and PEPE-B on the effects of lowering lipid accumulation in Murine RAW 264.7 macrophages. Cells were pretreated with 70 μg oxLDL per ml medium for 12 h, then treated with PEPE-A and PEPE-B at the final concentration of 200 μg/ml for additional 24 h. Cells were fixed, and stained with oil red O. Bar, 20 μm. The quantification data of lipid content in B was shown in lower right corner. Data represent mean ± SD from three independent experiments. The significance was set at the levels $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the control group.

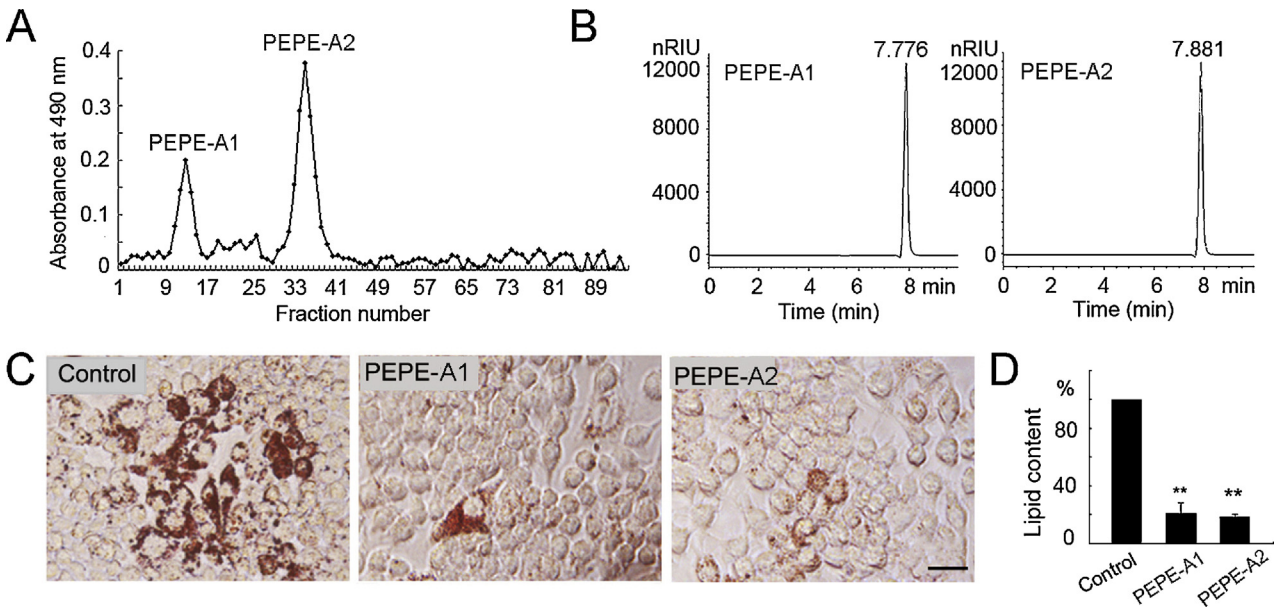

Fig. 5. (A) Elution profiles of PEPE-A on a Sephadex G-100 gel column (60 cm × 1.6 cm, GE Healthcare) eluted with 0.1 mol/L PBS at 0.5 ml/min. (B) HPLC chromatograms of PEPE-A1 and PEPE-A2 using RI (refractive index) detector with 5 mM H_2SO_4 at 0.5 ml/min (Bio-Rad, Aminex HPX-87H). (C) Comparison of PEPE-A1 and PEPE-A2 on the effects of lowering lipid contents in Murine RAW 264.7 macrophages. Cells were pretreated with 70 μg oxLDL per ml medium for 12 h, then treated with PEPE-A1 and PEPE-A2 at the final concentration of 200 μg/ml for additional 24 h. Cells were fixed, and stained with oil red O. Bar, 20 μm. (D) Quantification data of lipid content correlated to (C). Data represent mean ± SD from three independent experiments. The significance was set at the levels $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the control group.

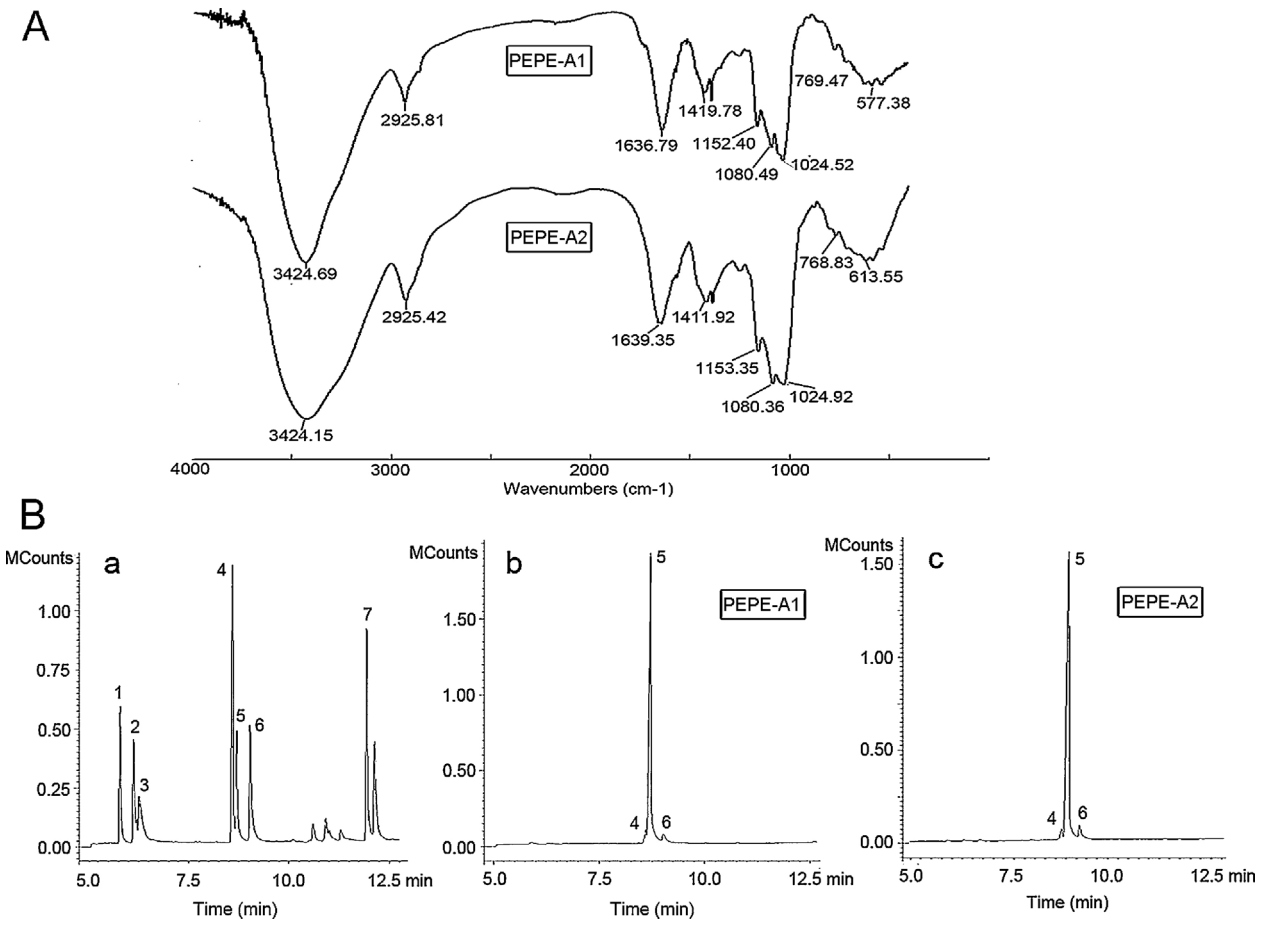

Fig. 6. (A) IR spectra of the polysaccharides of PEPE-A1 and PEPE-A2 recorded on an infrared spectrometer (Nicolet, USA). (B) Gas chromatograms of acetylated monosaccharides. (a): standard, the number represents different standard monosaccharides. (1) L-rhamnose, (2) D-arabinose, (3) D-xylose, (4) D-mannose, (5) D-glucose, (6) D-galactose, (7) D-fructose. (b): PEPE-A1, (c): PEPE-A2. All samples were hydrolyzed, and then to be acetylated for further analysis by gas chromatography.

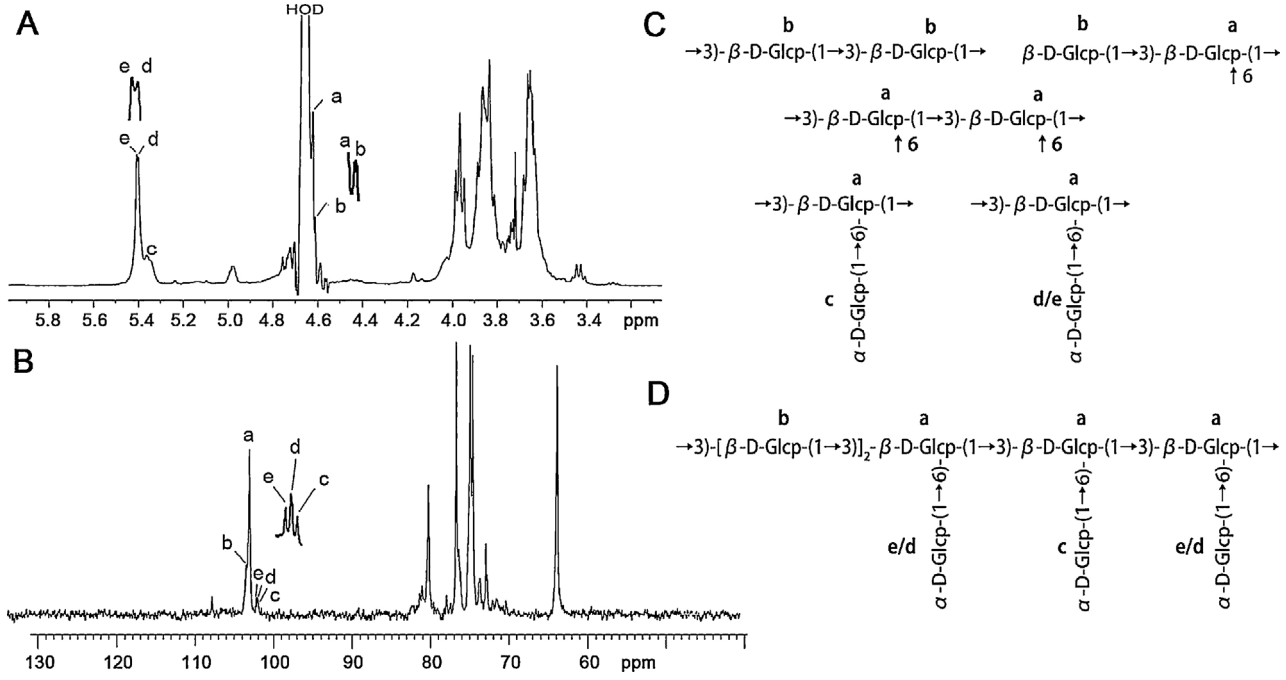

Fig. 7. NMR spectrum analysis of PEPE-A1. (A) ^{1}H NMR spectrum (500 MHz, D_2O, 27 °C) for PEPE-A1. Five anomeric signals appeared at 4.63, 4.61, 5.36, 5.40 and 5.41 ppm, were labeled as "a", "b", "c", "d" and "e", respectively. (B) ^{13}C NMR spectrum (125 MHz, D_2O, 27 °C) for PEPE-A1. Five anomeric signals appeared at 103.9, 103.8, 102.4, 102.1 and 101.7 ppm, were labeled as "b", "a", "e", "d", and "c", respectively. (C) The possible glycosidic linkage of molecular structure for purified polysaccharides. "b"-β(1 → 3) "b"; "b"-β(1 → 3) "a"; "a"-β(1 → 3) "a"; "c"-α(1 → 6) "a"; "d"/"e"-α(1 → 6) "a". (D) The predicted octa-saccharide repeating unit of purified polysaccharides.

of methyl glycosides referred that residue "a" was linked at C-6 and C-3. Thus, residue "a" was a (1 → 3,6)-linked-β-D-glucopyranosyl moiety. With the same analysis method, we concluded that residue "b" was a (1 → 3)-linked-β-D-glucopyranosyl moiety, and residues "c", "d", and "e" were (1 →)-linked-α-D-glucopyranose. Furthermore, the molar ratio of β-configuration and α-configuration of monosaccharides was determined according to the area integration of carbon NMR (103–106 ppm and 98–103 ppm) and proton NMR spectra (4.4–4.9 ppm and 4.9–5.8 ppm) using MestReNova (USA) software. The results suggested that PEPE-A1 and PEPE-A2 had similar molar ratios of β-configuration and α-configuration: (roughly) 5:3.

Based on all of the above data and as shown in Fig. 7C, the possible glycosidic linkage in the molecular structure of these polysaccharides has been identified as the following: "b"-β(1 → 3) "b"; "b"-β(1 → 3) "a"; "a"-β(1 → 3) "a"; "c"-α(1 → 6) "a"; "d"/"e"-α(1 → 6) "a". In comparison, PEPE-A2 had similar NMR spectra to PEPE-A1 as shown in Fig. S4. Thus, the possible main glycosidic linkage in the molecular structure of these purified polysaccharides was similar: β (1 → 3)-glucan as the main chain accompanied by the O-6 branched α-glucopyranosyl side chain as indicated in Fig. 7D.

4. Discussion

Many lines of previous evidence have demonstrated that polysaccharides from edible mushrooms have the potential to prevent or attenuate human diseases including cancers, inflammatory diseases, hyperlipidemia, diabetes, and atherosclerosis, etc. (Lindequist, Niedermeyer & Julich, 2005; Zhang, Cui, Cheung & Wang, 2007). Some mushroom polysaccharides have already been integrated into products for human health products and supplements for strengthening the body's immunity; these include *Lentinus edodes* (lentinan) (Chihara, Hamuro, Maeda, Arai & Fukuoka, 1970; Maeda & Chihara, 1971; Yu, LiHua, Qian & Yan, 2009), *Ganodema lucidum*, *Trametes versicolor* (krestin), and *Schizophyllum commune* (schizophyllan) (Rout, Mondal, Chakraborty, Pramanik & Islam, 2005). In our study, polysaccharides extracted from different species of edible mushrooms have been shown to have diverse abilities on lipid accumulation. Of polysaccharides from the eight mushroom species that we tested, PEPE derived from the group *P. eryngii* had the strongest inhibitory effect on lipid accumulation. Most importantly, the purified polysaccharides PEPE-A1 and PEPE-A2 from *P. eryngii* displayed the similar function to crude extracts-PEPE in the ability to inhibit the lipid accumulation. Moreover, we found PEPE-A1 and PEPE-A2 shared nearly the same structural features including molecular weight, monosaccharide composition, configuration and position of glycosidic linkages. Thus, based on above described characterization, possibly PEPE-A1 maybe is identical to PEPE-A2. The reason for the difference between them for their molecular weights (37.50 kDa and 30.00 kDa) is just simply degradation.

In addition, the results of monosaccharide composition suggest that except for D-glucose, these purified polysaccharides contained small amounts of D-mannose and D-galactose. It is likely that the inhibitory effect of lipid deposition may be due to this specific polysaccharide structural feature. Nevertheless, it is worthy noting that polysaccharides except for *P. eryngii* and *L. edodes* almost showed very weak effects on the lipid deposition of macrophage-derived foam cells. However, our unpublished data indicated that some polysaccharides such as *G. lucidum* polysaccharide could significantly inhibit the growth of human MCF-7 breast cancer cells (data not shown), although it hardly decreased the lipid content in foam cells, suggesting that polysaccharides from different species may have their own components and the specific structural features, resulting in the characteristic bioactive abilities.

Besides, several studies have demonstrated that cytokine interferon-gamma (IFN-g), as a part of the innate immune response, also plays the undeniably complex role during the pathology of atherosclerosis. Nevertheless, it has been reported that IFN-g is able to inhibit foam-cell formation by binding to the very low-density lipoprotein receptor (Harvey & Ramji, 2005). Meanwhile, previous studies have verified that the glucans from many kinds of

mushrooms possess the immuno-stimulating and the anti-tumor activities by inducing IFN-g production in monocytic cells (Harada, Miura, Adachi, Nakajima, Yadomae & Ohno, 2002). Therefore, it is not excluded that the polysaccharides from the mushrooms in this study also could affect the production of IFN-g, resulting in the inhibition of the foam cell formation to some extent. The detailed mechanisms are needed to be explored in future study.

Moreover, previous studies have verified that Simvastatin, as one of the most effective prescription statins, mainly has the function in suppressing the hydroxy methylglutaryl coenzyme A (HMG-CoA) reductase to control cholesterol biosynthesis (Hernández-Perera et al., 1998). Our results showed Simvastatin also decrease the lipid accumulation in foam cells. It is possible that Simvastatin may have more unexplored biological functions in addition to suppressing the action of HMG-CoA reductase. Collectively, our findings suggest that the polysaccharide from *P. eryngii* is a potentially effective reagent with biological activity that disrupts the formation of foam cells and possibly as a good drug candidate to prevent the process of atherosclerosis.

5. Conclusion

In the present study, using HPLC assay and the macrophage-derived foam cell model, we successfully set up an effective system to screen and identify the bioactive polysaccharide component with biological activity capable of disrupting the foam cells formation. Moreover, through fractionation of DEAE-52 cellulose anion exchange column and Sephadex G-100 column, the single polysaccharide component from *P. eryngii*, has been purified and identified, respectively. Our results indicated that crude polysaccharide from *P. eryngii* had the strongest inhibitory effect on lipid accumulation among these tested polysaccharides from eight types of mushrooms. Most importantly, the purified polysaccharides PEPE-A1 and PEPE-A2 showed the similar biological activity to crude polysaccharide from *P. eryngii*. Thus, our findings presented here firstly provide the direct evidence that links the purified polysaccharide with lipid accumulation in foam-cell model.

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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.2013.05.028.

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