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The excreted polysaccharide of *Pleurotus eryngii* inhibits the foam-cell formation *via* down-regulation of CD36

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ABSTRACT

Previous study has verified the polysaccharide from the fruiting body of *Pleurotus eryngii* (PEPE) is capable of decreasing the lipid content in both of cell-line and mouse model. However, little is known about underlying mechanisms and whether this bioactive polysaccharide exists in submerged culture. Here, we verified the excreted polysaccharides EP and EP-1 from submersion culture of *P. eryngii* have the remarkable inhibitory effects on lipid accumulation in macrophage-derived foam cells. Structure analysis indicates EP-1 consists of D-types of glucose, galactose and mannose with the main β(1 → 3)-glucan glycosidic linkage branched at O-6 by α-D-glucose while EP digested by β-1,3-glucanase fails to decrease the lipid accumulation, suggesting that the special structure is essential for its function. Expression analysis suggests that EP is able to cause the down-regulation of the scavenger receptor-CD36 on both transcription and protein levels. Most importantly, EP can be obtained by fermentation in a mass-production.

1. Introduction

For decades, mushrooms as the macro-fungi have been collected, cultivated and valued as edible and medicinal resources in most of the Asian countries (Royse, 2002). Various mushrooms are popular, not only for their texture and flavor but also for medicinal and nutritional characteristics (Mallavadhani et al., 2006). To date, there are more than 300 species of mushrooms have been verified to possess numerous therapeutic properties. Thus, the term "medicinal mushroom" has received increasing attention worldwide. Indeed, extensive studies have revealed that these fungi own their specific bioactive activities, including anti-oxidant, anti-tumor, hypoglycemic, hypolipidemic, and immunostimulating activities. Moreover, some of bioactive components from these medicinal fungi have been isolated and characterized, including polysaccharides, steroids, triterpenoids, polyphenol, *etc.* (Lin & Sung, 2006). Among them, one kind of the most important components belongs to the complex carbohydrates known as polysaccharides, which have cancer-fighting and immune-boosting abilities. Previous studies have indicated the pharmaceutically bioactive polysaccharides in medicinal mushrooms can be derived from the fruiting bodies, fermented mycelia, and submerged culture (Koh, Kim, Chang, & Suh, 2003; Wasser, 2011). However, the excreted polysaccharides from submerged culture have received more and more attentions mainly due to their rapid completion by fermentation. Most importantly, since excreted polysaccharides can be extracted easily and purified efficiently, it makes the industrialized production feasible.

As a popular type of edible mushroom, *Pleurotus eryngii* (*P. eryngii*) which belongs to *Pleurotus*, *Pleurotaceae*, *Agaricales*, *Basidiomycetes*, may have potential bioactive functions as described in Chinese traditional medicine. In our previously study, we have identified that the polysaccharide from the fruiting body of *P. eryngii* could remarkably decrease the lipid content in both of the high-fat-load mouse model and the macrophage-derived foam cell model (Chen et al., 2013; Chen, Mao, Yong, Li, Wei, & Lu, 2012a). However, little is known about whether this bioactive polysaccharide could be obtained by submerged fermentation. Especially, underlying mechanisms behind biological ability are still elusive.

It is well known that the development of the macrophage-derived foam cells plays the integral role in all stages of atherosclerosis (Zou, Lu, & Wei, 2005). The formation of foam cells is mainly due to uncontrolled uptake of oxidized low density lipoprotein (oxLDL) or impaired cholesterol efflux in macrophages, which leads to excessive lipid accumulation inside the cells (Chu, Tai, Beer, & Hill, 2013; Park et al., 2011). It is generally accepted

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that scavenger receptors (SRs) (SR-A and CD36) are responsible for the internalization of oxLDL (Voloshyna et al., 2013). By contrast, the efflux of accumulated cholesterol in macrophages is mediated through reverse cholesterol transporters (RCTs) including ATP-binding cassette transporter A1 and G1 (ABCA1 and ABCG1) and their upstream transcription factors *etc.* (Chen et al., 2011). Based on above information, we wonder to ask whether the bioactive polysaccharide affects the uptake of oxLDL or the efflux of excessive cholesterol through regulating the expression of SRs or RCTs in macrophage-derived foam cells.

In this study, we have verified the biological activity of the excreted polysaccharide from submerged culture of *P. eryngii*, along with the underlying mechanisms for its bioactive function. Our results clearly indicate that the excreted polysaccharide, which comprises of D-glucose, D-mannose, and D-galactose, is a potentially effective reagent with biological activity that lowers the lipid accumulation in foam cells. Most importantly, the excreted polysaccharide inhibits the formation of foam cells through the down-regulation of CD36 (a scavenge receptor), which is responsible for the uptake of oxLDL.

2. Materials and methods

2.1. Materials and reagents

The *P. eryngii* strain (ACCC 52700) isolated for the excreted polysaccharide was obtained from Jiangsu provincial academy of agricultural sciences. DEAE-52 cellulose was purchased from GE Healthcare (England, UK). Standard monosaccharides, trichloroacetic acid and standard dextrans (molecular weight 500, 70, 40, 20 and 10 kDa) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Oxidized low density lipoprotein (oxLDL) was purchased from Yiyuan biotechnology Ltd. Co. (Guangzhou, China). All other un-described reagents used in this study belonged to the analytical grade category.

2.2. Extraction and preparation of the excreted polysaccharide from P. eryngii

For preparation of the excreted polysaccharide (EP) from *P. eryngii*, the mycelium was initially grown on the medium of potato dextrose agar (PDA, 200 g of potato/L, 20 g of dextrose/L, and 20 g of agar/L) in a Petri dish cultured for 7 days at 25 °C, then punched out the mycelium and transferred them to the liquid medium, and incubated at 25 °C on a rotary shaker incubator at 150 rpm. After 9 days, the liquid culture was centrifuged at 6000 × *g* for 5 min and the resulting supernatant was precipitated by adding ethanol to a final concentration of 80% overnight at 4 °C. After that, the precipitates were collected and re-dissolved in distilled water. Then, above polysaccharide solution was further dialyzed by a dialysis membrane MD34 (Solarbio, China) for 36 h in ultra-pure water to discard small molecules less than 3.5 kDa. Lastly, polysaccharide solution was lyophilized in vacuum freeze dryer to obtain EP.

2.3. Purification of the excreted polysaccharide from P. eryngii

EP was dissolved in the distilled water and passed through a DEAE-52 cellulose anion-exchange chromatography column (30 × 2.6 cm, GE Healthcare, UK), and then eluted with 0.05 mol/L PBS, followed with gradient elution of 0.05–1.0 mol/L NaCl at a flow rate of 1 mL/min. After that, all elution fractions (each with 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). One major fraction referred EP-1 was collected, dialyzed and then lyophilized to pellets. For the evaluation for the purity of polysaccharide, high-performance liquid chromatography (HPLC) with the analysis column (Bio-rad Aminex HPX-87H, USA) was performed according to previously described (Chen et al., 2012a).

2.4. IR spectrum, molecular weight measurement, monosaccharide composition analysis and NMR spectrum of the purified fraction

Above purified polysaccharide was ground with KBr powder and then pressed into pellets for IR measurement in the frequency range of 4000–500 cm^{-1}. Infrared (IR) spectrum of polysaccharide was recorded on an infrared spectrometer (Nicolet, USA). The molecular weight determination, monosaccharide composition and NMR spectrum analysis of the purified fraction were performed according to previously described (Chen et al., 2013).

2.5. Cell Culture

The detailed procedures for cell culture, oil-red O staining, lipid content measurement, MTT assay, RNA isolation, semi-quantitative and real-time quantitative reverse transcription-PCR were described in the Supplemental materials.

2.6. Flow cytometry and immuno-staining

Cultured cells (RAW264.7) were harvested by centrifugation at 1200 × *g*, rinsed with 0.01 M PBS (pH 7.4), and then fixed with 4% (v/v) paraformaldehyde for 30 min. After that, the precipitated cells were washed by PBS (0.01 M, pH 7.4) for four times and suspended in PBS buffer with 0.4% (v/v) Tween. Next, after four times washing by PBS, the samples were incubated with the ABCA1 antibody (Abcam ab18180, Cambridge, UK) and CD36 antibody (Novus biologicals NB400-144, Littleton, USA) (0.1 mg/mL in PBS buffer) as the primary antibody separately for at least 3 h at room temperature, Alexa Fluor 546 goat anti-mouse IgG antibody (Invitrogen 52296A, USA) or Alexa Fluor 555 goat anti-rabbit IgG antibody (Invitrogen 645146, USA) was used as the secondary antibody incubated for at least 2 h at room temperature in the dark. Fluorescent signal was quantified using a Becton Dickinson FACSort (Fluorescence activated cell sorter), 488 nm was used as excitation wavelength and 546 nm or 555 nm was used as emitted light detector adjusted to a fixed channel using standard Brite Beads (Coulter, USA) prior to determining fluorescence. Data acquisition and manipulation were performed with Cell Quest and FACSExpress v3 and fluorescence was measured for 20,000 cells.

The immunostaining of ABCA1 was carried out according to previously described (Chen et al., 2012b). Differential interference contrast (DIC) images of the cells were collected with a Zeiss Axio Imager A1 microscope (Zeiss, Jena, Germany). These images were then collected and analyzed with a Sensicam QE cooled digital camera system (Cooke Corporation, Germany) with MetaMorph/MetaFluor combination software package (Universal Imaging, West Chester, PA), and the images were assembled in Adobe Photoshop 7.0 (Adobe, San Jose, CA).

2.7. Western blotting analysis

Cultured cells were collected and rinsed with PBS and subsequently lysed with RIPA buffer containing 1% Triton X-100, 0.1% SDS, 1% deoxycholate and 1 mM phenylmethylsulfonyl fluoride for seconds on ice. The lysed solution was centrifuged at 10,000 × *g*, and then the supernatant was collected for Western blotting assay. For the quantification of the protein concentration, BCA protein assay kit (Beyotime P0012, China) was used according to the manufacturer's instructions. Finally, aliquots (60 μg for total protein) of cell lysates were separated on 10% SDS-PAGE, and immunoblotting

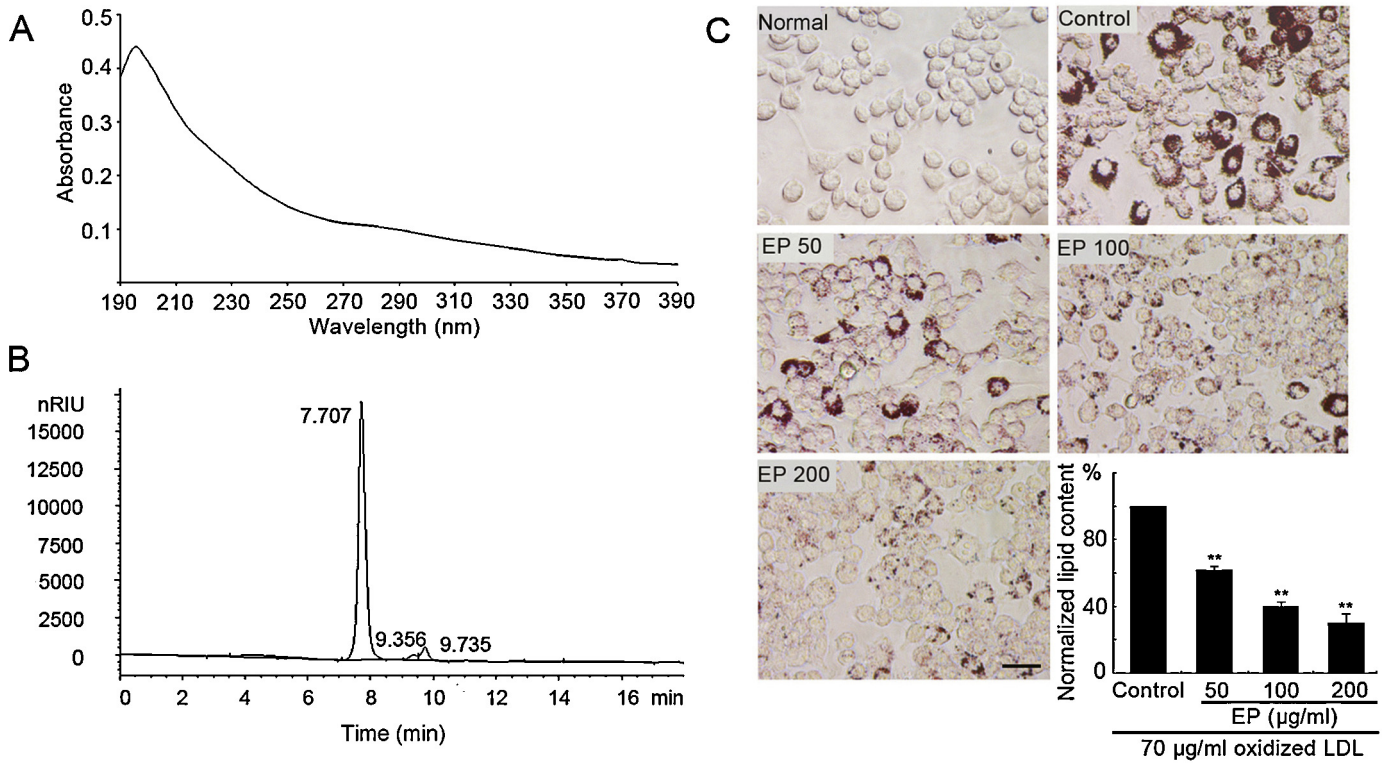

Fig. 1. The excreted polysaccharides from submerged culture of *P. eryngii* lowered the lipid content in a dose-dependant manner in oxLDL-induced foam cells. (A) Full wavelength scanning diagram of EP using a spectrophotometer from 190 to 400 nm. (B) HPLC chromatograms of EP using refractive index detector with 5 mM H_2SO_4 at 0.5 mL/min. (C) Murine RAW 264.7 macrophages were incubated with oxLDL, and amended with the indicated concentrations of EP to the cultured media for 24 h. Cells were fixed, and stained with oil red O. Bar, 20 μm. The quantification data of lipid content in C was shown in lower right panel. 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. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

was performed as described previously (Gao et al., 2011) with CD36 antibody (1:1000, Novus biologicals, NB400-144) and anti-rabbit IgG conjugated to alkaline phosphatase (1:2000, Vector laboratories 94010, Burlingame, CA). The protein bands were visualized by NBT/BCIP alkaline phosphatase color development kit (Beyotime C3206, China) according to the manufacturer's instructions.

2.8. Optimization of submerged culture condition

Optimization of submerged culture condition was described in the Supplemental materials.

3. Results

3.1. Inhibitory effect of EP on lipid accumulation in macrophage-derived foam cells

The excreted polysaccharide (referred as EP) in submerged culture was collected and extracted as described in Section 2. Full wavelength scanning result showed that there was no detectable absorbance peak at 260 or 280 nm for EP, suggesting that nucleic acids or proteins are excluded in EP (Fig. 1A). HPLC analysis showed that EP was composed of two or three different components; the main fraction (94.05%) appeared at 7.7 min of retention time (Fig. 1B). To test the effect of EP on lipid accumulation, macrophage-derived foam cells induced by oxLDL were used as the cell model. For the treatment groups, the murine RAW 264.7 macrophages were incubated with oxLDL, and amended with the different concentrations of EP (50, 100, and 200 μg/mL) to the cultured media for 24 h. In comparison, the same volume of media was added to the control group. For a normal group, no oxLDL was added in the media during all the cultured time. Consequently, and probably most excitingly, when at the concentrations of 50, 100, and 200 μg/mL, EP was able to lower the lipid content significantly, resulted in the lipid content of cells to 61.54 ± 2.34%, 40.06 ± 2.24%, and 29.90 ± 5.70% compared to 100% in the control, respectively ($p < 0.01$) (Fig. 1C). In addition, to get rid of the side effect of EP on the activity of cellular enzyme, cell viability assay-MTT assay was carried out in the murine RAW 264.7 macrophages. As predicted, EP had no detectable effect on the cell viability (Fig. S1). These results clearly indicate that the bioactive polysaccharide which can decrease accumulation in foam cells also exists in submerged culture of *P. eryngii*.

3.2. Isolation and purification of bioactive component-EP-1 in EP

To isolate the single and bioactive component, dissolved EP was fractionated on a DEAE-52 cellulose anion exchange column. As shown in Fig. 2A, the main fraction of EP (at about 7.7 min of retention time) was obtained; we refer to this as EP-1 hereafter. HPLC analysis of EP-1 displayed a single and symmetric peak, indicating that EP-1 is comprised of only one component (Fig. 2B). To investigate the ability for decreasing the lipid content, EP-1 was added with media as described above. As we expected, EP-1 was also able to significantly reduce the amount of lipid droplets in a dose-dependent manner (Fig. 2C). At the concentration of 50, 100, and 200 μg/mL, EP-1 was able to decrease the content of lipid to 61.79 ± 5.85%, 47.73 ± 3.32%, and 23.58 ± 1.98% ($p < 0.01$) in the murine RAW 264.7 macrophages compared to the control group induced by oxLDL (Fig. 2D). These results suggest that EP-1 is a main bioactive component in EP that attenuates the lipid accumulation of oxLDL-induced foam cells.

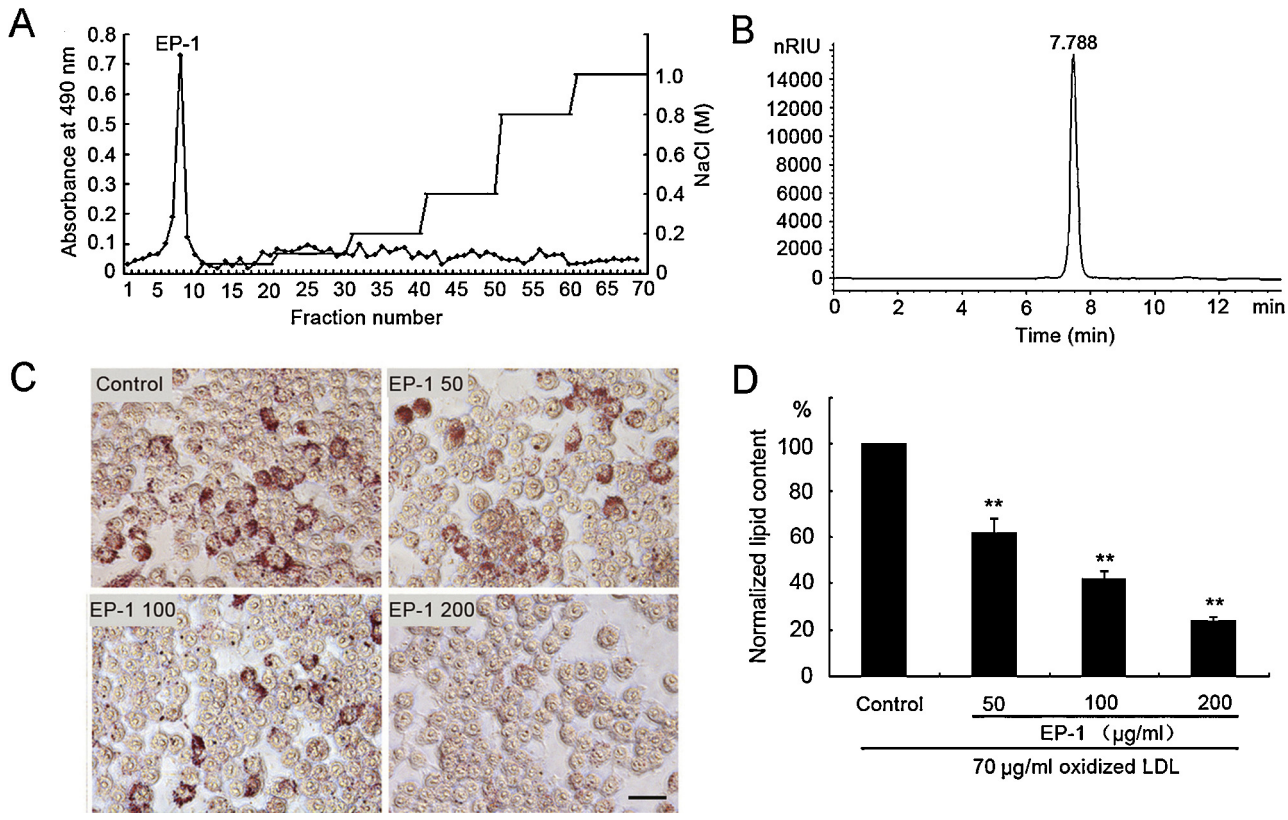

Fig. 2. The purified fraction EP-1 lowered the lipid content in a dose-dependant manner in oxLDL-induced foam cells. (A) Elution profiles of EP on DEAE-cellulose column (30 × 2.6 cm, GE Healthcare) eluted with 0.05 mol/L PBS and followed with the gradient solution of 0.05, 0.1, 0.2, 0.4, 0.8 and 1.0 mol/L NaCl at 1 mL/min. (B) HPLC chromatograms of EP-1 using refractive index detector with 5 mM H_2SO_4 at 0.5 mL/min. (C) Murine RAW 264.7 macrophages were incubated with oxLDL and amended with the indicated concentrations of EP-1 for 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. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. IR spectrum, molecular weight, monosaccharide composition and NMR spectrum of purified fraction

To analyze the structural characteristics of the aforementioned functional component EP-1, IR spectrum, molecular weight measurement, monosaccharide composition analysis and NMR spectrum were conducted as previously described. IR spectrum of the purified EP-1 was analyzed with a Nicolet 560 IR spectrometer that was equipped with a DTGS detector. The result of IR spectrum in Fig. 3A displayed a broadly-stretched intense peak at 3424 cm^{-1} characteristic of hydroxyl groups and a weak C—H band at around 2926 cm^{-1}. The relatively strong absorption peak at around 1650–1600 cm^{-1} indicates the characteristic of C=O. The peak near 1430–1390 cm^{-1} also suggests the presence of carboxyl groups. The peaks at 1200–950 cm^{-1} illustrate the presence of C—O—C and C—O—H link bonds (Luo et al., 2010). Chen et al. (2008) also reported that the strong band between 1160 and 950 cm^{-1} attributed to the stretching vibrations of pyranose ring. Collectively, these analytical data indicate that purified fraction-EP-1 has a typical absorption pattern of the polysaccharide.

The average molecular weight (Mw) of EP-1 which evaluated using HPLC as previously described (Chen et al., 2013) was estimated to be 36.67 kDa. Furthermore, EP-1 was hydrolyzed and acetylated as described in Section 2, and GC was performed to further analyze the monosaccharide composition. As shown in Fig. 3B, the main monosaccharide composition of EP-1 is 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. 3B), and EP-1 is composed of 96.39% glucose, 2.26% galactose and 1.35% mannose. In addition, to characterize the molecular structure for the excreted polysaccharide, NMR spectrum analysis was performed. As shown in Fig. 3C and D, the proton NMR spectrum (500 MHz) and the carbon NMR spectrum (125 MHz) of EP-1 displayed the five different signals in the anomeric region, which labeled as “a”, “b”, “c”, “d”, and “e”, respectively. With the same analytical methods as described before, we conclude that the NMR spectrum of EP-1 is similar to that of PEPE-A1, which was extracted from fruiting bodies of *P. eryngii* (Chen et al., 2013). Residue “a” is a (1 → 3,6)-linked-β-D-glucopyranosyl moiety, “b” indicates a (1 → 3)-linked-β-D-glucopyranosyl moiety, and residues “c”, “d” and “e” represent (1 →)-linked-β-D-glucopyranose. The possible structure of main glycosidic linkage in EP-1 is as followed: β(1 → 3)-glucan works as the main chain accompanied by branched O-6 and side chain of α-glucose.

To further confirm the biological effect of excreted polysaccharide was due to its specific structural feature, we incubated EP with β-1,3-glucanase (Sigma G4423) to digest the main glycosidic linkage in EP and then observe whether its activity would be changed. As shown in Fig. 4A and B, after the treatment of β-1,3-glucanase, HPLC analysis data showed that EP was really digested into several fractions, at the retention time 7.802, 8.494, 9.250 and 10.949, respectively, which was totally different from the original component at the retention time about 7.651 min. After getting rid of excess β-1,3-glucanase by thermal treatment and centrifugation,

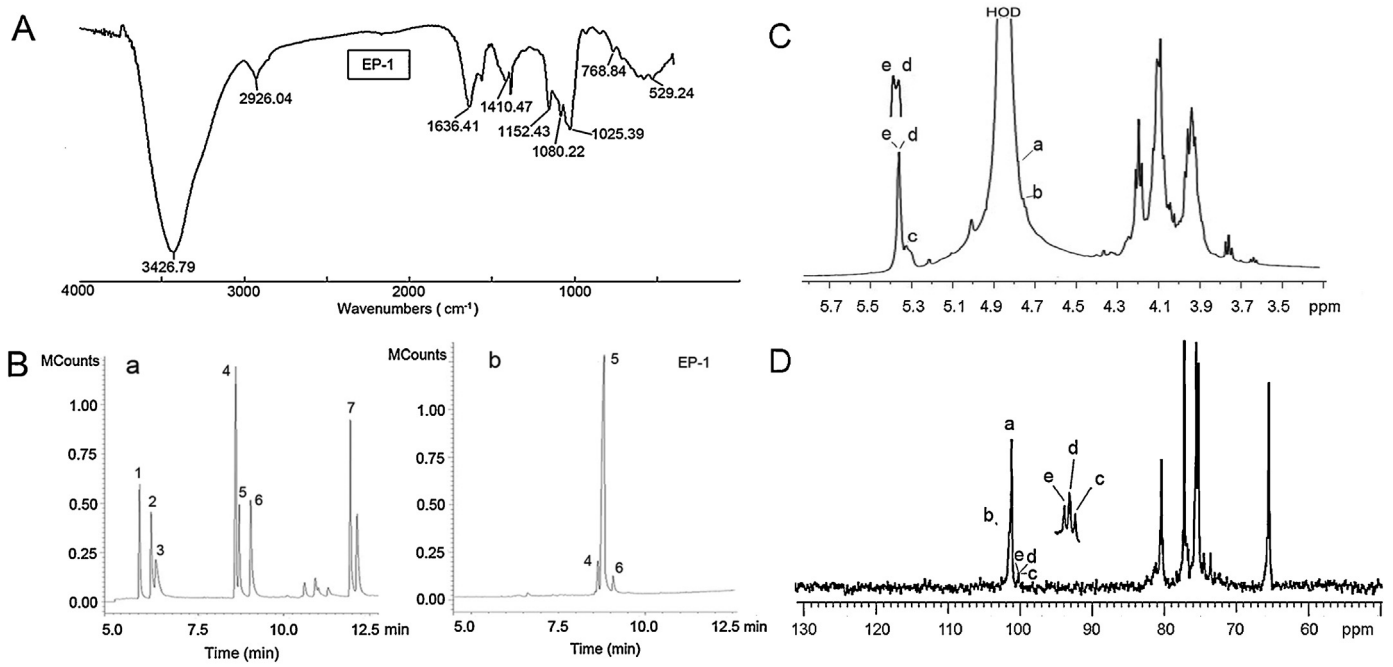

Fig. 3. (A) IR spectrum of EP-1 recorded on an infrared spectrometer (Nicolet, USA). (B) Gas chromatograms of acetylated monosaccharides. (a) Standard, the number represents different standard monosaccharides. (1) L-rhamnose, (2) D-arabinose, (3) D-xylose, (4) D-mannose, (5) D-glucose, (6) D-galactose, (7) D-fucose. (b) EP-1. All samples were hydrolyzed, and then to be acetylated for further analysis by gas chromatography. (C) ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectrum for EP-1. (D) Five anomeric signals were labeled as "a", "b", "c", "d" and "e", respectively.

we referred above digested EP as EP-D which was then used to detect for the function for lowering the lipid content in foam cells. As shown in Fig. 4C and D, EP-D failed to decrease the lipid accumulation mostly in foam cells compared to the treatment by EP such that the foam cells treated by EP-D still included the lipid content of 66.12 ± 6.16% compared to treatment of EP with 25.41 ± 4.92% detected at the same time both at the concentration of 200 μg/mL. The results suggest that the special structure is essential for the activity of excreted polysaccharide to decrease the lipid accumulation in foam cells.

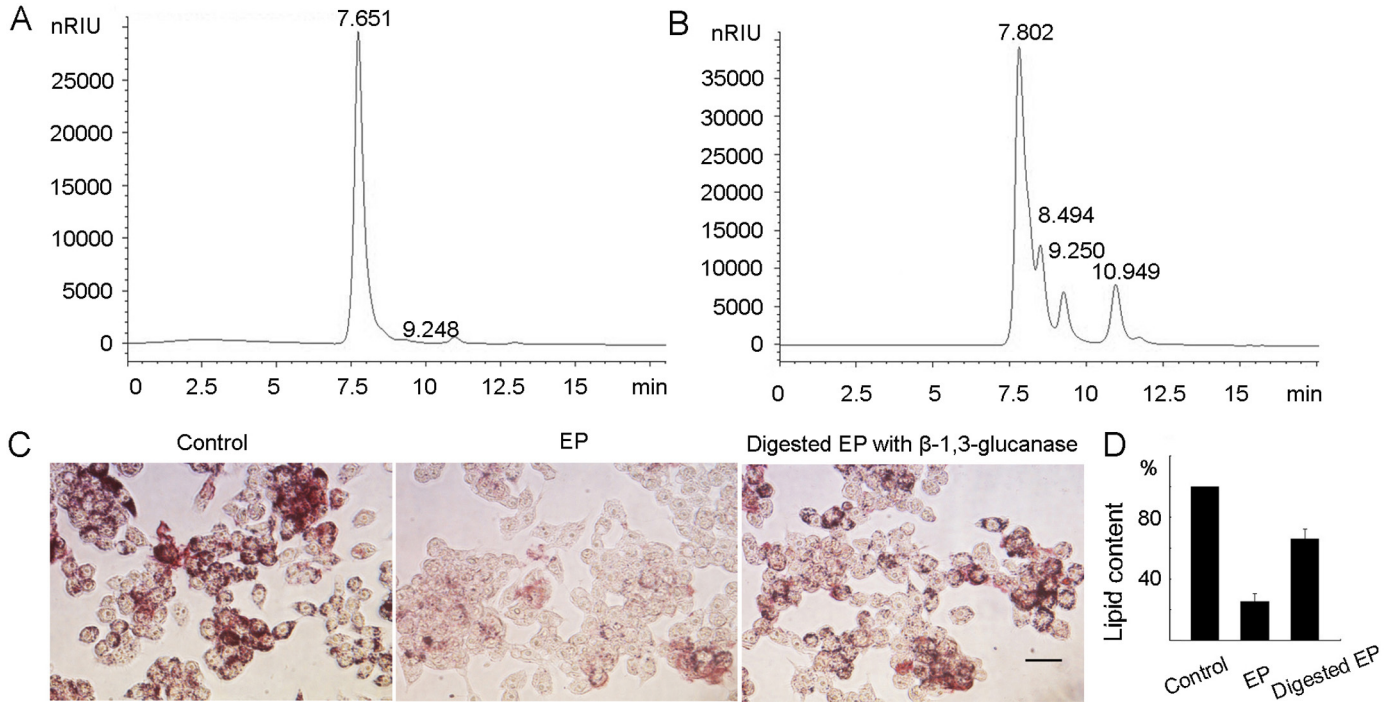

Fig. 4. Digested EP with β-1,3-glucanase was unable to decrease the lipid accumulation mostly in foam cells. HPLC chromatograms of EP (A) and digested EP (1 mg/mL) by β-1,3-glucanase (60 mg/mL) (B) using refractive index detector with 5 mM H_2SO_4 at 0.5 mL/min. (C) Murine RAW 264.7 macrophages were incubated with oxLDL, and treated with EP and digested EP at the final concentration of 200 μg/mL for 24 h. Cells were fixed, and stained with oil red O. Bar, 20 μm. (D) The quantification data of lipid content correlated to (C). Data represent mean ± SD from three parallel experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Correlation between the expression of key players in oxLDL uptake and the role of excreted polysaccharide in lipid accumulation

Next, we explored the mechanisms behind the excreted polysaccharide inhibited lipid accumulation in foam cells. Previous studies have reported that SRs (SR-A and CD36) are responsible for the internalization of oxLDL, and the efflux of accumulated lipid in macrophages is mediated through ABCA1 and ABCG1 and their upstream transcription factors LXRα, RXRα, and PPARγ (Voloshyna et al., 2013). Thus, to determine whether EP can affect the related lipid metabolism by interfering with these genes, we analyzed the mRNA expression levels of these candidate genes with real-time quantitative reverse transcription PCR assay (RT-qPCR). As shown in Fig. 5A, among the tested genes, there were no significant changes for *sr-a, abcg1*, *lxrα*, *rxrα* and *pparγ* between before and after treatment with oxLDL or EP. Notably, the mRNA expression of *cd36* had been dramatically increased (by about 6 folds) in oxLDL-treated cells compared to that of normal cells. Most interestingly, EP could significantly suppress the up-regulation of *cd36* mRNA expression induced by oxLDL. This result was further confirmed by semi-quantitative reverse transcription-PCR, as shown in Fig. 5B. Meanwhile, the mRNA expression of *sr-a*, which also belonged to SRs was not statistically changed when treated by oxLDL or EP. However, the mRNA expression of *abca1*, which belonged to the cholesterol efflux-related gene, was up-regulated by oxLDL as previously reported (Tang et al., 2004), but could not be changed under the EP treatment condition (Fig. 5A and B).

To further validate whether the polysaccharide can affect the expression of these genes at the protein levels, we used the flow cytometry analysis and the immuno-staining assay to evaluate the protein expression level of ABCA1. As shown in Fig. 6A and C, consistent with the above described mRNA data, there was no significant difference in the protein expression of ABCA1 when treated with EP compared with the oxLDL group. However, under the same condition, the flow cytometry analysis revealed that treatment of EP could decrease the oxLDL-induced CD36 protein expression by 37.83% (Fig. 6B). Moreover, Western blotting data further confirmed that CD36 expression was up-regulated by oxLDL treatment for 24 h in cells. Perhaps the most excitingly, this up-regulation could be substantially suppressed by amending with EP in the cultured media until for 24 h but not for 12 h (Fig. 6D). Thus, these data suggest that the bioactive polysaccharide from *P. eryngii* may be able to lower the lipid accumulation in foam cells *via* down-regulation of CD36 to inhibit the uptake of oxLDL.

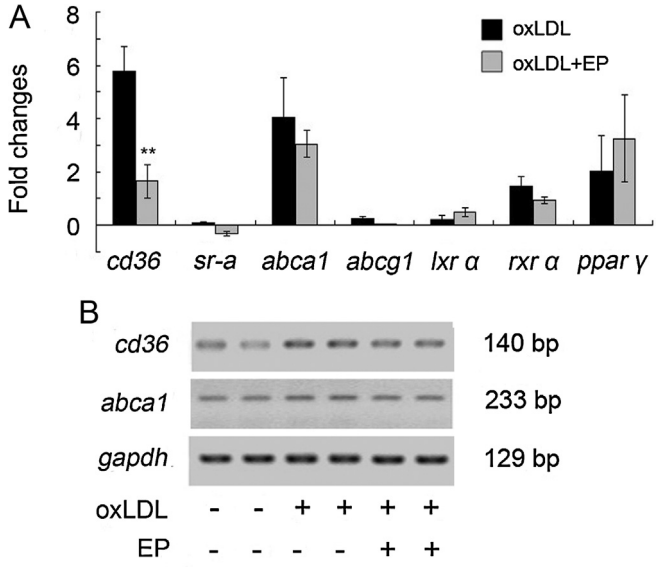

Fig. 5. Effects of excreted polysaccharide on the mRNA expression of lipid-metabolism-related candidate genes during the formation of macrophage-derived foam cells. (A) Murine RAW 264.7 macrophages were incubated with oxLDL (70 μg/mL) alone and EP (200 μg/mL) simultaneously combined with oxLDL (70 μg/mL) for 1 h. mRNA levels including *cd36*, *sr-a*, *abca1*, *abcg1*, *lxr α*, *rxr α*, and *ppar γ* were determined using real-time quantitative PCR. The relative mRNA level for each of the treated samples was normalized using C_T values obtained for the gapdh mRNA amplifications running in the same plate. The data were the means ± SD of three sets of experiments. The significance was set at level * $p < 0.05$ and ** $p < 0.01$ compared with the oxLDL-treated group. (B) mRNA levels including *cd36* and *abca1* were shown by semi-quantitative RT-PCR in 1% agarose gel.

3.5. Optimization of submerged cultural condition for the production of EP

The optical condition for EP production is 3.5% galactose, 0.3% KH_2PO_4, 0.5% peptone and at pH 6.0, and maximum EP content can reach 5.92 g/L, which is about 2.55 times higher than that in the basal medium. The detailed analysis was described in the supplemental materials.

4. Discussion

Numerous evidences have verified that polysaccharides from edible mushrooms possessed pharmaceutical properties and could be used to prevent or remedy human diseases including cancers, inflammatory diseases, hyperlipidemia and diabetes, *etc.* (Lindequist, Niedermeyer, & Julich, 2005; Zhang, Cui, Cheung, & Wang, 2007). In addition, the active polysaccharides can be not only derived from the fruiting body bust also from the submerged culture of mycelium. Findings presented in this report provided direct evidence about that the bioactive polysaccharide existed in submerged culture of *P. eryngii*. The results showed the excreted polysaccharide (EP) and its purified fraction (EP-1) from submerged culture of *P. eryngii* possessed the inhibitory effects on lipid accumulation in foam cells. Treatment with EP and EP-1 at the concentration of 200 μg/mL could result in only about 29.90% and 23.58% of lipid content left inside foam cells compared to 100% with the control (Figs. 1 and 2). Most importantly, the excreted polysaccharide can be purified in a much easier way from submerged culture than that from the fruiting body of *P. eryngii*, and also can be obtained by fermentation in a mass-production setting. However, the best formulation of the cultural condition in fermentation and the material recipe at low-cost in industrial production are needed to be explored in future study.

Moreover, the result of structural analysis as determined *via* gas chromatograph and NMR analysis shows the excreted polysaccharide of *P. eryngii* is mainly comprised of D-glucose and the main chain of β(1 → 3)-glucan (Fig. 3). In addition, EP digested by β-1,3-glucanase fails to decrease the lipid accumulation remarkably in foam cells. It is most likely that the inhibitory effect of lipid deposition may simply be due to this specific structural feature of polysaccharide. Furthermore, it is worth noting that our results suggest that the excreted polysaccharide (EP-1) is similar to some extent to the polysaccharides from the fruiting body of *P. eryngii* (PEPE-A1 and PEPE-A2) in molecular size and some of characterization such as IR and NMR *etc.*, especially in function of decreasing the lipid content (Chen et al., 2013). However, these bioactive polysaccharides are not exactly the same. For example, the average molecular weight of EP-1 is 36.67 kDa while PEPE-A1 and PEPE-A2 are 37.50 and 30.00 kDa, respectively. The percentage ratio for monosaccharide composition also shows variable to some extent. Probably, these differences may be caused by the structural variation in these bioactive polysaccharides. However, we still could

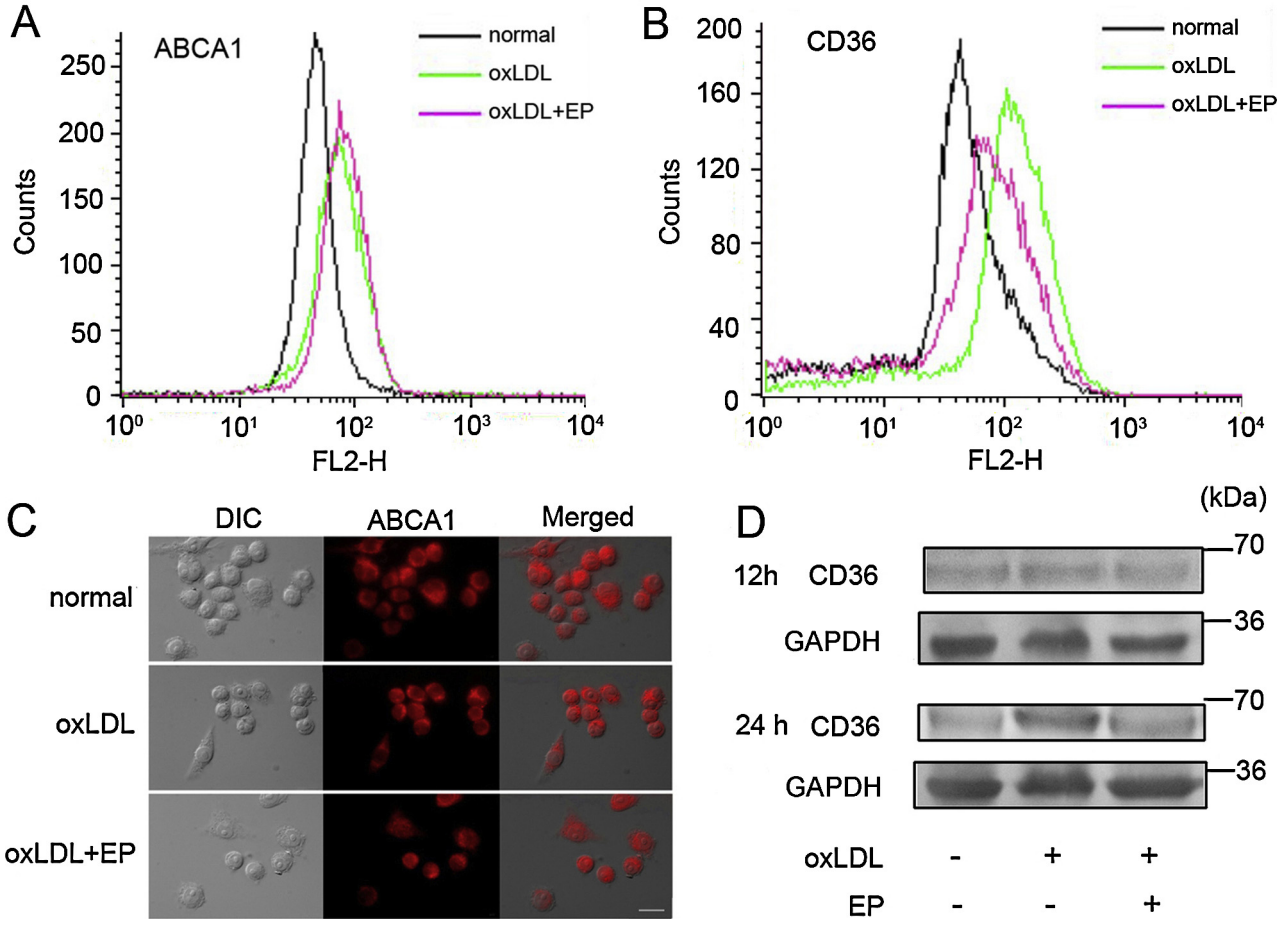

Fig. 6. Effects of excreted polysaccharide on the protein expressions of ABCA1 and CD36 during the formation of macrophage-derived foam cells. Murine RAW 264.7 macrophages were incubated with oxLDL (70 μg/mL) alone, EP (200 μg/mL) simultaneously combined with oxLDL (70 μg/mL) separately for 24 h. The ABCA1 (A) and CD36 (B) protein expression levels were monitored using ABCA1 and CD36 antibody by flow cytometry. (C) The ABCA1 protein expression level was also monitored using ABCA1 antibody by immuno-staining. Bar, 20 μm. (D) Western blot analysis with an anti-CD36 antibody in murine RAW 264.7 macrophages incubated with oxLDL (70 μg/mL) alone, EP (200 μg/mL) simultaneously combined with oxLDL (70 μg/mL) for 12 h or 24 h separately. GAPDH was used as a loading control.

not exclude the possibility that some of differences may be from the detection error. Future studies will further confirm the detail.

In addition, previous reports have demonstrated that the formation of foam cells is mainly due to the dysregulation of intercellular lipid homeostasis, including uncontrolled uptake of oxLDL or impaired cholesterol efflux in macrophages. Indeed, these two key processes are tightly controlled by SRs and RCTs, respectively. Among them, SR-A and CD36 are the key regulators of oxLDL internalization (Kunjathoor et al., 2002; Nicholson, Han, Febbraio, Silversterin, & Hajjar, 2001; Rahaman et al., 2006) whereas transporters ABCA1 and ABCG1 play the main roles in cholesterol efflux (Chu et al., 2013; Voloshyna et al., 2013). Consistent with this understanding, inactivation of ABCA1 or ABCG1 impairs lipid clearance, which in turn augments lipid deposition in macrophages. In addition, over-expression of ABCG1 enhances cholesterol efflux and subsequently reduces lipid accumulation in foam cells (Joyce et al., 2002; Out et al., 2006). By contrast, macrophages that had their oxLDL uptake disrupted by defective SR-A or CD36 are less prone to form foam cells (Kao, Tseng, Lee, Chan, & Wang, 2009; Osto et al., 2008). Moreover, additional evidence has verified that the reduced expression of SRs or the improved function of RCTs in macrophages leads to reduced lipid accumulation in macrophages (Chen et al., 2011; Park et al., 2011). These effects are consistent with our findings: *P. eryngii* excreted polysaccharide can down-regulate the expression of CD36 at both mRNA and protein levels. However, there is no significant, quantitative difference in ABCA1 expression between the treatment of polysaccharide and the control (Figs. 5 and 6). Thus, *P. eryngii* polysaccharide is able to lower lipid accumulation in foam cells possibly *via* down-regulation of the expression of CD36 to inhibit the uptake of oxLDL. Even though *P. eryngii* polysaccharide is unable to up-regulate the expression of ABCA1, it is not excluded that the polysaccharide probably can affect the lipid efflux by increasing the ABCA1 pump vitality.

Besides, complications of atherosclerosis cause most morbidity and mortality in patients with diabetes mellitus. In type 2 diabetes, there is characteristic abnormality in the lipid profile including the increased level of LDL whose particles are proatherogenic and readily undergo oxidative modification. Meanwhile, monocytes ingested oxidized LDL *via* scavenger receptors could become foam cells. Thus, understanding the formation of foam cells and their roles during the developing process of diabetes and atherogenesis may have important therapeutic implications for these diseases (Beckman, Creager, & Libby, 2002)

Collectively, our findings suggest that the excreted polysaccharide inhibits the lipid accumulation by down-regulating a scavenger receptor, which resulting in the remarkable deceasing of the lipid uptake. Most importantly, the bioactive polysaccharide exists in the submerged culture of *P. eryngii* and can be obtained easily by fermentation in a mass-production than that from the fruiting body of *P. eryngii*.

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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.2014.05.068.

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