

Purification, characterization and *in vitro* bile salt-binding capacity of polysaccharides from *Armillaria mellea* mushroom

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Abstract: The crude polysaccharides from *Armillaria mellea* were obtained with an ultrasound assisted enzymatic extraction and ethanol precipitation. Two polysaccharide fractions were obtained by ethanol precipitation, which were named AMP-1 and AMP-2. The results of the monosaccharide composition analysis indicated that AMP-1 was composed of mannose, rhamnose, glucose, galactose, arabinose and fucose and that AMP-2 was composed of mannose, rhamnose, glucose, galactose and fucose. Glucose and galactose were the main monosaccharide fractions. The protein and nucleic acid contents in AMP-1 and AMP-2 were detected by using ultraviolet and infrared spectroscopy. The bile salt-binding capacities of the polysaccharide samples were studied *in vitro*. In comparison with lentinan (LP), AMP-1 and AMP-2 showed increased bile salt-binding capacity. AMP-1 showed the highest binding capacity against all the bile salts. The findings presented in this study highlight the potential of the *A. mellea* polysaccharides as a natural hypolipidaemic agent.

Keywords: *Armillaria mellea*; bile salt-binding; hypolipidemic; polysaccharide

Edible mushrooms are used as a source of food, and they have been used as natural medicine for thousands of years in China because of their multiple bioactivities (ALAM *et al.* 2009; REIS *et al.* 2017). In recent years, *Armillaria mellea*, a new and a special species of edible mushroom, has successfully received increasing attention due to its high nutritional value (KOSTIĆ *et al.* 2017). *A. mellea* belongs to the Basidiomycetes in the Armillaria family and is a traditional Chinese edible fungus with an extensive distribution in northeastern China. Although *A. mellea* has received a high level of attention, it can be artificially cultivated, and all *A. mellea* available on the marketplace are wild and natural. Therefore, *A. mellea* was also named ‘the fourth treasure of northeastern China’. Over the years, *A. mellea* has drawn the attention of many researchers due to the abundance of functional components and biological properties

present in these mushrooms. Polysaccharides have been recognized as the important bioactive components. According to previous studies, *A. mellea* polysaccharides have potential biological activities, such as anti-tumour activity (WU *et al.* 2012), antioxidant activity (ZHANG *et al.* 2015), and anti-inflammatory activity (SUN *et al.* 2009).

Hyperlipidaemia has become a major health ailment and a serious social problem in recent years. Many studies have proven that the blood concentration of total cholesterol (TC), triglycerides (TG) and low-density lipoprotein cholesterol (LDL-C) were the major risk factors in the pathogenesis of hyperlipidaemia. Polysaccharides isolated from different mushrooms have shown good hypolipidaemic activity, such as polysaccharides from *Pholiota nameko* (LI *et al.* 2010), *Termitomyces albuminosus* (ZHAO *et al.* 2016), *Pleurotus eryngii* var. *tuolensis* (XU *et al.* 2017)

and lentinan (WANG *et al.* 2005). Bile salt is a kind of amphoteric macromolecule with a steroid nucleus structure that plays an important role in the digestion and absorption of fat and cholesterol and the metabolism of fat-soluble vitamins. Previous studies have shown that the reduction of bile salts in the intestine plays an important role in reducing blood cholesterol (GALLAHER *et al.* 2000; CAMIRE & DOUGHERTY 2003; LI 2012). However, as far as our literature survey could ascertain, few reports have evaluated the bile salt binding capacity of *A. mellea* polysaccharides. Therefore, in this paper, we intend to describe the purification and characterization of two polysaccharide fractions isolated from *A. mellea* by ethanol grading extraction with two ethanol concentrations (40 and 70%). In addition, the hypolipidaemic activity was investigated by testing the bile salt-binding capacity *in vitro*.

MATERIAL AND METHODS

Material and chemicals. The dried fruiting bodies of *A. mellea* were purchased from a local market (Harbin, China). The sample powders were obtained by passing the samples through a 60-mesh screen and stored in a desiccators at room temperature (25°C) for further use. The monosaccharide standards (L-arabinose, D-xylose, L-rhamnose, L-fucose, D-mannose, D-glucose and D-galactose), Sephadex G-150, Sodium cholate, sodium taurocholate and sodium glycocholate were obtained from Sigma Chemical Co. (USA). All other reagents were of analytical grade.

Preparation of crude polysaccharide. Crude polysaccharides were prepared from *A. mellea* according to the method described by our previous study (CHEN *et al.* 2017). Briefly, the dried *A. mellea* powder was mixed with complex enzymes (cellulose and papain; 2:1) at 1.9% concentration. The enzyme-catalysed reactions were carried out in a shaking bath (50°C) with extraction time of 138 min and a ratio of water to raw material of 30:1 ml/g.

The extraction was performed and supplemented with ultrasound at a designated power (360 W) for 20 minutes. After centrifugation at 4000 rpm for 30 min, the supernatant was collected and concentrated with a rotary evaporator at 45°C under vacuum.

Isolation and purification of the crude polysaccharide. The crude polysaccharides were dissolved in the deionized water and the protein was removed using the Sevag method (SEVAG *et al.* 1938).

The decolorization of the crude polysaccharide was performed by hydrogen peroxide method with temperature 50°C, pH 8 and time 3 h and the hydrogen peroxide concentration 40%. The crude polysaccharide was dissolved in distilled water and further purified by ultrafiltration (membrane MW cut-off: 10 000) under pressure (0.5 MPa). Different volumes of anhydrous ethanol were successively added to (40 and 70%, v/v) ethanol solutions. The precipitate was obtained by centrifugation at 4000 rpm for 15 minutes. The precipitates were then freeze dried, and two different fractions (AMP-1, and AMP-2) were obtained.

The two polysaccharide fractions (AMP-1 and AMP-2) were dissolved in deionized water and separated by a Sephadex G-150 gel column (2.6 × 30 cm), respectively. The column was eluted with 0.05 mol/l NaCl (5 ml/tube).

Analysis of monosaccharide composition. The monosaccharide composition was measured by the method of SIU *et al.* (2015) with some modification. In brief, the polysaccharide fractions (2 mg) was hydrolysed with 2M trifluoroacetic acid (0.5 ml) at 120°C for 2 hours. The hydrolysate was then dried under nitrogen in a hot water bath. The hydrolysate solution (450 l) was mixed with 0.5 ml of a 0.5M 1-phenyl-3-methyl-5-pyrazolone (PMP) solution in methanol, and 0.5 ml of a 0.3M NaOH solution and then incubated at 70°C for 30 minutes. The reaction was stopped by neutralizing with 0.5 ml of 0.3M HCl, and the product was partitioned with chloroform three times. The aqueous layer was collected and filtered through a 0.22 mm membrane and applied to HPLC instrument. HPLC was performed with Agilent SHISEIDO C18 column (4.6 × 250 mm, 5 µm) on an Agilent 1260 instrument at 25°C with potassium phosphate buffered saline (0.1M, pH 7) and acetonitrile (82:18, v/v) as mobile phases, and UV detection at 245 nm.

UV and IR spectroscopy. The ultraviolet spectra of the polysaccharide solutions were determined with an ultraviolet spectrophotometer (TU-1800; Tianjin Taisite Instrument Co. Ltd., China) in the range of 190–400 nm.

The IR spectra of the polysaccharides were determined using a Shimadzu 8400S Fourier transform IR spectrophotometer (FTIR) (Japan). The polysaccharides were crushed with KBr powder and then pressed into pellets for FT-IR measurement in the range of 400–4000 cm⁻¹ (YAN *et al.* 2010).

Bile-salt binding capacity *in vitro*. The bile salt stock solutions (0.3 mmol/l) were prepared by dis-

solving sodium cholate, sodium taurocholate and sodium glycocholate in phosphate buffer solutions (1 mol/l, pH 6.3). Seven volumes of bile salt stock solutions (0, 0.1, 0.5, 1.0, 1.5, 2.0, and 2.5 ml) were added to the plug tube (10 ml). Then, the solutions were diluted to 2.5 ml with a phosphate buffer solution and 7.5 ml sulfuric acid solution (60%, w/v) was subsequently added to the tube. The mixture was incubated at 70°C for 20 min in water bath and cooled for 5 min in an ice bath. Then, the spectra were measured at 387 nm. The standard curves were plotted by using the concentration of bile salts as the abscissa and the absorbance as ordinate.

At total of 100 mg of each AMP-1, AMP-2 and lentinan were treated with 1 ml HCl (0.1 mol/l) and incubated at 37°C for 1 h with continuous shaking. The pH of the mixture was adjusted to 6.3 using NaOH (0.1 mol/l) and 5 ml pancreatin (10 mg/ml) was added and mixed, with subsequent incubation at 37°C for 1 hour. Each polysaccharide sample was treated with 4 ml of a bile salt solution (sodium cholate, sodium taurocholate and sodium glycocholate 0.3 mol/l). The mixture was incubated for one hour at 37°C, and centrifuged at 4000 rpm for 20 minutes. The supernatant was collected for bile salt determination.

Statistical analysis. All experiments were performed in triplicate, and the data were expressed as the mean $\pm$ standard deviation. Differences were considered significant when the $P < 0.05$. The SPSS/11.5 software was used for data analysis.

RESULTS AND DISCUSSION

Isolation and purification of crude polysaccharides. The water-soluble crude polysaccharides (AMP), with a 40.56% dried raw material yield, were obtained from *Armillaria mellea* by ultrasonic-assisted enzymatic extraction, ethanol fractional precipitation, and deprotein by Sevag method, followed by decolorization with hydrogen peroxide

and dialysis against water. As shown in Table 1, two different crude polysaccharide fractions named AMP-1 and AMP-2 were obtained with different ethanol concentrations (40 and 70%, respectively). The polysaccharide contents of AMP-1 and AMP-2 were 38.66 and 30.60%, respectively.

The elution profile of AMP-1 and AMP-2 with a Sephadex G-150 gel chromatography column is illustrated in Figure 1. As shown in Figure 1, the two fractions had only one symmetrical peak, indicating that they were relatively homogeneous samples (WANG *et al.* 2014). Following concentration, dialysis, and freeze-drying, the AMP-1, and AMP-2 powders were obtained.

Monosaccharide composition analysis. The monosaccharide composition of AMP-1 and AMP-2 was analysed by comparing the retention time against standards by GC (gas chromatography) (Figure 2A and B). As shown in Figure 2A and B, AMP-1 was composed of mannose, rhamnose, glucose, galactose, arabinose and fucose, while AMP-2 was composed of mannose, rhamnose, glucose, galactose and fucose. The molar ratio of monosaccharides is shown in Table 1. Glucose and galactose were the main monosaccharide fractions, which is in agreement with the results of SUN *et al.* (2009). Compared with AMP-1,

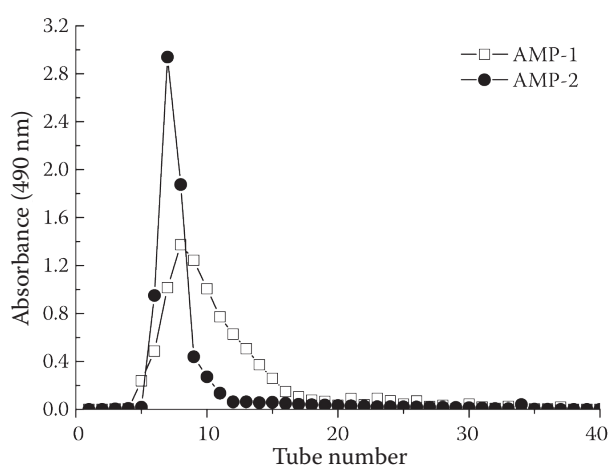


Figure 1. Elution profile of AMP-1 and AMP-2 on Sephadex G-150 gel chromatography column

Table 1. Monosaccharide composition of purified AMP-1 and AMP-2

Samples	Monosaccharide composition (mol %)					
	mannose	rhamnose	glucose	galactose	arabinose	fucose
AMP-1	8.97	0.26	51.60	20.40	0.22	12.88
AMP-2	9.51	0.49	59.87	21.79	nd	10.95

Individual components are presented as mol% for each sugar; nd – not detected

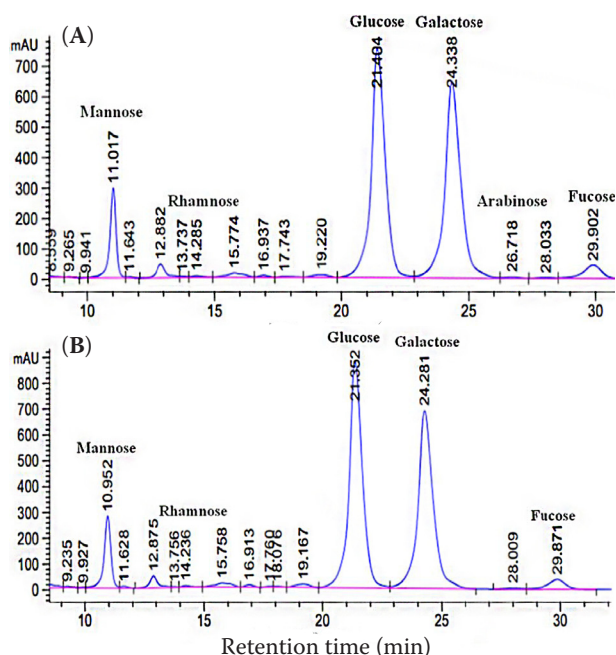


Figure 2. High performance liquid chromatography (HPLC) analysis of (A) AMP-1 and (B) AMP-2 derivatized standard monosaccharides

no arabinose was detected in AMP-2. These results revealed that there are some differences in the monosaccharide composition of the two polysaccharide fractions. From the composition and molar ratio, we speculate that the two polysaccharides with a glucan as the backbone chain (MA *et al.* 2014).

UV spectrum analysis. The UV spectra of AMP-1 and AMP-2 in the range of 190–400 nm was shown in Figure 3. The UV spectra of AMP-1 and AMP-2 showed no absorptions at 260 nm and 280 nm, indicating the absence of nucleic acid and protein (MA *et al.* 2014).

IR spectra analysis. The FT-IR spectra of AMP-1 and AMP-2 ranging from 400–4000 cm^{-1} are shown in Figure 4. All samples displayed the same broad

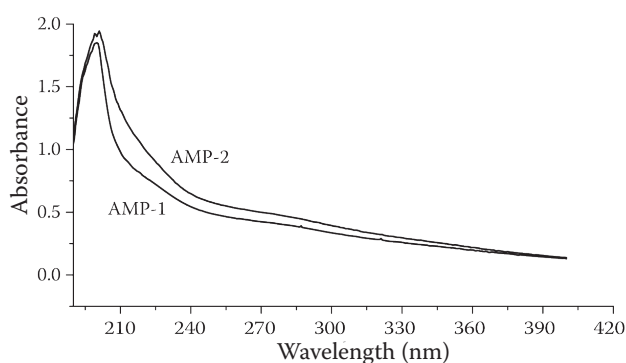


Figure 3. UV spectra of AMP-1, AMP-2 in the range of 190–400 nm

stretching band near 3420 cm^{-1} , with AMP-1 at 3405 cm^{-1} and AMP-2 at 3373 cm^{-1} , which is characteristic for O-H groups; A weak C-H stretching vibration near 2932 cm^{-1} was also observed in all samples (JIANG *et al.* 2013). A strong transmitting angle band at approximately 1620 cm^{-1} , with AMP-1 at 1637 cm^{-1} and AMP-2 at 1654 cm^{-1} as well as a weak symmetrical band at approximately 1420 cm^{-1} , with AMP-1 at 1374 cm^{-1} and AMP-2 at 1375 cm^{-1} were also observed, implying the presence of carboxyl groups. Absorption bands were observed within the range of 1100–1010 cm^{-1} , with AMP-1 at 1070 cm^{-1} and AMP-2 at 1074 cm^{-1} , indicating the possible presence of a furanose ring in AMP-1 and AMP-2 (LI & SHAH 2014). The FT-IR spectra of the two *A. melaleuca* polysaccharide fractions (AMP-1 and AMP-2) were similar.

Bile salt-binding capacity. Bile salt is a kind of amphoteric macromolecule with a steroid nucleus structure that plays an important role in the digestion and absorption of fat and cholesterol and the metabolism of fat-soluble vitamins in humans. Previous studies have proposed that an alternative mechanism to explain the reduction in blood cholesterol levels by polysaccharides is the prevention of lipid absorption (ZHOU *et al.* 2006; YOKOYAMA *et al.* 2011), which can be related to bile acid-binding mechanisms (QI *et al.* 2012). The synthesis of bile acid can be stimulated by polysaccharides and the bile acids are reabsorbed in the small intestine and return to the liver. Bile salt-binding plays an important role in interrupting the enterohepatic circulation and increasing the faecal excretion of bile salts, resulting in reduction of bile acids returning to the liver (LIU *et al.* 2012).

The binding capacities of AMP-1 and AMP-2 against sodium cholate, sodium taurocholate and sodium glycocholate were examined. Lentinan, a traditional and widely used polysaccharide (ZHANG *et al.* 2011), that has been proven to have hypolipidemic activity, was used as a control in this study (Xu *et al.* 2008; Li *et al.* 2015). As shown in Figure 5, all the polysaccharide samples differed in their capacity to bind the selected bile salts. The binding capacities of polysaccharides against the bile salts were generally arranged as follows: AMP-1 > AMP-2 > LP. The highest binding capacities against sodium glycocholate and sodium cholate were detected in AMP-1 at a level of 2.234 $\mu\text{mol/g}$ and 1.331 $\mu\text{mol/g}$, respectively, which was significantly higher than the AMP-2 and LP samples. The binding capacity against sodium taurocholate was lower than those against sodium cholate and sodium glycocholate for the three

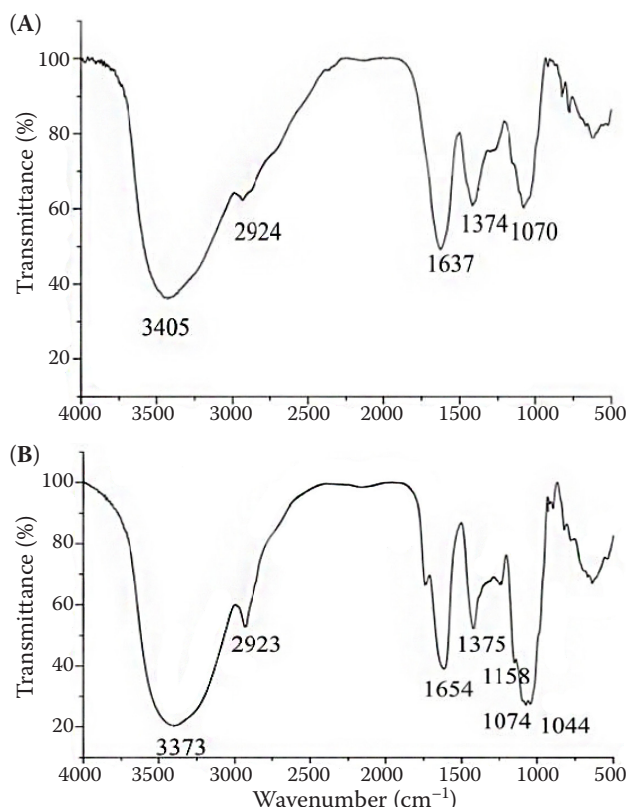


Figure 4. FT-IR spectra of AMP-1 (A), AMP-2 (B) in the range of 400–4000 cm⁻¹

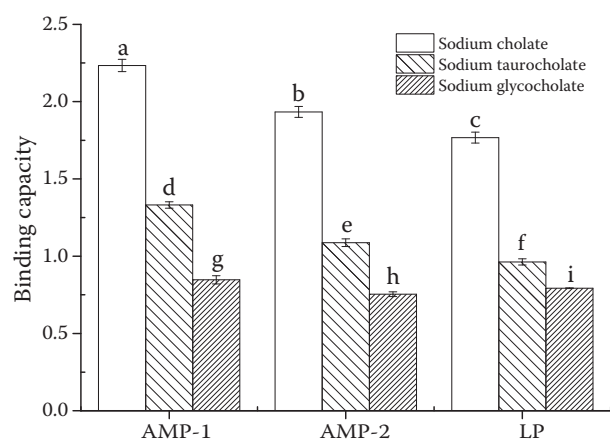


Figure 5. Bile salt-binding capacity of polysaccharide samples

polysaccharide samples. The results indicated that both AMP-1 and AMP-2 showed a stronger bile salt-binding capacity than LP, especially against sodium glycocholate. As shown in Table 1, the content of fucose in AMP-1 was significantly higher than in AMP-2, therefore, we hypothesized that fucose had a significant effect on the bile salt-binding capacity, which is in an agreement with the results of WANG *et al.* (2016).

CONCLUSIONS

Two purified polysaccharides, AMP-1 and AMP-2, were obtained from crude *Armillaria mellea* polysaccharides through ethanol fractionation precipitation and purified by Sephadex G-150 gel chromatography column in this study. The affinity of these two fractions to bile acids was evaluated in an *in vitro* bile salt binding capacity assay. Monosaccharides analysis revealed that both AMP-1 and AMP-2 mainly contain the glucose and galactose. In addition, AMP-1 contains more arabinose than AMP-2. The results of the UV and FT-IR spectra showed that there was no nucleic acid or protein in these two fractions. The *in vitro* hypolipidemic activity of these two fractions has been revealed via bile salt-binding capacity *in vitro*. The results showed that AMP-1 and AMP-2 exhibited significantly higher bile salt-binding capacity than lentinan. In particular, both AMP-1 and AMP-2 showed strong bile salt-binding capacity against sodium glycocholate, which could be related to their structural characteristics.

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