**Supplementary materials**

**Emulsification Stability of *Auricularia auricula* Polysaccharides and its Effect on Steady-state Properties of β-carotene Embedding**

Xiaqing Lva1, Dexi Zhanga1, Xiangyang Zhua,b, Dapeng Lia, Chen Zhanga\*

a. College of Food Science and Engineering, Shandong Agricultural University, Key Laboratory of Food Nutrition and Human Health in Universities of Shandong, Taian 271018, People’s Republic of China.

 b. Institute of Quality Standards and Testing Technology for Agro-Products of Chinese Academy of Agricultural Sciences, Key Laboratory of Agro-food Safety and Quality, Ministry of Agriculture and Rural Affairs, Beijing 100081, P. R. China

1 contributed equally to this work.

\* Corresponding authors:

 Dr. Chen Zhang, Tel: +868246029; E-mail: zhangchensdau@163.com;

**Supplementary methods**

**2. Materials and methods**

2.2 Extraction of AAP

AAP are extracted by three methods: hot water extraction, acid extraction or alkali extraction.

Hot water extraction: A certain amount of powdered black fungus is weighed and mixed with deionized water in a ratio of 1:30 (m:v). The mixture is then placed in a water bath and stirred at a constant temperature of 95℃ for 4 hours. After the extraction, the mixture is centrifuged at 6000 rpm for 15 minutes. The residue is then subjected to three repeated extraction and centrifugation cycles. The supernatants are combined. The combined supernatants are transferred to a rotary evaporator and concentrated to one-third of the original volume. Then, three times the volume of anhydrous ethanol is added, and the mixture is allowed to stand overnight at 4℃. After the ethanol precipitation is complete, the mixture is centrifuged at 6000 rpm for 15 minutes, and the precipitate is taken out. After the ethanol evaporates, the precipitate is re-dissolved in water. It is then subjected to dialysis for 72 hours using a 3.5 kDa dialysis bag and flowing deionized water. Finally, the sample is freeze-dried to obtain water-extracted black fungus polysaccharide (AAP-W).

Hot acid extraction: Mix the black fungus powder with deionized water in a ratio of 1:30 (m:v). Adjust the pH of the solution to 2 using 2 M citric acid. Then, perform hot water extraction to obtain acid-extracted black fungus polysaccharide (APP-A).

Hot alkali extraction: Mix the black fungus powder with deionized water in a ratio of 1:30 (m:v). Adjust the pH of the solution to 9 using 2 M sodium hydroxide. Then, follow the hot water extraction method to obtain alkali-extracted black fungus polysaccharide (APP-AL).

The yields ofAAP were calculated as follows:

$$Auricularia auricula polysaccharide \left（\%\right）=\frac{Auricularia auricula polysaccharide dry weight \left（g\right）}{Auricularia auricula dry weight (g)}$$

2.3 Physicochemical properties of AAP

2.3.1 Total sugar content of AAP

The total sugar content in AAP was determined by phenol-sulfuric acid method with reference to the standard NY/T 1676-2008, with slight modifications.

To prepare a glucose standard curve, first, dry the glucose standard in an oven at 105°C until its weight is constant. Then, accurately weigh out 0.1000 g of the dried glucose standard and dissolve it in deionized water with stirring until fully dissolved. Transfer this solution to a 100 mL volumetric flask and bring it to volume to create the stock solution. Pipet 10 mL of the stock solution into another 100 mL volumetric flask and dilute to volume to create the working standard solution. Next, use a pipet to transfer varying volumes (0.0 mL, 0.2 mL, 0.4 mL, 0.6 mL, 0.8 mL, and 1.0 mL) of the glucose working standard solution into separate test tubes plugged with stoppers, adding deionized water to each tube to make a total volume of 1.0 mL. Add 1.0 mL of a 6% phenol solution and 5.00 mL of concentrated sulfuric acid to each tube, shaking gently to mix. Place the tubes in a boiling water bath for 15 minutes, then cool to room temperature. Transfer the contents to a 1 mL cuvette and measure the absorbance at 490 nm, repeating measurements three times for each sample and calculating the average absorbance value.

For sample analysis (AAP-W, AAP-A, AAP-AL), weigh 10 mg of each sample precisely, dissolve in deionized water, and transfer to a 100 mL volumetric flask, bringing it to volume. Take 0.1 mL of the sample solution and follow the same procedure as for the glucose standard curve, adding phenol and concentrated sulfuric acid sequentially. Heat in a water bath, cool, and measure absorbance at 490 nm. Use the standard curve to calculate the total sugar content of the samples.

2.3.2 Protein content of AAP

Determination of the protein content in AAP using the Coomassie brilliant blue methods. To prepare a Bovine Serum Albumin (BSA) standard curve, first weigh 10 mg of Coomassie Brilliant Blue G-250 and dissolve it in 5 mL of 90% ethanol, followed by adding 10mL of 85% phosphoric acid. Transfer the mixture to a 100 mL volumetric flask and bring it to volume with deionized water, then store the dye reagent at 4°C, protected from light. Next, weigh 0.01 g of BSA and dissolve it completely in deionized water, transferring the solution to a 100 mL volumetric flask and diluting to achieve a concentration of 100 μg/mL (the standard protein solution), which should be stored at 4°C, away from light. Pipet varying volumes (0.0, 0.2, 0.4, 0.6, 0.8, and 1.0 mL) of the BSA standard solution into separate test tubes, bringing each tube to a total volume of 1.0 mL with deionized water to create concentrations of 0.0, 20.0, 40.0, 60.0, 80.0, and 100.0 μg/mL, respectively. Add 4.0 mL of the prepared Coomassie Brilliant Blue G-250 dye reagent to each tube, mix well, and incubate for 3 minutes. Measure the absorbance of each solution at 595 nm using a spectrophotometer, taking three readings per sample and calculating the average. Plot the standard curve using the BSA concentration as the x-axis and the measured absorbance as the y-axis, fitting the best linear equation to this data.

For sample analysis (AAP-W, AAP-A, AAP-AL), weigh 0.50 g of each freeze-dried sample separately, dissolve completely in deionized water, transfer the solutions to separate 10 mL volumetric flasks and bring up to volume. Pipet 1.0 mL of each sample solution into separate test tubes, add 4.0 mL of the prepared Coomassie Brilliant Blue G-250 reagent to each tube, mix immediately, incubate for 3 minutes, measure the absorbance at 595 nm, and record the average of three measurements. Use the obtained absorbance values and the standard curve to calculate the protein content of each sample.

2.3.3 Alduronic acid content

The content of alduronic acid in AAP with reference to the method of Taylor *et al*. (1992), with slight modifications. To prepare a glucuronic acid standard curve, accurately weigh 25.00 mg of dried-to-constant-weight galacturonic acid, dissolve it in water, and transfer the solution to a 25 mL volumetric flask, bringing it to volume. Pipet varying volumes (0, 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 mL) of the standard solution into separate test tubes, adding water to each tube to achieve a final volume of 1 mL. Place the tubes in an ice bath, add 5 mL of sodium tetraborate/sulfuric acid solution to each tube, shake uniformly, incubate in a 65°C water bath for 10 minutes, rapidly cool in an ice-water bath, add 0.1 mL of 0.15% m-hydroxybiphenyl solution to each tube, vortex for 1 minute, and measure the absorbance at 520 nm, taking three measurements per sample and calculating the average. Plot the standard curve with the concentration of uronic acid as the x-axis and the absorbance value as the y-axis, fitting the best linear equation to this data.

For sample analysis (AAP-W, AAP-A, AAP-AL), take 1 mL of each solution at appropriate concentrations, add 5 mL of sodium tetraborate/sulfuric acid solution on ice, shake uniformly, incubate in a 65°C water bath for 10 minutes, measure the absorbance following the same method used for the standard solution, and use the standard curve to calculate the content of uronic acid in the polysaccharides.

2.3.5 Monosaccharide composition of AAP

The principle of high-performance liquid chromatography (HPLC) for determining monosaccharide composition involves hydrolyzing polysaccharides into monosaccharides, followed by derivatization and separation on the column based on retention time.

To analyze the monosaccharide composition of AAP-W, AAP-A, and AAP-AL, 5 mg of each sample is weighed into separate test tubes with plugs, hydrolyzed with 2 M trifluoroacetic acid at 120°C for 4 hours to release monosaccharides, dried using nitrogen gas, and then re-dissolved in 5.0 mL of ultra-pure water. The samples are analyzed using a Thermo Field U3000 HPLC system with an Xtimate C18 column (4.6×200 mm, 5 μm). Standard solutions of Ara, Rib, Fuc, Glc, GlcA, Rha, Xyl, Gal, GalA, and Man are prepared, and the molar mass ratio of each monosaccharide in the samples is obtained by calculating the peak area ratio using the area normalization method.

2.3.7 Fourier transform infrared (FT-IR) spectroscopic analyses

The structure of AAP were analyzed and identified by FT-IR according to Qiu *et al*. (2019), with slight modifications. Take 2 mg of each polysaccharide sample and add 150 mg of KBr powder. Grind them together in an agate mortar to obtain a homogeneous fine powder. Then, use a pellet press to compress the powder into thin pellets. Measure the samples at a wavelength range of 4000-400 cm-1, using 32 scans.

2.3.8 Thermogravimetric (TG) analysis

The TG analysis of AAP was measured using the method of Chen *et al*. (2018) with slight modifications. Weigh approximately 3.0mg of AAP-W, AAP-A, and AAP-AL and place them in a crucible. After removing any outer impurities, set the measurement parameters as follows: test temperature range of 25-600℃, heating rate of 10℃/min, and nitrogen gas as the protective gas.

2.3.9 X-ray diffraction (XRD)

Crystallization properties of AAP determined by XRD. Set the 2θ scanning range for the sample from 5° to 80°, with a voltage of 40 kV, current of 40 mA, and using the step scan mode.

2.3.10 Three-phase contact angle

To determine the three-phase contact angles of *Auricularia auricula* polysaccharide, approximately 0.1 g of each type of polysaccharide powder is weighed and compressed into evenly thick flakes using a tablet press. The flakes are then carefully placed into a transparent container filled with corn oil. The table top and lens are adjusted to ensure the samples are level in the field of vision. An injection needle filled with water, free of bubbles, is positioned directly above the sample sheet, and water is allowed to drip slowly into the oil and onto the sample plane by slowly turning the knob. An optical contact angle measuring instrument is used to take pictures and observe changes in the droplet contour, and the images of the balanced droplet contours are analyzed to determine the three-phase contact angles of the *Auricularia auricula* polysaccharide.

2.4 Effect of different conditions on the stability of AAP-W stabilized emulsions

The effects of polysaccharide concentration, oil-water ratio, pH value and salt ion concentration on emulsion stability were investigated.

2.4.1 Effect of Polysaccharide Concentration on Emulsion Stability

Weigh a certain amount of AAP-W powder and dissolve it in 10 mL of deionized water containing 0.02% sodium azide. Stir until fully dissolved, resulting in final concentrations of 0.1%, 0.25%, 0.5%, 1%, 2%, and 3%. Then, add an equal volume of corn oil to prepare emulsions.

2.4.2 Effect of Oil-to-Water Ratio on Emulsion Stability

Set the concentration of AAP-W to 1% (containing 0.02% sodium azide) and adjust the oil phase fraction to 0.2, 0.3, 0.4, 0.5, and 0.6 (v/v) to prepare emulsions.

2.4.3 Effect of pH on Emulsion Stability

Set the concentration of AAP-W to 1% (containing 0.02% sodium azide) and adjust the solution pH to 3, 5, 7, 9, and 11. Keep the oil phase fraction at 0.5 (v/v) to prepare emulsions.

2.4.4 Effect of Salt Ion Concentration on Emulsion Stability

Set the concentration of AAP-W to 1% (containing 0.02% sodium azide) and adjust the salt ion concentration in the solution to 50 mM, 100 mM, 200 mM, 300 mM, 400 mM, and 500 mM. Keep the oil phase fraction at 0.5 (v/v) to prepare emulsions.

2.6 AAP emulsion loaded with β-carotene

2.6.3 Retention of β-carotene

β-carotene was dissolved in chloroform and prepared into solutions with concentrations of 0.5, 1, 2, 3, 4, 5 μg/ml. Absorbance was measured at 450 nm wavelength and the standard curve of β-carotene was drawn. The retention rate of β-carotene was measured by accelerated oxidation method. The emulsion loaded with β-carotene was stored at 25 ℃ and 60 ℃ for 8 days, and samples were taken every two days. Take out 1ml sample, add 2ml n-hexane and 1ml ethanol for extraction, repeat extraction for 3 times, combine with supernatant, transfer to a volumetric bottle with a fixed volume of 10ml, and then determine the absorbance at 450 nm wavelength to calculate the concentration C of β-carotene. The retention rate of β-carotene was calculated using C/C0 (C0 is the initial β-carotene content of the emulsion).

2.6.4 *In vitro* digestion of beta-carotene loaded emulsion

Perform *in vitro* simulated digestion experiments according to Brodkorb *et al*. (2019), with slight modifications.

Oral Digestion Stage: Simulate a saliva solution containing 0.896 mg/mL of KCl, 0.6 mg/mL of α-amylase solution, and 0.298 mg/mL of NaCl. Mix the initial sample with the prepared simulated saliva in a 1:1 (w/w) ratio, adjust the pH to 6.8, and heat the mixture in a water bath to 37℃. Stir the mixture at 37℃ for 5 minutes.

Gastric Digestion Stage: Simulate a gastric fluid containing 3.2 mg/mL of pepsin solution and 2 mg/mL of NaCl. Mix the sample after oral digestion with the simulated gastric fluid in a 1:3 (w/w) ratio, and heat the mixture in a water bath to 37℃. Stir the mixture at 37℃ for 2 hours, and adjust the pH to 3.0 every 15 minutes.

Intestinal Digestion Stage: Simulate an intestinal fluid containing 218.7 mg/mL of NaCl, 1.5 mL of 36.7 mg/mL CaCl2, 24 mg/mL of lipase and pancreatic protease solution, and 54 mg/mL of bile salt solution. Mix the sample after gastric digestion with the simulated intestinal fluid in a 1:2 (w/w) ratio, and heat the mixture in a water bath to 37℃. Stir the mixture at 37℃ for 2 hours, and adjust the pH to 7.0 every 15 minutes.

After digestion, β-carotene enters the micelle layer, which has smaller particle size compared to other components of the digested material. Micelles are spherical aggregates of amphiphilic molecules that spontaneously form in aqueous solutions. They have a hydrophobic core and a hydrophilic shell, allowing them to solubilize lipophilic substances. At the end of the *in vitro* simulated digestion, the digestion fluid is transferred to a centrifuge tube and centrifuged at 14000 rpm for 25 minutes. The micelle phase is separated from the other components, and the sample is extracted three times to completely release β-carotene from the micelle phase. The content of β-carotene is then determined. Bioaccessibility (B\*) and Digestive Stability (S\*) of β-carotene were calculated by the following formula:





CMicelle: Concentration of β-carotene in micelles. CDigesta: Concentration of β-carotene in the mixture after digestion in the small intestine stage. CInitial: Starting concentration of β-carotene in emulsions.