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Purification, characterization and antioxidant activities of polysaccharides from thinned-young apple

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ABSTRACT

Purification, characterization and antioxidant activities of polysaccharides from thinned-young apple (TYA) were investigated in the study. A water-soluble polysaccharide (TYAP) was obtained with hot water extraction, which was further purified by chromatography of *Cellulose* DEAE-52 and *Sephadex* G-150 to get three purified fractions of TYAP-1, TYAP-2 and TYAP-3. HPLC analysis showed that the three fractions were mainly composed of galactose and arabinose with the average molecular weights of 115 kDa, 479 kDa and 403 kDa, respectively and the monosaccharide compositions of TYAP-2 and TYAP-3 were more complicated than that of TYAP-1. Moreover, TYAP fractions of three had no absorptions at 260 nm and 280 nm in the UV spectrum, indicating the absence of protein and nucleic acid. Their structure properties were further confirmed by FT-IR. *In vitro* antioxidant activities of three fractions were characterized by DPPH•, HO•, $O_2^{\bullet-}$ and ferric-reducing antioxidant power systems. The results indicated that TYAP-1, TYAP-2 and TYAP-3 possessed significant antioxidant effects in a concentration-dependant manner in the tested concentration range of 0.25–5.0 mg/mL. Among the fractions tested, TYAP-3 showed significantly higher antioxidant activity than that of TYAP-1 and TYAP-2. All of these findings provide a scientific basis for the further use of polysaccharides from thinned-young apple.

1. Introduction

Apple is one of the most widely cultivated tree fruit in the world [1]. The pomaceous fruit is a main contributor of nutritional phytochemicals in the human diet [2]. Fruit thinning is to remove excessive fruitlets from apple trees, which is an effective measure to improve the size, color and quality of fruit at apple production and to balance the yield of apple the following year [3]. During the process, large quantities of young fruits are removed. For example, approximately 1.9 million tonnes of young apples are thinned annually in China alone. The thinned-young apples (TYA) are usually abandoned on the ground of the orchard [4]. The deserted young fruits can become the host of pathogens which accelerate the spread of fruit diseases. In addition, the practice is a waste of resources, since the unripe fruit contains biologically active compounds such as low-molecular weight flavonoids which show anticancer, antioxidant, antibacterial and antiallergenic activities [5,6].

Another type of substance contained in the young fruit is water-soluble polysaccharides which play important roles as dietary free radical scavengers for oxidative damage prevention and can be exploited as novel antioxidants [7]. The antioxidants are important in the preservation of food products, and they are effective in preventing and treating numerous free radical-mediated chronic diseases [8]. Therefore the water-soluble polysaccharide extracted from TYA can be a valuable choice since its polysaccharide is relatively low in toxicity and thus few side effects [9]. Although the simultaneous separation and purification of total polyphenols, chlorogenic acid and phlorizin from TYA have been thoroughly investigated [4], little work has been done regarding the water-soluble polysaccharides from TYA and their antioxidant activities. In this regard, detailed work is needed to carry out the structural characterization and their antioxidant capacities of polysaccharides from TYA.

The objectives of this study were to purify the crude polysaccharides from TYA by *Cellulose* DEAE-52 and *Sephadex* G-150 chromatography, and characterize the purified fractions by chemical analysis, Fourier transform infrared spectroscopy (FT-IR) and high performance liquid chromatography (HPLC). Furthermore, their *in vitro* antioxidant activities were also investigated. This study will provide a clue to elucidate the structure–function

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relationship, which would contribute to the sustainable use of TYA agricultural resource.

2. Materials and methods

2.1. Materials and reagents

TYA were collected 30 days after blossom in Liquan, Shaanxi province of China in May 2013. All samples were stored in a refrigerator and washed before experiments. The specimen of the plant materials was identified and deposited at College of Food Engineering and Nutritional Science, Shaanxi Normal University, China. *Cellulose* DEAE-52 and *Sephadex* G-150 were purchased from Whatman Co. (Maid-stone, Kent, UK) and Pharmacia Co. (Sweden), respectively. D-mannose, D-ribose, L-rhamnose, D-glucuronic acid, D-galacturonic acid, D-glucose, D-xylose, D-galactose, L-arabinose, D-fucose, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and trifluoroacetic acid (TFA) used were obtained from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). Triethylamine (TEA) and 1-phenyl-3-methyl-5-pyrazolone (PMP) were purchased from Merck (Darmstadt, Germany). Other chemicals used in this study were of analytical grade.

2.2. Preparation of TYA polysaccharides

The polysaccharides of TYA were isolated according to the reported method with modifications [10]. Briefly, the fruiting bodies of TYA were sliced and bleached to suppress the activities of polyphenol oxidase to improve the color value of polysaccharides. Then, the sliced TYA were dried at 55 °C and crushed into powder (60 mesh) by a disintegrator. The powder was extracted with 95% ethanol (1:10, w/v) refluxing at 80 °C for 8 h to remove impurities and small lipophilic molecules. Subsequently, the degreased powders (100 g) were dried, and extracted with hot water (1:40, w/v) at 90 °C for 2 h for three cycles. The combined aqueous extract was concentrated to 25% of the original volume by rotary vacuum evaporator at 60 °C and then centrifuged at 4500 g for 20 min. At this time, the supernatant was collected and precipitated for three cycles by adding five times of volume of 95% (v/v) ethanol at 4 °C for 24 h. After centrifuging, the separated precipitate was completely dissolved in appropriate volume of water, and intensively dialyzed for 3 days against ultrapure water (cut-off Mw 8000 Da) to remove the small molecular compounds (*e.g.*, flavonoids or polyphenols). The remaining portion was deproteinized by the freeze–thaw process for repeating 10 times in a plastic bottle, followed by filtration. Finally, the extracts were centrifuged at 3000*g* for 10 min to remove insoluble material and the supernatant was lyophilized in the freeze-dry apparatus (Sihuan Co., China) to obtain the refined thinned-young apple polysaccharides (TYAP) with a brown fluffy shape.

2.3. Isolation and purification of crude TYAP

Crude TYAP was purified sequentially by *Cellulose* DEAE-52 and *Sephadex* G-150 chromatography according to previous studies with slight modification [11,12]. Briefly, 10 mL of crude polysaccharide solution (0.3 g/10 mL) was centrifuged at 4000*g* for 8 min and then subjected to the DEAE-52 column ($2.5 \times 60\ cm^2$). Subsequently, the column was eluted stepwise with deionized water, followed by a NaCl gradient (0.1–0.5 M) at a flow rate of 1.0 mL/min. The obtained elution (8 mL/tube) was collected automatically and the carbohydrate contents were quantified by the phenol–sulfuric acid method [6]. As a result, three relevant fractions were obtained, concentrated, dialyzed against ultrapure water and further purified on a *Sephadex* G-150 column ($1.6 \times 60\ cm^2$) eluted with deioned water at a flow rate of 0.5 mL/min. The fractions obtained were combined and monitored by the phenol–sulfuric acid method. Each fraction generated a single elution peak representing TYAP-1, TYAP-2 and TYAP-3. Finally, three purified fractions (TYAP-1, TYAP-2 and TYAP-3) with high polysaccharides content were collected, concentrated, dialyzed and lyophilized for further study, respectively.

2.4. Characterization of TYAP fractions

2.4.1. Determination of content of carbohydrates

The total carbohydrate compounds in TYAP fractions were estimated by phenol–sulfuric acid colorimetric method with glucose as a standard [13]. The absorbances of five calibration solutions of glucose (10–50 μg/mL) were determined at 490 nm using a 2100 spectrophotometer (Unico, Shanghai), and the standard curve was drawn with absorbance as ordinate and concentration as abscissa.

2.4.2. Determination of content of total polyphenols

The content of total polyphenols was determined based on the Folin–Ciocalteu colorimetric method as previously described [14]. Briefly, 1.0 mL of appropriately diluted samples, 1.0 mL of Folin–Ciocalteu reagent (2 N, Sigma) and 12.0 mL of 7.5% sodium carbonate solution were added into a 25 mL volumetric flask and diluted with distilled water to volume. Then, the absorbance of the reaction mixtures was measured using a Unico 2100 spectrophotometer at 765 nm wavelength after incubation for 2 h at room temperature. A calibration curve ($Y = 9.9343X + 0.0035$, $R^2 = 0.9995$) was generated using a gallic acid standard in the range of 10–50 μg/mL.

2.4.3. Determination of content of protein and nucleic acid

TYAP fractions were dissolved in distilled water with magnetic stirring until complete solubilization [15]. UV spectrum was recorded on a Cary 50 UV–vis spectrophotometer (Puxi Co., China) in the range 190–400 nm taking distilled water as control.

2.4.4. Analysis of monosaccharides composition

Monosaccharides composition of TYAP fractions were analyzed with high performance liquid chromatography (HPLC) and the sample was prepared according to the method described by Lv et al. [10]. Briefly, 2.0 mL of 3 M TFA was added in 5 mL sealed ampoule to hydrolyze 20 mg TYAP to release constitutive monosaccharides at 95 °C for 8 h. After being cooled to room temperature, the reaction mixture was centrifugated at 4000 rpm for 5 min. The supernatant was transferred to a 5 mL micro-round-bottomed flask and was dried under reduced pressure before dissolving with 1.0 mL ultrapure water. Subsequently, the hydrolyzed TYAP solutions (100 μL) were spiked with 0.5 M methanol solution of PMP (200 μL) and 0.3 M aqueous NaOH (300 μL) for derivatization to attain a strengthen UV absorption. The mixture solution was incubated at 70 °C for 1 h, followed by cooling to room temperature. Next, the mixture solution was neutralized by adding 300 μL of 0.3 M HCl. The resulting solution was extracted with chloroform and the process was repeated three cycles. Finally, the aqueous layer was filtered through a 0.45 μm membrane for HPLC analysis.

The analysis of PMP-labeled monosaccharide was performed on a Shimadzu LC-2010A HPLC system which equipped with an UV detector fixed at 250 nm, an autosampler and Shimadzu Class-VP 6.1 workstation (SHIMADZU, Kyoto, Japan). The analytical column used was a reversed-phase C_{18} column (4.6 mm i.d. × 250 mm, 5 μm, Venusil, USA) at 37 °C. Acetonitrile was used as mobile phase A, and mobile phase B was 3.3 mM KH_2PO_4–3.9 mM TEA buffer containing 10% acetonitrile. The gradient program was as follows: 0–4 min, 94% B hold; 4–9 min, linear gradient to 88% B; 9–30 min,