

Physi-Chemical Property Research of Polysaccharides from Pomegranate Flowers

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Abstract

Pomegranate flowers as raw materials were used for extraction of polysaccharides by water-extraction and alcohol-precipitation method. After purification, the physical and chemical properties, structure, monosaccharide composition and molecular weight were studied. The results showed that the polysaccharides from pomegranate flowers mainly contained two kinds of water soluble acidic polysaccharides, and monosaccharide composition were arabinose and galactose, both contained hydroxyl, carboxyl, amino, hydroxyl radical, sulfate, beta glycosidic bond and alpha glycosidic bond structure. The molecular weight of PP1 and PP2 were 6.16×10^4 ($\pm 6.6\%$) and 9.01×10^4 ($\pm 3.2\%$), respectively. The results of this study laid the foundation for further development and application of polysaccharides from pomegranate flowers.

Keywords

Polysaccharides, Pomegranate Flowers, Physical and Chemical Properties

1. Introduction

Pomegranate flower was the floral organ of *Punica granatum* L., which contained polyphenols, flavonoids, three terpenoids and other components [1]. The pomegranate flowers could be used for the treatment of otitis media, epistaxis, and trauma hemorrhage as a traditional Chinese medicine [2], which had astringent taste. They were called “Gulina” in Uygur Medicine, played a role in treating slight corneal opacity, neurasthenia and nausea [3]. In addition, they also had been used in the treatment of diabetes and its complications in India [4].

Polysaccharides widely existed in nature and possessed a variety of biological activities, could be used as one of the effective constituents of Chinese herbal

medicine. They were ideal immune enhancers [5] [6] [7], and improved the immune system's function and had no toxic or side effects on normal cells. Polysaccharides were used for the drug research of antitumor, antiviral, anti-aging, which was one of the important directions of the new medicine [8]. Therefore, the studies of extraction and biological activity of plant polysaccharides had attracted more and more attention. Currently, the research indicated that pomegranate flowers contained polysaccharide components [9], but there were few researches on polysaccharide from pomegranate flowers. In this paper, we studied the physicochemical properties, structure, molecular weight and monosaccharide fractions of purified polysaccharides from pomegranate flowers, which laid a foundation for the development and utilization of polysaccharides from pomegranate flower.

2. Materials and Methods

2.1. Materials and Reagents

Pomegranate flower was collected from Zaozhuang University campus in June 2014. Pomegranate variety is "Gangliu". The sample was dried at constant temperature of 60 °C and stored in the refrigerator.

Phenol, sulphoacid, chloroform, isopropyl alcohol, ethanol, methanol, Coomassie brilliant blue G-250, sodium tetraborate, iodine, potassium iodide, ethyl acetate, acetic acid, acetone, ethyl ether, aniline, diphenylamine, phosphoric acid (AR); potassium bromide (SP); m-hydroxydiphenyl, bovine serum albumin (BSA), d-galacturonic acid, glucose, rhamnose, xylose, mannose, galactose, L-Arabinose, fructose were used without further purification. Blue dextran 2000 (BR); dextran T10, T20, T50, T70, T100, T200 (RT); 44 - 3500 dialysis bag, D101 resin, DEAE cellulose DE-52, Board silica gel GF254, Sephadex G-75 were used according to the instructions.

2.2. Test Methods

2.2.1. Measurement of the Polysaccharide Content

The content of polysaccharide was determined by phenol sulfuric acid method [10]. The specific steps of making the glucose standard curve were as follows: firstly, preparing 16 - 72 µg/mL of gradient standard solution of glucose, each gradient was 2.0 mL. After adding 1.0 mL of 5% phenol, quickly added 5.0 mL of concentrated sulfuric acid and shook well. The absorbance at 490 nm was measured after placing 30 min at room temperature. The regression curve was $A = 0.0168c - 0.0441$ ($R^2 = 0.9999$).

2.2.2. Determination of Protein Content

The content of protein was determined by Coomassie blue staining [11]. The specific steps of making protein standard curve were as follows: firstly, preparing 5 - 30 g/mL of gradient BSA standard solution, each gradient was 0.1 mL. Then added 5 mL Coomassie brilliant blue G-250 and shook well. The absorbance at 595 nm was measured after placing 10 min at room temperature. The regression

curve was $A = 0.9311c - 0.0055$ ($R^2 = 0.9972$).

2.2.3. Detection of Polysaccharide Purity

Two kinds of polysaccharides from pomegranate flowers were separated and purified. Determine the purity of polysaccharides samples by 190 - 400 nm UV spectrum.

2.3. Methods of Extracting and Purifying Polysaccharides from Pomegranate Flowers

2.3.1. Extraction Method of Crude Polysaccharide

Mixed pomegranate flower powder with distilled water at 1:20 of solid-liquid ratio, and extracted 3 h at 95°C. After anhydrous ethanol were added to get the volume fraction of 80%, kept overnight static polysaccharide precipitation, filtered and dried to obtain crude polysaccharide.

2.3.2. Seavage Protein Removal

Preparing 5 mg/mL crude polysaccharide solution, and added seavage reagent (chloroform: n-butanol = 4:1) according to the proportion of 1:1. Mixing reacted 20 min in a separatory funnel, released the underlying seavage reagent and intermediate denatured protein. Repeated 5 times until the denatured protein did not appear and determined the protein content. The polysaccharide with low protein content was obtained by adding 2 times volume of ethanol and dried in vacuum [12].

2.3.3. Removal of Pigment by Macroporous Resin D101

Preparing 10 mg/mL polysaccharide solution, then added the pretreated D101 resin with 10:1 (mL/g) ratio. After being stirred for 1 h, the resin was filtered out, and the polysaccharide solution was stored at 4°C.

2.3.4. Removal of Small Molecular Impurities by Dialysis

Transferred polysaccharides filtrate to 44 - 3500 Da dialysis bags, dialysed against distilled water for at least 24 h.

2.3.5. Classification and Purification of Polysaccharide Components

DEAE-52 cellulose was fully dissolved in pure water, and activated by washing with hydrochloric acid of 0.5 M and sodium hydroxide of 0.5 M. A chromatographic column of $\Phi 1.6 \times 50$ cm was adopted, and the volume of the packed bed was 56 mL. Balanced chromatographic column with 0.05 M sodium chloride and added 2 mL sample of polysaccharide solution. Then eluted with a gradient of 0.05 to 1 M sodium chloride. Controlled the flow rate was 0.5 mL/min, collected 1 tube every 10 min. The collection solution was detected by phenol sulfuric acid method and the elution curve was drawn. Six tube eluate with centered peak were collected, then freeze-dried after dialysis.

2.4. Analysis of Physicochemical Properties

Weighed each polysaccharide component 10.0 mg and mixed with 10 mL ultra-

pure water, dissolved at 4 degrees overnight.

2.4.1. Solubility Test

The polysaccharides were dissolved in cold water, hot water, ethyl acetate, ethanol, acetone, ether, n-butanol, isopropanol, and observed the dissolution.

2.4.2. Phenol Sulfuric Acid Test

1 mL polysaccharide solution, added phenol and sulfuric acid according to the method in 1.3.1, and observed the change of solution color.

2.4.3. Determination of Uronic Acid Content

The m-hydroxydiphenyl method [13] was adopted to determine the uronic acid content. 1 mL polysaccharide solution was cooled in ice bath, 6 mL $B_4Na_2O_7$ of sulfuric acid was added. Boiling 12 min, then cooled to room temperature, 80 μ L of 0.15% m-hydroxydiphenyl solution was added. After 40 min, OD_{525} was tested and calculated the content of uronic acid, d-galacturonic acid as standard sample.

2.4.4. I-KI Reaction

Absorbed 5 drops of polysaccharide samples solution in the small hole of the drip plate and two drops of I-KI solution were added. Contrasted color with distilled water and starch solution.

2.4.5. Infrared Spectrum Analysis of Polysaccharides

Mixed and grinded the dried polysaccharide solids with KBr at a ratio of 1.5 to 100 and compressed to tablet. After deducting the background, the tablets were scanned infrared spectrum within the range of 397 - 4001 cm^{-1}

2.4.6. Thin Layer Analysis of Monosaccharide Composition

Weighed 2.00 mg sample in 5 mL ampoules bottle, continuous 4 h of acid hydrolysis reaction at 110°C, then dried with nitrogen at 50°C, and added 3 mL methanol, repeated 3 - 4 times to remove TFA. Lastly, the precipitation was dissolved in 200 mL ultrapure water [14].

The silica gel plate was activated at 110°C for 30 min, and 5 mg/mL standard solution or the solution of PP1, PP2 were spotted. The sample volume was 0.5 mL. After drying, chromatography was carried out in the expansion agent. The proportions of liquid were ethyl acetate:methanol:acetic acid:water = 12:3:3:2. After spraying the chromogenic agent, the silica gel plate was placed at 150°C for 5 min, the migration distance was measured according to the spots, and the mobility (RF) was calculated. The chromogenic agent was 1% hydrochloric acid, 2% aniline, 2% of diphenylamine, and 8.5% of phosphoric acid in acetone.

2.4.7. Determination of Molecular Weight of Polysaccharides by Gel Method

With the Sephadex G-75 column, the mobile phase was 0.1 M of NaCl. The 10 t, 20 t, 50 t, 70 t, 100 t and 200 t of dextran standard samples were entered the chromatographic column, and measured the elution volume, named V_e , then

determined water column volume V_0 by measuring the blue glucose with 2 million molecular weight. The standard curve was made out, with V_e/V_0 as the ordinate and logarithm of molecular weight as the abscissa. The polysaccharide components were used to determine V_e of elution volume under the same conditions, and the molecular weight was obtained according to the standard curve.

3. Results and Analysis

3.1. Purification of Polysaccharides from Pomegranate Flowers

After the extraction of crude polysaccharide by sewage method, the content of protein was 1.03% in polysaccharide, and the retention rate was 58.14%. Using macroporous resin to remove pigment, and dialysis method to remove small molecular impurities, DEAE-52 cellulose column to separate polysaccharides, the gradient elution curve was shown in **Figure 1**. As shown in **Figure 1**, the two prominent symmetric peaks indicated that polysaccharides from pomegranate flowers were separated into two components. According to the order, the two peaks recorded as polysaccharide 1 and 2 (PP1; PP2). The protein contents of two components closed to 0, determined by coomassie brilliant blue G250 method. Furthermore, in the UV absorption spectra of PP1 and PP2 shown in **Figure 2**, the obvious absorption peaks can be observed around 200 nm, which were characteristic absorption peaks of polysaccharides. These results indicated that PP1 and PP2 were pure, without pollution of nucleic acid or protein.

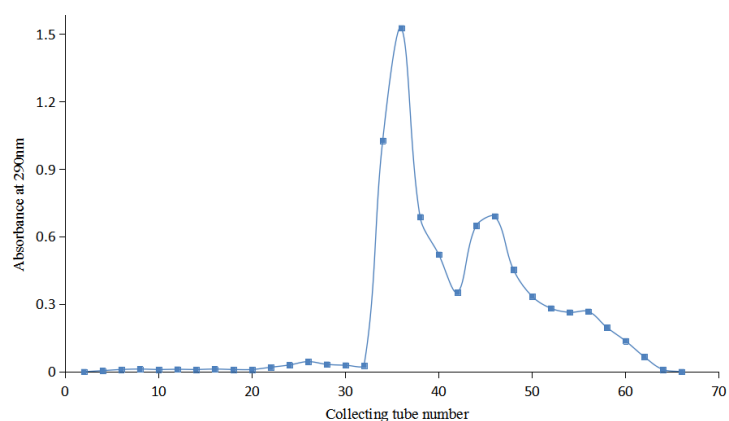


Figure 1. The elution curve of polysaccharides from pomegranate flowers.

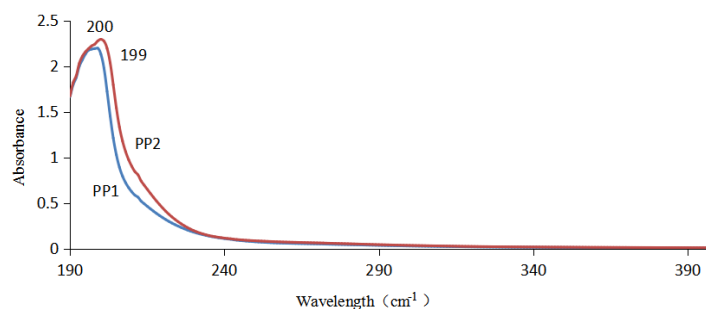


Figure 2. The UV scanning curve of PP1 and PP2.

3.2. Basic Physicochemical Properties of PP1 and PP2

PP1 was white flocculent solids, soluble in water, aqueous solution was colorless clear liquid; PP2 was popcorn flocculent solids, slightly soluble in water, aqueous solution was colorless popcorn clear liquid. The two compounds were not soluble in ethyl acetate, ethanol, acetone, ether, n-butanol and other organic solvents. The phenol sulfuric acid reaction was positive in both of them, and the contents of uronic acid were 57.4% in PP1 and 27.5% in PP2, which indicated that both of them were acidic polysaccharides. The iodine potassium iodide reactions of PP1 and PP2 were negative, indicating that the samples were different with starch. The data are shown in **Table 1**.

3.3. Monosaccharide Composition of PP1 and PP2

Analysing the results of silica gel chromatography, the specific migration rate of each monosaccharide standard and the acidolysis products of PP1 and PP2 were shown in **Table 2**. From the color spots and migration speed, it can be concluded that the monosaccharide components of PP1 and PP2 contained arabinose and galactose.

3.4. Infrared Spectrum Analysis of PP1 and PP2

The infrared spectra of PP1 and PP2 were shown in **Figure 3**. The functional groups in monosaccharide molecules were mainly hydroxyl and carbon hydrogen bonds, the absorption peaks of the whole spectrum were less, and most of them were wide and blunt.

As shown in **Figure 3**, the infrared absorption spectra of the two components were consistent, but with different absorption intensity. In 3400 - 3200 cm^{-1} range, the absorption of PP1 was strong and broad, the large and intense bands near 3380 cm^{-1} were the absorption peak of O-H which overlaps with the stretching vibration absorption peak of N-H bond [1]; 2927 cm^{-1} and 2857 cm^{-1} were the stretching vibration absorption peaks of C-H, which were called characteristic absorption peaks of polysaccharides together with the OH absorption peak at 3380 cm^{-1} [15]; C=O stretching vibration absorption peak of COOH at 1722 cm^{-1} ; 1621 cm^{-1} was the N-H variable angle vibration absorption peak of first and second class amino; the absorption peak at 1406 cm^{-1} (PP1) and 1423 cm^{-1} (PP2) were attributed to C-O stretching vibration absorption peaks of carboxyl;

Table 1. Basic physicochemical properties of polysaccharides.

Groups	Surface	Solubility		Phenol sulfuric acid reaction	The uronic acid content/%	I-IK reaction
		Water	Organic solvent			
PP1	White flocculent	Soluble in cold water	Insoluble	Positive	57.4	Negative
PP2	Pale yellow flocculent	Slightly soluble in hot water	Insoluble	Positive	27.5	Negative

The absorption peak at 1142 cm^{-1} was the C-O absorption peak on the ring; At 1102 cm^{-1} and 1020 cm^{-1} were two variable angle vibrational absorption peaks of the alcohol hydroxyl radical; Absorption peaks at 895 cm^{-1} indicate that both PP1 and PP2 have beta glycosidic bonds; The absorption peak at 855 cm^{-1} was characteristic of alpha difference structure [16], so it was presumed that PP1 and PP2 also contained alpha glycosidic bonds. The absorption peak of D grape sugar ring [17] was 778 cm^{-1} , so it could be confirmed that the monosaccharide component contained galactose; the absorption peaks at 1240 cm^{-1} - 1.850 cm^{-1} and 820 cm^{-1} indicated that the two components contained sulfuric acid group, proved that the PP1 and PP2 were acidic polysaccharides.

3.5. Molecular Weight Identification of PP1 and PP2

The standard curve of dextran standard was $y = -2.8061 \times 16.199$ ($R^2 = 0.9999$). The outer water volume of dextran column was 14.4 mL, and the elution volume and the molecular weight range of PP1 and PP2 were shown in **Table 3**. The elution peaks of PP1 were wider, and there were some miscellaneous peaks in the corresponding PP2 peaks. The elution peaks of PP2 were relatively narrow, and with miscellaneous peaks corresponding to PP1. This showed that the two components could not be separated completely, and PP1 was the main component.

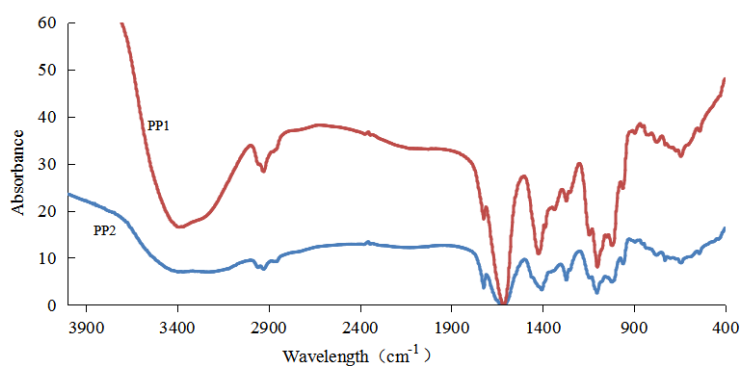


Figure 3. The IR scanning curve of PP1 and P2.

Table 2. The color and chromatographic mobility of each component.

Monosaccharide standard							PP1		PP2	
rhamnose	xylopyranose	mannose	arabinose	galactose	fructopyranose	glucose	Spot 1	Spot 2	Spot 1	Spot 2
light green	sky blue	Pale Yellow-Green	light blue	Pale Yellow-Green	brick red	Pale Yellow-Green	Pale Yellow-Green	light blue	Pale Yellow-Green	light blue
0.7857	0.6963	0.6296	0.6489	0.5797	0.6187	0.6141	0.5928	0.6446	0.5974	0.6439

Table 3. Molecular weight of polysaccharides from pomegranate flowers.

Component	elution volume (ml)	molecular weight
PP1	39.71 mL	6.16×10^4 ($\pm 6.6\%$)
PP2	33.06 mL	9.01×10^4 ($\pm 3.2\%$)

4. Conclusion

In this study, the physical and chemical properties of polysaccharides from pomegranate flowers were studied by various physical and chemical methods. The results showed that PP1 and PP2 were acidic polysaccharides, soluble in water, insoluble in organic solvents, without starch, nucleic acids, proteins and other substances; which mainly consisted of the arabinose and galactose; contained hydroxyl, carboxyl, amino, hydroxyl, sulfate base, beta glycosidic bond and glycosidic bond structure. The molecular weight of PP1 and PP2 was 6.16×10^4 ($\pm 6.6\%$), and 9.01×10^4 ($\pm 3.2\%$), respectively. The results laid a foundation for the further development and application of polysaccharides from pomegranate flowers.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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