Food Quality and Safety, 2023, **7**, 1–8
https://doi.org/10.1093/fqsafe/fyad029
Advance access publication 3 July 2023
Article

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Identification of carbohydrate in *Polygonatum sibiricum*: fructo-oligosaccharide was a major component

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Abstract

Polygonatum sibiricum, famous for its physiological activities, is a popular Chinese food and a traditional Chinese herb containing many carbohydrates as the main component. However, except for most reported polysaccharides, other detailed compositions of carbohydrates are still not clear. To verify the controversial existence of starch and investigate other components, especially oligosaccharides, we used iodine–potassium iodide colorimetric detection and enzymatic hydrolysis to determine starch. Then, oligosaccharides were analyzed by thin-layer chromatography, gel permeation chromatography, high-performance anion exchange chromatography with pulsed amperometric detection, and hydrophilic interaction chromatography–electrospray tandem mass spectrometry. The results showed that the rhizome of *P. sibiricum* lacked starch, and fructo-oligosaccharides were the main component, accounting for approximately 28.95%. Oligosaccharides with degrees of polymerization above 10 were the most abundant components. This study clearly illustrated the unknown carbohydrate components of the *Polygonatum* rhizome, promoting its functional value with new evidence.

Keywords: *Polygonatum sibiricum*; starch determination; fructo-oligosaccharides; HPAEC-PAD; HILIC-ESI-MS/MS.

Introduction

Polygonatum is a perennial plant belonging to the Asparagaceae family (Bremer *et al.*, 2009). It is a traditional Chinese herb that can be eaten as food or used medicinally. The rhizomes of *P. sibiricum* (PS), *P. cyrtonema* Hua (PC), and *P. kingianum* (PK) are the three species recorded in pharmacopeia. With regard to its components and function, it was commonly reported that *Polygonatum* contains polysaccharides, flavonoids, saponins, and alkaloids, thus it has a variety of pharmacological effects, including anti-aging, antibacterial (Cui *et al.*, 2018), antiglycation (Zhao *et al.*, 2020), immunomodulatory (Chen *et al.*, 2020), and antidiabetic activities (Shi *et al.*, 2023).

Numerous studies have been done to demonstrate that polysaccharides were the primary components of *Polygonatum* with a content of approximately 5%–20% (He *et al.*, 2022; Hu *et al.*, 2022). The polysaccharides from *P. sibiricum* were mainly composed of mannose (Man), galactose (Gal), glucose (Glc), rhamnose (Rha), arabinose (Ara), and galacturonic acid (GalA) (Sun *et al.*, 2020). However, the total sugar content in the rhizomes is approximately 40%–60% (Yu *et al.*, 2019; Wei *et al.*, 2022). From the above result of the existence of glucose, supposing there is much starch seems reasonable, but it also remains controversial as follows: one study showed that the starch content accounted for approximately 2.2%–5.9% in PC from different origins (Wang *et al.*, 2021a), and the soluble starch content was approximately 0.04%–0.09%, as determined by the iodine colorimetric method (Shen *et al.*, 2020). Using enzymatic hydrolysis, starch content in PK was approximately 3% (Li *et al.*, 2018). Moreover, through acid hydrolysis, the starch content of PK, PC, and PS was 9.98%–10.89% (Zhang *et al.*, 2022), and up to 22.8% in *P. sinopubescens*. In addition, one study noted that the thin-walled cells of this product contained starch granules, and dropping the iodine test solution onto the cross-section of PK with a purple-colored appearance (Zhu *et al.*, 2019) also indicated the presence of starch. On the other hand, when comparing the microscopic characteristics of several Chinese Materia Medica, the *Polygonatum* rhizome did not contain starch granules but contained other water-soluble sugars (Wong *et al.*, 2012), and a recent study using iodine staining and cytological observation demonstrated the absence of starch in PC seeds and rhizomes (Si and Zhu, 2021).

Received 23 March 2023; Revised 9 June 2023; Editorial decision 16 June 2023

We hypothesize that the *Polygonatum* rhizome probably contains many oligosaccharides. A few studies found oligosaccharides in PC, but the content was not determined (Jin *et al.*, 2018). Therefore, in addition to polysaccharides, approximately 30%–40% of carbohydrate components have not been clearly identified. In the present study, we used the rhizome of PS to investigate the unknown part. The carbohydrates were extracted by hot water. Then, alcohol precipitation was conducted to separate the polysaccharides fraction (which precipitated) and the other fraction. The unprecipitated fraction was identified by thin-layer chromatography (TLC), gel permeation chromatography (GPC), high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), and hydrophilic interaction chromatography–electrospray tandem mass spectrometry (HILIC-ESI-MS/MS). This study systematically analyzed the 30%–40% unknown carbohydrate components of PS and demonstrated a new prebiotic natural source that is rich in fructo-oligosaccharide, which will represent a revolutionary breakthrough in *Polygomatum*'s nutrients and its product development in the future.

Materials and Methods

Materials and chemicals

PS dried rhizomes from Ninghai, Zhejiang Province, were cleaned, dried at 101 °C for 1.5 h in a forced fan oven, and then ground to an average particle size of 250 μm. Amylopectin from maize, amylose from maize, α-amylase and amyloglucosidase from *Aspergillus niger* were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China); glucose, fructose, sucrose, fructo-oligosaccharides from chicory, dextran standards with the weight-average molecular weights (M_w) of 1000 and 5000 were purchased from Sigma-Aldrich (St. Louis, MO, USA), and the above monosaccharide mass percentage was greater than 98%; 1-kestose and 1F-fructofuranosylnystose were purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China); other reagents were analytically pure reagents.

Extraction of polysaccharides and the low molecular weight fraction

PS powder was infiltrated, respectively, with 95% ethanol and anhydrous ether to remove fat, pigment and other substances. The residue was collected and dried, and ultrapure water was added for extraction at 90 °C for 2 h. After centrifugation, the unprecipitated fraction was the PS extract solution.

Absolute ethanol was added to the PS extract solution to bring its ethanol concentration to 80% and placed at 4 °C overnight for alcoholic precipitation. The precipitated fraction was crude polysaccharides. The unprecipitated fraction was concentrated under reduced pressure until there was no ethanol, and then diluted with ultrapure water to obtain the low molecular weight fraction from PS.

Carbohydrate content analysis

Determination of total carbohydrate content

The total carbohydrate content was calculated by the subtraction method of the Association of Official Analytical Chemists (AOAC, USA), total carbohydrate (%)=1–(protein+fat+moisture+ash). The protein content was determined by the Kjeldahl method (AOAC, 2001), which was based on total nitrogen content multiplied by a conversion factor (6.25). Crude fat was extracted using petroleum ether by continuously refluxing for 6 h, and the liquid was collected and weighed after it was dry. Moisture content was determined by oven dehydration at 105 ℃ for 12 h, then cooled and weighed. The total ash content was determined by a muffle furnace at 650 ℃ for 4 h, and then the weight of the ash content was recorded (Gupta *et al.*, 2016).

Determination of dietary fiber content

Total dietary fiber (TDF), soluble dietary fiber (SDF), and insoluble dietary fiber (IDF) were calculated by the AOAC enzymatic-gravimetric method, TDF=SDF+IDF.

Determination of total sugar content

The total sugar content was analyzed by the phenol–sulfuric acid method (DuBois *et al.*, 1956). Using sucrose as a standard, dilute the solution to an appropriate concentration, and then add 1 mL of 6% phenol solution and 5 mL of H_2SO_4. The absorbance was measured at 620 nm. All contents were calculated and expressed on a dry-weight basis.

Determination of starch content

Iodine–potassium iodide colorimetric detection

Starch and iodine can combine to form a spiral structure known as a complex, which has a special color reaction depending on their branching. PS powder was incubated with 0.5 mol/L KOH in boiling water for 10 min, and then iodine–potassium iodide (I_2-KI) was added dropwise for color development reaction while comparing it with amylopectin and amylose. The absorbance curve was measured between 400 nm and 800 nm.

Enzymatic hydrolysis reaction

Starch content was determined by the increase in glucose content after enzymatic hydrolysis reactions. The powder was mixed with hot water at 90 °C and kept warm for 30 min (pH 6–7). Next, α-amylase was added and kept warm for an additional 90 min. After cooling to 60 °C, the solution was incubated in acetate buffer containing amyloglucosidase (pH 4.5) for 60 min (Man *et al.*, 2013). After centrifugation, the supernatant was precipitated with 80% ethanol, the unprecipitated fraction was collected, and ethanol was removed. Solutions were passed through 0.22 μm aqueous phase filter membranes.

Analysis of glucose content by HPAEC-PAD

Glucose was used as a standard product and dissolved in ultrapure water to prepare standard sugar solutions of different concentrations. Samples were analyzed on a Dionex ICS 5000+ chromatographic system (Thermo Scientific, Waltham, MA, USA) and a Dionex pulsed amperometric detector equipped with a Dionex Carbopac PA-10 column (250 mm×4 mm). The mobile phase comprised solutions A (deionized water) and B (200 mmol/L NaOH), and the gradient elution procedure was as follows: 0–15 min, 24% B; 15–19 min, 24%–100% B; 19–27 min, 100%–24% B. The flow rate of the mobile phase was 1.0 mL/min, and the

column temperature was 35 °C. All contents were calculated and expressed on a dry-weight basis.

Analyze the composition of the low molecular weight fraction

TLC analysis

TLC analysis was performed on a 5 cm ×7 cm silica gel plate. A solution of glacial acetic acid:chloroform:absolute ethanol:water at a ratio of 10:11:11:2 (volume fraction) was used as a developer for good separation. Diphenylamine (2.5 g), 5 mL of aniline and 25 mL of phosphoric acid were dissolved in 220 mL of acetone to prepare the colorants. Glucose, fructose, sucrose, 1-kestose, 1F-fructofranosylnystose, and dextran standards (M_w 1000 and M_w 5000) were used as standards at a concentration of 3 mg/mL. When the spots had sufficiently expanded, wet the silica gel plate with a colorant, dry quickly, and further heat at 105 °C for 10 min.

Quantitative analysis by HPAEC-PAD

Glucose, fructose, and sucrose were dissolved in ultrapure water to prepare mixed standard sugar solutions of different concentrations. The low molecular weight fraction was hydrolyzed with 2 mol/L trifluoroacetic acid at 110 °C for 30 min, and then blown dry using nitrogen, and the excess acid was removed by adding methanol and drying. Then, it was redissolved in ultrapure water to obtain acidolysis products. The low molecular weight fraction and the acidolysis products were passed through 0.22 μm aqueous phase filter membranes. The composition of oligosaccharides and monosaccharide content were analyzed by HPAEC-PAD. The conditions were the same as the analysis of glucose content by HPAEC-PAD.

Molecular weight distribution analysis by GPC

The molecular weight was measured by GPC using Waters 1525 HPLC (Waters, Milford, MA, USA), equipped with Ultrahydrogel 250 column (7.8 mm×300 mm) and Ultrahydrogel 120 column (7.8mm×300 mm). In addition, glucose (M_w 180), 1-kestose (M_w 504), 1F-fructofranosylnystose (M_w 829), and dextran standards (M_w 1000 and M_w 5000) were used to calibrate the standard curve by Waters Breeze GPC Software. The injection volume was 50 μL with 0.15 mol/L NaCl as the mobile phase (Wu *et al.*, 2010).

According to the proportion of the peak area, the content of the corresponding substance of each peak was calculated, each peak content (%)=peak area×total oligosaccharide content.

Analysis of the structure of oligosaccharides by HILIC-ESI-MS/MS

The structure of oligosaccharides was analyzed by HILIC-ESI-MS/MS with a BEH Amide column (Waters) and an AB SCIEX Triple-TOF 5600+ mass spectrometer (AB SCIEX, Framingham, MA, USA). Mass spectrometry data were obtained in negative ion mode using TOF MS-Product Ion-IDA acquisition mode. Fructo-oligosaccharides from chicory was used as a standard substance. Data were processed by PeakView® software (Version 1.2.0.3; AB SCIEX).

Results and Discussion

Carbohydrate contents

The contents of moisture, protein, fat, ash, and total carbohydrates are shown in Figure 1. Dietary fiber is considered an important nutrient, which refers to carbohydrates such as polysaccharides and oligosaccharides that are difficult to digest and absorb (An *et al.*, 2022). According to the calculation, the total dietary fiber content in PS was 19.4%, including 6.5% soluble dietary fiber (SDF) and 12.9% insoluble dietary fiber (IDF), which showed that the *Polygonatum* rhizome is a good source of dietary fiber.

In addition, the total carbohydrate content reached 80.11% by subtraction method, indicating that carbohydrates were the main component of PS. The total sugar content was 48.07%±1.06%, approximately equal to the water-soluble carbohydrate content of PS, and the content of the low molecular weight fraction was 41.63%±3.16%, indicating that small molecule sugars are the main carbohydrates of PS.

Starch content

Figure 2A shows the color change before and after the reaction with I_2-KI. It is clear that amylose turned blue and amylopectin turned red when combined with iodine, but the PS solution showed no noticeable changes, the same as the blank control. Amylose and amylopectin both showed absorption peaks between 500 nm and 600 nm when scanned at 400–700 nm (Figure 2B), but there were no absorption peaks for the PS solution, as with the blank. Analysis revealed that

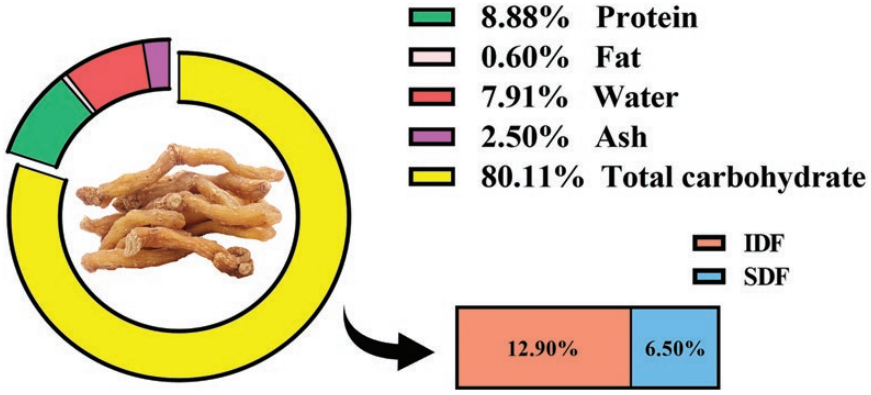

Figure 1. Nutrient contents (as a percentage of raw powder weight).

amylose and amylopectin were missing in PS. Meanwhile, it was found that the glucose content hardly changed after acidolysis as shown in Figure 2C. These results indicated a lack of starch in PS, and neither polysaccharides nor starch were the major carbohydrates of PS.

Chemical composition of low molecular weight fraction

As shown in Figure 3A, the TLC analysis showed that the low molecular weight fraction contained fructose and sucrose; however, fructose could not be detected after 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatization (Wang *et al.*, 2021b). Therefore, the HPAEC-PAD method was used to determine the contents of fructose, glucose, and sucrose.

According to the HPAEC-PAD analysis (Figures 3B and 3C), the main free monosaccharides contained in the PS extract solution were glucose (0.34%±0.13%) and fructose (4.46%±0.81%), and the main disaccharide was sucrose (3.51%±0.07%), which is a representative disaccharide and plays an important role in photosynthesis (Geng *et al.*, 2022).

After acidolysis, glucose and fructose were the main monosaccharides, accounting for 8.27%±1.26% and 28.99%±2.87%, respectively. These data implied that approximately 6.18% of glucose and 22.78% of fructose came from oligosaccharides, which indicated that the oligosaccharide in PS was mainly glucofructan, which can significantly enhance the growth and functionality of gut microflora (Singh *et al.*, 2017). The glucose and fructose contents were added together to calculate the total content of 28.95% glucofructan.

Identification of the low molecular weight fraction

In Figure 4A, the molecular weight distribution of the low molecular weight fraction from PS showed seven peaks. The weight-average molecular weights (M_w) were calculated depending on the calibration curve, and the content of each

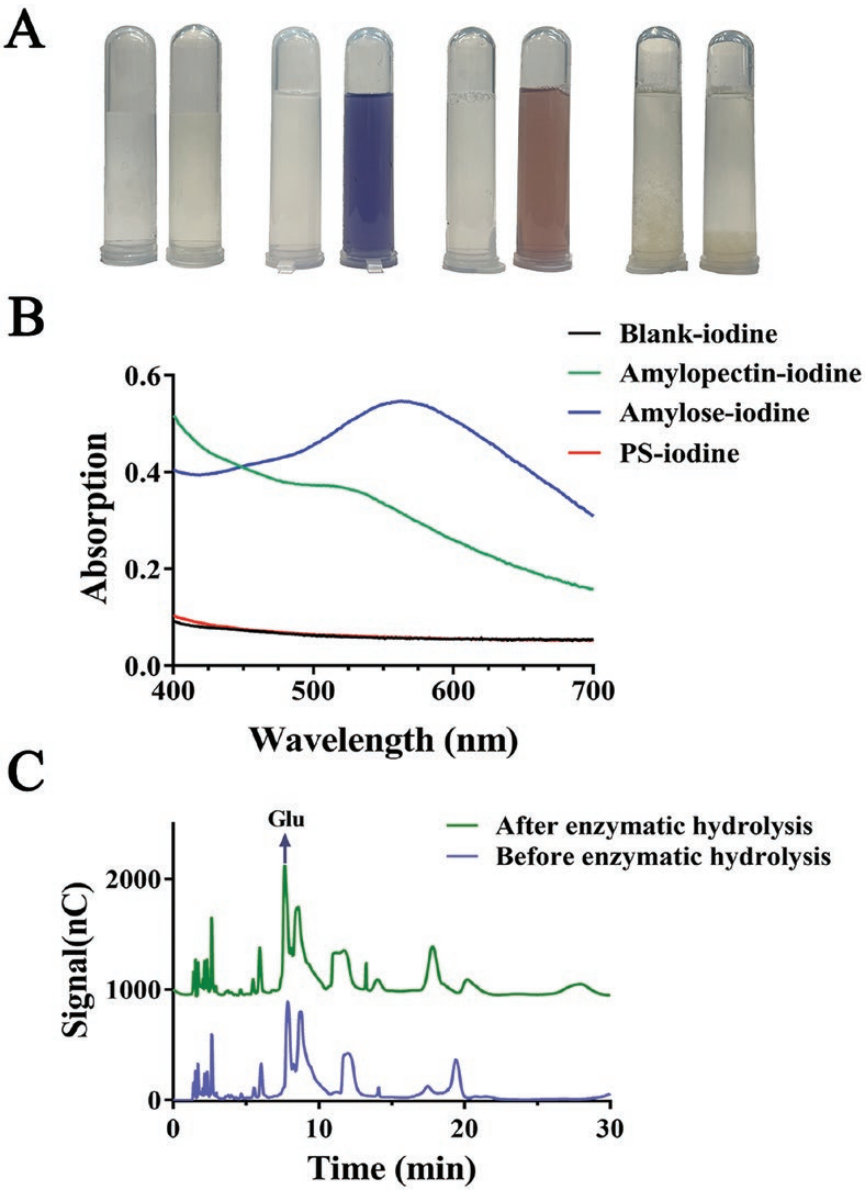

Figure 2. (A) Color development with the addition of I_2-KI. From left to right: blank, amylose solution, amylopectin solution, and PS solution. (B) Light absorption scan (400–700 nm). (C) Signal of glucose before and after the enzymatic hydrolysis under HPAEC-PAD analysis.

peak is shown in Table 1. The molecular weights of Peaks 6 and 7 were sharp, and the molecular weights of Peak 1 were wide with the 1853 Da, which indicates the presence of oligosaccharides with a degree of polymerization (DP) greater than 10. According to the peak area ratio and the content of Peak 1 (52.45% and 15.18%), and combined with the results of the chemical composition above, it was speculated that the main oligosaccharide in the low molecular weight fraction from PS was mainly glucofructan, of which DP was greater than 10.

Recently, it was reported that HILIC was used for the analysis of milk oligosaccharides, galactooligosaccharides, and linear oligosaccharides from plant cell walls (Kailemia *et al.*, 2014). HILIC-ESI-MS/MS provided more structural information, such as the precise molecular weight and the degree of polymerization of oligosaccharides. Based on the composition of PS oligosaccharides, fructo-oligosaccharides from chicory were used as a standard, and they were inulin-type oligosaccharides, which were usually produced from root chicory group (Lenzi *et al.*, 2022). Inulin-type oligosaccharides can form fructose chains with terminal glucose units (GF_n; Van Laere and Van Den Ende, 2002), which is similar to sucrose.

Total ion chromatograms (TIC) of the low molecular weight fraction from PS and the standard are shown in Figure 4B. Because chromatographic peaks could not be unambiguously identified by TIC, identities had to be confirmed by comparing retention time and molecular ions for each peak.

In this study, the mass spectral conditions were optimized in negative-ion mode, and oligosaccharides had obvious peaks and good separation in mobile phase conditions. By comparing the retention time and the MS^2 data of the standard and low molecular weight fraction from PS, the main fructo-oligosaccharide structures in PS were determined.

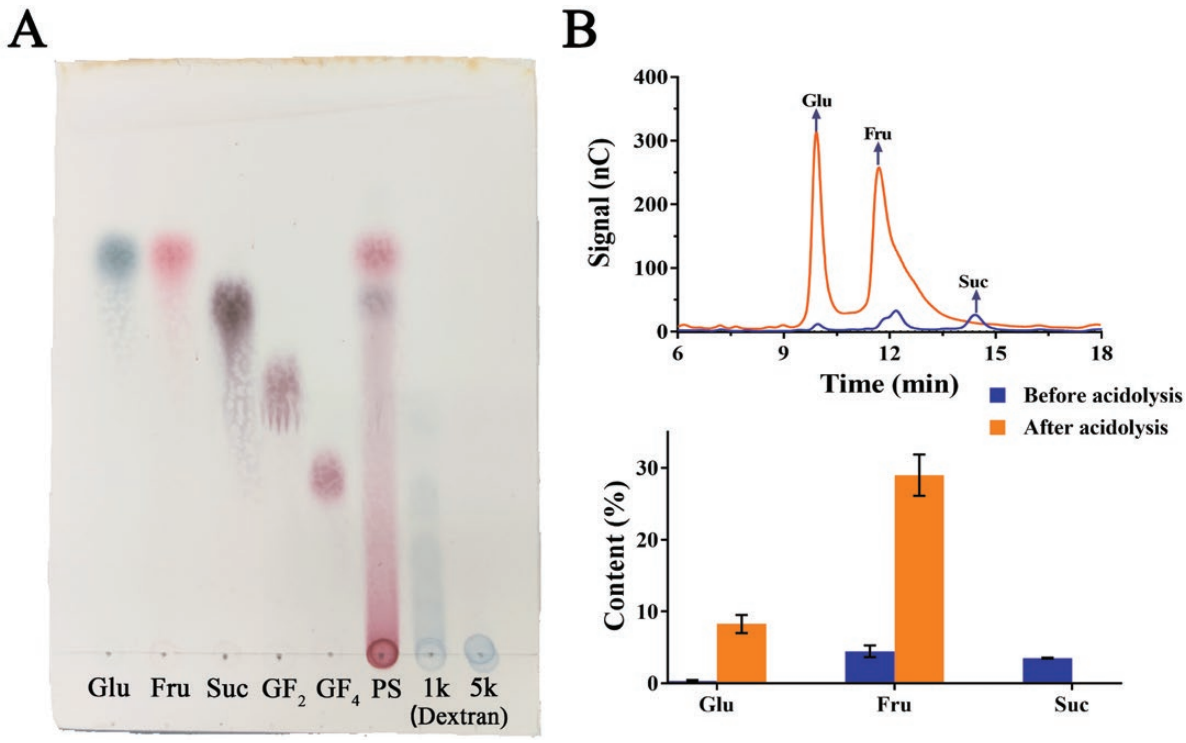

Figure 3. (A) TLC of glucose (Glu), fructose (Fru), sucrose (Suc), 1-kestose (GF_2), 1F-fructofranosylnystose (GF4), low molecular weight fraction of PS and dextran standards with M_w 1000 and M_w 5000 (1k and 5k) in TLC experiments. (B) Signal of glucose, fructose and sucrose under HPAEC-PAD analysis, and changes in their contents (as a percentage of raw powder weight).

Table 1. The molecular weights and peak area ratios of seven peaks in the low molecular weight fraction from PS

Peak	M_w	M_n	M_p	Peak area ratio (%)	Content (%)
1	1858	1619	1750	52.45	15.18
2	787	783	890	6.06	1.75
3	604	601	681	4.54	1.31
4	451	447	447	5.83	1.69
5	363	362	385	1.69	0.49
6	263	261	278	25.07	7.26
7	178	177	163	4.36	1.26

M_n, number-average molecular weight; M_p, peak molecular weight.

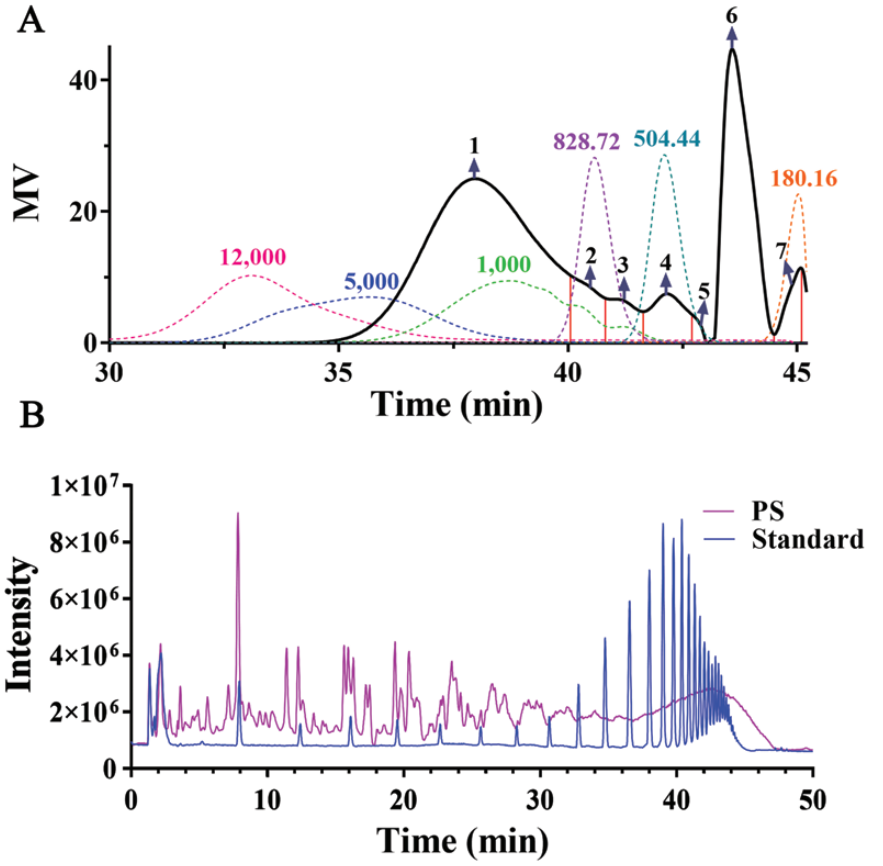

Figure 4. (A) GPC profiles with molecular weight distribution of low molecular weight fraction from PS. (B) Total ion chromatograms (TIC) of standards and low molecular weight fraction from PS.

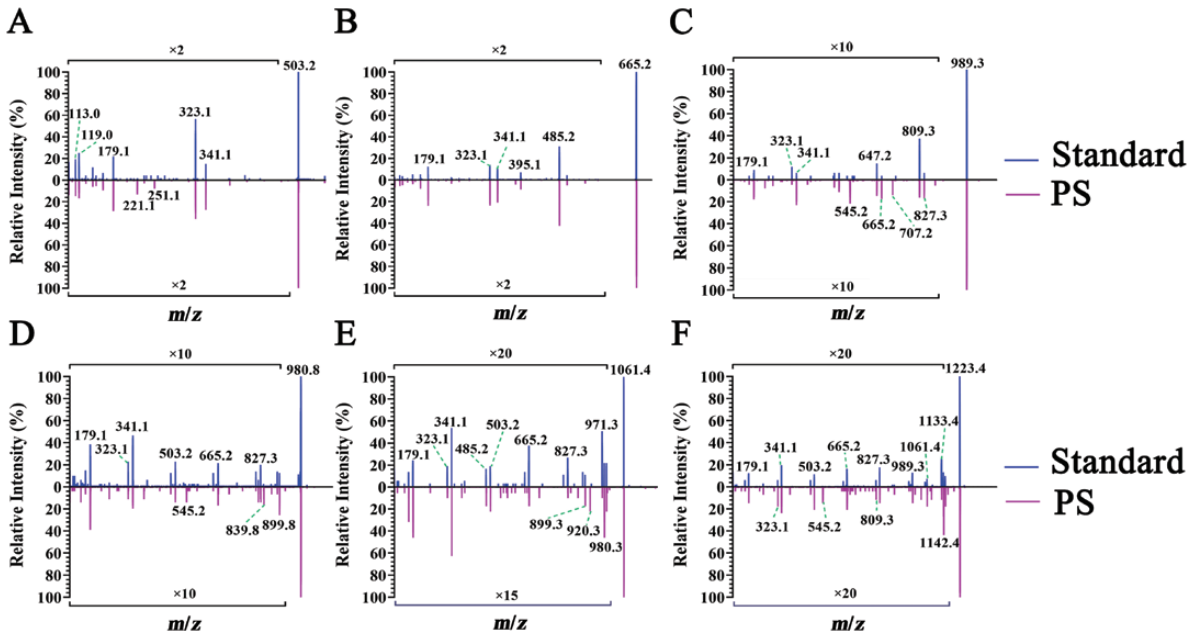

Figure 5. MS^2 spectrums of fructo-oligosaccharides. (A) MS^2 spectrum of the ion at *m/z*=503.2. (B) MS^2 spectrum of the ion at *m/z*=665.2. (C) MS^2 spectrum of the ion at *m/z*=989.3. (D) MS^2 spectrum of the ion at *m/z*=980.8. (E) MS^2 spectrum of the ion at *m/z*=1061.4. (F) MS^2 spectrum of the ion at *m/z*=1223.4.

As shown in Figure 5, the MS^2 data of the standard and PS appeared at a consistent retention time, and then the data were placed on the same abscissa, and their fragment ion peaks were compared. When DP is 2–7 ($GF–GF_6$), fructo-oligosaccharides exhibit quasi-molecular ions $[M-H]^-$: dimer (peak 1) (M_w 342) *m/z*=341, 3-mer (M_w 504) *m/z*=503, 4-mer

(M_w 666) *m/z*=665; when DP is 12–20 (GF_{11}–GF_{19}), fructo-oligosaccharides exhibit quasi-molecular ions $[M-2H]^{2-}$: 13-mer (M_w 1962) *m/z*=989, 14-mer (M_w 2124) *m/z*=1061, 15-mer (M_w 2448) *m/z*=1223 (Chen *et al.*, 2021).

The comparison found that although there were slight differences in the relative intensity of some fragments, they were almost identical overall, and it was confirmed that the PS contained fructo-oligosaccharides with DPs of 3, 4, 6, 12, 13, and 15, and the structure was the same as fructo-oligosaccharides from chicory. As a result, PS contained six inulin-type oligosaccharides: GF_2, GF_3, GF_5, GF_{11}, GF_{12}, and GF_{14}. Therefore, PS offers a new prebiotic natural source containing many fructo-oligosaccharides.

Conclusions

Polygonatum has been used for thousands of years for dietary treatment and is considered to be moderately successful in Chinese folklore. However, carbohydrates, as the primary component of *Polygonatum*, are more than 50%, but the composition is not clearly identified. Although many studies have reported that PS contains a large amount of starch, this study verified a completely different result that PS contains little starch, and the main sugar is fructo-oligosaccharides (approximately 30%), which is a well-known prebiotic that promotes human gut health. In particular, fructo-oligosaccharides above DP10 have been identified for the first time. These new meaningful findings explore a new natural prebiotic source that is rich in fructo-oligosaccharides, showing great health value.

Author Contributions

Jiabei Xia: drafted the manuscript and completed the experimental section. Cenrong Zhang: collected data and contributed to the conception of the study. Kai Zhu: performed the data analyses. Xingyu Mei: collected the reference. Huan Cheng, Shiguo Chen, Xingqian Ye, and Jianle Chen: review & editing, supervision, funding acquisition.

Funding

This work was financially supported by the grants from Ningbo Municipal Bureau of Science and Technology (2022Z174), The Major Agricultural Technology Collaborative Promotion Plan of Zhejiang Province (2022XTTGZYC03), and The Key Research and Development Program of Zhejiang Province (2021C02001), China.

Conflict of Interest

The authors declare no conflict of interest.

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