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Purification and structural characterization of an α-glucosidase inhibitory polysaccharide from apricot (*Armeniaca sibirica* L. Lam.) pulp

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ABSTRACT

In this study, the crude polysaccharide (APPS) from the fruiting bodies of apricot (*Armeniaca sibirica* L. Lam.) was isolated and fractionated by ultrafiltration and Sephadex G-75 gel chromatography. The hypoglycemic activities of all fractions were determined by α-glucosidase inhibitory activity in vitro. The fraction APPS1-2 showed the best activity with an IC_{50} of 6.06 mg/mL. The properties and chemical compositions of this fraction were analyzed with high-performance liquid chromatography, gel permeation chromatography-eighteen angle laser light scattering instrument, UV spectroscopy, infrared spectroscopy, and NMR spectroscopy (^1H). The results demonstrated that APPS1-2 was a neutral glycoconjugate with a molecular weight of 25.93 kDa. It comprised rhamnose, glucose, mannose, and galactose, with a relative molar ratio of 1.34:2.01:0.48:0.35. The backbone of APPS1-2 may consist of rhamnose and glucose, but its branches may consist of mannose and galactose. The IR and UV spectrum of APPS1-2 revealed the typical characteristics of heteropolysaccharide. ^1H NMR spectrum showed that APPS1-2 contained α-configurations.

1. Introduction

Apricot (*Armeniaca sibirica* L. Lam.) is rosaceae apricot genus. The fruit pulp of it is so thin and dry that its mature pulp is easy to separate from the kernel. Moreover, the taste of apricot pulp is inedible because of acidity (Flora of China, 2004). The existing cultivated area of apricot accounts for approximately 1/8 of the Three-North Shelter forest preservation area. Its fruit production is approximately 200,000–300,000 t per year, approximately 100,000–150,000 t of which are kernel and pulp, respectively. Although the development and utilization of apricot resources has gradually deepened, its sour pulp with poor palatability is often directly discarded, which caused serious waste of resources and environmental pollution. A previous study found that the dietary fiber content of apricot pulp is more than 50% (Yao, Luo, & Zhang, 2009). Water-soluble dietary fiber from apricot pulp contains polysaccharides, which are known to have good biological activities. Nevertheless, only a few studies have focused on the bioactive functions and disposal of polysaccharides from apricot pulp (Leccese, Bartolini, & Viti, 2012). Therefore, comprehensive development and utilization of apricot pulp not only reduces the production costs of apricot related products but also promotes environmental protection.

Diabetes mellitus is a chronic metabolic disease with the highest rates of prevalence and mortality worldwide. Oxidative stress and damage to tissues are common end points of chronic diseases, such as diabetes, atherosclerosis and rheumatoid arthritis. Although the leading mechanism of diabetic complications remains unclear, considerable attention has been given to the role of oxidative stress

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(Forlenza & Rewers, 2011; Jemai, Feki, & Sayadi, 2009; Liu, Chang, & Chiang, 2010). Several natural and synthetic antioxidants have been proposed to treat human diseases.

In previous studies, we revealed that water-soluble dietary fiber from apricot pulp has excellent antioxidant activity, which was examined by a series of models in vitro (Cui et al., 2012). Antioxidant polysaccharides from plants and fungi, including *Cordyceps mycelia* (Li et al., 2006), *Lycium barbarum* (Luo, Cai, Yan, Sun, & Corke, 2004), and *Tremella aurantia* (Kiho, Morimoto, Sakushima, Usui, & Ukai, 1995), exhibit hypoglycemic activity. Evaluation of α-glucosidase inhibitor activity in vitro is a fast, simple, and accurate method of determining hypoglycemic activity. In the present study, an antidiabetic polysaccharide from apricot pulp (APPS) was isolated and characterized by ultrafiltration, gas chromatography, high-performance liquid chromatography (HPLC), multi-angle laser light scattering instrument (GPC/MALLS), UV spectroscopy, infrared (IR) spectroscopy, and ^{1}H NMR spectroscopy.

2. Materials and methods

2.1. Materials and reagents

Apricot pulp provided by Chengde in Hebei Province (China) was dried at 45 °C, milled, passed through an 80 mesh sieve with a pore diameter of 180 μm, and then stored at room temperature. α-Glucosidase (≥10 units/mg), p-Nitrophenyl-α-D-glucopyranoside (PNPG), L-rhamnose, D-glucose, D-xylose, D-mannose, and D-galactose were purchased from Sigma (USA). Unless otherwise noted, all other reagents and solvents were of analytical grade and used without further purification. All aqueous solutions were prepared using double-distilled water.

2.2. Analytical methods

The polysaccharide was determined through the anthrone–sulfuric acid method, using glucose as standard (Roe, 1955). Briefly, a concentrated sulfuric acid (sp.gr. 1.84) solution containing 0.05% (w/v) anthrone was used. Each 1 mL sample solution was placed in a matched colorimeter tube which was in an ice water bath, and added 5 mL of anthrone reagent. The tubes were shocked to mix thoroughly, and set in a tap water bath for 3–5 min to bring the contents of each tube to the same temperature. Then they were placed in a boiling water bath for 7 min and removed to a tap water bath until they were at room temperature. The absorbance of different tubes was detected by using a UV-visible spectrophotometer at 620 nm.

2.3. Production and isolation of the crude polysaccharide

The dried apricot pulp powder (100 g) was defatted with 95% ethanol at room temperature for 24 h under stirring to remove most of the polyphenols, pigments, and monosaccharide; the treatment was repeated thrice (Wang, Yu, Zhang, Xiao, & Wei, 2010). The polysaccharide was extracted 15 times (m/v) with deionized water and 2 g cellulase (1000 units/mg) for 6 h at 45 °C and pH 6 under stirring. After the hydrolysis, the mixture was heated in a 90 °C water bath for 30 min to deactivate the cellulase and centrifuged at 5000 rpm for 20 min at 4 °C. The residue was washed thrice with water, which was then mixed with the supernatant. The combined aqueous extracts were concentrated in a rotary evaporator under a reduced pressure at 50 °C and then filtered. The filtrate was precipitated by adding anhydrous ethanol (four times the volume of aqueous extract) at room temperature for 12 h and then centrifuged at 8000 rpm for 15 min (Buksa, Ziobro, Nowotna, Praznik, & Gambuś, 2012; Gan, Ma, Jiang, Xu, & Zeng, 2011; He et al., 2012; Sun, Liu, Yang, & Kennedy, 2010). The precipitate was dissolved in 400 mL of water and then deproteinized thrice with 100 mL of 4:1 $CHCL_3$–MeOH following the method described by Sevag (Staub, 1965). The resulting aqueous fraction was extensively dialyzed against double-distilled water for 3 d and precipitated again by adding a fourfold volume of anhydrous ethanol. After centrifugation, the precipitate was washed thrice with anhydrous acetone, dissolved in defined amount water, and then freeze-dried to obtain the crude polysaccharide (5.4398 g).

2.4. In vitro α-glucosidase inhibitor activity (AGA) analysis

The α-glucosidase inhibition activity was determined by ELISA reader in a 96-well plate using PNPG as substrate (Cremonesi, Torti, Pecile, Groppetti, & Biondi, 2003; Kima, Hyunb, & Kim, 2011). The assay mixture (240 μL) contained 120 μL of 0.5 mol/L phosphate buffer (pH 6.7), 50 μL of substrate solution (0.9133 mg/mL PNPG in 0.5 mol/L phosphate buffer), 50 μL of enzyme solution (25 mg/mL α-glucosidase in 0.5 mol/L phosphate buffer containing 0.2% BSA), and 20 μL of the indicated concentration gradient of the samples. The reaction was processed at 37 °C for 1 h and then stopped by adding 50 μL of 0.67 mol/L Na_2CO_3. The amount of PNP released was quantified on a microplate reader model 680 (Bio-Rad, CA, USA) at 405 nm. One set of the reaction mixture prepared by an equivalent volume of water instead of the samples was used as blank. Another set of reaction mixture prepared by 100 μL phosphate buffer instead of the substrate and enzyme was used as background. The inhibitory rates (AGA) were calculated according to the following formula:

$$\mathrm{AGA} = \left(1 - \frac{A_1 - A_2}{A_3}\right) \times 100\%$$

where:

A_1 – the absorbance values of the sample
A_2 – the absorbance values of background
A_3 – the absorbance values of blank
IC_{50} was calculated by the modified Bliss method.

2.5. Separation and purification of polysaccharide

The crude polysaccharide was separated into different molecular weight fractions using an ultrafiltration membrane (Millipore Pellicon XL Biomax 5, Biomax 10, Biomax 30, Millipore Co.) at 4 °C. After the crude polysaccharide aqueous solution was passed through a 0.45 μm membrane filtration system, the filtrate was ultrafiltered by different molecular cut-off ranged 30, 10, and 5 kDa series of Pellicon Biomax membrane in a Millipore Labscale TFF System (Toledano, García, Mondragon, & Labidi, 2010). Different molecular weight ranges of the filtrate (>30 kDa, 10–30 kDa, 5–10 kDa, and <5 kDa) were collected and lyophilized to determine their α-glucose inhibition rates. The component with the highest inhibition rate was subjected to Sephadex chromatography for further purification.

The active component was subjected to gel chromatography using a Sephadex G-75 column (16 mm × 600 mm, Pharmacia, USA). The column was eluted with degassed distilled water at a flow rate of 0.7 mL/min, whereas the volume of the effluent was collected by an automatic fraction collector (BS-100A, Shanghai Huxi Analytical Instrument Factory Co.) at 10 min/tube. The total carbohydrate content of each tube was measured at 620 nm by the anthrone–sulfuric acid method, and protein absorption at 280 nm was measured for each fraction (Zhu, Sheng, Yan, Qiao, & Lv, 2012). The main polysaccharide fractions were collected, lyophilized, and used for detecting α-glucose inhibitory activity. The fraction with the best inhibitory effect was utilized for structural analysis.

2.6. Molecular weight determination

The molecular weight and molecular weight distribution of the sample were measured using GPC/MALLS (Coto, Suárez, & Caballero, 2007; Liu, Bo, Zhang, & Zhu, 2003; Suárez, Caballero, & Coto, 2010). This process was performed on an infusion system (Waters 515 HPLC Unit Pump) fitted with one gel column (Shodex SB-806M) and the combined detectors (Wyatt-DAWN HELEOS® eighteen-angle laser light scattering and Wyatt-Optilab rex refractive index detector). The mobile phase was a 0.1 mol/L sodium nitrate solution with 0.02% sodium azide at a flow rate of 0.5 mL/min, and the column temperature was 40 °C. The injection volume was 0.2 mL. The molecular weight and molecular weight distribution of the sample were determined.

2.7. Analysis of monosaccharide composition

The polysaccharide sample (25 mg) was hydrolyzed with 2 mol/L trifluoroacetic acid at 100 °C in a sealed tube for 12 h (Selim & Shawky, 2010; Xie et al., 2013). Excess acid was removed by flash evaporation on a water bath at 40 °C and then codistilled with methyl alcohol thrice. The polysaccharide hydrolysate was dissolved with 1 mL distilled water and then passed through a 0.45 μm microporous filter. The monosaccharide contents were quantified by HPLC (LUMTECH/K501, Lumiere Tech., Germany) on a Waters Spherisorb® 5 μm NH_2 analytical column (4.6 mm × 250 mm) using acetonitrile and water (80:20, v/v) as the mobile phase at a flow rate of 0.8 mL/min. The column temperature was 30 °C, and a 10 μL sample was injected.

2.8. Spectrometric analysis

The spectral data were obtained on a UV-visible spectrophotometer (T6 New Century, Pgenenal, Beijing, China) at 25 °C in the range of 200–400 nm using a quartz cuvette with a 1 cm path length. Each UV spectrum determination was repeated thrice (Attal, Thiruvengadathan, & Regev, 2006).

The IR spectrum of the polysaccharide fraction was recorded on a Fourier-transform infrared spectrophotometer (Nesus 670-IR, Nicolet, USA) by grinding a mixture of the sample (1 mg) with dry KBr (100–200 mg for 48 h at 120 °C) and pressing in a mold (Ray, 2006). The experiment conditions were 400–4000 cm^{-1} frequency, 8 cm^{-1} resolution, and 32 scans.

^1H NMR spectra were recorded with a Bruker BioSpin GmbH spectrometer (600 M), and the HOD signal fixed at 4.790 ppm. The polysaccharide sample (30 mg) was dissolved in 1.2 mL of D_2O (Dueñas-Chasco et al., 1997).

3. Results and discussion

3.1. Isolation and purification of α-glucosidase inhibitory polysaccharides

The crude polysaccharide was isolated from the fruiting bodies of apricot following the method which was mentioned in Section 2.2. It was diluted with distilled water and passed through a 0.45 μm membrane with vacuum filtration. Utilizing the ultrafiltration instrument, we intercepted the polysaccharide aqueous solution into four fractions according to molecular weight: >30 kDa (APPS1), 10–30 kDa (APPS2), 5–10 kDa (APPS3), and <5 kDa (APPS4). The above components were then assayed for α-glucosidase inhibitor activity. The inhibitor activity rates at the different molecular weight ranges are shown in Fig. 1. The >30 kDa fraction (APPS1) had the highest activity. Under the same concentration, the α-glucosidase inhibition rate of this fraction was 89.76% ± 0.03, which is significantly higher than that of the other fractions.

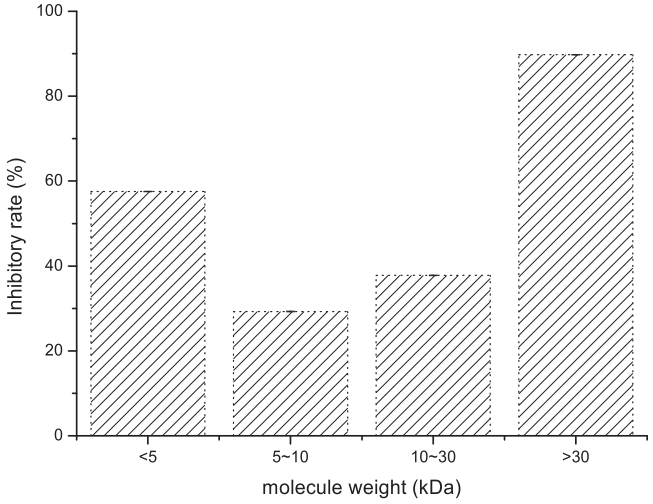

Fig. 1. α-Glucosidase inhibition rate of 50 mg/mL of different molecular weight fractions.

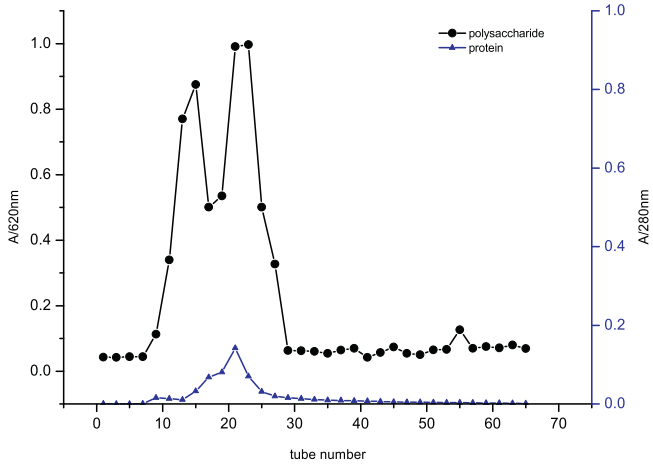

Fig. 2. Gel chromatography of the polysaccharide fraction APPS1 on Sephadex G-75. Absorbance at 620 nm after the color reaction of the elution with anthrone–sulfuric acid; absorbance of the protein contained in APPS1 at 280 nm.

Table 1
Monosaccharide composition and molar ratio of APPS1-2 by high-performance liquid chromatography.

Monosaccharide	Equation	R^2	S.D.	Molar ratio
Rhamnose	$y = 82.064x + 12.578$	0.9995	0.0170	1.34
Glucose	$y = 69.907x - 11.804$	0.9996	0.0096	2.01
Mannose	$y = 71.080x - 17.424$	0.9997	0.0140	0.48
Galactose	$y = 54.027x - 7.8159$	0.9963	0.0079	0.35

The active fraction APPS1 was further purified using gel filtration by applying Sephadex G-75 gel chromatography. As shown in Fig. 2, APPS1 was separated into two fractions: APPS1-1 and APPS1-2. The IC_{50} values of the α-glucosidase inhibitor activity of these two fractions are 7.66 ± 0.04 mg/mL and 6.06 ± 0.02 mg/mL respectively. The α-glucosidase inhibitory activity of fraction APPS1-2 exhibited higher than that of APPS1-1.

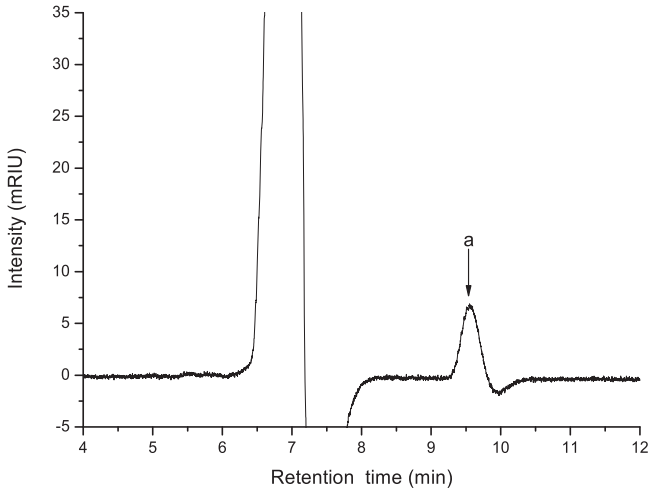

Fig. 3. High-performance liquid chromatography of polysaccharide fraction APPS1-2 (a – the peak of APPS1-2).

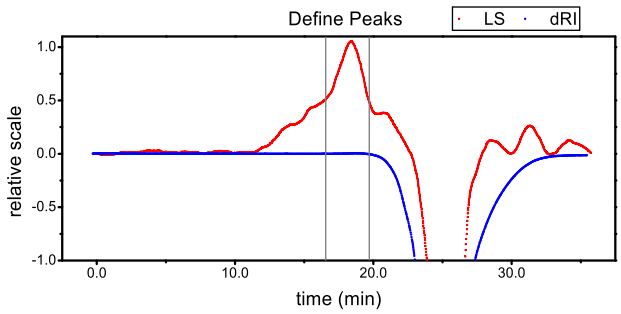

Fig. 4. Gel permeation chromatography-MALLS of the polysaccharide fraction APPS1-2.

3.2. Monosaccharide composition and molecular weight determination

Fig. 3 showed that the HPLC chromatogram of APPS1-2 was one sharp peak (Peak a), implying it was a single substance. HPLC analysis (Table 1) demonstrated that APPS1-2 was composed of rhamnose, glucose, mannose, and galactose, with a relative molar ratio of 1.34:2.01:0.48:0.35. The molar ratio of the four types of monosaccharides indicated that rhamnose and glucose were the major components of APPS1-2.

The molecular weight of the purified active fraction APPS1-2 determined by GPC/MALLS was 25.93 ± 0.03 kDa. The GPC/MALLS profile (Fig. 4) demonstrated that APPS1-2 showed a single, symmetrical sharp peak, revealing that it was a homogeneous polysaccharide. The polydispersity Mw/Mn 1.240 (Table 2) that approached 1.00 also indicated that APPS1-2 was a narrow distribution sample, and the relative molecular weight distribution was regarded as homogeneous.

3.3. Structural characterization of APPS1-2

The UV spectrum of APPS1-2 was recorded on a T6 New Century spectrophotometer. A weak peak at 280 nm was attributable to the absorption of protein (Fig. 5), implying that APPS1-2 was a glycoprotein containing polysaccharide chains and protein residues according to our previous study (Fig. 2).

The IR spectrum of APPS1-2 is shown in Fig. 6. The absorption bands within the range of 3600 cm^{-1} to 3200 cm^{-1}, 3000 cm^{-1} to 2800 cm^{-1}, 1400 cm^{-1} to 1200 cm^{-1}, and 1200 cm^{-1} to 1000 cm^{-1} were the characteristic absorption peaks of polysaccharides. The IR spectrum of APPS1-2 exhibited a broadly stretched intense peak at 3430.07 cm^{-1} and a weak peak at 1007 cm^{-1}, which corresponded to hydroxyl stretching vibration and deformation vibration, respectively. The peaks at 2928, 2855, 1400.70, and 1385.17 cm^{-1} corresponded to a weak C—H stretching vibration. The peak at approximately 1606.59 cm^{-1} was attributed to the N—H vibration or C—O asymmetric vibration of the carboxyl group, which was also the characteristic IR absorption of polysaccharides. The bands in the 3430.07 and 1606.59 cm^{-1} regions corresponded to acylamino, which showed that APPS1-2 was a glycoconjugate containing polysaccharide and protein. The wave numbers between 800 and 1200 cm^{-1} represent the fingerprint region for carbohydrates (Kumar, Joo, Choi, Koo, & Chang, 2004; Matsuhiro, Osorio-Roman, & Torres, 2012; Wu et al., 2012). The absorption band at 1130.86 cm^{-1} corresponded to the C—O—C stretching vibration. The strong absorption peaks at 3172.40 and 1400.70 cm^{-1}

Table 2
Molecular weight and relative molecular weight distribution of APPS1-2 (mean ± RSD).

Molar mass moments (g/mol)				Polydispersity	
Mn[a]	Mp[b]	Mw[c]	Mz[d]	Mw/Mn	Mz/Mn
20,910 ± 0.03	14,870 ± 0.04	25,930 ± 0.03	35,820 ± 0.07	1.240 ± 0.08	1.713 ± 0.03

[a] Number-average molecular weight.
[b] Peak-position molecular weight.
[c] Weight-average molecular weight.
[d] Z-average molecular weight.

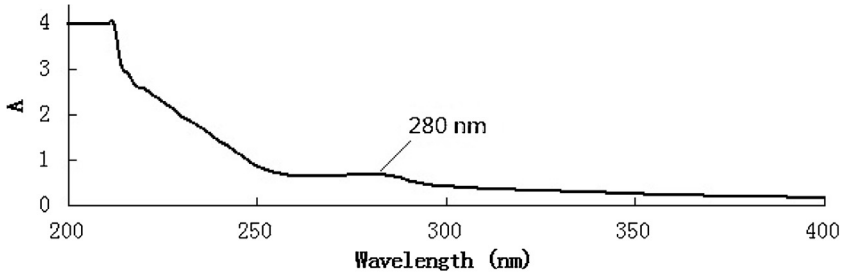

Fig. 5. UV spectrum of the polysaccharide fraction APPS1-2.

were the stretching vibration of the furan ring. The weak band at 852 cm^{-1} was ascribed to α-pyranoses in the polysaccharide (Nakanishi & Solomon, 1977).

The ^1H NMR spectroscopy of APPS1-2 (Fig. 7) was complex due to the convergence of most sets of the signals in the region 3.45–4.25 ppm. There were two partially overlapping signals for protons at 5.421 and 5.427, suggesting α-configurations, consistent with IR data.

The structure of the glycoconjugate is complicated, but the above results only explain a part of the structural characteristics.

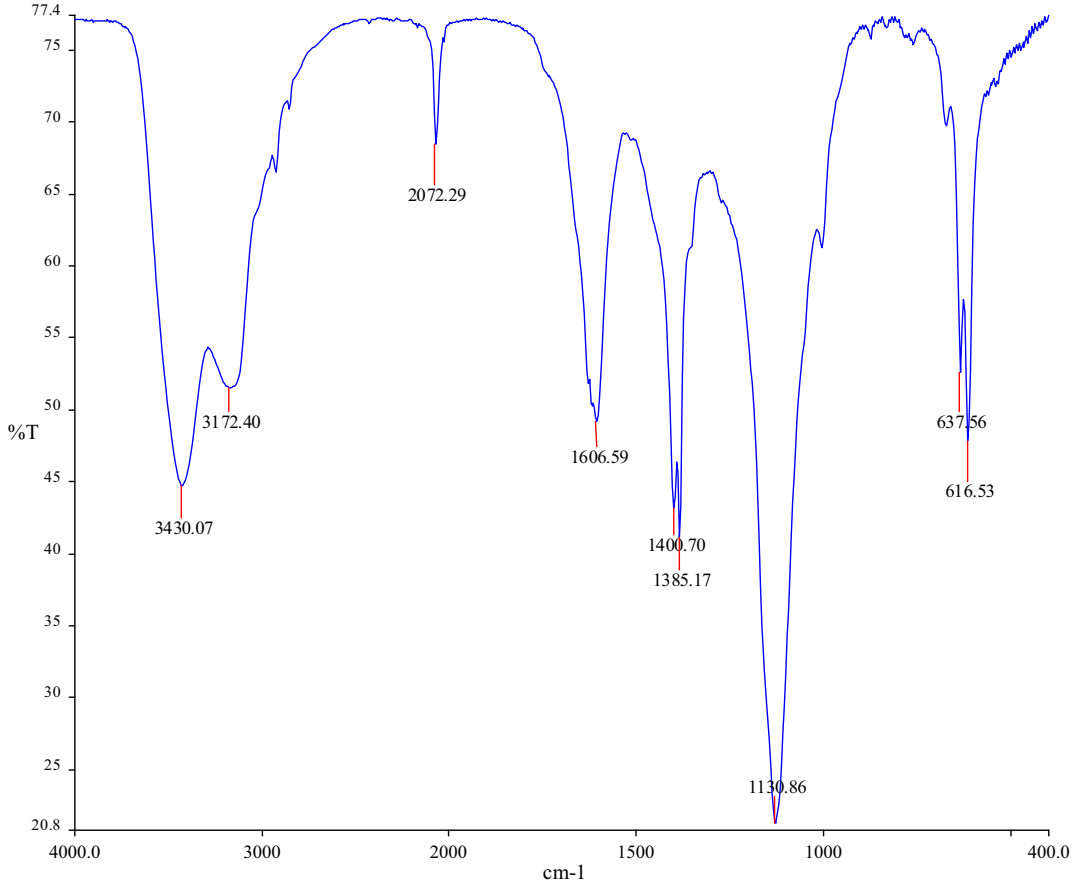

Fig. 6. IR spectrum of the polysaccharide fraction APPS1-2.

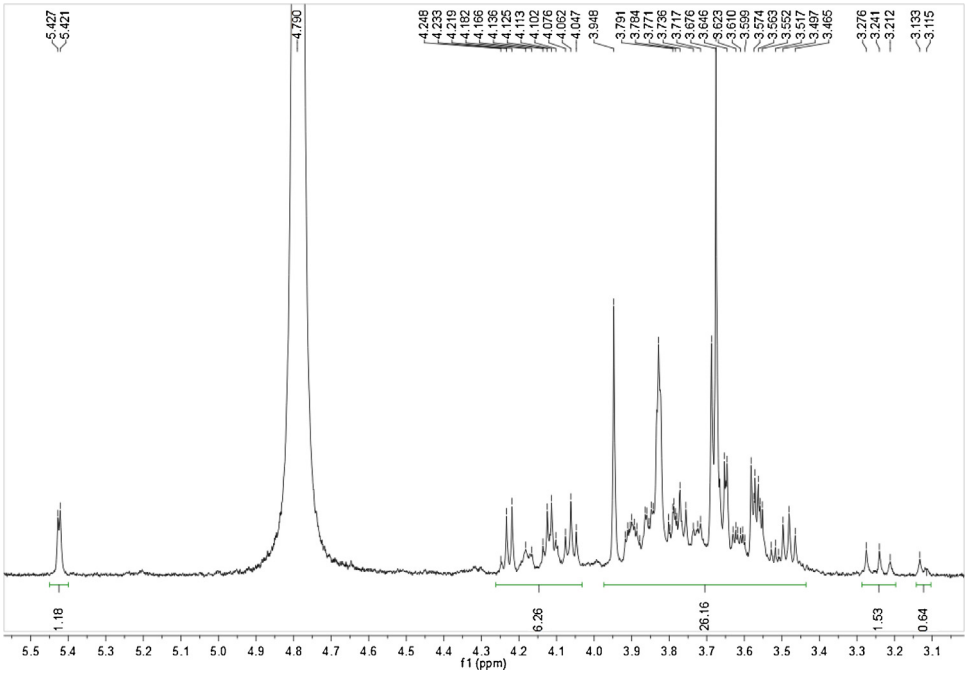

Fig. 7. ^1H NMR (400 M) spectrum of polysaccharide fraction APPS1-2 recorded in D_2O.

Further studies must perform methylation analysis and other spectroscopy to provide detailed structural information.

4. Conclusion

In this study, a polysaccharide conjugate with hypoglycemic activity was isolated from apricot pulp. The crude polysaccharide was separated into four fractions by ultrafiltration according to different molecular weight values. The fraction APPS1 demonstrated the highest hypoglycemic activity. APPS1 was then purified to two fractions with Sephadex G-75 gel chromatography. APPS1-2, which showed better activity (IC_{50} = 6.06 mg/mL) than APPS1-1, was predominantly composed of the monosaccharides rhamnose, glucose, mannose, and galactose with a ratio of 1.34:2.01:0.48:0.35. The Mw of APPS1-2 was 25.93 ± 0.03 kDa, as determined by GPC/MALLS. APPS1-2 can be explored as a novel antidiabetic healthcare product with hypoglycemic activity and antioxidant ability. Future works should focus on its structure and functions.

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