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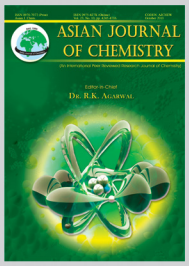

A Bioactive Polysaccharide Isolated from the Fruits of Chinese Jujube

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A polysaccharide JuBP-4 with mean molecular mass of 1600KDa, was isolated from fruits of *Ziziphus jujuba* Mill. by fractionation on DEAE column and gel column chromatographies. Chemical and spectroscopic studies indicated that it is composed of rhamnose, arabinose, glucose, galactose and galacturonic acid in a molar ratio of 1:8.83:2.08:7.44:33.79. The main backbone chain in JuBP-4 is mostly composed with 1,4-linked α-D-galacturonic acid interspersed with 1,2-linked α-L-rhamnose and the side chains was mainly 1,5-linked α-arabinose residues and 1,6-linked β-galactose. The polysaccharide was demonstrated mitogenic activity properties.

Key Words: *Ziziphus jujuba* Mill. 'jinsixiaozao', Fruit, Polysaccharide, Immunological activity.

INTRODUCTION

Ziziphus jujuba Mill. belongs to the family of rhamnaceae and is widely distributed in China. Its fruits are not only good taste food but traditional Chinese medicine. Its fruits were found to be generally much richer in calories, carbohydrates, minerals (calcium, iron), vitamin C and cAMP than other fruits[1]. As an important component, the structural characterization and immunological activity of polysaccharide have been studied from many plants, but the polysaccharides of Chinese jujube (*Ziziphus jujuba* Mill.) have received minor attention. Our study indicated that polysaccharides from *Ziziphus jujuba* Mill. 'jinsixiaozao' have immunological activities[2-4]. In this paper, we reported structural characterization and immunological activity of one polysaccharide from fruits of *Zizizphus jujuba* Mill. 'jinsixiaozao'.

EXPERIMENTAL

The fruits of *Ziziphus jujuba* Mill. 'jinsixiaozao' were collected from Cangzhou, Hebei province (P. R. China) in 2003. The cultivar was identified by Mengjun Liu. The stone was removed and the remained flesh was dried overnight at 60 ºC in the presence of silica gel. All treatments were repeated in triplicate and all analyses were performed in triplicate.

General analysis: Homogeneity and molecular mass of JuBP-4 was evaluated and estimated from the calibration curve of the elution volume of standard dextrans (Dextran T-2000, T-500, T-70, T-40, T-10 and glucose; Pharmacia Co.), which was performed on an Agilent 1100 series apparatus equipped with a Shodex KS-805 column (Shoko Co. Ltd., Japan) and an ELSD detector. Distilled water was used as the solvent and eluent and the flow rate was kept at 1.0 mL/min. All gel permeation chromatography was eluted with H_2O.

Polysaccharide sample was deproteined with Sevage method[5]. Protein content was analyzed by Lowry method[6]. Optical rotations were measured on a Perkin-Elmer 243B polarimeter. IR spectra were determined with an AVATER-360 spectrometer. The ^1H, ^{13}C spectra were recorded on an INOVA-500 instrument operating at 28 ºC in D_2O. GC-MS was performed on a Finnigan Trace GC-MS instrument equipped with DB-5 column and detected by a FID detector (temperature: 250 ºC). The column temperature was increased from 160-250 ºC in a rate of 5 ºC/min.

Analysis of carbohydrate composition: Neutral sugars composition analysis was conducted by reported method[4]. Uronic acid determined by *m*-hydroxyl diphenyl method[7] with D-glucoronic acid as standard; D-galacturonic acid was reduced by reported method[8].

Partial acid hydrolysis of JuBP-4: JuBP-4 was hydrolyzed with 0.3 M TFA at 100 ºC for 1 h. The products were evaporated to dryness and the residues were dialyzed against distilled water. The nondialysate was purified by Sepharose CL-6B chromatography to give JuBP-4-sp1 (determinated as a single peak by HPLC).

Degree of esterification of pectic substances and determination of O-acetyl groups: The degree of esterification (DE) of pectic substances was determined by the release of MeOH after treatment of the samples with 1M HCl by GC on an Agilent 6890N series with a flame ionization detector[9]. Nitrogen was used as carrier gas. The column temperature was 60 ºC while the injector and detector temperature was 150 and 190 ºC, respectively.

The content of acetyl groups in polysaccharide was determined by ^{1}H NMR spectrum[10].

Methylation analysis: The methylation analysis was conducted by reported method[4].

Measurement of immuno-modulating activity: Mice (6-8 weeks) were sacrificed and their spleens were removed and passed through a sterilized iron sieve to obtain single cell suspensions. The single cell suspension was washed with PBS and then the red blood cells were lyzed with ACK lyse buffer (0.15M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM EDTA) for 3 min. The spleen cells were washed and then cultured in U-bottom well plates (2×10^5/well) in a volume of 200 mL per well in the presence of 10, 30 and 100 μg/mL of JuBP-4, negative control and positive control (LPS, 2.5 μg/mL) groups, respectively. After a 3 day drug treatment, DNA synthesis was measured by H3-thymidine (Du Pont) incorporation (1 μCi / well) in the final 6 h of the cultured period. The data were tested for statistical differences using the T test.

RESULTS AND DISCUSSION

Structure investigation of JuBP-4: The dried fruits of Chinese jujube were refluxed with ethanol for 3 times (1.5, 1.5 and 1.0 h) to remove monosaccharides and lipids. The boiling water extract of the residue was precipitated with 4 vols. EtOH to give the crude polysaccharide Ju. After successive separation by DEAE cellulose anion exchange and Sepharose CL-6B permeation chromatographic steps, it afforded the carbohydrate fraction JuBP-4. JuBP-4 was determined as a single peak by size exclusive chromatography in a HPLC analysis.

Its molecular weight was determined to be over 1600KDa by HPLC, in reference to standard dextrans. It had a specific rotation of $[\alpha]^{20}_D$ + 154 (c = 1.05, H_2O) and was free of protein according to the Lowry method. The IR spectrum of JuBP-4 in the frequency range 4000-400 cm^{-1} showed the characteristics of a pectic polysaccharide with absorption at 1614 cm^{-1} (carboxylate group) and1743 cm^{-1} (ester bonds) of the carboxyl group and 829 cm^{-1} of the pyranose ring.

The presence of 67.18 % uronic acid was inferred by the *m*-hydroxydiphenyl method. After reduction with CMC-$NaBH_4$ procedure, the carboxyl-reduced JuBP-4 was obtained. A combination of sugar composition analysis of native JuBP-4 and carboxyl-reduced JuBP-4 revealed that JuBP-4 contained of rhamnose (Rha), arabinose (Ara), glucose (Glu), galactose (Gal) and galacturonic acid (GalA) in the molar ratios 1: 8.83: 2.08: 7.44: 33.79. After partial acid hydrolysis of JuBP-4, the hydrolysate was dialyzed. The nondialysate was subjected to Sepharose CL-6B chromatography and gave JuBP-4-sp1, which showed homogeneity with a molecular weight of about 1400 kDa. TLC and compositional analysis of JuBP-4-sp1 suggested that it was composed of rhamnose, arabinose, glucose, galactose and galacturonic acid in a molar ratio of 1:1.26:0.67:3.56:32.53. In comparison with the composition of the native polysaccharide (1:8.83:2.08:7.44:33.79), the proportion of arabinose, glucose and galactose significantly decreased, while the ratio of rhamnose and galacturonic acid remained almost unchanged. These data indicated that the main chain of JuBP-4 was composed of rhamnose and galacturonic acid.

The results of methylation of native and reduced were summarized (Table-1). As shown, galacturonic acid was dominant in the complex and the rhamnose residues occupied the least. The data of methylation indicated that the main backbone chain in JuBP-4 was mostly composed with 1,4-linked α-D-GalA interspersed with 1,2-linked α-L-Rha. The side chains were attached to the backbone at the O-4 position of rhamnose residues, as shown by the presence of the 3-O-methyl rhamnitol and 24.8 % of the a-L-Rha possess side chains. Arabinose residues were present as furanosyls. Most of arabinose (67.7 %) was 1,5-linked and 32.3 % was 1,3-linked. Galactose was present in the pyranose form and was predominantly 1,6-linked in the complex. In addition, terminal-linked Galp and some 1,3,6-linked Galp were detected. Glucose was present in pyranose form and all of them was terminal-linked.

TABLE-1
RESULT OF METHYLATION ANALYSIS OF JuBP-4

Methylated sugars	Linkage	Molar ratio
2,3-Me_2-L-Araf	1,5-Araf	30.35
2,5-Me_2-L-Araf	1,3-Araf	14.45
3,4-Me_2-L-RhaP	1,2-Rhap	4.36
3-Me-L-Rhap	1,2,4-Rhap	1.08
2,3,4,6-Me_4-D-Galp	1-Galp	10.35
2,3,4-Me_3-D-Galp	1,6-Galp	25.98
2,4-Me_2-D-Galp	1,3,6-Galp	3.31
2,3,4,6-Me-D-Glup	1-Glup	8.09
2,3,6-Me_3-D-GalP*	1,4-GalAp	132.5

*Uronic acids were detected after the polysaccharide being reduced.

The ^{1}H NMR spectrum of JuBP-4 in D_2O (Fig. 1) showed anomeric proton signals from 4.96-5.15 ppm. Signals at 3.80, 3.99, 4.41 and 4.77 ppm were assigned to H-2, H-3, H-4 and H-5 of α-D-galacturonic acid[4]. Signals at 1.29 and 1.24 ppm assigned to H-6 of α-L-Rhap. There were numerous signals from 3.34 to 4.61 ppm corresponded to H-2 to H-6 of α-D-Galp, H-2 to H-6 of β-D-Glup and H-2 to H-5 of α-L-Araf. The signal at 3.75 ppm was assigned to methoxyl group. The signals at 2.07 and 2.18 ppm suggested that JuBP-4 contained two kinds O-acetyl groups.

In the ^{13}C NMR spectrum of JuBP-4 (Fig. 2), the signals at δ 21.0 and 53.6 ppm confirmed the presence of O-acetyl and O-methyl groups in JuBP-4. The content of acetyl group was estimated to be 4.5 % and the degree of methyl esterfication of JuBP-4 was 42.02 %. Signals at 171.5-174.1 ppm were assigned to the carboxyl group of α-D-GalpA, methyl ester carboxyl group of α-D-GalpA and O-acetyl group[11]. Signals at 100.2-100.9 ppm assigned to anomeric carbons. The signals at 68.7, 69.4, 78.6 and 72.1 ppm were assigned to C-2 to C-5 of α-D-(1,4)-GalpA[4]. Signal at 17.3 ppm assigned to C-6 of α-L-Rhap.

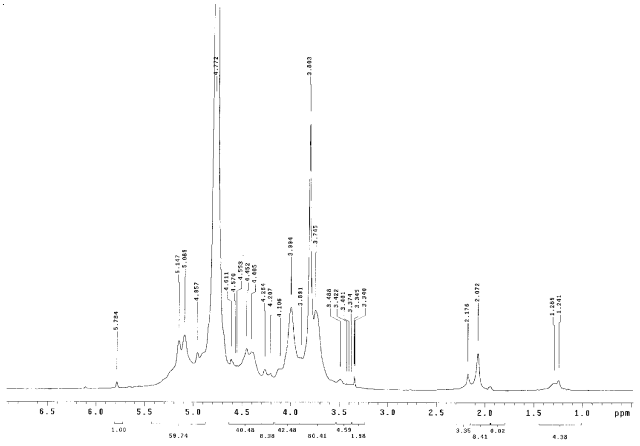

Fig. 1. ^{1}H NMR spectrum of JuBP-4

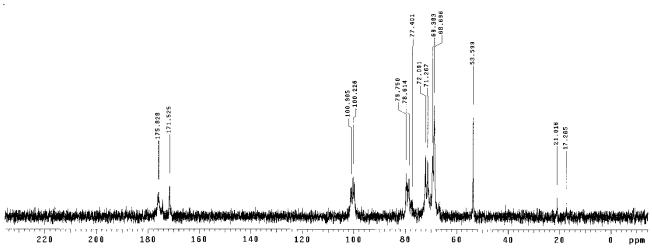

Fig. 2. ^{13}C NMR spectrum of JuBP-4

The dominant feature of pectins is a linear chain of α-1,4-linked D-galacturonic acid in which varying proportions of the acid groups are present as methoxyl esters. These homogalacturonan sequences may be interspersed at intervals with α-L rhamnopyranosyl residues carrying the major part of neutral sugar side chains, mainly arabinans, galactans or arabinogalactans[4].

Thus, JuBP-4 was proposed that its main backbone chain was mostly composed with 1,4-linked α-D-GalA interspersed with 1,2-linked α-L-Rha and the side chains was main composed with 1,5 linked α-Araf residues and 1,6 linked β-Galf.

Immunological activities: In cultured spleen cells, JuBP-4 was applied from 10-100 μg mL^{-1} for 3 days. In order to quantify the proliferation rate, the DNA synthesis as revealed by the incorporation of [^{3}H] thymidine was determined in the treated spleen cells. Application of JuBP-4 induced the spleen cells proliferation in a dose-dependent manner (Fig. 3). Using the stimulation effect on spleen cell proliferation, higher dose of JuBP-4 revealed significant ($p < 0.01$) immunoenhancing activities in a dose-dependent manner.

Conclusion

A polysaccharide JuBP-4 was isolated from *Ziziphus jujuba* Mill. 'jinsixiaozao' and its structures was characterized by acid hydrolysis, methylation, partial acid hydrolysis and

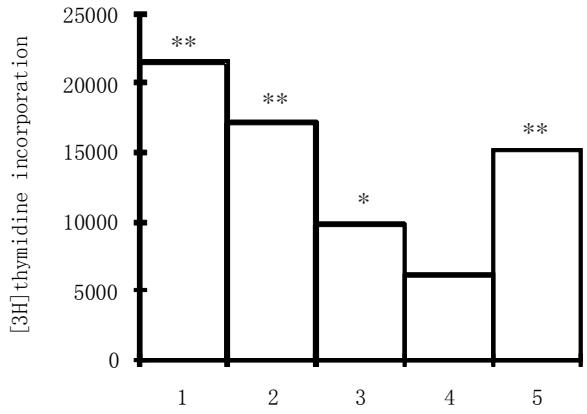

Fig. 3. Immunological activity of JuBP-4. Each data point represents mean ± SD, n = 6; each has triplicates. Control and LPS indicates absence of sample and positive control, respectively. Sample group vs control group is $^{**}p < 0.01$, $^{*}p < 0.05$. 1-100 mg/mL, 2 -30 mg/mL, 3-10 mg/mL, 4-control, 5-LPS

NMR spectroscopy. JuBP-4 was proposed that its main backbone chain was mostly composed with (1→4)-linked α-D-galacturonic acid interspersed with 1,2-linked α-L-Rha and the side chains was mainly 1,5 linked α-Araf residues and 1,6 linked β-Galf. *In vitro* test revealed that JuBP-4 (30-100 μg/mL) had significant ($p < 0.01$) immunoenhancing effect on spleen cells proliferation in a dose-dependent manner.

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