

穿山龙多糖 DMA 的结构和抗氧化活性研究

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摘要: 采用热水煮提法和 DEAE-琼脂糖凝胶 CL-6B 凝胶层析对穿山龙多糖 DMA 进行提取和分离纯化。通过高效液相色谱法和气相色谱法鉴定 DMA 的纯度和糖原组成。采用红外光谱法, 高碘酸氧化-Smith 降解法, 甲基化分析以及核磁共振法对多糖 DMA 的结构进行分析。利用羟自由基实验研究 DMA 的抗氧化活性。研究发现多糖 DMA 显示一个单一对称的色谱峰, 主要由葡萄糖组成, 是由 $\rightarrow 6$ - α -D-Glcp-(1 \rightarrow) 组成的多糖。DMA 具有清除羟自由基的活性。

关键词: 穿山龙; 多糖; 抗氧化活性; 核磁共振

中图分类号: Q53

文献标识码: A

DOI: 10.16333/j.1001-6880.2015.08.003

Structure and Antioxidant Activities of Polysaccharide from Fraction-DMA of *Dioscorea nipponica* Makino

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Abstract: Boiling-water extraction and a DEAE-Sepharose CL-6B column chromatography were used to isolate and purify the major polysaccharide DMA from *Dioscorea nipponica*. Purification and compositional analysis of DMA were carried with HPLC and GC-FID analysis. IR analysis, periodate oxidation-smith degradation, methylation analysis and NMR studies (^1H , ^{13}C , ^1H - ^1H COSY and HSQC) were used to identify its structure. On the basis of hydroxyl radical assay, its antioxidant activity was investigated. The results showed that the purified fraction DMA was a single peak, and it mainly comprised of glucose. The repeating unit of DMA was $\rightarrow 6$ - α -D-Glcp-(1 \rightarrow). DMA exhibited a scavenging activity for hydroxyl radical.

Key words: *Dioscorea nipponica* Makino; polysaccharide; antioxidant activity; NMR spectroscopy

Oxidation is essential to many organisms for the production of energy to fuel biological processes. However, the uncontrolled production of oxygen derived free radicals is involved in onset of many diseases such as cancer, rheumatoid arthritis and atherosclerosis as well as in degenerative processes associated with aging^[1]. Many synthetic antioxidants are used as medical materials at the present time. However, most commonly have been suspected of being responsible for liver damage and carcinogenesis^[2]. Thus, it is essential to develop and

utilize effective and natural antioxidants^[3]. Published data indicated that plant polysaccharides in general had strong antioxidant activities and can be explored as novel and potential antioxidants^[4].

Dioscorea nipponica Makino is well known as a traditional edible plant in oriental countries. It has been widely used as Chinese herbal medicine and health food for hundreds of years. We reported on the extraction and purification of a major polysaccharide of *D. nipponica* using a DEAE Sepharose CL-6B column chromatography and a Sephadex G-100 column chromatography^[5]. Structural identification of polysaccharides is necessary to better study the relationship between structures and functional properties of polysaccharides and effectively exploit these potential substances. However, there are only a few studies on its chemical composition and structures, and its detailed

Received: April 10, 2015 Accepted: June 24, 2015

Foundation Item: National Nature Science Foundation of China (31201314); High Level Scientific Research Foundation of Huaqiao University (11BS428); Fundamental Research Funds for the Central Universities (JB-ZR1222); Cultivation Project Funds for Postgraduates Innovation Ability of Huaqiao University

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structure is not precisely known.

In this study, we reported on the extraction and purification of a major polysaccharide DMA of *D. nipponica* Makino using a DEAE Sepharose CL-6B column chromatography. In addition, the structural characteristics and antioxidant activities of the major polysaccharide DMA were also identified.

Material and Methods

Materials and chemicals

Dried underground part of *D. nipponica* was purchased from a local drugstore (Quanzhou, Fujian Province, China). The material was identified by Professor J. F. Liu, Huaqiao University, Fujian, China. Monosaccharide standard samples (G-8270, Sigma Co., St Louis, MO, USA), Nitro Blue Tetrazolium (NBT, 3069B117, Amresco Inc., Solon, OH, USA), Nicotinamide Adenine Dinucleotide Hydrogen (NADH, 6109B08, Amresco Inc., Solon, OH, USA) and Phenazine Methosulfate (PMS, 101413172, Fluka Inc., St Louis, MO, USA) were purchased from a local agent (Taijing Co., Xiamen), while DEAE Sepharose CL-6B was from the Pharmacia Co. (Sweden). All other reagents used were of analytical grade.

Instruments

High performance liquid chromatography (HPLC) was performed on a HP1100 instrument (Agilent Component, USA). Fourier transform infrared spectrophotometer (FT-IR) was Nicolet Nexus 470 (NICOLET Component, USA). GC-FID and GC-MS analyses were performed on a HP5890 GC instrument and a HP5890-5972 GC-MS instrument, respectively (Agilent Component, USA). NMR spectrometer was from Bruker Inc. (DRX500, Switzerland).

Methods

Preparation of polysaccharide DMA

D. nipponica sample was extracted with distilled water at 90 °C for 2.5 h. The soluble polymers were separated from residues by filtration, and extracts were concentrated. The supernatant was collected and precipitated by the addition of four times volume ethanol. After overnight precipitation at 4 °C, the sample was centrifuged at 3,500 rpm for 15 min. The sample was washed

successively with ethanol, and dried at reduced pressure, giving a crude polysaccharide (DM). The yield was calculated using the equation: DM weight/sample weight $\times 100\%$. DM (1 g) was dissolved in 10 mL of distilled water, centrifuged and then the supernatant was injected to a column (4.6 cm \times 30 cm) of DEAE-Sepharose CL-6B equilibrated with distilled water for 500 mL at 4 mL/6 min. A major polysaccharide fraction was collected, named DMA.

Total carbohydrate and protein of these polysaccharides were determined by the phenol-sulfuric acid and Bradford, respectively [6]. GC-FID was used for identification and quantification. First, the polysaccharide (10 mg) was dissolved in 1 mL of a 1 M HCl-Methanol, removed air with nitrogen, hydrolyzed at 80 °C for 20 h, and then neutralized by KOH-Methanol. Derivation was then carried out using the trimethylsilylation reagent. The monosaccharide compositions of DMA were determined by gas chromatography equipped with a column HP-5 (30 m \times 0.25 mm \times 0.25 μ m) and the flame-ionization detector. Temperatures-programmed GC-FID measurements were carried out from 180 °C to 220 °C (held for 2 min) at a rate of 8 °C/min, and then to 250 °C (held for 2 min). The molecular weight of the polysaccharide DMA was determined by a gel-filtration chromatographic column of Shodex Sugar KS-804 (SHOWA DENKO K. K., Japan) with a high-performance liquid chromatography instrument (Agilent 1100, USA) maintained at a temperature of 50 °C, eluted with the distilled water at a flow rate of 1.0 mL/min and detected by a refractive index detector. Preliminary calibration of the column was conducted using several standard samples of different molecular weight (T2000, T10, T40, T70, T500 and Glucose) [6].

Structural analysis

IR spectrum of the polysaccharide was determined using a Fourier transform infrared spectrophotometer (Nicolet Nexus 470, NICOLET Component, USA). The purified polysaccharide was ground with KBr powder and then pressed into pellets for FTIR measurement in the frequency range of 4000-400 cm^{-1} [7].

For analytical purpose of Periodate oxidation-Smith degradation, 25 mg of DMA was dissolved, and four

samples were obtained and dried out for the GC-FID analysis [5].

For methylation analysis and GC-MS analysis, polysaccharide (40 mg) was dissolved in dimethylsulfoxide (DMSO, 12 mL), removed air with nitrogen, stirred for 24 h at 25 °C, and then sodium hydroxide-DMSO combined solution (12 mL) was added. After blending well 30 min and reacting with 7.2 mL of methanol iodide for 7 min exactly, the product was dialyzed against running water and distilled water for 24 h, respectively. The solution was concentrated to 10 mL, treated thrice with 10 mL chloroform, and fully shocked to extract the polysaccharide, after watered, dried over Na_2SO_4 for 24 h, filtered and evaporated to 1 mL, then examined by IR spectrum. The desiccated sample was subjected to 1 mL of 88% formic acid, suffused with nitrogen, kept at 100 °C for 4 h, got rid of excess formic acid by methanol, and dried in vacuum desiccators. The above sample was hydrolyzed in 2 M trifluoroacetate (TFA) for 6 h at 100 °C, treated with ethanol then distilled water, reduced with NaBH_4 for 24 h, and then acetylated with acetic anhydride-pyridine(1:1) at 100 °C for 2 h. The resulting acetates were subjected to GC-MS analyses. GC-MS was performed on a HP5890 instrument (Agilent Component, USA) with a Rtx-5ms column (30 m \times 0.25 mm \times 0.25 μm), and at temperatures programmed from 160 to 250 °C at 8 °C/min. Linkages were identified on the basis of relative retention time and fragmentation pattern. Peaks of methylated products

were identified by mass spectra. The molar ratios for each sugar were calibrated using the peak areas and response factor [8].

For NMR measurements, 30 mg DMA was dried in a vacuum over P_2O_5 for several days, exchanged with deuterium by lyophilizing with D_2O for several times, and then dissolved in D_2O (99.9%). The ^1H and ^{13}C NMR spectra experiments were recorded at 500 MHz and 125 MHz on a spectrometer (BRUKER DRX500, BRUKER Component, Swiss), respectively. The 2D COSY and HSQC NMR were performed using standard software. The ^1H NMR chemical shifts were referenced to residual HOD at δ 4.71 ppm. The ^{13}C spectrum was recorded at δ 31.05 ppm. HSQC data was recorded after a 50 ms delay to allow for the evolution of long-range couplings.

Antioxidant activities assay

The superoxide radical assay and the hydroxyl radical ability were measured by the methods of Ghiselli and Robak with a minor modification, respectively [5].

The scavenging ability for DPPH of DMA was investigated. Briefly, samples or Vc were dissolved in distilled water at 0, 2.5, 5, 10, 20, 40, 80 and 100 $\mu\text{g}/\text{mL}$. The sample solution (0.1 mL) or distilled water (0.1 mL, control) was mixed with 2 mL of 0.3 mM DPPH, and incubated for 20 min at 25 °C. The absorbance of the mixture was measured at 517 nm. The scavenging ability for DPPH of DMA was calculated using the following equation: $(1 - \text{absorbance of sample} / \text{absorbance of control}) \times 100\%$.

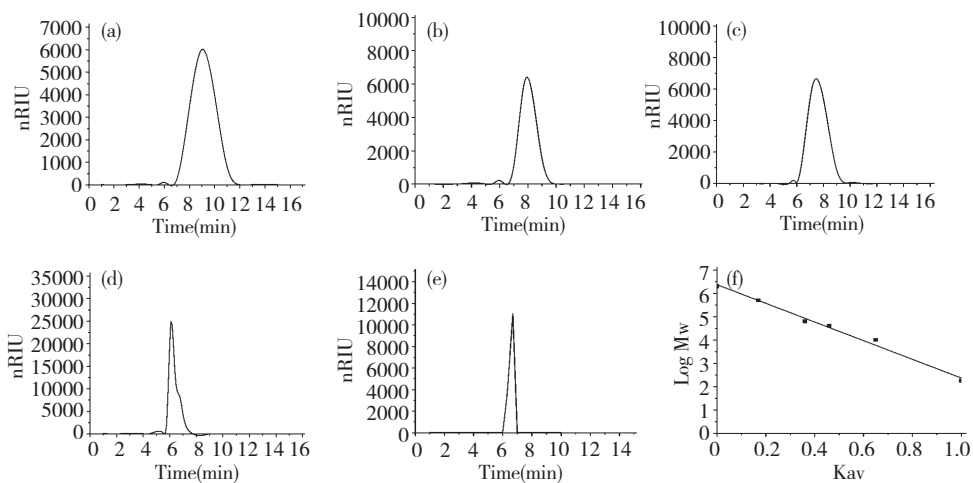


Fig. 1 HPLC chromatogram of T10 (a), T40 (b), T70 (c), T500 (d), DMA (e) and standard curve of molecular weight (f)

Results and Discussion

Isolation, purification and composition analysis of DMA

The crude polysaccharide was isolated from the hot-water extract of *D. nipponica* with a yield of 8.05%. After fractioned on DEAE-Sephacrose CL-6B column, DMA (30.6%) was obtained. DMA was one peak and a kind of polysaccharide by HPLC with Sugar KS-804

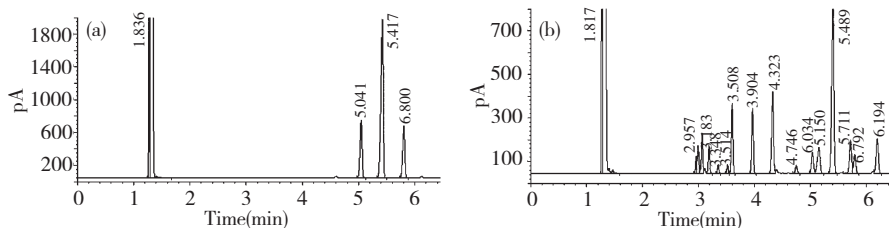


Fig. 2 GC-FID chromatograms of DMA hydrolyzed by TFA (a) and standard samples (b)

Table 1 Retention time of standard samples and DMA

Samples	Retention time		
	Isomer 1	Isomer 2	Isomer 3
Glc	5.034	5.792	
Gal	4.745	5.150	
Xyl	3.599	3.964	
Rha	3.060	3.514	
Man	4.323	5.150	
Ara	2.992	3.183	3.348
Gluc	5.717	6.194	
Mannitol	5.409 ^a /5.417 ^b		
DMA	5.041	5.800	

^aMannitol in GC-FID of standard samples

^bMannitol in GC-FID of DMA

Structural characterization of DMA

The polysaccharide of DMA showed abundance HIO₄ uptake, while it was oxidized. The consumption of HIO₄ (0.293 m mol) was two times than the amount of formic acid (0.143 mmol) that was produced after 36 h of the treatment, indicating the existing of large amounts of monosaccharide which were 1→linked or 1→6 linked of DMA. In addition, it should be concluded that sugar residues oxidized and linkages of (1→)-glycosidic linkages accounted for 97.42%. The oxidized products were hydrolyzed and examined by GC-FID. Glucose was absent, then it may be inferred that glucose was all linkages that can be oxidized, namely (1

column (Fig. 1), which was also confirmed by NMR. The homogeneity of DMA was not good for the asymmetric peak. The average molecular weight of DMA was 22.9×10^4 according to standard curve ($y = -3.67x + 6.356$). The polysaccharide content of DMA was 98.2% by the phenol-sulfuric acid, and DMA was mainly consisted of glucose by GC-FID analysis of DMA hydrolyzed by TFA and standard samples (Fig. 2 and Table 1).

→)-glycosidic linkages or (1→6)-glycosidic linkages. Results of smith-degradation analysis of fractions were obtained. There was no precipitation in the sack and no substances in the supernatant of sack, indicating that the backbone of DMA should be all oxidized by HIO₄. In the same time, the linkages of backbone could be arrayed by (1→6)-glycosidic linkages, (1→2)-glycosidic linkages or (1→2,6)-glycosidic linkages, according to the presence of glycerin out of sack.

The IR spectrum of DMA displayed a broad stretching intense characteristic peak at around 3407 cm⁻¹ for the hydroxyl group (Fig. 3a). The IR spectrum of methylated DMA (Fig. 3b) showed that no absorption peak of hydroxyl group identified the complete methylation. The analysis of fraction DMA showed the presence of 2,3,4-Me₃-Glc (Table 2). The methylation analysis of DMA indicated that 2,3,4-Me₃-Glc was the major component of the main-chain structure. On the basis of the results obtained above, it was possible to conclude that a repeating unit of DMA contained a main-chain composed of (1→6)-linked-glucose.

The ¹H (Fig. 4a) and ¹³C (Fig. 4b) NMR experiments were carried out. The 500 MHz ¹H NMR spectrum showed only one signal at δ 5.30 ppm. Since it did not provide enough information about the anomeric configuration, a one-bond C-1-H-1 heteronuclear NMR

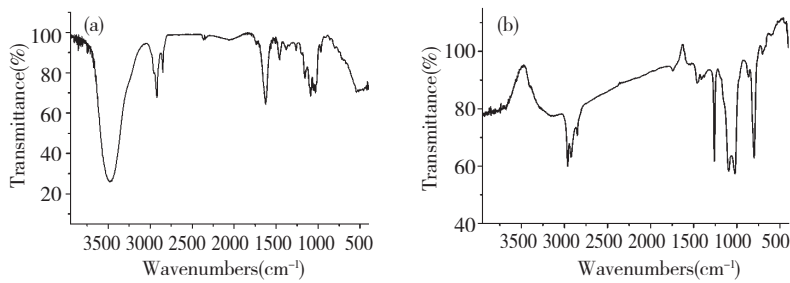


Fig. 3 IR spectra of DMA (a) and methylated DMA (b)

Table 2 Methylation analysis of DMA

Retention time (min)	Methylation product	Linkage pattern	Major mass fragment (m/z)
4.368	2,3,4,6-Me ₄ -Glc _p	1-linked Glc _p	43. 45. 71. 87. 101. 117. 129. 145. 161. 205
5.373	2,3,4-Me ₃ -Glc _p	1,6-linked Glc _p	43. 87. 99. 101. 117. 131. 161. 191

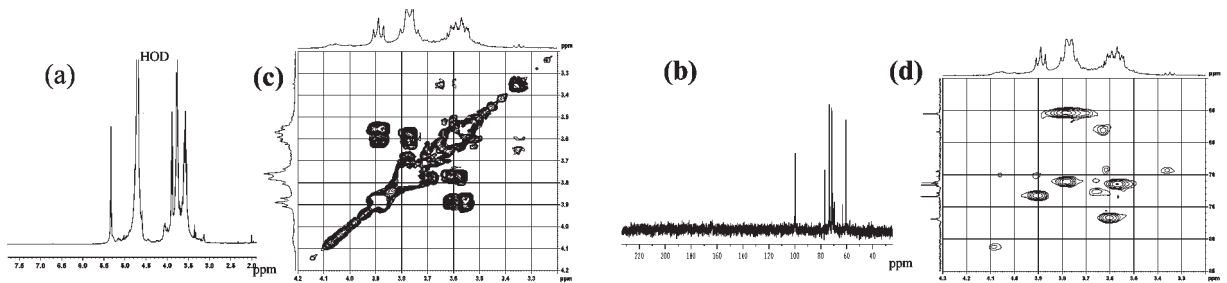


Fig. 4 ¹H NMR (a), ¹³C NMR (b), ¹H-¹H COSY (c) and Part of HSQC (d) spectra of DMA

experiment was carried out. All the ¹H and ¹³C signals were assigned using 2D-COSY (Fig. 4c) and HSQC (Fig. 4d) NMR experiments. The residue was designated as A according to its increasing anomeric shift. The proton chemical shifts of A were assigned from H-1 to H-6 by 2D-COSY spectra [9,10]. The carbon chemical shifts from the C-1 to C-6 for A were assigned from the HSQC spectrum, and these correspond nearly to the standard values of methyl glycosides of D-glucose. The anomeric signals for moiety A at δ 5.30 > 5.0 ppm and the C-1 signal of A at 99.64 ppm confirmed by the HSQC experiment indicated that A residue was α-D-pyranose [11]. The downfield shifts of C-6 carbon signal with respect to standard values indicated that the moiety A was linked at these positions [11]. Thus, considering the results of methylation analysis and NMR experiments, it may be concluded that A was pyranoid→6)-α-D-Glc_p-(1→. Based on all these evidences, the repeating unit in polysaccharide DMA was assigned as →6)-α-D-Glc_p-(1→.

Antioxidant activities

DMA had the scavenging effect for hydroxyl radicals. As shown in Fig. 5, the scavenging effects increased with increasing concentration. Scavenging effects of DMA were 0% to 28.9% at amount of 0.25 to 4 mg among the reaction system, respectively, and that of Vitamin C was about 0 to 43.09%. This result proved that polysaccharide DMA from *D. nipponica* had significant effect on scavenging hydroxyl radical, and that was lower than Vc. DMA had no abilities to scavenge superoxide radical and DPPH.

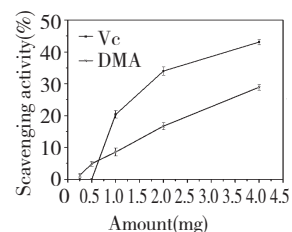


Fig. 5 Scavenging effects of DMA and Vc on hydroxyl radical

Conclusion

According to the results above, it was concluded that the crude water extracting polysaccharide of *D. nipponica* contained the water extractable polysaccharide (DMA) purified by DEAE Sepharose CL-6B. The IR, periodate oxidation-smith degradation, methylation and NMR analyses results indicated that DMA mainly composed of $\rightarrow 6$)- α -D-Glcp-(1 \rightarrow . In addition, DMA had a scavenging activity of hydroxyl radical.

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