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Structural investigation of a polysaccharide (DMB) purified from *Dioscorea nipponica Makino*

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ABSTRACT

A water-soluble polysaccharide was isolated from the aqueous extract of *Dioscored nipponica Makino*. Compositional analysis, IR analysis, methylation analysis, and NMR studies (^{1}H, ^{13}C, ^{1}H—^{1}H COSY, HSQC, and HMBC) revealed the presence of the following repeating unit in the polysaccharide:

→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→6)-2-*O*-*Me*-β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→

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↑

1-β-D-Glc*p*/2-D-Fru*f*

1. Introduction

Many synthetic antioxidants are used as medical materials at the present time. However, the most commonly have been suspected of being responsible for liver damage and carcinogenesis (Qi et al., 2005). Thus, it is essential to develop and utilize effective and natural antioxidants (Nandita & Rajini, 2004). Published data indicate that plant polysaccharides in general have strong antioxidant activities and can be explored as novel potential antioxidants (Hu, Xu, & Hu, 2003; Jiang, Jiang, Wang, & Hu, 2005).

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 the major polysaccharide of *D. nipponica Makino* using a DEAE Sepharose CL-6B column chromatography and a Sephadex G-100 column chromatography. In addition, antioxidant activities of the major polysaccharide had been also identified (Luo, 2008). Structural identification of polysaccharides is necessary to better study the relationship between structural and functional properties of polysaccharides and effectively exploits these potential activity substances. However, there are only few studies on its chemical composition and structure, and its detailed structure is not precisely known. The aim of this work is to study in detail the structure of the polysaccharide isolated from *D. nipponica Makino*.

2. Experimental

2.1. Materials and chemicals

Dried underground part of *D. nipponica Makino* was purchased from a local drugstore (Quanzhou, Fujian Province, China). Monosaccharide standard samples were purchased from Sigma Chemical Co. (St Louis, MO, USA). All other reagents used were analytical grade.

2.2. Preparation of polysaccharide DMB

The *D. nipponica Makino* 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 3500 rpm for 15 min. The sample was washed successively with ethanol and aether, and dried at reduced pressure, giving a crude polysaccharide (DM). DM (1 g) was dissolved in 10 mL distilled water, centrifuged, and then the supernatant was injected to a column (4.6 cm × 30 cm) of DEAE-Sepharose CL-6B equilibrated with distilled water for 500 mL at 4 mL/6 min, followed stepwise by NaCl aqueous solution (0 and 2 M) for 400 mL, respectively, at 45 mL/h. A major polysaccharide fraction was collected,

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and then purified by gel filtration chromatography on a column of Sephadex G-100, named DMB.

Total carbohydrate and protein of these polysaccharides were determined by the phenol–sulfuric acid and Bradford, respectively. Paper chromatography (Wang, Luo, & Liang, 2004) and gas chromatography (GC) were used for identification and quantification. The molecular weight of the polysaccharide DMB 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) (Luo & Fang, 2008).

2.3. IR analysis

The IR spectrum of the polysaccharide was determined using a Fourier transform infrared spectrophotometer equipped (Nicolet Nexus 470, NICOLET Component, USA). The purified polysaccharide was grounded with KBr powder and then pressed into pellets for FTIR measurement in the frequency range of 4000–400 cm^{-1} (Kumar, Joo, Choi, Koo, & Chang, 2004).

2.4. Methylation study

Polysaccharide (40 mg) was dissolved in dimethylsulfoxide (12 mL), removed air with nitrogen, stirred for 24 h at 25 °C, and then sodium hydroxide–dimethylsulfoxide combined solution (12 mL) was added. After blending well 30 min, the mixture was methylated with 7.2 mL methanol iodide, reacting 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 methylated polysaccharide, after watered, dried over Na_2SO_4 for 24 h, filtered and evaporated to 1 mL, then examined by IR spectrum. No absorption peak of hydroxyl identified the complete methylation.

The desiccated methylated polysaccharide was subjected to 1 mL 88% formic acid, suffused with nitrogen, kept at 100 °C for 4 h, got rid of excess formic acid by methanol, dried in a vacuum desiccator. The above sample was hydrolyzed in 2 M trifluoroacetic acid for 6 h at 100 °C, treated with ethanol then distilled water, reduced with $NaBH_4$ for 24 h, followed by acetylation with acetic anhydride–pyridine(1:1) at 100 °C for 2 h. The resulting alditol acetates were subjected to GC–MS analyses.

Gas chromatography–mass spectrometry (GC–MS) was performed on a HP5890 instrument (Agilent Component, USA) with a column Rtx-5ms (30 m × 0.25 mm × 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. The molar ratios for each sugar were calibrated using the peak areas and response factor of the flame-ionization detector in GC (Wang et al., 2001).

2.5. NMR spectroscopy

For NMR measurements, 30 mg DMB 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 and 125 MHz on a spectrometer (BRUKER DRX500, BRUKER Component, Swiss), respectively. The 2D COSY, HSQC, and HMBC NMR were performed using standard software. The ^1H NMR chemical shifts were referenced to residual HOD at δ 4.51 ppm as internal standard. The ^{13}C spectrum was recorded at δ 31.05 ppm. HSQC and HMBC data were recorded after a 50 ms delay to allow for the evolution of long-range couplings.

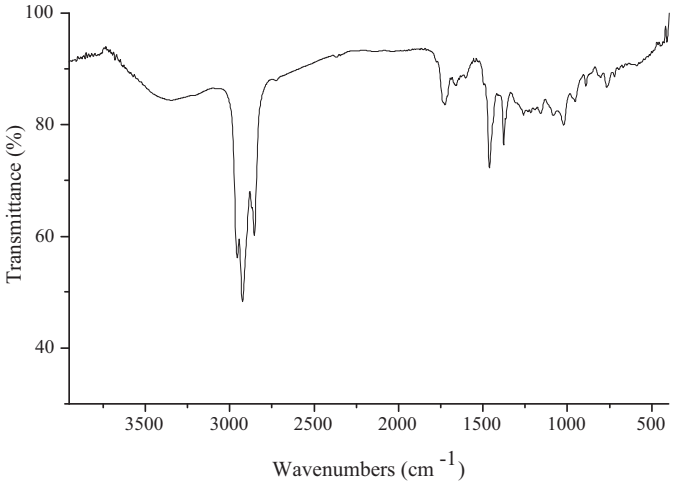

Fig. 1. IR spectra of methylated DMB.

3. Results and discussion

3.1. Some characterizations of DMB

The crude polysaccharide was isolated from the hot-water extract of *D. nipponica Makino* by a yield of 8.05%. After fractioned on DEAE-Sepharose CL-6B column and purified by gel chromatography on Sephadex G-100 column, DMB (18.6%) was obtained. DMB is homogeneous by gel-filtration chromatography on Sephadex G-100 column (1.6 cm × 80 cm) and HPLC with Sugar KS-804 column.

The polysaccharide content of DMB is 98.9%, average molecular weight is 3.8×10^4, and DMB is mainly consisted of glucose and fructose (45:1). According to the results of partial acid hydrolysis we come to conclusion that glucose is the component of main-chain structure of DMB, and fructose and a part of glucose may be in the position of branched structure of DMB (Luo, 2008).

3.2. Methylation analysis

The infrared spectrum of DMB displayed a broad stretching intense characteristic peak at around 3407 cm^{-1} for the hydroxyl group. The IR spectrum (Fig. 1) of methylated DMB showed that no absorption peak of hydroxyl group identified the complete methylation. The methylation analysis of fraction DMB shows the presence of four components, namely 2,3,4,6-Me_4-Glc, 2,3,6-Me_3-Glc, 2,3,4-Me_3-Glc and 2,3-Me_2-Glc in molar ratios of 3.81:31.97:7.2:7.65 (Table 1). This shows a good correlation between terminal and branched residues. This assay did not detect fructose due to its low content in the sample. By methylation linkage analysis, glucose is the linkage those all can be oxidized by HIO_4.

Both results of partial acid hydrolysis and methylation linkage analysis of DMB indicate that 2,3,6-Me_3-Glc and 2,3,4-Me_3-Glc are the major components of the main-chain structure. The relative amounts of (1→4,6)-linked-glucose indicates that approximate branch ratios may theoretically be 16%, namely on average one branching point for each six residues of main-chain. On the basis of the results obtained above, it is possible to conclude that a repeating unit of DMB contains a main-chain composed of (1→6)-linked-glucose and (1→4)-linked-glucose with branches attached to O-6 or O-4 of some glucose.

Table 1
Methylation analysis of DMB.

Retention time (min)	Methylation product	Linkage pattern	Molar ratio	Major mass fragment (*m*/*z*)
4.377	2,3,4,6-Me_4-Glc*p*	1-linked Glc*p*	3.81	43.45.71.87.101.117.129.145.161.205
5.219	2,3,6-Me_3-Glc*p*	1,4-linked Glc*p*	31.97	43.45.87.99.101.113.117.233
5.377	2,3,4-Me_3-Glc*p*	1,6-linked Glc*p*	7.20	43.87.99.101.117.131.161.191
6.485	2,3-Me_2-Glc*p*	1,4,6-linked Glc*p*	7.65	43.101.117.261

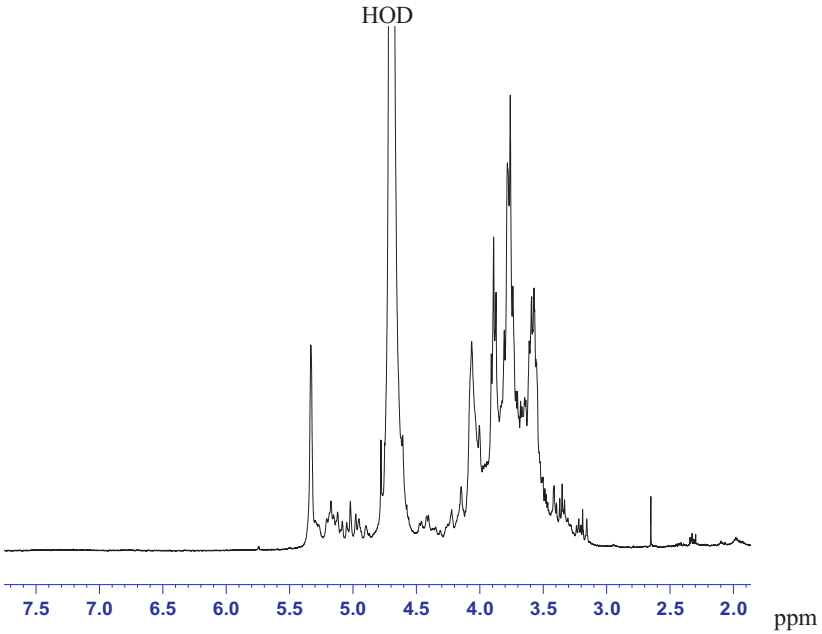

Fig. 2. ^{1}H NMR spectrum of polysaccharide DMB.

Table 2
^{1}H chemical shift data for DMB.

Residue	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
A →4)-α-D-Glc*p*-(1→	5.33	3.51	3.27	3.64	3.99	–	–
B →6)-2-*O*-*Me*-β-D-Glc*p*-(1→	5.19	4.10	3.91	3.55	3.41	3.65	–
C β-D-Glc*p*-(1→	4.99	4.02	3.67	3.39	3.65	–	–
D →4,6)-β-D-Glc*p*-(1→	4.46	3.61	3.90	4.12	3.91	3.55	3.65

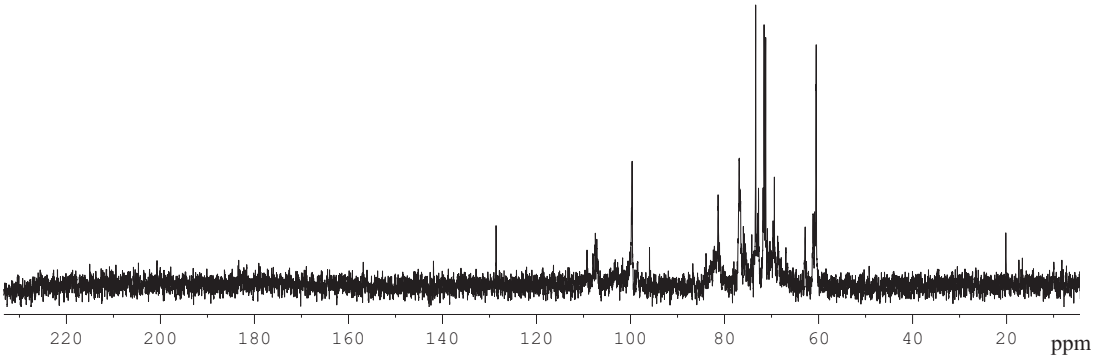

Fig. 3. ^{13}C NMR spectrum of polysaccharide DMB.

3.3. NMR spectroscopy

The ^{1}H (Fig. 2, Table 2) and ^{13}C (Fig. 3, Table 3) NMR experiments were carried out. The 500 MHz ^{1}H NMR spectrum showed only one signal at δ 5.33 ppm. Since they did not provide enough information about the anomeric configuration, a one-bond C-1—H-1 heteronuclear NMR experiment was carried out. All the ^{1}H and ^{13}C signals were assigned using 2D-COSY (Fig. 4) and HSQC

Table 3
^{13}C chemical shift data for DMB.

Residue	C-1	C-2	C-3	C-4	C-5	C-6
A →4)-α-D-Glc*p*-(1→	99.96	72.10	73.1	77.10	70.12	–
B →6)-2-*O*-*Me*-β-D-Glc*p*-(1→	107.17	81.91	77.20	71.21	76.92	72.1
C β-D-Glc*p*-(1→	107.48	76.21	77.12	70.11	77.11	–
D →4,6)-β-D-Glc*p*-(1→	103.00	76.10	77.28	81.09	76.96	71.78

(Fig. 5) NMR experiments. The four glycosyl moieties were designated as A, B, C and D according to their increasing anomeric shifts.

The proton chemical shifts of A were assigned from H-1 to H-6 by 2D-COSY (Fig. 4) spectra. 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 5.33 > 5.0 ppm and the C-1 signal of A at 99.96 ppm confirmed by the HSQC experiment indicate that A residue is α-D-pyranose (Corsaro et al., 2005; Mondal, Chakraborty, Rout, & Islam, 2006; Rout, Mondal, Chakraaborty, & Islam, 2006). Thus, considering the results of methylation analysis and NMR experiments, it may be concluded that A is pyranoid→4)-α-D-Glc*p*-(1→.

All the proton chemical shifts of B (H-1 to H-6, Table 3) were identified from COSY spectra. The chemical shifts of the C-1 to C-6 of moiety B were assigned from the HSQC spectrum. The anomeric chemical shift for moiety B at δ 5.19 ppm and $J_{1,2}$ (8.1 Hz) indicate that B is β-D-pyranose (Faber, Van Haaster, Kamerling, & Vliegenthart, 2002; Omarsdottir et al., 2006; Yang et al., 2007). The downfield shift of C-6 (δ 72.1) indicates that B is a 6-linked D-glucose moiety.

The presence of signals at δ 3.3–3.5 ppm in ^{1}H NMR and at δ 55–61 ppm in ^{13}C NMR indicates that there is O-methyl residue in B. The δ C-2 shifted to low field, which corresponded to →6)-2-O-Me-β-D-Glc*p*-(1→ (Sun, Shan, Cui, Tang, & Gu, 2009). Residue C and D had an anomeric proton signal at δ < 5 ppm, and the ^{13}C signal for the anomeric carbon of C and D were observed at δ > 102 ppm indicate that C and D are β-D-pyranose. The proton signals (Table 2) of C from H-1 to H-6 were assigned using the 2D-COSY experiments. It can be confirmed from Tables 2 and 3 that C may be β-D-Glc*p*-(1→. The carbon signals (Table 3) from C-1 to C-6 for residue D were identified from the HSQC spectrum. The downfield shifts of C-6 (δ 71.78) and C-4 (δ 81.09) carbon signals with respect to standard values indicate that the moiety D is linked at these positions (Agrawal, 1992; Pramanik, Mondal, Chakraborty, Rout, & Islam, 2005). These observations also supported the GC–MS data for this linkage. Hence 1,4,6-linked D-glucose is present in the DMB polysaccharide.

Long-range ^{13}C—^{1}H correlations were obtained from an HMBC (spectrum not shown). The cross-peaks of both anomeric protons and carbons of each of the sugar moieties were examined, and intra- and inter-residual connectivity were observed from the HMBC experiment. Cross-peaks are found between H-1 of A and C-6 of B, C-6 of D and H-1 of B.

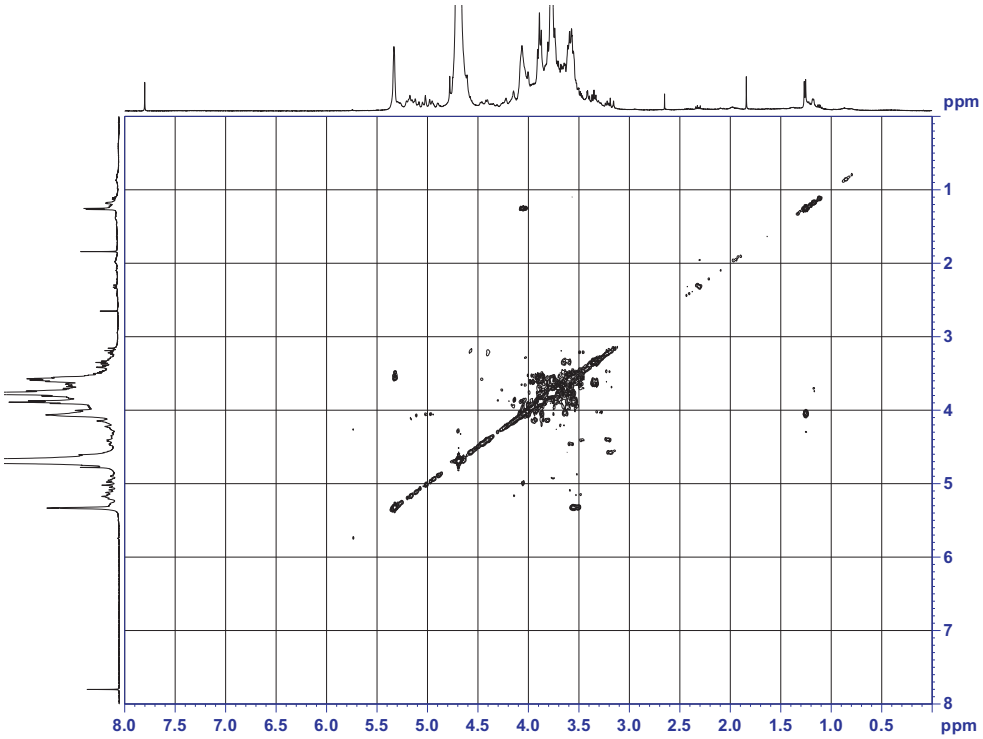

Fig. 4. ^{1}H—^{1}H COSY spectrum of polysaccharide DMB.

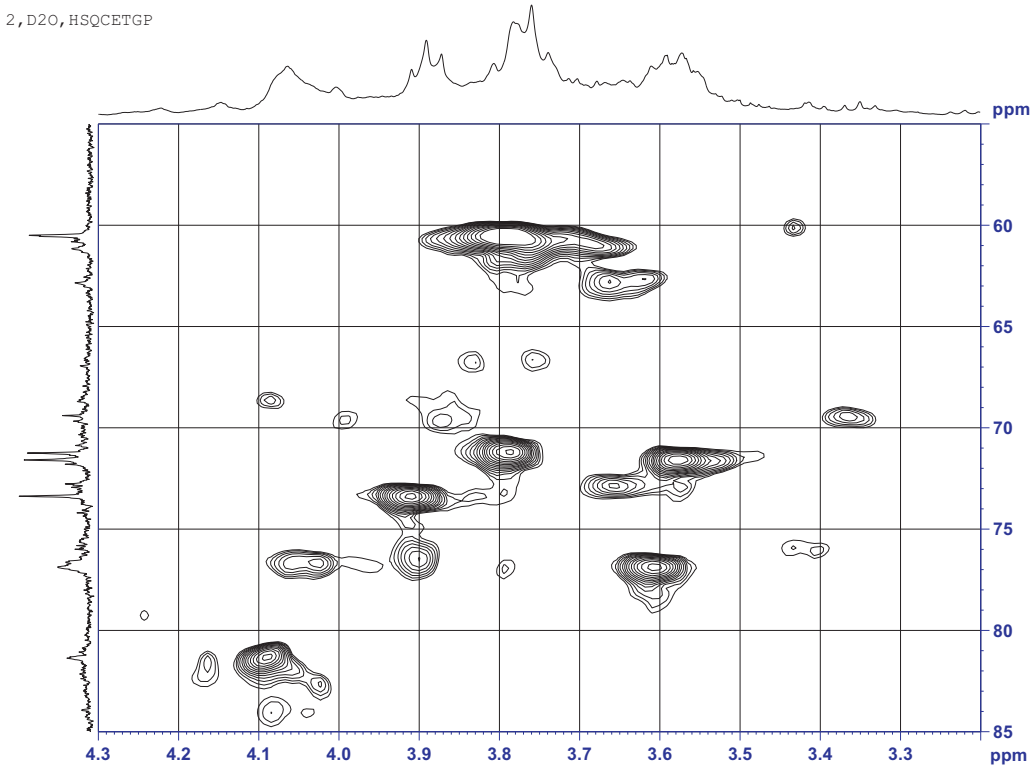

Fig. 5. Part of HSQC spectrum of polysaccharide DMB.

Based on all these evidences the structure of the repeating unit present in polysaccharide, DMB is assigned as:

→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→6)-2-*O*-*Me*-β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→

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1-β-D-Glc*p*/2-D-Fru*f*

3.4. Antioxidant activities

On the basis of superoxide radical assay, hydroxyl radical assay and self-oxidation of 1,2,3-phentriol assay, antioxidant activities of DMB were investigated. DMB had a higher scavenging effect than Vc. Scavenging effects of DMB for hydroxyl radical were 3.35–43.73% at amount of 0.25–4 mg, and that of vitamin C was about 0–43.09%. At the amount of between 0.25 and 2 mg, the effects on scavenging superoxide of DMB were 27.5–35.52%, and the scavenging activity of vitamin C was 0 at 0.25–2 mg.

This purified fraction of polysaccharide exhibits equivalent inhibiting power for self-oxidation of 1,2,3-phentriol to vitamin C, a little higher scavenging activity of superoxide radical and hydroxyl radical than vitamin C, and should be explored as a novel potential antioxidant (Luo, 2008).

4. Conclusion

According to the results above, it is concluded that the water extracting crude polysaccharide of *D. nipponica Makino* contains predominantly water extractable polysaccharide (DMB) purified by DEAE Sepharose CL-6B and Sephadex G-100 column chromatography. In addition, DMB has a little higher scavenging activity of hydroxyl radical than vitamin C, a higher activity at scavenging superoxide radical than vitamin C in low amounts, and equivalent inhibiting ability to vitamin C on self-oxidation of 1,2,3-phentriol. Analysis by methylation analysis and NMR studies indicate that DMB is mainly composed of →4)-α-D-Glc*p*-(1→ and →6)-2-O-Me-β-D-Glc*p*-(1→, and the linkage of branch was →4,6)-β-D-Glc*p*-(1→.

Acknowledgments

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