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Carbohydrate Polymers 72 (2008) 376–381
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Structural identification of ginseng polysaccharides and testing of their antioxidant activities

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Received 10 July 2007; received in revised form 11 August 2007; accepted 4 September 2007
Available online 14 September 2007

Abstract

A large number of polysaccharides were presented in boiling-water extraction of ginseng. A DEAE-Sepharose CL-6B column chromatography was used to isolate the major polysaccharides from ginseng. Two fractions were obtained, named GPII and GPIII. Both GPII and GPIII were a neutral polysaccharide and a single peak in HPLC with Sugar KS-804 column, with a molecular weight of 3×10^5 and 4×10^5, respectively, comprised mainly of glucose. Analysis by Periodate oxidation–Smith degradation indicated that GP was composed of 60.06% (1→)- or (1 → 6)-glycosidic linkages and 39.94% (1 → 3)-glycosidic linkages, and GPIII 16.23% (1→)- or (1 → 6)-glycosidic linkages, 25.98% (1 → 2)-glycosidic linkages, and 57.79% (1 → 3)-glycosidic linkages. On the basis of superoxide radical assay, hydroxyl radical assay and self-oxidation of 1,2,3-phentriol assay, their antioxidant activities were investigated. GPII exhibited equivalent inhibiting power for self-oxidation of 1,2,3-phentriol to Vc, a little higher scavenging activity of superoxide radical and hydroxyl radical than Vc, and should be explored as a novel potential antioxidant.

Keywords: Ginseng; Purification; Periodate oxidation–Smith degradation; Antioxidant activity

1. Introduction

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 (Mau, Lin, & Song, 2002). In order to reduce damage to the human body, synthetic antioxidants are used for industrial processing at the present time. However, the most commonly have been suspected of being responsible for liver damage and carcinogenesis (Grice, 1988; Qi et al., 2005). Thus, it is essential to develop and utilize effective and natural antioxidants so that they can protect the human body from free radicals and retard the progress of many chronic diseases (Kinsella, Frankel, German, & Kanner, 1993; 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; Ramarahnam, Osawa, Ochi, & Kawaishi, 1995).

Ginseng (*Panax ginseng* C.A. Meyer, ginseng) has been the most precious and renowned tonic drug in traditional Chinese medicine. To date, no investigation has been carried out on polysaccharides that may account for antioxidant activities of ginseng. Identification of the polysaccharides is necessary to better effectively exploit the structure and functional properties of these substances. In this study, we report on the extraction and purification of the major polysaccharides of ginseng using a DEAE-Sepharose CL-6B column chromatography. In addition, the structure properties and antioxidant activities of these major polysaccharides are also identified.

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doi:10.1016/j.carbpol.2007.09.006

2. Materials and methods

2.1. Materials and chemicals

Dried underground part of ginseng was purchased from a local drugstore (Quanzhou, Fujian Province, China). Nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), dihydronicotineamidadenine dinucleotide (NADH), thiobarbituric acid (TBA), deoxyribose, L-rhamnose, D-glucose, D-arabinose, D-xylose, D-fructose, D-galactose and D-mannose were purchased from Sigma Chemical Co. (St. Louis, MO, USA), while DEAE-Sepharose CL-6B was from the Pharmacia Co. (Sweden). All other reagents used were of analytical grade.

2.2. Isolation and purification of polysaccharide

The ginseng (250 g) was extracted with 80% ethanol at 50 °C for 6 h, dried, and then extracted with distilled water at 80 °C for 2 h twice. After each extraction, the soluble polymers were separated from residues by filtration, and extracts were combined, concentrated and dialyzed against running water for 48 h. The above extract was submitted to graded precipitation with four volumes of ethanol and the mixture was kept overnight at 4 °C to precipitate the polysaccharides. The precipitate was collected by centrifugation, washed successively with ethanol and a ether, and dried at reduced pressure, giving a crude polysaccharide (GP). Size-exclusion and anion-exchange chromatography were used for the fractionation of this preparation. GP (500 mg) was dissolved in 10 mL distilled water, centrifuged, and then the supernatant was injected to a column (4.6×30 cm) of DEAE-Sepharose CL-6B equilibrated with distilled water. After loading with sample, the column was eluted with distilled water for 1000 mL at 4 mL/6 min, followed stepwise by NaCl aqueous solution (0 and 1 M) for 400 mL respectively at 40 mL/h. The major polysaccharide fractions were collected with a fraction collector, dialyzed against tap water and distilled water for 48 h, respectively.

2.3. Monosaccharide composition and properties

Total carbohydrate and protein of these polysaccharides were determined by the phenol–sulfuric acid (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and (Bradford, 1976), respectively. Paper chromatography (PC, Wang, Luo, & Liang, 2004) and gas chromatography (GC) were used for identification and quantification. GC was performed on a 6890N instrument (Angilent, USA) with a column HP-5 ($30\ m \times 0.32\ mm \times 0.25\ \mu m$). First, the polysaccharide (10 mg) was dissolved in 10 ml of a 2-M TFA and hydrolyzed at 120 °C for 6 h, and then hydrolyzed products were evicted TFA by ethanol and dried. Derivation was then carried out using the trimethylsilylation reagent according to the method of Guentas et al. (2001) with some modifications (Wang & Luo, 2007).

The IR spectrum of the polysaccharide was determined using a Fourier transform infrared spectrophotometer (FTIR, Bruker, Germany) equipped. The purified polysaccharide was ground with KBr powder and then pressed into pellets for FTIR measurement in the frequency range of 4000 to 500 cm^{-1} (Kumar, Joo, Choi, Koo, & Chang, 2004).

2.4. Determination of the molecular weight

The molecular weight of the fractions was determined by gel-permeation chromatography, in combination with a high-performance liquid chromatography instrument (Angilent1100, USA). Sample (2.0 mg) was dissolved in distilled water (2 mL) and passed through a 0.45-μm filter, applied to a gel-filtration chromatographic column of Shodex Sugar KS-804 (SHOWA DENKO K.K, Japan), 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 dextrans of different molecular weight (Dextran Blue, Dextran T10, T40, T70, T500 and Glucose). The molecular weight was calculated by the calibration curve obtained by using various standard dextrans (Wang, Liang, & Zhang, 2001).

2.5. Periodate oxidation–Smith degradation

For analytical purpose, 50 mg of sample was dissolved in 25 ml of distilled water and 25 ml of 30 mmol/L $NaIO_4$ were added. The solution was kept in the dark at RT, 0.1 ml aliquots were withdrawn at 6 h intervals, diluted to 25 ml with distilled water and read in a spectrophotometer at 223 nm. Glycol (2 ml) was added, and then the experiment of periodate oxidation was over. The solution of periodate product (2 ml) was sampled to calculate the yield of formic acid by 0.005 M sodium hydroxide, and the rest was extensively dialyzed against tap water and distilled water for 24 h, respectively. The content inside was concentrated and reduced with sodium borohydride (160 mg), and the mixture was left for 24 h at room temperature, neutralized to pH 6.0 with 50% acetic acid, dialyzed as described above, and concentrated to a volume (10 ml). One-third of solution described above was freeze-dried and analyzed with GC. Others were added to the same volume of 1 M sulfuric acid, kept for 40 h at 25, neutralized to pH 6.0 with barium carbonate, and filtered. The filtrate was dialyzed as foresaid, and the content out of sack was desiccated for GC analysis; the content inside was diluted with ethanol, and after centrifugation, the supernatant and precipitate were also dried out for the GC analysis.

2.6. Assay for antioxidant activity

2.6.1. Hydroxyl radical assay

The hydroxyl radical assay was measured by the method of Ghiselli, Nardini, Baldi, and Scaccini (1998) with a

minor modification. Samples were dissolved in distilled water at 0 (control), 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.3, 12.5, 25 or 50 mg/mL. The sample solution (0.1 mL) was mixed with 0.6 mL of reaction buffer [0.2 M phosphate buffer (pH 7.4), 2.67 mM deoxyribose, and 0.13 mM EDTA], 0.2 mL of 0.4 mM ferrous ammonium sulfate, 0.05 mL of 2.0 mM ascorbic acid, and 0.05 mL of 20 mM H_2O_2 was then added to the reaction solution. The reaction solution was incubated for 15 min at 37 °C and then 1 mL of 1% thiobarbituric acid and 1 mL of 2.0% trichloroacetic acid were added to the mixture. The mixture was boiled for 15 min and cooled on ice. The absorbance of the mixture was measured at 532 nm. Percent inhibition of hydroxyl radical was calculated as (1 − absorbance of sample/absorbance of control) × 100%.

2.6.2. Superoxide radical assay

The superoxide radical assay was measured by the method of Robak and Gryglewski (1988) with a minor modification. Samples were dissolved in distilled water at 0 (control), 0.78, 1.56, 3.125, 6.25, 10, 20, 40, 50, 80 or 100 mg/mL. A 0.1-mL aliquot of each sample solution was mixed with 1 mL of 16 mM Tris–HCl (pH 8.0) containing 557 μm NADH, 1 mL of 16 mM Tris–HCl (pH 8.0) containing 45 μm PMS, and 1 mL of 16 mM Tris–HCl (pH 8.0) containing 108 μm NBT. After 5 min of incubation at 25 °C, the absorbance was measured at 560 nm. The superoxide radical effect was calculated as scavenging activity (%) = (1 − absorbance of sample/absorbance of control) × 100%.

2.6.3. Self-oxidation of 1,2,3-phentriol assay

The scavenging ability for self-oxidation of 1,2,3-phentriol of all different contents were investigated according to the method of Marklund and Marklund (1974) with a minor modification. Briefly, samples were dissolved in distilled water at 0 (control), 5, 10, 40 or 80 mg/mL. The sample solution (0.1 mL) was mixed with 2.8 mL of 0.05 M Tris–HCl buffer (pH 8.0) containing 1 mM EDTA and 1,2,3-phentriol (0.2 mL, 6 mM), was shaken rapidly at room temperature. The absorbance of the mixture was measured at 325 nm per 30 s for 4 min against a blank, and a slope was calculated as absorbance of per min. The ability of different scavenging ability for self-oxidation of 1,2,3-phentriol of all fractions was calculated using the equation (1 − slope of sample/ slope of control) × 100%.

3. Results and discussion

3.1. Isolation, purification and composition of fractions

The crude polysaccharide was isolated from the hot-water extract of ginseng by a yield of 28.05%. After fractioned on DEAE-Sepharose CL-6B column, GPII (3.7%) and GPIII (7.78%) were obtained from distilled water elute (Fig. 1) and NaCl elute (Fig. 2), respectively.

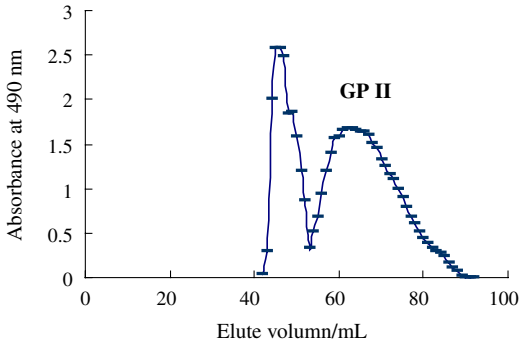

Fig. 1. DEAE-Sepharose CL-6B column chromatogram of GP from distilled water elute.

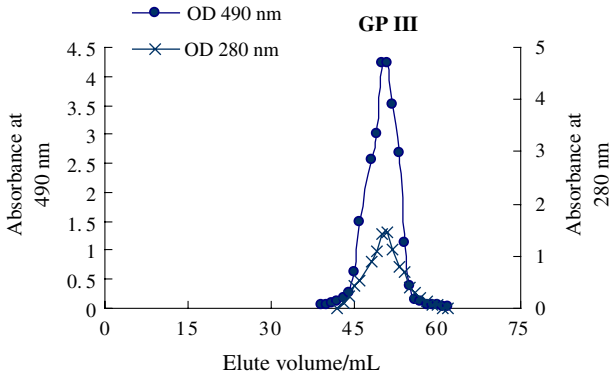

Fig. 2. DEAE-Sepharose CL-6B column chromatogram of GP from NaCl stepwise elute.

We came to a conclusion that GPII and GPIII were homogeneous by the following tests. It showed only one symmetrical peak from gel-filtration chromatography on HPLC with Sugar KS-804 column (Fig. 3) which was equilibrated in distilled water at 1 mL/min, indicating that no other polysaccharide was present in the sample.

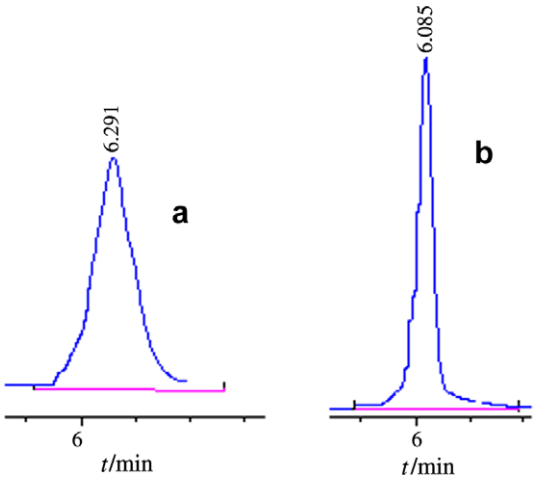

Fig. 3. HPLC of the polysaccharide purified from ginseng: (a) GPII; (b) GPIII.

The polysaccharide content of GPII and GPIII were 99.8% and 96.5%, protein content were 0% and 3.4%, and average molecular weight were about 3×10^5 and 4×10^5, respectively. Both GPII and GPIII were mainly consisted of glucose. Both infrared spectrums of GPII and GPIII displayed a broad stretching intense characteristic peak at around 3407 cm^{-1} for the hydroxyl group, and a weak C–H stretching band at 2931 cm^{-1}. Two stretching peaks at 1077 and 1154 cm^{-1} suggest the presence of C–O bonds.

3.2. Structural characterization of GPII and GPIII

Both GPII and GPIII showed abundance HIO_4 uptake, while it was oxidized (Table 1). The consumption of HIO_4 (0.368 mmol) in GPII was about two times than the amount of formic acid (0.183 mmol) that was produced after 23 h of periodate treatment, indicating the existing of large amounts of monosaccharide which are 1 → linked or 1 → 6 linked or more branch. In addition, it should be concluded that sugar residues oxidized or linkages of (1→)-glycosidic linkages account for 60.06%. The periodate-oxidized products were hydrolyzed and examined by gas chromatography. The presence of glucose indicating a part of glucose are (1 → 3)-glycosidic linkages, namely linkages that cannot be oxidized. Results of Smith-degradation analysis of fractions were summarized in Table 2. There were no precipitation in the sack and no substances in the supernatant of sack, indicating that the backbond of GPII should be all oxidized by HIO_4. So the linkages of backbond could be alternate arrayed by (1 → 3)-glycosidic linkages and (1 → 6)-glycosidic linkages, or the linkages of backbond could be the (1 → 6)-glycosidic linkages and the linkages of branch (1 → 3)-glycosidic linkages, according to the presence of glucose out of sack.

The consumption of HIO_4 (0.18 mmol) in GPIII was more two times than the amount of formic acid (0.05 mmol) that was produced after 23 h of periodate treatment, indicating the existing of little amounts of monosaccharide which are 1→ linked or 1 → 6 linked or little branch. It could be concluded that sugar residues oxidized account for 42.21% and linkages of (1→)-glycosidic linkages 16.23%. The presence of glucose indicating a part of glucose are (1 → 3)-glycosidic linkages in gas chromatography. There were glucose in the sack and no substances in the supernatant of sack (Table 2), indicating that the backbond of GPIII should be not oxidized by HIO_4. So the linkages of backbond could be arrayed by (1 → 3)-glycosidic linkages. The linkages of branch could be (1 → 2)-glycosidic linkages, according to the presence of glycerol out of sack. According to results above, (1 → 2)-glycosidic linkages should account for 25.98%.

Table 1
The results of periodate oxidation

Results	Samples	
	GPII	GPIII
Reaction time (h)	23	23
Amount of sample (mmol)	0.308	0.308
Consumption of HIO_4 (mmol)	0.368	0.18
Consumption of HIO_4/hexose (mol/mol)	1.195	0.584
Amount of formic acid (mmol)	0.183	0.05
Amount of formic acid/hexose (mol/mol)	0.594	0.162

Table 2
GC results from fractions of Smith-degradation of GPII and GIII

Fractions	GPII			GPIII		
	Glu[a]	Gly[b]	Ery[c]	Glu	Gly	Ery
Full acid hydrolysis	+[d]	−[e]	−	+	+	−
Smith-degradation						
Out of sack	+	−	−	−	+	−
Precipitation in the sack	−	−	−	−	−	−
Supernatant in the sack	−	−	−	+	−	−

[a] Glucose.
[b] Glycerol.
[c] Erythritol.
[d] Detectable.
[e] Undetectable.

3.3. Scavenging activity of hydroxyl radical

Hydroxyl radicals, generated by reaction of iron–EDTA complex with H_2O_2 in the presence of ascorbic acid, attack deoxyribose to form products that, upon heating with 2-thiobarbituric acid under acid conditions, yield a pink tint. Added hydroxyl radical scavengers compete with deoxyribose for the resulted hydroxyl radicals and diminish tint formation (Cheng, Ren, Li, Chang, & Chen, 2002). The above mentioned model was used to measure inhibitory activities of all fractions on hydroxyl radicals.

Both GPIII and GPII had a higher scavenging effect than Vc (Fig. 4). Their scavenging effects increased with increasing concentration. Scavenging effects of GPIII and GPII were 3.17–98.99% and 0–59.9% at amount of 0.01–5 mg, respectively, and that of Vitamin C was about 0–58.84%. This result proved that polysaccharides from ginseng had significant effect on scavenging hydroxyl radical, and GPIII and GPII were more pronounced than that Vitamin C.

3.4. Scavenging activity of superoxide radical

Superoxide radicals were generated in a PMS/NADH system for being assayed in the reduction of NBT. Fig. 5 shows that the inhibitory effect of GPII and GPIII extracted and purified from ginseng indicated a concentration-dependent, radical-scavenging activity at all tested concentrations of GPII.

As shown in Fig. 5 GPII was found to have more scavenging activities of superoxide radical than GP and Vc. The purification fraction GPIII showed no scavenging activities. At the amount of between 0.156 and 5 mg, the effects on scavenging superoxide of GPII were 1.4–84.05%. How-

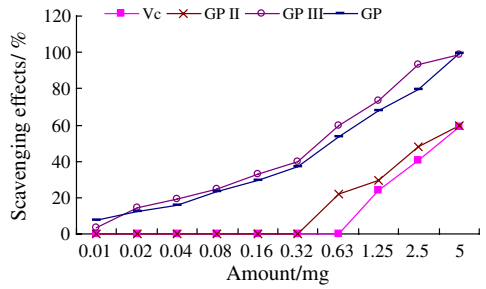

Fig. 4. Scavenging activities of hydroxyl radical by GP, GPII, GPIII and Vc.

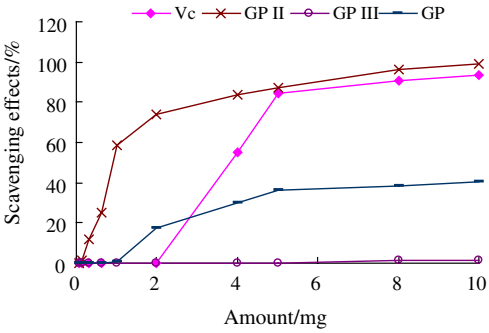

Fig. 5. Scavenging effects of GP, GPII , GPIII and Vc on super oxide radicals.

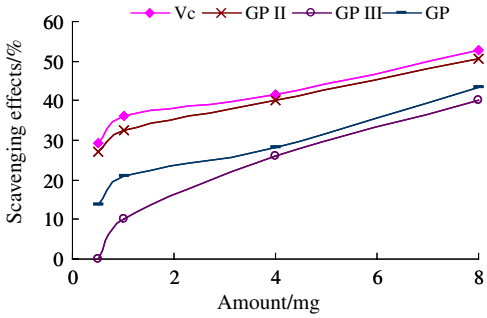

Fig. 6. Inhibitory effects of GP, GPII , GPIII and Vc on self-oxidation of 1,2,3-phentriol.

ever, the scavenging activity of Vitamin C for superoxide radical was 0–55.25%. Compared to these results, GPII had stronger scavenging activity for superoxide radical than Vitamin C. Our data on the activity of scavenging superoxide radical of GPII suggested that it was likely to contribute towards the observed antioxidant effect.

3.5. Scavenging activity of self-oxidation of 1,2,3-phentriol

Fig. 6 depicted the scavenging power of self-oxidation of 1,2,3-phentriol of GPIII and GPII extracted and purified from ginseng. Their scavenging powers correlated well with increasing concentrations. Moreover, the scavenging power of GPII was more stronger than GP and GPIII, and correspond to that of Vc. These results indicate that GPII has strong scavenging power for self-oxidation of 1,2,3-phentriol and should be explored as novel potential antioxidants.

4. Conclusions

According to the results above, it was concluded that the water extracting crude polysaccharide of ginseng contained predominantly two fractions (GPII and GPIII) purified by DEAE-Sepharose CL-6B column chromatography. In addition, Both GPII and GPIII exhibited strong antioxidant activities. Among GPII and GPIII, they had a little higher scavenging activity of hydroxyl radical than Vc, and GPII had a higher activity at scavenging superoxide radical than Vc and equivalent inhibiting ability to Vc on self-oxidation of 1,2,3-phentriol. Analysis by Periodate oxidation -Smith degradation indicated that GPII was composed of 60.06% (1→)- or (1 → 6)-glycosidic linkages and 39.94% (1 → 3)-glycosidic linkages, and GPIII 16.23% (1→)- or (1 → 6)-glycosidic linkages, 25.98% (1 → 2)-glycosidic linkages, and 57.79% (1 → 3)-glycosidic linkages.

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