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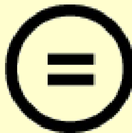

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약학석사학위논문

The antioxidant activity of neutral and acidic polysaccharides from *Panax ginseng* on *Caenorhabditis elegans*

고려인삼에서 추출한 중성 및 산성다당체의 항산화 효과

2018 년 8 월

서울대학교 대학원

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Abstract

The antioxidant activity of neutral and acidic polysaccharides from *Panax ginseng* on *Caenorhabditis elegans*

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Ginseng polysaccharide is a kind of polymer in ginseng. According to the composition, it can be divided into neutral polysaccharide and acidic polysaccharide. It has been reported that, ginseng polysaccharide has lot of pharmacological effects, such as immunomodulation, anti-tumor, anti-oxidant activity, and so on. Because of the large molecular weight and complex structure, many effects have not been fully studied. In this study, we focused on the antioxidant activity comparison between neutral and acidic polysaccharide from *Panax ginseng*.

A neutral polysaccharide (WGPN) and an acidic polysaccharide (WGPA) were extracted from *Panax ginseng* by DEAE cellulose ion-exchange chromatography. Their structures were characterized by chemical composition

analysis, high performance gel permeation chromatography (HPGPC), Fourier transform infrared spectrometry (FT-IR), and liquid chromatography with diode-array detector (LC-DAD). Further, their antioxidant activities were investigated both in vitro and in vivo. Results showed that WGPA had higher uronic acid content and larger average molecular weight than WGPN. Compared with WGPN, WGPA exhibited higher scavenging activities against ABTS and hydrogen peroxide free radical, higher reducing power and higher metal chelating activity in vitro, as well as preferably inhibitory effect on lipid peroxidation in the mode of *Caenorhabditis elegans*.

Keywords: Neutral polysaccharide; Acidic polysaccharide; antioxidant activities; *Caenorhabditis elegans*; *Panax ginseng*;

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1. Introduction

Panax ginseng has been used in Korea, China, and Japan as a traditional medicine for over 2000 years. It is believed to be a panacea and to promote longevity[1]. As we all know that, *Panax ginseng* is widely used in our daily life, such as medicines, health products, food, drinks, daily necessities, and cosmetics. That is because ginseng contains many active components, including ginsenosides, and ginseng polysaccharides. In the past, many researchers focused on ginsenosides, while because of the complex structure of polysaccharides, it is difficult to separated, but recently, the polysaccharide is under the spotlight. It has been reported that ginseng polysaccharide have immunomodulation, anti-fatigue, anti-hyperlipidemic effects.

Reactive oxygen species (ROS), the generic term for chemically reactive molecules containing oxygen, are continuously produced as byproducts of normal mitochondrial electron transport and other metabolism processes[2]. It is believed that low levels of ROS play a critical role in maintaining homeostasis, delivering signal and regulating development. However, high concentrations of ROS are causing damage to proteins, lipids and nucleic acids[3-5]. Furthermore, a large amount of in vitro and in vivo evidences suggest that high levels of ROS result in metabolic disorders such as inflammation, neurodegeneration, cancer and aging[6-8].

Normally, enzymatic and non-enzymatic antioxidant defense systems maintain ROS under damage threshold in organism. Antioxidant emzyme system contains superoxide dismutases, catalases, and glutathione peroxidases. Non-enzymatic antioxidant system consists of low-molecular weight

compounds, such as ascorbic acid, ubiquinone, and uric acid[9, 10]. Moreover, natural products, vitamins, polyphenols and polysaccharides can potently scavenge ROS[11-13].

To our knowledge, a comparative study of the properties and antioxidant activities between neutral and acidic polysaccharides purified from *Panax ginseng* has not been studied so far. Therefore, in this study, we focused on the anti-oxidant activity of *Panax ginseng* polysaccharide, and compare the different between neutral and acidic polysaccharide[14]. he neutral and acidic polysaccharides from *Panax ginseng* were obtained by ion-exchange and gel filtration chromatography, Their physicochemical properties were characterized by chemical composition, Fourier transform infrared spectroscopy, high performance gel permeation chromatography, and high performance liquid chromatography. Moreover, in vitro antioxidant abilities and in vivo antioxidant abilities based on *Caenorhabditis elegans* model were also investigated.

2. Experiment

2. 1. Sample collection and pretreatment

The roots of *Panax ginseng* were purchased from local markets in Korea. After cut the ginseng roots into slices, put them in oven to dry. Then the roots were ground to fine powder and stored in conical tubes at room temperature.

2. 2. Chemicals and materials

2. 2. 1. Chemicals

Chemicals used in extraction and purification of polysaccharides

- Water (J.T. Baker, Phillipsburg, NJ, USA)
- n-Butanol (Sigma-Aldrich, USA)
- Ethyl Alcohol (Samchun, Korea)
- Ether (J.T. Baker, Phillipsburg, NJ, USA)
- Chloroform (J.T. Baker, Phillipsburg, NJ, USA)
- DEAE-cellulose (Toronto research chemicals, Canada)

Chemicals used in Chemical properties analysis

- Water (J.T. Baker, USA)
- Glucose (Sigma-Aldrich, USA)
- Sulfuric acid (Sigma-Aldrich, USA)
- Phenol (Sigma-Aldrich, USA)
- Galacturonic acid (Sigma-Aldrich, USA)

- m-hydroxydiphenyl (Sigma-Aldrich, USA)
- Sodium tetraborate (Sigma-Aldrich, USA)

Chemicals used in vitro antioxidant activity assay

- Water (J.T. Baker, USA)
- Ascorbic acid (Sigma-Aldrich, USA)
- ABTS (Sigma-Aldrich, USA)
- Potassium persulfate (Sigma-Aldrich, USA)
- Disodium phosphate (Sigma-Aldrich, USA)
- Sodium dihydrogen phosphate (Sigma-Aldrich, USA)
- Potassium ferricyanide (Sigma-Aldrich, USA)
- Trichloroacetic (Acros Organics, USA)
- Ferric chloride (Sigma-Aldrich, USA)
- Ferrozine (Sigma-Aldrich, USA)
- Ferrous chloride (Sigma-Aldrich, USA)
- EDTA (Amresco)
- Ammonium ferric sulfate (Sigma-Aldrich, USA)
- Hydrogen peroxide (Sigma-Aldrich, USA)
- 1,10-Phenanthroline (Sigma-Aldrich, USA)

Chemicals used in vivo antioxidant activity assay

- Sodium Chloride (Sigma-Aldrich, USA))
- Agar (Becton, Kickinson and company, France)
- Bactopeptone (Sigma-Aldrich, USA)
- Magnesium sulfate (Sigma-Aldrich, USA)

- Calcium chloride (Duksan pure chemical, Korea)
- Potassium dihydrogen phosphate (Samchun, Korea)
- Dipotassium phosphate (Samchun, Korea)
- Cholesterol (Sigma-Aldrich, USA)
- Ethyl Alcohol (Samchun, Korea)
- Malonaldialdehyde (Sigma-Aldrich, USA)
- Thiobarbituric acid (Sigma-Aldrich, USA)
- Trichloroacetic acid (Sigma-Aldrich, USA)

2. 2. 2. Experimental supplies

- Straw cutter
- Drying oven (FO-600M, JEIO TECH)
- Blender (Wonder Blender, WB-1, Japan)
- Measuring cylinder (100 mL, 500 mL)
- Erlenmeyer flask (1000 mL)
- Round bottomed flask (500 mL)
- Water bath (Buchi, B-480)
- Rotavapor (Buchi, R-124)
- Vacuum controller (Buchi, B-800)
- Nitrogen Purge (EYELA MG-2200)
- Centrifuge (Eppendorf AG, Hamburg, Germany)
- Conical Tube (50 mL, SPL Life Sciences Co. Ltd)
- Electronic scale (XPE205, Mettler Toledo)
- Adjust Pipette (0.5 ~ 10 μL, 20 ~ 200 μL, 100 ~ 1000 μL, Eppendorf AG, Hamburg, Germany)

- Pipette Tips (0.5 ~ 10 μL, 20 ~ 200 μL, 100 ~ 1000 μL, Eppendorf AG, Hamburg, Germany)
- Vortex Mixer (Vortex Genie 2)
- Glass open column (2×35 cm)
- Ultrasonicator (Sonics & Material, Inc., USA)
- Safe-lock Tube (1.5 mL, 2 mL, Eppendorf AG, Hamburg, Germany)
- 96 well plate (Nunclon Delta Surface, Thermo Scientific)
- Oven (ON-02GW, Jeio tech)
- Chemical-free Freeze Dryer (-120°C, Operon)
- Ultrasonic Processor (VCX-130)

2. 2. 3. Analytical instruments

- Multi reader (Molecular devices, SpectraMax M5)
- High performance gel permeation chromatography (Dionex HPLC Ultimate 3000 RI System, Thermo scientific, USA)

  Ultrahydrogel column (Ultrahydrogel 120, 500, 1000, Waters, USA)
- Fourier transform infrared spectrometry (4200, Jasco, USA)
- Liquid chromatography with diode-array detector (Agilent 1260 infinity, Agilent, USA)

  ODS column (Ecilipse Plus C18, 3.5 μm, 4.6 ×150 mm)

2. 3. Extraction and purification of white ginseng polysaccharide

The method of sample extraction and preparation was in accordance with previously developed study[1], as shown in Figure 1. To be specific, 40 g ginseng powder were extracted with 560 mL distilled water at 100 ℃ for 4 h then centrifuged to separate the solid fraction and the liquid fraction. The solid material was extracted twice again under the same conditions. The liquid extraction were combined, concentrated to a sticky state. Then precipitated by the addition of 4 volumes of ethanol. After centrifugation, the precipitate was dried by solvent exchange method, using ethanol twice and ether once, finally, dry the extraction with nitrogen blow. In this way, 27 g of crude ginseng polysaccharide was obtained. The crude ginseng polysaccharide was re-dissolved in distilled water and treated with 4 volumes of Sevag reagent (1:4 n-butanol : chloroform, v/v) to remove proteins[15]. After precipitating by ethanol and drying by solvent exchange method again, the deproteined white ginseng polysaccharide fraction (WGP) was obtained.

Dissolved WGP 250 mg in 5 mL distilled water, and loaded on a DEAE-Cellulose column (2.0×30 cm), which pre-equilibrated with distilled water. The column was eluted first with distilled water to obtain the neutral fraction (WGPN), and then eluted with 0.5 M NaCl to obtain acidic fraction (WGPA). The eluate was collected at 5 mL per tube and assayed for the distribution of total sugars and uronic acids. The appropriate neutral fractions were combined, concentrated and freeze dry to obtain the neutral polysaccharide (WGPN). While the appropriate acidic fraction were combined, concentrated, dialysis to

discard the NaCl, which contained in acidic fraction and freeze dry to obtain the acidic polysaccharide (WGPA).

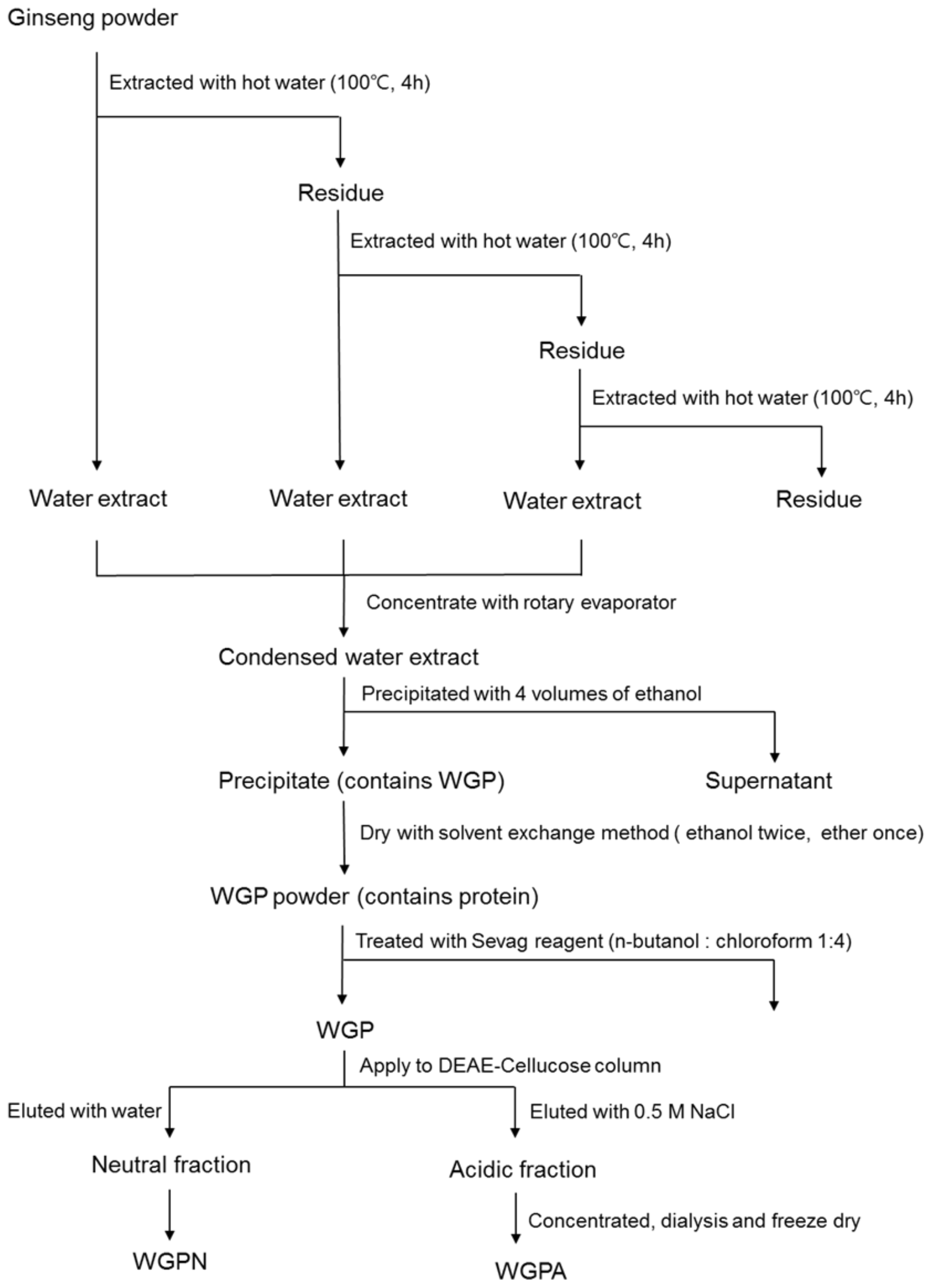

Figure 1. Preparation of WGPN and WGPA.

2. 4. Preliminary characterization of WGPN and WGPA

2. 4. 1. Chemical properties

Total carbohydrate content of polysaccharides was determined by phenol-sulfuric acid colorimetric method, with glucose as standard[16]. Carbohydrates react in the presence of strong acid and heat to generate furan derivatives that condense with phenol to form stable orange compounds that can be measured by Spectrophotometry. To be specific, add 60 μL 5% phenol and 300 μL sulfuric acid into 100 μL standard sample (20~100 ppm) and polysaccharide sample (0.2 mg/mL). After incubate at 90 ℃ for 20 min, measure the absorbance at 490 nm. After calculation, total carbohydrate content was obtained.

The uronic acid content was assessed by m-hydroxydiphenyl method, with d-galacturonic acid as the standard[17]. Sample containing uronic acid is heated in sulfuric acid and sodium tetraborate ($Na_2B_4O_7$), after cooling down the mixture is allowed to interact with the m-hydroxydiphenyl reagent to produce a pinkish product with maximum absorbance at 520 nm. To be specific, add 1.2 mL sodium tetraborate which dissolved in sulfuric acid to 200 μL standard sample (20~100 ppm) and polysaccharide sample (0.2 mg/mL), After incubate at 100℃ for 10 min and cool down with ice-water bath, add 20 μL m-hydroxydiphenyl, then measure the absorbance at 520 nm. After calculation, uronic acid content was obtained.

The protein content was quantified using the BCA method, with bovine serum albumin as the standard. The BCA assay primarily relies on two reactions. First, the peptide bond in protein reduce copper ion (Cu^{2+}) to cuprous ion (Cu^{+}) under a certain temperature. Next, BCA acid chelate with Cu^{+} ion, forming a purple complex with maximum absorbance at 562 nm. To be specific, mix 1.0 mL standard sample (0~200 ppm) and polysaccharide sample (100 ppm) with working reagent separately, after incubate at 60 ℃ for 1 h, Measure the absorbance at 562 nm. The protein content was obtained by calculation.

The average molecular weight of polysaccharides was estimated by high performance gel permeation chromatography system. The column is Waters Ultrahydrogenl 120, 500, 1000 column which were serial connected. The mobile phase was 0.1M sodium azide in water. Flow rate was 1 mL/min. Injection volume was 50 μL. Temperature was 40℃. And we use pullulan as standard.

Instrument	High performance gel permeation chromatography (Dionex HPLC Ultimate 3000 RI System, Thermo scientific, USA)
Column	Waters Ultrahydrogenl 120, 500, 1000 column (Serial connection)
Mobile phase	Sodium azide 0.1M in water
Flow rate	1 mL/min
Injection volume	50 μL
Temperature	40℃
Standard	Pullulan
Detecter	RI
Software	Chromeleon 6.8 Extention-pak

Table 1. HPGPC analysis conditions.

2. 4. 2. Fourier-transform infrared spectroscopy (FT-IR)

The FT-IR spectrum of the polysaccharides was carried out using a FT-IR spectrophotometer. To be specific, add samples drop by drop to a demountable liquid cell, after it was totally dried, a film of sample was appeared, then measure absorbance at 4000~500 cm^{-1}. Resolution is 4 cm^{-1}. Accumulation is 16 times.

2. 4. 3. Monosaccharide composition

Monosaccharide composition of polysaccharides was performed by Liquid chromatography with diode-array detector. To be specific, added sample 10 mg and 1M methanolic HCl 10 mL into tight cap vial, incubated at 80℃ for 16 h, nitrogen blow to evaporate all the methanolic HCl, then added 2 M trifluoroacetic acid 10 mL, incubated at 120℃ for 1 h. Transferred 1 mL to another vial, dried with nitrogen blow, then added 0.3M NaOH 0.5 mL, dissolved it at an ice-water bath condition, with sonication. After that, added 0.5 M PMP (1-phenyl-3-methyl-5-pyrazolone) 0.5 mL, incubated at 70℃ for 30 min. Then added 0.3 M HCl to neutralize the sample to suitable pH. The PMP which was left in sample was discarded by liquid-liquid extraction with chloroform. The samples were applied to high performance liquid chromatograph system with diode-array detector. The column is reversed phase column (Ecllipse Plus C_{18}, 3.5 μm, 4.6×150 mm, Agilent, USA). The temperature is 30℃, Flow rate of mobile phase is 1.0 mL/min. Mobile phase A

consists of 20% acetonitrile in 20 mM phosphate buffer, and mobile B consists of 30% acetonitrile in 20 mM phosphate buffer. 0 ~30 min (0~100% B), 30~40 min (100% A). Injection volume is 10 μL, wavelength of detector is 245 nm. Use glucose, galactose, rhamnose, mannose, arabinose, galacturonic acid, glucuronic acid as standards.

Insturment	Liquid chromatography with diode-array detector (Agilent 1260 infinity, Agilent, USA)
Column	Eclipse Plus C18 (4.6 x 150 mm)
Mobile phase	A: 20% Acetonitrile in water with 20 mM phosphate buffer (pH = 7.0)
	B: 30% Acetonitrile in water with 20 mM phosphate buffer (pH = 7.0)
Gradient	0 – 30 min: Eluent A to B, linear gradient
	30 – 40 min: Eluent A 100%
Flow rate	1.0 mL/min
UV absorbance	245 nm

Table 2. LC-DAD analysis conditions.

2. 5. In vitro antioxidant activity assay

2. 5. 1. ABTS radical scavenging activity

The ABTS radical scavenging activities of polysaccharide fractions were determined according to a previous method[18], with slight modifications. Briefly, 192.05 mg ABTS and 33.1 mg $K_2(SO_4)_2$ were dissolved in 50 mL water, and incubated at room temperature for 12~16 h in dark as stock solution. The stock solution including 7.0 mM ABTS and 2.45 mM $K_2(SO_4)_2$ was diluted with 0.01 M pH 7.4 PBS to the right concentration (0.7 absorbance at 734). In the reaction system, 0.5 mL H2O and 1 mL ABTS solution were added into 0.5 mL of polysaccharide sample (0~1.4 mg/mL), and then the absorbance was measured at 734 nm after incubate at room temperature away from light for 6 min. Use ascorbic acid as standard. The ABTS scavenging activity was calculated using the following formula:

$$\text{ABTS scavenging activity (\%)} = (1-A_S/A_C) \times 100\%$$

Where A_S is the absorbance of sample or standard and A_C was blank

2. 5. 2. Reducing power method

The reducing power of polysaccharide fractions were determined according to a previous method[19], with slightly modifications. Briefly, 2.5 mL of 0.2 M pH 6.6 PBS and 2.5 mL 1% (w/v) of $K_3Fe(CN)_6$ were added into 1.0 mL of sample (0~1.4 mg/mL). The mixture was incubated at 50°C for 20 min, followed by the addition of 2.5 mL 10% (w/v) trichloroacetic acid. The mixture was centrifuged at 3000 rpm for 10 min to collect 2.5 mL of upper layer of the

solution, mixed with 2.5 mL distilled water and added 0.5 mL 0.1% (w/v) of $FeCl_3$. The absorbance was measured at 700 nm. The Reducing power was calculated using the following formula:

Reducing power (%) = $(1-A_S/A_C) \times 100\%$

Where A_S is the absorbance of sample or standard and A_C was blank.

2. 5. 3. Metal chelating activity method

The metal chelating activity of polysaccharide fractions were determined according to a previous method[19], with slightly modifications. Briefly, 0.1 mL of polysaccharide sample (0~1.4 mg/mL) is added to a solution of 0.5 mL, 0.2 mM ferrous chloride. The reaction is started by the addition of 0.2 mL, 5 mM of ferrozine and incubated at room temperature for 10 min and then the absorbance is measured at 562 nm. EDTA was used as a standard. The Metal chelating activity was calculated using the following formula:

Metal chelating activity (%) = $(1-A_S/A_C) \times 100\%$

Where A_S is the absorbance of sample or standard and A_C was blank.

2. 5. 4. Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging activity of polysaccharide fractions were determined according to a previous method[19], with slightly modifications. Briefly, added 1 mM 0.25 mL (NH4)2Fe(SO4)2 into 1.5 mL polysaccharide sample (0~1.4 mg/mL), and added 30 μL 5 mM hydrogen peroxide, then incubated room temperature for 5 min in dark, added 1.5 mL 1 mM 1,10-phenanthroline, then measure the absorbance at 510 nm after incubated at room

temperature for 10 min. The hydrogen peroxide scavenging activity was calculated using the following formula:

Hydrogen peroxide scavenging activity (%) = $(1-A_S/A_C) \times 100\%$

Where A_S is the absorbance of sample or standard and A_C was blank.

2. 6. In vivo biological activity assay

2. 6. 1. *C. elegans* strain and culture conditions

The *C. elegans* were grown and maintained under standard laboratory conditions at 20℃ on nematode growth medium (NGM) agar plates fed with Escherichia coli OP50. The synchronized populations were obtained by sodium hypochlorite treatment of gravid hermaphrodites.

2. 6. 2. Lethality assay

The toxicity of polysaccharides in *C. elegans* was performed according to a previously described method[20], with some modification. Briefly, synchronized *C. elegans* were grown to L4 larva stage, Then, young adult *C. elegans* were transferred to fresh NGM plates containing 0.5, 1.0, 1.5, 2.0 mg/mL polysaccharides continue to train 24 h. Then we can count the number of living worms, dead worms and living ratio. *C. elegans* were judged to be dead if they failed to respond to stimulus using a platinum wire.

2. 6. 3. Experimental design

In order to evaluate the in vivo antioxidant effect of WGPN and WGPA, juglone, an intracellular ROS generator was used to induce oxidative stress in *C. elegans*. Briefly, synchronized *C. elegans* were grown to L4 larva stage, Then, young adult *C. elegans* were transferred to fresh NGM plates containing juglone, juglone with 1.0 mg/mL WGPN, and juglone with 1.0 mg/mL WGPA, continue to train 24 h. After that, the worms was applied to ROS level assay and Lipid peroxidation assay.

2. 6. 4. ROS level assay

ROS level was measured by 2, 7-dichlorodihydrofluorescein diacetate (H_2DCFDA) dye using fluorescence. The H_2DCFDA can transfer into cells through cell membrane, after cellular esterase activity, it generated to H_2DCF without fluorescent, H_2DCF further reacted with ROS, finally generated to DCF with fluorescent. Then measure the fluorescent with SpectroMax M5 multi-reader, the excitation wavelength was 485 nm and the emission wavelength was 525 nm. After that, protein contents were calculated for mormalization.

2. 6. 5. Lipid peroxidation assay

The lipid peroxidation level in the *C. elegans* was estimated by using the thiobarbituric acid (TAB) colorimetric method[21]. To be specific, perform synchronization and breed until L4 stage in the NGM plate, using M9 buffer transfer worms to NGM or juglone media and incubate for 24 h. To measuring

the malondialdehyde (MDA) content. The absorbance of thiobarbituric acid reactive substance (TBARS) was measured at 532 nm.

3. Result and discussion

3. 1. Isolation and purification of polysaccharides

The crude polysaccharides were extracted from the root of *Panax ginseng* by water, and separated by DEAE-cellulose anion-exchange chromatography column. Two fraction named WGPN and WGPA were collected, concentrated, dialyzed, dried respectively.

3. 2. Preliminary characterization

3. 2. 1. Chemical compositions and molecular weight of WGPN and WGPA

The carbohydrate content, protein content, uronic acid content and molecular weight of WGPN and WGPA were summarized in Table 3. There were significant differences between WGPN and WGPA. Especially, Uronic acid content of WGPN and WGPA were 0.7% and 25.7% respectively, indicating that WGPN was a neutral polysaccharide while WGPA was an acidic polysaccharide. And WGPN had a quite high carbohydrate content while WGPA's carbohydrate content was much lower. In addition, based on HPGPC results, the molecular weights of WGPA (79.99 kDa) was higher than that of WGPN (16.11 kDa). However, there was still a similar point between WGPN and WGPA, both of them had an extremely low protein content.

Sample	WGPN	WGPA
Carbohydrate (%)	77.4	28.2
Uronic acid (%)	0.78	25.7
Protein (%)	1.82	5.07
Molecular weight (KDa)	16.11	79.99

a: Phenol-sulfuric acid method with glucose as standard

b: m-HDP method with galacturonic acid as standard

c: BCA method with BSA as standard

d: HPGPC system

Table 3. Chemical characteristics and molecular weight of WGPN and WGPA.

Fractions	Monosaccharide composition (%)						
	Galactose	Glucose	Arabinose	Rhamnose	Mannose	GalA	GluA
WGPN	1.1	97.9	1	-	-	-	-
WGPA	24.4	24.0	18.1	-	-	32.2	1.3

Table 4. Monosaccharide composition of WGPN and WGPA

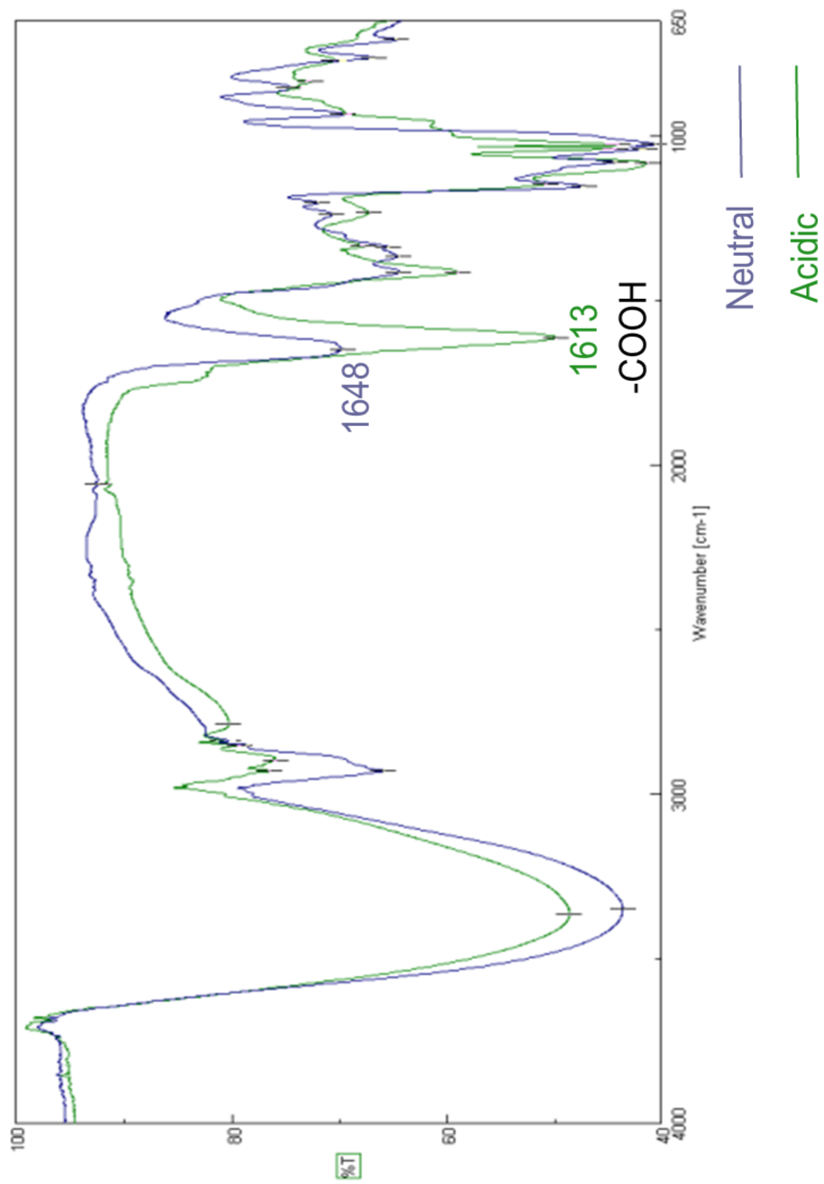

Figure 2. Flowchart of sample preparation for HPLC-MS analysis

3. 2. 2. FT-IR spectrum

The FT-IR results indicated that two samples had the polysaccharides typical peaks within the range of 3600~3000, 3000~2800, 1800~1000 cm^{-1}. As shown in Figure 2, the strong and broad intense peak around 3600~3000 cm^{-1} was attributed to O-H stretching vibration while the weak peak around 3000~2800 cm^{-1} was assigned to C-H[22]. The band in the range of 1800~1000 cm^{-1} were attributed to –COOH functional group, which indicated that WGPA was acidic polysaccharide. These results were consistent with the fact that WGPA had relative high uronic acid content.

3. 2. 3. Monosaccharide composition of WGPN and WGPA

The monosaccharide composition results of WGPN and WGPA were summarized in Table 4. WGPN was compose of galactose, glucose and arabinose, with the molar percentages of 1.1%, 97.9% and 1%, respectively. WGPA was composed of galactose, glucose, arabinose, galacturonic acid and gluconic acid, with the molar percentages of 24.4%, 24.0%, 18.1%, 32.2%, 1.3%, respectively. Glucose was the major monosaccharide in WGPN, which indicated that WGPN is a neutral polysaccharide. While, galacturonic acid was the major monosaccharide of WGPA, which indicated that WGPA is an acidic polysaccharide. This result was consistent with the results of uronic acid quantification.

3. 3. In vitro antioxidant activities of polysaccharide fractions

3. 3. 1. ABTS radical scavenging activity

ABTS is a stable radical. ABTS can be oxidize to a green ABTS+ under the action of appropriate oxidants. The production of ABTS+ is inhibited in the presence of antioxidants, and the absorbance of ABTS+ is measured at 734 nm to determine and calculate the total antioxidant capacity of the sample. As shown in Figure 3, WGPN and WGPA displayed concentration-dependent ABTS radical scavenging effects. At 1.4 mg/mL, the scavenging activities for WGPN and WGPA were 13.03% and 40.94%, respectively. Previous reports showed that the antioxidant activity of polysaccharide correlated with their monosaccharide composition, uronic acid content, molecular weight. WGPA exhibited higher antioxidant activity than WGPN, which might be attribute to its higher uronic acid content and larger molecular weight of WGPA.

3. 3. 2. Reducing power method

Antioxidants reduce the ferric ion (Fe^{3+}) of potassium ferricyanide ($K_3Fe(CN)_6$) to ferrous iron in potassium ferrocyanide, and it further react with ferric chloride ($FeCl_3$) which forms Prussian blue with maximum absorbance at 700 nm. Determination of the absorbance level of 700 nm can reflect the antioxidant capacity, the greater absorbance, the stronger antioxidant capacity. As shown in Figure 4, the reducing power of WGPN and WGPA increased with

sample concentration. At 1.4 mg/mL, the reducing power were 21.78% and 41.68%, respectively. These results indicated WGPA has higher reducing power than WGPN.

3. 3. 3. Metal chelating activity

Ferrozine is able to form a purple complex with ferrous ions. Antioxidants are thought to have the ability of chelating metal ions, so it has competition with ferrozine. And the absorbance of the purple complex is measured at 562 nm. As shown in Figure 5, the result demonstrated that the polysaccharides exhibited metal chelating activity in a concentration-dependent manner. At 1.4 mg/mL, the metal chelating rates for WGPN and WGPA were 26.23% and 58.34%. The strong metal chelating activity of WGPA might be partly due to its high content of carboxylic groups, which was in conformilty with the result of uronic acid content.

3. 3. 4. Hydrogen peroxide scavenging activity

Hydrogen peroxide will oxidize ferrous ion to ferric ion, but antioxidants can reduce ferric iron to ferrous iron. Ferrous ions can react with 1,10-Phenanthroline, and form an orange-red complex with maximum absorbance at 510 nm. As shown in Figure 6, WGPA displayed concentration-dependent hydrogen peroxide scavenging activity, while WGPN show much lower hydrogen peroxide scavenging effects. At 1.4 mg/mL, the hydrogen peroxide scavenging activity of WGPA and WGPN were 19.57% and 42.01%, respectively.

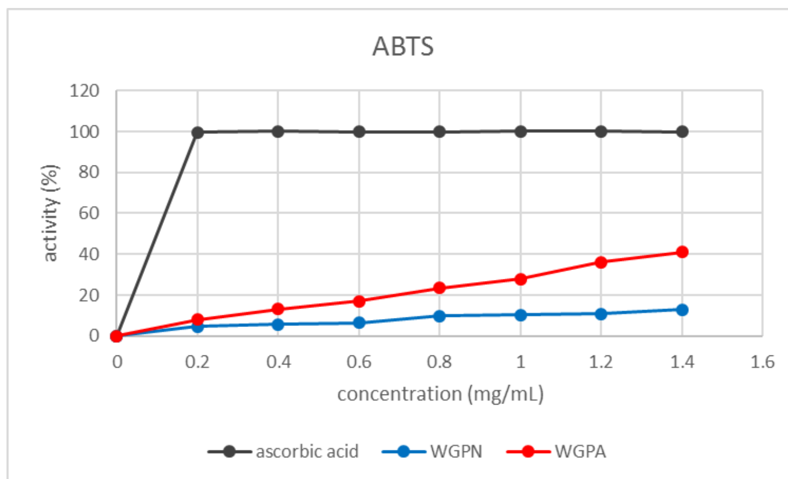

Figure 3. ABTS scavenging activity.

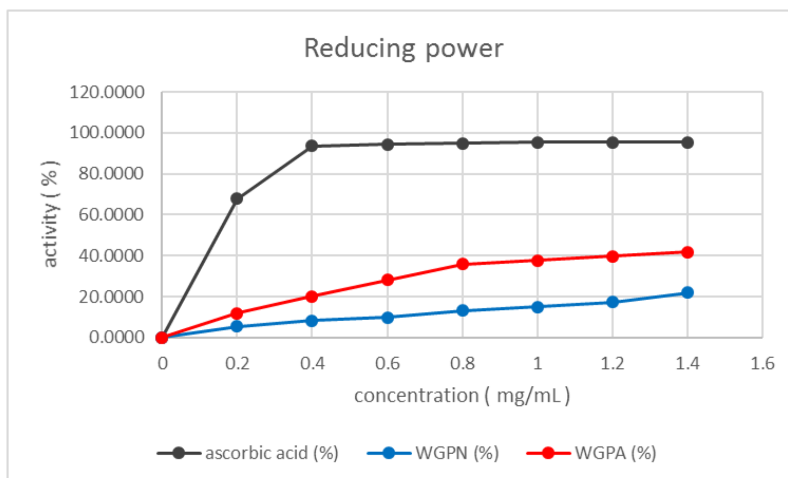

Figure 4. Reducing power.

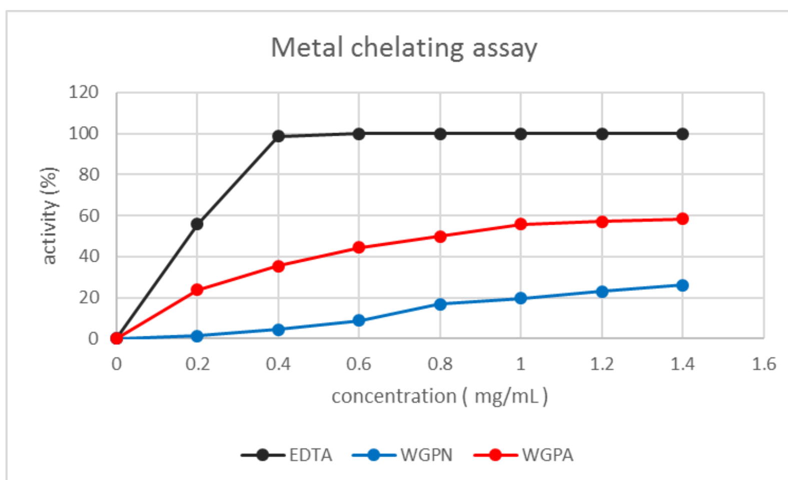

Figure 5. Metal chelating activity

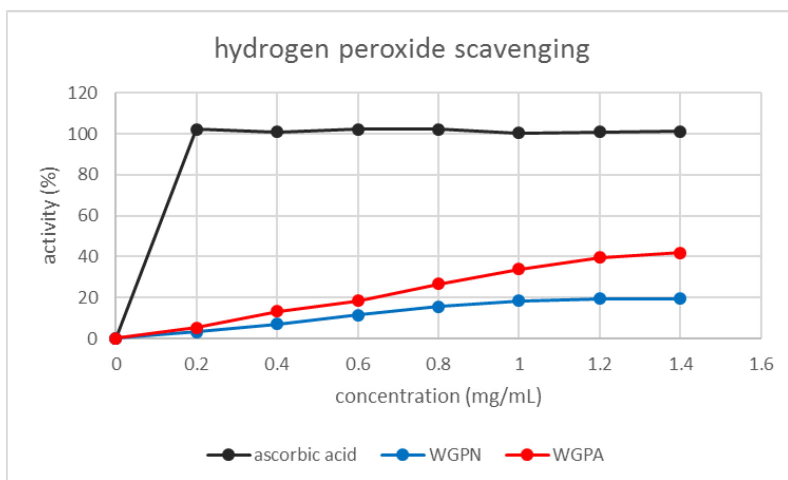

Figure 6. Hydrogen peroxide scavenging

3. 4. In vivo biological activity analysis

3. 4. 1. Lethality assay of polysaccharides on *C. elegans*.

Pretreatment with crude polysaccharide for 24 h, the safety property of polysaccharide for L4 *C. elegans* in vivo were observed. The results showed that treatment with 1.0 mg/mL of polysaccharides did not influence their lifespan. However, once polysaccharides concentrations were higher than 1.0 mg/mL, *C. elegans* lifespan been affected. Thus, 1.0 mg/mL was selected for the following *C. elegans* feeding tests.

Concentration (mg/mL)	Alive	Dead	Ratio (%)
0.5	20	0	100
1	20	0	100
1.5	19	1	95
2	16	4	80

Table 5. Lethality assay.

3. 4. 2. ROS level assay.

Reactive oxygen species (ROS), such as ABTS radical and Hydrogen peroxide, are highly reactive molecules produced by cellular aerobic metabolism. In vivo, the excessive ROS may cause DNA mutation, protein and membrane lipid degradation, and ultimately lead to cancer, aging and many chronic diseases. 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA) is a non-fluorescent substance, it can go through the cell membrane easily, then it can react with cellular esterase, generate to another non-fluorescent substance H_2DCF, and then it will be oxidized by ROS in cell, and generate to a fluorescent substance DCF. By measuring the intensity of fluorescence, the level of ROS in cell could be obtained. The excitation wavelength and Emission wavelength were 485 nm and 525 nm respectively.

Juglone can lead to oxidative stress.Therefore, juglone is used as positive group, and the effects of WGPN and WGPA on the antioxidant defense system of *C. elegans* are investigated. As shown in Figure 7, compared to control group, treatment with juglone significantly increased the ROS level in *C. elegans*. However, compared to juglone alone, the supplementation of polysaccharides (1 mg/mL) with juglone remarkably decreased the ROS level. The result suggested that both WGPN and WGPA has antioxidant activity.

3. 4. 3. Lipid peroxidation assay.

Lipid peroxidation can commonly reflect the oxidation activity intensity in vivo. Fatty, especially polyunsaturated fatty acids, is easily attacked by ROS, and thus yields an amount of by products that may be responsible for secondary

damage to cells[23]. Malondialdehyde (MDA), the final product of lipid peroxidation, can be considered as an indicator of fatty damage. ROS are responsible for the lipids peroxidation, therefore, we quantitated the level of lipid peroxidation in terms of MDA. As shown in Figure 8, compared with juglone group, the exogenous supplementation of polysaccharides (1 mg/mL) along with juglone could decrease the levels of MDA. The results shown that, the exogenous supplementation of polysaccharides could overcome oxidative injury of *C. elegans*. The decrease of the level of lipid peroxidation might be results further suggested that WGPA presented a stronger antioxidant capacity in vivo than WGPN, which revealed that the antioxidant activities of polysaccharides in vivo were mainly associated with their uronic acid content, molecular weight, monosaccharide composition. However, as was known to all, numerous factors also influence the antioxidant activities in vivo. Namely, the biological properties of polysaccharides were usually influenced by multi-factor, and the detail mechanism should be further investigated.

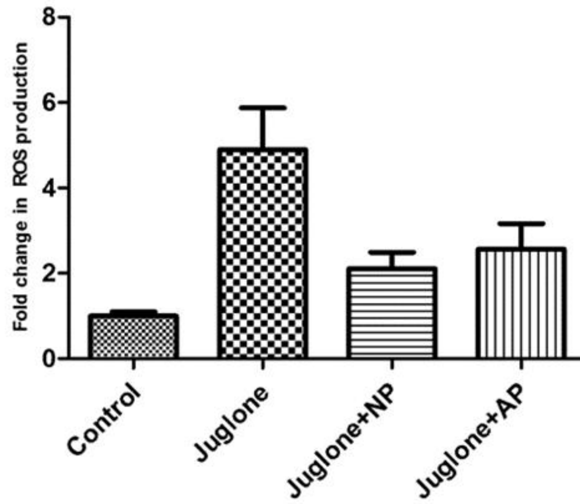

Figure 7. ROS level in *C. elegans*

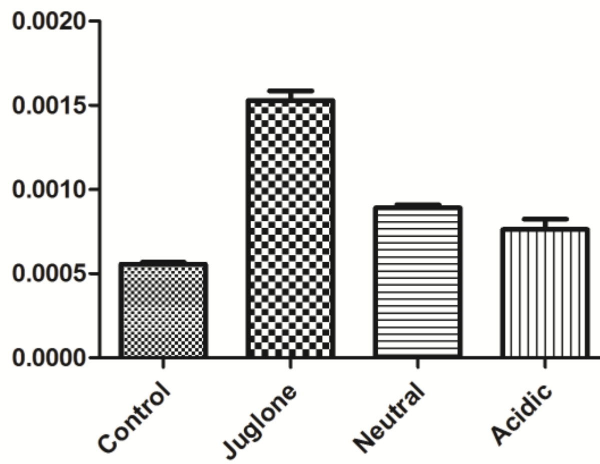

Figure 8. Lipid peroxidation assay

4. Conclusion

In this study, we purified a neutral polysaccharide (WGPN) and an acidic polysaccharide (WGPA) from *Panax ginseng*. The essential characteristic analytic results showed that WGPN and WGPA were quite different in their chemical composition, especially for uronic acid content, molecular weight, and monosaccharide composition. Compared with WGPN, WGPA exhibited stronger scavenging activities against ABTS and hydrogen peroxide free radical, higher reducing power and higher metal chelating activity in vitro, as well as preferably inhibitory effect on lipid peroxidation in the mode of *Caenorhabditis elegans* in vivo. The antioxidant activities of WGPA might be correlated with its high uronic acid content and large molecular weight. The findings suggested that WGPA might have promising potential for application prospect in pharmacology and functional food.

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국문초록

고려인삼에서 추출한 중성 및 산성다당체의 항산화 효과

인삼 다당체는 인삼 내 중합체의 한 종류이다. 다당체는 구성 성분에 따라 중성과 산성 다당체로 분류할 수 있다. 인삼 다당체는 면역 조절, 항암 작용 및 항산화 효과 등의 약리학적 효과를 가지고 있는 것으로 보고되었다. 복잡한 구조와 큰 분자량으로 인해 이러한 효과에 대해 많은 연구가 수행되지 않았다. 이에 따라 본 연구에서는 고려 인삼의 중성과 산성 다당체의 항산화효과를 비교하였다. 중성 다당체와 산성다당체는 DEAE 셀룰로오스 이온교환 크로마토그래피를 통해 인삼으로부터 분리하였다. 분리된 다당체의 조성은 화학성분분석과 젤투과크로마토그래피, 푸리에변환 적외분광분석, 다이오드어레이검출기가 연결된 액체크로마토그래피를 이용하여 확인하였다. 분리된 인삼다당체는 in vitro 와 in vivo 에서 항산화 효과를 확인하였다. 산성다당체는 중성다당체에 비해 높은 우론산 함량을 나타냈으며, 평균 분자량도 높게 나타났다. ABTS 와 과산화수소 프리라디칼, 환원력, 메탈킬레이팅 등의 항산화효과 시험에서도 산성다당체가 높은 효과를 나타냈다. 또한, 예쁜꼬마선충에서 항산화효과를 확인하기 위해 지질 과산화의 저해 작용을 확인하였고, 산성다당체가 더 높은 효과를 나타냈다.

주요어: 중성다당체, 산성다당체, 항산화효과, 예쁜꼬마선충,

고려인삼.

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