Components and Antioxidant Activity of the Polysaccharide from *Streptomyces virginia* H03

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A polysaccharide was isolated from the broth of cultured *Streptomyces virginia* H03 which was treated by ethanol deposition and savage method to remove the protein, and was purified using Sephadex G-150 column chromatography. The components of the polysaccharide were determined by gas chromatography. The purified polysaccharide was made up of mannose, glucose and galactose, in a 2:1:1 proportion. Its average apparent molecular weight was $3.76 \cdot 10^4$ Da which was determined by gel permeation chromatography. In addition, several antioxidant assays were adopted to investigate the antioxidant activity of the polysaccharide *in vitro*. The results indicated that the purified polysaccharide showed significant antioxidant activity against superoxide anion, hydrogen peroxide and 1,1-diphenyl-2-picrylhydrazyl radical, and lipid peroxidation as with standard antioxidants such as vitamin C. Furthermore, the polysaccharide had a better heat stability than vitamin C, which suggested that the polysaccharide might be a potent useful antioxidant.

Key words: Polysaccharide, Antioxidant Activity, *Streptomyces virginia* H03

Introduction

Polysaccharides, important natural compounds mainly from plant, microbe and animal sources, play an essential role in many molecular processes affecting eukaryotic biology and diseases, and exhibit varied biological activities, such as antitumour, immunostimulant, anticancer, anticomplementary, anti-inflammatory, anticoagulant hypoglycaemic, antiviral and immunological activities, which makes them usable in many fields including food, cosmetics, biomedicine, agriculture, environmental protection and wastewater management (Majeti and Kumar, 2000).

Streptomyces are gram-positive bacteria, characterized by a complex morphologic differentiation cycle, accompanied by the production of many kinds of bioactive secondary metabolites having great structural and functional diversity including antibiotics, antifungal, antiviral, anticancer, and immunosuppressant agents, insecticides and herbicides (Williams *et al.*, 1983). About 70% of all known drugs had been obtained from actinomycetes of which 75% and 60% were used in medicine and agriculture, respectively (Tanaka and Mura, 1993). In recent years, heredity, growth and metabolic regulation of *Streptomyces* are research hotspots. Therefore, *Streptomyces* are considered as the most familiar bacteria in fermentation manufacturing of active pharmaceutical compounds.

In our laboratory, over 1000 strains, mostly unusual genera of actinomycetes were isolated, purified and identified from all kinds of soils by the method of selective plating technique, construction of genetic marker systems, a combination of chemical markers and 16S rRNA sequence analysis. Among these strains, a special *Streptomyces* strain named *Streptomyces virginia* H03 was found, which can produce a polysaccharide. In the present study, several antioxidant assays were adopted to investigate the antioxidant activity of the polysaccharide *in vitro*, which might be beneficial for more extensive biological and medical studies.

Experimental

Materials

Streptomyces virginia H03 was obtained from soil of Dabieshan, China. It was identified and conserved in China Center for Type Culture Collection (CCTCC), Wuhan, and the serial number is M 207049. It was fermented in a 20 l auto-control bioreactor (C20–3 type, B. Braun Biotech In-

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ternational, Melsungen, Germany) with sensors for pH value, dissolved oxygen (DO) and temperature. The growth medium consisted of 2% corn flour, 0.1% yeast extract, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% NaCl and distilled water. The initial pH value was 7.2. 10% seed culture was inoculated in a bioreactor with 16 l fermentation medium (pH 7.2), then cultured for 6 d at 28 °C with a speed of 200 rpm.

Isolation and purification of the polysaccharide from the broth of Streptomyces virginia H03

After centrifugation with a speed of $5000 \times g$ (model RS-2III, Tomy Seiko, Tokyo, Japan) for 10 min at 0 °C, 1 l of the broth of *Streptomyces virginia* H03 was condensed to 200 ml under reduced pressure at 60 °C, then extracted five times with 200 ml savage reagent such as $CHCl_3$/BuOH (v/v, 4:1) to deproteinize (Navarini *et al.,* 1999). After the savage reagent was removed, four times of the volume of alcohol was added to the water phase and kept at 4 °C overnight to precipitate carbohydrate compounds. After filtration with a Buchner funnel under reduced pressure, washing with alcohol, the sediment was dialyzed for 3 d by a dialysis tube (∅ 36) with a 2 KD membrane to remove small molecular substance. Finally, the residue in the dialysis tube was lyophilized as crude polysaccharide and stored for analysis.

Crude polysaccharide extract (400 mg) was dissolved in distilled water and applied to a Sephadex G-150 gel column (1.6 cm × 80 cm). The column was eluted with water (500 ml) firstly and then with 0.5 mol l^{-1} NaCl (500 ml) successively, at a flow rate of 0.1 ml min^{-1}, with collection of 3 ml outflow fractions for each tube. The fractions were detected by the phenol sulfuric acid method (Dubois *et al.*, 1956). Then the main fraction representing the polysaccharide was lyophilized for further purification on another Sephadex G-150 gel column under the same conditions.

Partial characterization of the polysaccharide

The purified sample was analyzed by the method of ninhydrin test to exclude the proteins (Moore and Stein, 1948). Distilled water and calf serum were regarded as the controls, respectively. Ultraviolet spectra were recorded with a spectrophotometer (Lambda Bio-40, Perkin Elmer, USA) from 190 to 400 nm to determine whether the sample had proteins and nucleic acids or not. In addition, infrared spectra were measured (EQUIOX 55, Bruker, Germany and Switzerland). At last, the polysaccharide content was determined by the phenol sulfuric acid method (Dubois *et al.*, 1956). D-Glucose was used as a standard.

Analysis of the average molecular weight

The average molecular weight of the polysaccharide was determined by gel permeation chromatography (GPC) with a HPLC apparatus (Agilent, Waldbronn, Germany) equipped with a TSK G-3000 SW column (300 × 7.5 mm), a refractive index detector (model 410) and an Agilent Workstation used for the calculation of average molecular weights. The dextran standards T-10, 40, 70, 270, 500, 2000 were used for the calibration curve. The detailed experimental conditions were as follows: column temperature, 23 °C; column pressure, 5 MPa (model 600 pump); injection volume, 50.0 μl; mobile phase, water; mobile rate, 1.0 ml min^{-1}; and run time, 20 min.

Analysis of monosaccharide components

The analysis of neutral monosaccharides was based on the method of gas chromatography (GC) (Osborne *et al.*, 1999). After the polysaccharide sample (10 mg) was hydrolyzed thoroughly with 3.0 ml 2.0 mol l^{-1} trifluoroacetic acid (TFA) in a sealed tube for 3 h at 110 °C, the hydrolyzed product was evaporated under vacuum to dryness, and the residue was derivatized to alditol acetates according to Antoni *et al.* (1999), then submitted to GC analysis. Gas chromatography was performed on a HP-5988 instrument (Hewlett-Packard, Wilmington, USA) equipped with a C60-polysiloxane capillary column (13.2 mm × 0.25 mm). The GC program was as follows: the initial temperature was set at 80 °C; 1 min later, the column was heated to 140 °C with a speed of 30 °C/min, and then held for another 2 min; finally, the column was heated to 250 °C with a speed of 10 °C/min and held for another 7 min. D-Glucose, D-xylose, D-mannose, D-galactose and D-arabinose were used as controls. Peaks of neutral monosaccharides were identified by comparison of their retention time with monosaccharide standards. The amount of individual neutral monosaccharides was calculated by comparison of peak areas.

Determination of the antioxidant activity of the polysaccharide in vitro

Determination of the superoxide anion scavenging activity of the purified polysaccharide

Measurement of the superoxide anion scavenging activity of the purified polysaccharide was according to Liu *et al.* (1997) with slight modification. The reaction mixture including 1 ml nitroblue tetrazolium (NBT) solution, 1 ml NADH solution, 0.1 ml of purified polysaccharide and 100 ml phenazine methosulfate (PMS) solution was incubated at 25 °C for 5 min. Later, the absorbance was measured at 560 nm. The concentration of the sample was from 50 to 400 μg ml^{-1}. A reaction mixture in which the sample was replaced by 0.9% NaCl was the control. Vitamin C, a powerful antioxidant, was used as a standard. The percentage inhibition of the superoxide anion generation was calculated by the formula: % inhibition = $[(A_0 - A_1)/A_0] \cdot 100$, where A_0 is the absorbance of the control, and A_1 is the absorbance of the purified polysaccharide or vitamin C.

Determination of the hydrogen peroxide scavenging activity of the purified polysaccharide

The hydrogen peroxide scavenging activity was determined by the method of blood cell oxidation hemolysis. The blood that came from the eyeball vein of a rat was collected in an anticoagulant and centrifuged at $1200 \times g$ for 15 min. The buffy coat was removed, and the packed cells were washed three times with physiological saline, then diluted to a 0.5% suspension. The reaction system consisting of 1 ml blood suspension, 200 μl polysaccharide with different concentrations from 50 to 400 μg ml^{-1}, and 100 μl 100 mmol l^{-1} H_2O_2 was incubated at 37 °C in water for 1 h, then diluted to 6-fold by physiological saline. After centrifugation at $1200 \times g$ for 5 min, the absorbance of the supernatant representing the hemolysate was determined at 415 nm. In addition, the reaction system in which the polysaccharide sample was replaced by physiological saline was used as the positive control. The system only including 1 ml blood and 300 ml physiological saline was the blank control. Vitamin C was used as a standard.

The percentage of scavenging of hydrogen peroxide of both purified polysaccharide and standard was calculated by the formula: % scavenged hydrogen peroxide = $[1 - (A_0 - A_1)/A_2] \cdot 100$, where A_2, A_1 and A_0 are the absorbance of positive control, blank control and the reaction system of sample or vitamin C, respectively.

Determination of the 1,1-diphenyl-2-picrylhydrazyl ($DPPH^-$) radical scavenging activity of the purified polysaccharide

The free radical scavenging activity of the purified polysaccharide was measured by $DPPH^-$ as described by Shimada *et al.* (1992). 2 ml of 0.2 mmol l^{-1} $DPPH^-$ and 2 ml polysaccharide with different concentrations (50–400 μg ml^{-1}) were mixed thoroughly, then placed at room temperature. 30 min later, the absorbance of the mixture was measured at 517 nm. In addition, a reaction system in which the polysaccharide sample was replaced by 2 ml ethanol was used as the positive control. The system only including 2 ml polysaccharide or vitamin C and 2 ml ethanol was the blank control. Vitamin C was used as a standard.

The capability to scavenge $DPPH^-$ radicals was calculated by the equation: % $DPPH^-$ scavenging effect = $[1 - (A_0 - A_1)/A_2] \cdot 100$, where A_2, A_1 and A_0 are the absorbance of positive control, blank control and the reaction system of sample or vitamin C, respectively.

Determination of the antioxidative effect on lipid peroxidation of the purified polysaccharide

The antioxidative effect of polysaccharide on lipid peroxidation induced by carbon tetrachloride was determined by measuring the malondialdehyde-thiobarbituric acid (MDA-TBA) content as described by Buege and Aust (1978) with slight modification. The rat was executed by cervical vertebra dislocation after fasting for 16 h. The liver was homogenized with physiological saline in an ice-bath and the homogenate was filtered through 4 layers of gauze. In each tube, 1 ml of liver homogenate was mixed with 1 ml physiological saline, 50 μl polysaccharide with different concentrations and 50 μl of 80% CCl_4 solution. After the mixture was incubated at 37 °C for 40 min, the reaction was stopped immediately by the addition of 10% trichloroacetic acid (TCA). After centrifugation at $3300 \times g$ for 15 min, 2 ml of supernatant were mixed with 2 ml of 0.5% (w/v) thiobarbituric acid (TBA). The mixture was incubated at 100 °C for 15 min, and quickly cooled in an ice-bath. Then the absorbance of the mixture was determined at 535 nm. In addition, a reaction system in which the polysaccharide sample was replaced by physiological saline was used as the positive control. The sys-

tem without TBA was the blank control. Vitamin C was used as a standard.

The capability to inhibit MDA formation was calculated by the formula: % inhibition = $[1 - (A_0 - A_1)/A_2] \cdot 100$, where A_2, A_1 and A_0 are the absorbance of positive control, blank control and the reaction system of sample or vitamin C, respectively.

Heat stability of the antioxidant activity of the purified polysaccharide

The purified polysaccharide (300 μg ml^{-1}) was heated from 50 to 100 °C for 5 min to investigate the heat stability of the antioxidant activity. The antioxidant activity assay was according to the method as mentioned above. Vitamin C was used as a standard.

Statistical analysis

All data in the figures and Table I represent the mean ± standard error. Every experiment was repeated at least 3 times. The significant difference between the treatment and the control was statistically evaluated by analysis of variance (ANOVA).

Results and Discussion

Extraction, isolation and purification of the polysaccharide from the broth of Streptomyces virginia H03

Two peaks appeared when the sample was eluted from the first Sephadex G-150 column (Fig. 1a). Moreover, the area of solution corresponding to the first peak was far bigger than the second one. Detected by the phenol sulfuric acid method, the first peak was collected as a crude polysaccharide and lyophilized for further purification on another Sephadex G-150 gel column. The results showed a symmetrical single peak (Fig. 1b), which indicated that it was a homogeneous component. The purified polysaccharide was a yellow powder with good water solubility, and the pH value was about 6.5. Furthermore, the yield of the purified polysaccharide and the content of sugar were 15.6 g l^{-1} and 95.6%, respectively.

Partial characterization of the polysaccharide

The ninhydrin test was used to identify amino acids after the purified polysaccharide was completely hydrolyzed. Negative reaction was found in both the polysaccharide and distilled water,

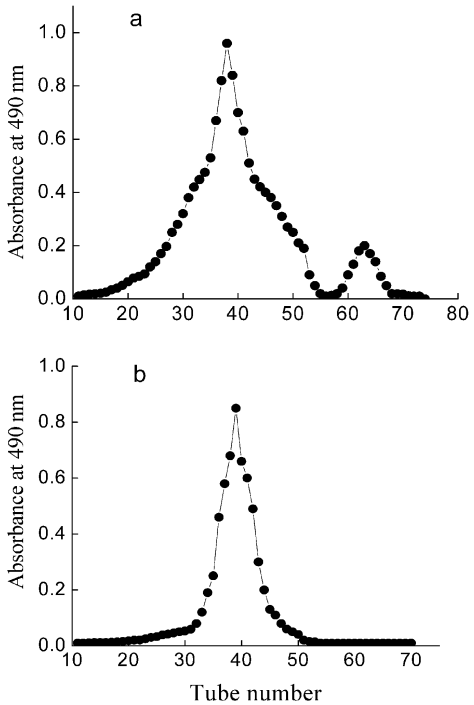

Fig. 1. Chromatograms of the polysaccharide after elution from (a) the first and (b) the second Sephadex G-150 column.

while a colour reaction was observed in calf serum, which suggested that the purified polysaccharide had no protein. In addition, the absorbance peaks of nucleic acid and protein were not detected between 200 and 400 nm in ultraviolet spectra. All this confirmed that the polysaccharide was of high purity, containing no nucleic acid or protein impurities (Qin *et al.*, 2002).

With the dextran T-10, 40, 70, 270, 500, 2000 standards used for calibration curve ($y = -1.1606x + 14.111$, $R^2 = 0.997$), the average apparent molecular weight of the polysaccharide was $3.76 \cdot 10^4$ Da.

Infrared spectrum of the purified polysaccharide

As it is well known, IR spectroscopy is a very useful tool for detecting the interaction in polymer blends. In this study, IR spectroscopy was used to examine the possible interactions between the blend components. The sample showed IR absorptions at 3409, 2932, 1660, 1402, 1185, 1113, 875, 618 cm^{-1} (Fig. 2). The IR spectrum displayed a

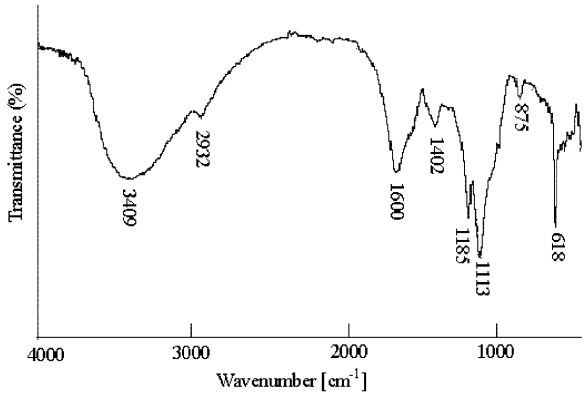

Fig. 2. IR spectrum of the purified polysaccharide.

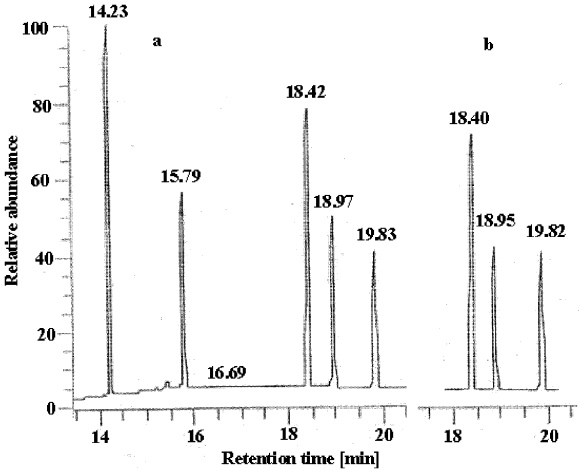

Fig. 3. Gas chromatograms of acetylated aldononitriles of the sample. (a) Gas chromatogram of acetylated aldononitriles of the standard including arabinose, xylose, mannose, glucose and galactose. (b) Gas chromatogram of acetylated aldononitriles of the purified polysaccharide hydrolyzed thoroughly.

broad intense stretching peak around 3400 cm^{-1} characteristic for a hydroxy group, and a weak C–H stretching band at 2932 cm^{-1}. The peak around 1660 cm^{-1} indicated aliphatic C=O bonds (Freddi *et al.*, 1999). Furthermore, the banding-like structure in the regions of 1185 and 1113 cm^{-1} is characteristic of a pyranoside. Additionally, missing absorption at 890 cm^{-1} for β-configuration indicated that the polysaccharide consisted of an α-pyranoside (Qin *et al.*, 2002).

Analysis of monosaccharide components

The retention times of the series of standard ramifications such as arabinose, xylose, mannose, glucose and galctose were shown by GC analysis (Fig. 3a), which were 14.23, 15.79, 18.42, 18.97 and 19.83 min, respectively. Compared to the control, it was deduced that the polysaccharide was mainly composed of mannose (18.40 min), glucose (18.95 min) and galctose (19.82 min) with a 2:1:1 proportion of the peak areas (Fig. 3b).

Scavenging activity of the purified polysaccharide against superoxide anion

Superoxide anion radical is generated firstly by the reactive oxygen species. Although it is a relatively weak oxidant, it decomposes to form stronger reactive oxidative species, such as singlet oxygen and hydroxyl radicals (Dahl and Richardson, 1978). Furthermore, superoxide anion radicals are also known to indirectly initiate lipid peroxidation as a result of H_2O_2 formation, creating precursors of hydroxyl radicals (Meyer and Isaksen, 1995). The inhibition (%) of superoxide radical generation by varying concentrations (50–400 $\mu g\ ml^{-1}$) of the purified polysaccharide is shown in Fig. 4a. The results showed that the inhibition rate increased with increasing concentration of the sample and vitamin C from 50 $\mu g\ ml^{-1}$ to 300 $\mu g\ ml^{-1}$, and reached a peak of 68.0% and 84.9% for the concentration of 300 $\mu g\ ml^{-1}$, which indicated that the inhibition (%) of superoxide radical generation of the polysaccharide or vitamin C increased rapidly in an concentration-dependent manner ($p < 0.05$). But no difference was observed in both the polysaccharide and vitamin C when the concentration was above 300 $\mu g\ ml^{-1}$ ($p > 0.05$). Though the inhibition of superoxide generation by the polysaccharide was weaker than by vitamin C at the same doses ($p < 0.05$), it was still deemed to have a good scavenging activity against the superoxide anion.

Scavenging activity of the purified polysaccharide on hydrogen peroxide

Except for the superoxide anion, the hydroxyl radical is considered to be a highly potent oxidant, which can react with all biomacromolecules functioning in living cells (Qi *et al.*, 2005). Thus, the removing of H_2O_2 is very important for antioxidant defence in cells or food systems (Aruoma, 1998). The scavenging ability of the purified polysaccharide on hydrogen peroxide is shown in Fig. 4b. The results showed that the inhibition rate

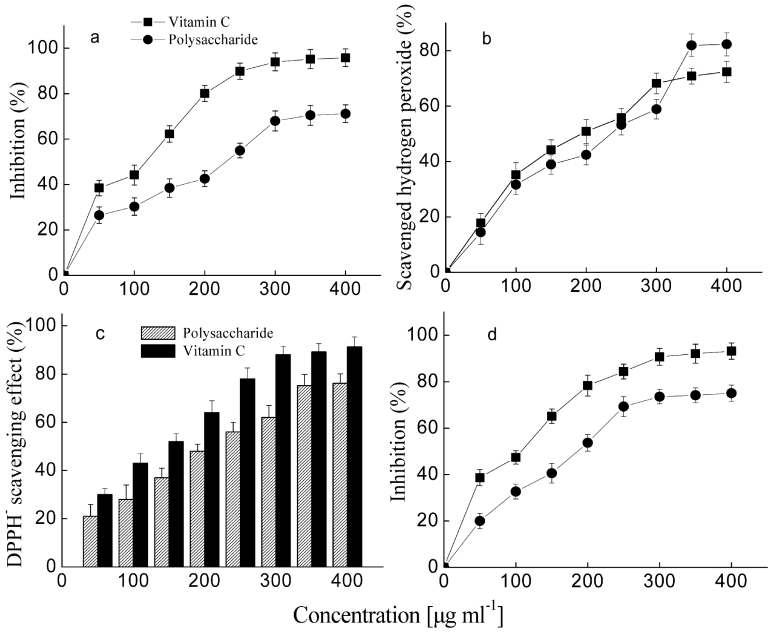

Fig. 4. Scavenging activity of the purified polysaccharide and vitamin C against (a) superoxide anion, (b) hydrogen peroxide, and (c) $DPPH^-$ radical and (d) inhibition rate on lipid peroxidation. Each data indicates the mean of three independent experiments (mean ± standard error).

increased with the increasing concentration of the sample and vitamin C from 50 μg ml^{-1} to 350 μg ml^{-1}, and reached a peak of 76.96% and 70.9% for the concentration of 350 μg ml^{-1}, which indicated that the scavenging activity of the purified polysaccharide on hydrogen peroxide was concentration-dependent ($p < 0.05$) as vitamin C did. When the concentration was between 350 to 400 μg ml^{-1}, the scavenging activity of the purified polysaccharide was markedly stronger than of vitamin C at the same dose ($p < 0.05$), which suggested that the purified polysaccharide had strong scavenging activity at high concentration.

Scavenging activity of the purified polysaccharide on $DPPH^-$ radical

The model of scavenging the $DPPH^-$ radical is a widely used method to evaluate the free radical scavenging ability of various samples (Leong and Shui, 2002; Nagai *et al.*, 2003). The effect of antioxidants on $DPPH^-$ radicals scavenging is due to their hydrogen-donating ability. From Fig. 4c it was found that the scavenging activity of the purified polysaccharide and vitamin C on $DPPH^-$ radicals increased dose-dependently, and reached 79.2% and 82.98% at the dose of 350 μg ml^{-1}, then it leveled off. The results indicated that the purified polysaccharide had a noticeable effect on scavenging free radicals as vitamin C has, which was also similar to the polysaccharide extracted from *Lycium barbarum* fruits (Li *et al.*, 2007) at the same dose.

Antioxidant effect of the purified polysaccharide on lipid peroxidation

Lipid peroxidation, a typical free radical oxidation, is an important event in cellular damage, which is strongly associated with aging, carcinogenesis and other diseases (Halliwell *et al.*, 1992). In addition, lipid peroxidation also plays an important role in the deterioration of foods during storage (Duthie, 1993). The antioxidative effect of the polysaccharide on lipid peroxidation induced by CCl_4 is shown in Fig. 4d. The inhibition rate of both the polysaccharide and vitamin C increased rapidly with increasing concentration from 50 to 300 μg ml^{-1}. But no significant difference was observed from 300 to 400 μg ml^{-1} ($p > 0.05$) both in the polysaccharide and vitamin C. These results were consistent with the observation of Chen *et al.* (2005), who found that tea polysaccharide conjugate from lower grade green tea had significant

Table I. Heat stability of the antioxidant activity of the polysaccharide (P) and vitamin C (V). 1, 2, 3 and 4 represent the scavenging activity against superoxide anion, hydrogen peroxide, $DPPH^-$ radical and the inhibition rate on lipid peroxidation, respectively. Each data indicates the mean of three independent experiments (mean ± standard error).

t [°C]	1		2		3		4	
	P	V	P	V	P	V	P	V
30	64.4 ± 0.3	90.4 ± 0.5	55.6 ± 0.7	70.4 ± 0.4	63.4 ± 0.5	87.4 ± 0.6	76.4 ± 0.4	89.3 ± 0.3
50	63.7 ± 0.4	63.7 ± 0.4	54.7 ± 0.4	65.7 ± 0.4	63.2 ± 0.2	65.9 ± 0.4	75.6 ± 0.3	55.7 ± 0.5
70	63.5 ± 0.2	56.5 ± 0.3	53.4 ± 0.5	36.5 ± 0.3	62.4 ± 0.3	36.5 ± 0.4	73.7 ± 0.2	36.5 ± 0.7
90	62.8 ± 0.5	23.8 ± 0.5	53.3 ± 0.6	20.8 ± 0.6	60.4 ± 0.5	18.8 ± 0.3	72.3 ± 0.5	21.6 ± 0.3
100	61.9 ± 0.3	11.2 ± 0.6	52.3 ± 0.2	10.2 ± 0.5	59.8 ± 0.5	8.9 ± 0.6	70.8 ± 0.2	9.2 ± 0.5

inhibitory effects on hydroxyl and superoxide radicals and lipid peroxidation using a deoxyribose assay, a photoreduction of nitroblue tetrazolium (NBT) assay and a lipid peroxidation inhibition assay. Similarly, a polysaccharide from the fern *Lygodium japonicum* was reported to show strong antioxidant activity against liposome peroxidation, $DPPH^-$ radical scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging, and metal chelating activities (Li *et al.*, 2006). Moreover, the inhibitory effect of the purified polysaccharide was weaker than of vitamin C ($p < 0.05$) at the same dose.

Heat stability of antioxidant activity of the purified polysaccharide

Heat stability of antioxidant activity of the purified polysaccharide is shown in Table I. The antioxidant activities remained almost unchanged after the purified polysaccharide was treated from 50 to 100 °C for 5 min, while the antioxidant activity of vitamin C decreased sharply from 50 to 100 °C, which indicated that the polysaccharide had a better heat stability than vitamin C.

Conclusion

Polysaccharides from mushrooms, plants and microbes were shown to be useful candidates as effective, non-toxic substances with antioxidant activity. The multifunctions of polysaccharides have been extensively investigated. Tsai *et al.* (2007) found that a water-soluble polysaccharide from the fermented filtrate and mycelia of *Antrodia cinnamomea* had antioxidant properties. Wang and Luo (2007) reported that a water-soluble crude polysaccharide obtained from *Gynostemma pentaphyllum Makino*, which was a well known edible and medicinal plant in oriental countries, has high scavenging effects on superoxide radicals and inhibitory effects on self-oxidation of pyrogallic acid (1,2,3-phentriol). In addition, the polysaccharide from *Gekko swinhonis* Guenther showed apoptosis-inducing effects on hepatocarcinoma cells (Wu *et al.*, 2006). Although the exactly molecular mechanisms of hepatoprotection effects of polysaccharides had not been yet revealed, both *in vitro* and *in vivo* studies showed that polysaccharides from naturally occurring ingredients have various bioactivities.

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