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Identification of structure and antioxidant activity of a fraction of polysaccharide purified from *Dioscorea nipponica* Makino

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Abstract

A large number of polysaccharides are present in boiling-water extraction of *Dioscorea nipponica* Makino. A DEAE-Sepharose CL-6B column chromatography was used to isolate the major polysaccharides from *D. nipponica* Makino. The largest amount of fraction of polysaccharide was subjected to further purification by gel-filtration on Sephadex G-100. The purified fraction was a neutral polysaccharide and a single peak in HPLC with Sugar KS-804 column, with a molecular weight of 38,000, and comprised mainly of glucose and fructose (45:1). Analysis by Periodate oxidation–Smith degradation indicated that there were 5.9%(1→)-glycosidic linkages, 4.94% (1 → 2)-glycosidic linkages, 61.16% (1 → 4)-glycosidic linkages, and 28% (1 → 3)-glycosidic linkages. On the basis of superoxide radical assay, hydroxyl radical assay, and self-oxidation of 1,2,3-phentriol assay, its antioxidant activity was investigated. This purified fraction of polysaccharide 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.
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Keywords: *Dioscorea nipponica* Makino; 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).

Dioscorea nipponica Makino is well known as a traditional edible plant in oriental countries. To date, no investigation has been carried out on polysaccharides that may account for the textural properties and antioxidant activities of *D. nipponica* Makino. 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 *D. nipponica* Makino using a DEAE Sepharose CL-6B column chromatography and a Sephadex G-100 column chromatography. In addi-

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tion, the structure properties and antioxidant activities of these major polysaccharides are also identified.

2. Experiment

2.1. Materials and chemicals

Dried underground part of *D. nipponica* Makino 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 and Sephadex G-100 were from the Pharmacia Co. (Sweden). All other reagents used were of analytical grade.

2.2. Isolation and purification of polysaccharide

The *D. nipponica* Makino (5.0 g) was extracted with 20 mL of distilled water at 90 ° C for 2.5 h once. After each extraction, the soluble polymers were separated from residues by filtration, and extracts were combined and concentrated. 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 (DM). Size-exclusion and anion-exchange chromatography were used for the fractionation of this preparation. 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. After loading with sample, the column was eluted 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 with a fraction collector, dialyzed against tap water and distilled water for 48 h, respectively, and then purified by gel-filtration chromatography on a column of Sephadex G-100 (2.6 cm × 70 cm), named DMB.

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 (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 HP5890 instrument (Hewlett-Packard Component, USA) with a column HP-5 (30 m × 0.32 mm × 0.25 μm). 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 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 4000–500 cm^{-1} (Kumar, Joo, Choi, Koo, & Chang, 2004).

2.4. Determination of the molecular weight

The molecular weight of the polysaccharide DMB was determined by gel-permeation chromatography (GPC), in combination with a high-performance liquid chromatography instrument (Angilent1100, USA). DMB (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. Partial hydrolysis with acid

Polysaccharide sample was hydrolyzed with 0.05 M trifluoroacetic acid, kept at 95 °C for 16 h, centrifuged and dialyzed the supernatant with distilled water for 48 h, and then diluted the solution in the sack with ethanol. After hydrolyzation, the precipitate and supernatant in the sack and the fraction out of sack were dried, and then GC analysis was carried out.

2.6. Periodate oxidation–Smith degradation

For analytical purpose, 25 mg of DMB was dissolved in 12.5 ml of distilled water and 12.5 ml of 30 mmol/l $NaIO_4$ was 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 0.001 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 (80 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 °C, 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.

2.7. 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), 2.5, 5, 10, 20, 50, 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%.

2.8. 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), 2.5, 5, 10, 20, or 40 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%.

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

The scavenging ability for self-oxidation of 1,2,3-phentriol of all different contents was investigated according to the method of Marklund and Marklund (1974) with a minor modification. Briefly, samples were dissolved in distilled water at 0 (control), 40, 60, 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 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, DMB (18.6%) was obtained from NaCl elute (Fig. 1). This fraction was purified by gel chromatography on Sephadex G-100 column.

We came to a conclusion that DMB was homogeneous by the following tests. It showed only one symmetrical peak from gel-filtration chromatography on Sephadex G-100 column (1.6 cm × 80 cm) which was equilibrated in 0.9% sodium chloride at 2.2 ml/12 min, and gave a single peak on HPLC with Sugar KS-804 column (Fig. 2), indicating that no other polysaccharide was present in the sample.

The polysaccharide content was 98.9%, average molecular weight was 3.8×10^4, and DMB was mainly consisted of

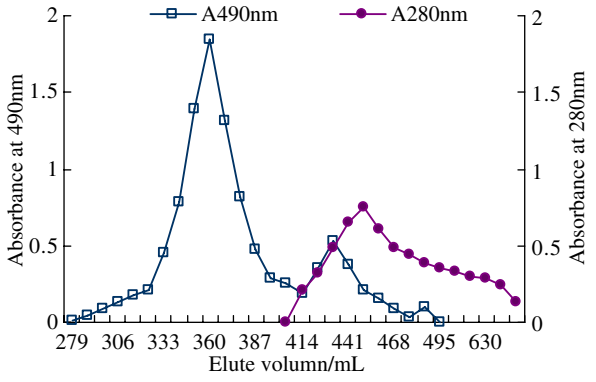

Fig. 1. DEAE-Sepharose CL-6B column chromatogram of DM from NaCl stepwise elute.

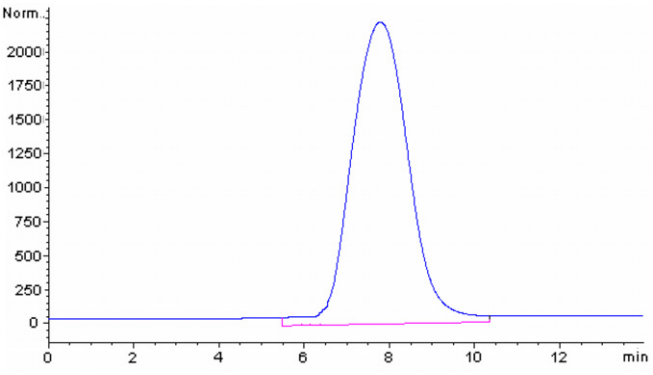

Fig. 2. HPLC chromatogram of DMB on Sugar KS-804 column.

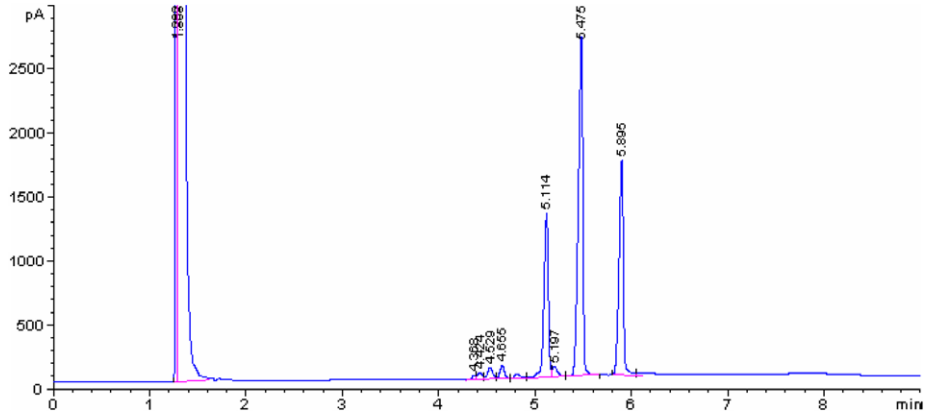

Fig. 3. Gas chromatogram of DMB hydrolyzed by TFA.

glucose and fru (45:1) (Fig. 3). The infrared spectrum of DMB 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 DMB

Four fractions obtained after partial acid hydrolysis of DMB, were subjected to GC analysis, yielding results shown in Table 1. Both $DMB_1$and DMB_2 contained the monosaccharide glucose, indicating that glucose is the components of backbond structure of DMB. This assay detected glucose in the DMB_3 and large amounts of fructose only in the fraction of DMB_4, indicating fructose and a part of glucose could be in the position of branched structure of DMB Table 2.

Table 1
GC analysis results of fractions from partial acid hydrolysis

Fractions	Molar ratios	
	Glu[a]	Fru[b]
DMB_1-precipitation.	+[c]	–[d]
DMB_2-precipitation in the sack	+	–
DMB_3-supernatant in the sack	+	–
DMB_4-fraction out of sack	+	+

[a] Glucose.
[b] Fructose.
[c] Detectable.
[d] Undetectable.

Table 2
The results of periodate oxidation

Reaction time (h)	36
Amount of DMB (mmol)	0.154
Consumption of HIO_4 (mmol)	0.120
Consumption of HIO_4/hexose (mol/mol)	0.779
Amount of formic acid (mmol)	0.0091
Amount of formic acid/hexose (mol/mol)	0.059

The polysaccharide of DMB showed abundance HIO_4 uptake, while it was oxidized. The consumption of HIO_4(0.120 mmol) was more two times than the amount of formic acid (0.0091 mmol) that was produced after 36 h of periodate treatment, indicating the existing of little amounts of monosaccharide which are 1→linked or 1→6 linked and little branch of DMB. In addition, it should be concluded that sugar residues oxidized and linkages of (1→)-glycosidic linkages account for 72% and 5.9%, respectively. The periodate-oxidized products were hydrolyzed and examined by gas chromatography (Table 3). The presence of glucose indicating a part of glucose are (1→3)-glycosidic linkages, namely linkages that cannot be oxidized. Fructose was absent, then it should be inferred that Fru is all linkages that can be oxidized, namely (1→)-glycosidic linkages, (1→2)-glycosidic linkages or (1→4)-glycosidic linkages. Results of Smith degradation analysis of fractions were summarized in Table 3. From molar ratios of glycerol and erythritol, and amount of formic acid, it could be obtained that (1→2)-glycosidic linkages account for 4.94%, and (1→4)-glycosidic linkages 61.16%. There was no precipitation in the sack and no substances in the supernatant of sack, indicating that the backbond of DMB should be all oxidized by HIO_4. At the same time, the branch of DMB was little, so the linkages of

Table 3
GC results from fractions of Smith degradation of DMB

Fractions	Molar ratios			
	Glu[a]	Fru[b]	Gly[c]	Ery[d]
Full acid hydrolysis	+[e]	–[f]	1	3.88
Smith degradation				
Out of sack	+	–	1	3.81
Supernatant in the sack	–	–	–	–
Precipitation in the sack	–	–	–	–

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

backbond could be alternately arrayed by (1→3)-glycosidic linkages and (1→2)-glycosidic linkages or (1→4)-glycosidic linkages, according to the presence of glycerin and erythritol out of sack, or the linkages of backbond could be the (1→2)-glycosidic linkages or (1→4)-glycosidic linkages and the linkages of branch (1→3)-glycosidic linkages.

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.

DMB had a higher scavenging effect than Vc (Fig. 4). The scavenging effects increased with increasing concentration. The purification fraction DMB showed scavenging activities of hydroxyl radicals, indicating that it was main contents to execute antioxidant functions from *D. nipponica* Makino. Scavenging effects of DMB were 3.35–43.73% at amount of 0.25–4 mg, and that of vitamin C was about 0–43.09%. This result proved that polysaccharides from *D. nipponica* Makino had significant effect on scavenging hydroxyl radical, and DMB was 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 DMB extracted and purified from *D. nipponica* Makino indicated a concentration-dependent, radical-scavenging activity at all tested concentrations.

As shown in Fig. 5, DMB was found to have the ability to scavenge superoxide radical. At the amount of between 0.25 mg and 2 mg, the effects on scavenging superoxide of DMB were 27.5–35.52%. However, the scavenging activity of vitamin C for superoxide radical was 0 at 0.25–2 mg. Compared to this results, DMB had stronger scavenging activity for superoxide radical than vitamin C at lower amounts. Our data on the activity of scavenging superoxide of DMB suggested that it was likely to contribute towards the observed antioxidant effect.

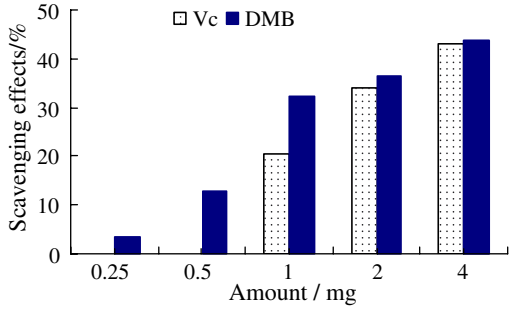

Fig. 4. Scavenging activities of hydroxyl radical by Vc and DMB.

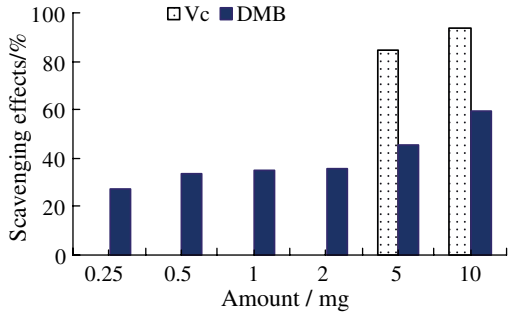

Fig. 5. Scavenging effects of Vc and DMB on superoxide radicals.

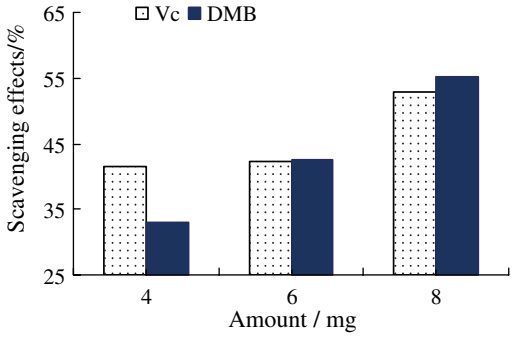

Fig. 6. Inhibitory effects of Vc and DMB on self-oxidation of 1,2,3-phentriol.

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 DMB extracted and purified from *D. nipponica* Makino. The scavenging powers of DMB correlated well with increasing concentrations. Moreover, the scavenging power of DMB corresponds to that of Vc. These results indicate that DMB have strong scavenging power for self-oxidation of 1,2,3-phentriol and should be explored as novel potential antioxidants.

4. Conclusion

According to the results above, it was concluded that the water extracting crude polysaccharide of *D. nipponica* Makino contained predominantly water extractable polysaccharide (DMB) purified by DEAE-Sepharose CL-6B and Sephadex G-100 column chromatography. In addition, DMB exhibited strong antioxidant activities. Among them, DMB had a little higher scavenging activity of hydroxyl radical than Vc, a higher activity at scavenging superoxide radical than Vc in low amounts, and equivalent inhibiting ability to Vc on self-oxidation of 1,2,3-phentriol, so it should be explored as a novel potential antioxidant.

Analysis by Periodate oxidation–Smith degradation indicated that there were 5.9% (1→)-glycosidic linkages, 4.94% (1→2)-glycosidic linkages, 61.16% (1→4)-glycosidic linkages, and 28% (1→3)-glycosidic linkages.

References

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Chemistry, 72*, 248–254.

Cheng, Z. Y., Ren, J., Li, Y. Z., Chang, W. B., & Chen, Z. D. (2002). Study on the multiple mechanisms underlying the reaction between hydroxyl radical and phenolic compounds by qualitative structure and activity relationship. *Bioorganic & Medicinal Chemistry, 10*, 4067–4073.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry, 28*, 350–356.

Ghiselli, A., Nardini, M., Baldi, A., & Scaccini, C. (1998). Antioxidant activity of different phenolic fractions separated from an Italian red wine. *Journal of Agricultural Food and Chemistry, 46*, 361–367.

Grice, H. C. (1988). Safety evaluation of butylated hydroxyanisole from the perspective of effects on forestomach and oesophageal squamous epithelium. *Food and Chemical Toxicology, 26*, 717–723.

Guentas, L., Pheulpin, P., Michaud, P., Heyraud, A., Gey, C., Courtois, B., et al. (2001). Structure of a polysaccharide from a *Rhizobium* species containing 2-deoxy-β-D-arabino-hexuronic acid. *Carbohydrate Research, 332*, 167–173.

Hu, Y., Xu, J., & Hu, Q. H. (2003). Evaluation of antioxidant potential of aloe vera (*Aloe barbadensis* Miller) extracts. *Journal of Agricultural and Food Chemistry, 51*, 7788–7791.

Jiang, Y. H., Jiang, X. L., Wang, P., & Hu, X. K. (2005). In vitro antioxidant activities of water-soluble polysaccharides extracted from *Isaria farinosa* B05. *Journal of Food Biochemistry, 29*, 323–335.

Kinsella, J. E., Frankel, E. N., German, J. B., & Kanner, J. (1993). Possible mechanisms for the protective role of antioxidants in wine and plant foods. *Food Technology, 4*, 85–89.

Kumar, C. G., Joo, H. S., Choi, J. W., Koo, Y. M., & Chang, C. S. (2004). Purification and characterization of an extracellular polysaccharide from haloalkalophilic *Bacillus* sp. I-450. *Enzyme and Microbial Technology, 34*, 673–681.

Marklund, S., & Marklund, G. (1974). Involvement of superoxide anion radicals in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *European Journal of Biochemistry, 47*, 469–471.

Mau, J. L., Lin, H. C., & Song, S. F. (2002). Antioxidant properties of several speciality mushroom. *Food Research International, 5*, 519–526.

Nandita, S., & Rajini, P. S. (2004). Free radical scavenging activity of an aqueous extract of potato peel. *Food Chemistry, 85*, 611–616.

Qi, H. M., Zhang, Q. B., Zhao, T. T., Chenc, R., Zhang, H., Niu, X. Z., et al. (2005). Antioxidant activity of different sulfate content derivatives of polysaccharide extracted from *Ulva pertusa* (Chlorophyta) in vitro. *International Journal of Biological Macromolecules, 37*, 195–199.

Ramarahnam, N., Osawa, T., Ochi, H., & Kawaishi, S. (1995). The contribution of plant food antioxidants to human health. *Trends in Food Science and Technology, 6*, 75–82.

Robak, J., & Gryglewski, R. (1988). Flavonoids are scavengers of superoxide anions. *Journal of Biochemical Pharmacology, 37*, 837–841.

Wang, G. Y., Liang, Z. Y., & Zhang, L. P. (2001). Studies on the structure of JS1 – the water soluble polysaccharide isolated by alkaline from *Hippophae rhamnoides* L.. *Chemical Journal of Chinese Universities, 22*, 1688–1690.

Wang, Z. J., & Luo, D. H. (2007). Antioxidant activities of different fractions of polysaccharide purified from *Gynostemma pentaphyllum* Makino. *Carbohydrate Polymers, 68*, 54–58.

Wang, Z. J., Luo, D. H., & Liang, Z. Y. (2004). Structure of polysaccharides from the fruiting body of *Hericium erinaceus* Pers. *Carbohydrate Polymers, 57*, 241–247.