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Antioxidant activities of different fractions of polysaccharide purified from pulp tissue of litchi (*Litchi chinensis* Sonn.)

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ABSTRACT

Three fractions of water-soluble polysaccharide fractions, coded as LFP1, LFP2 and LFP3, were isolated and purified from litchi pulp by DEAE-52 cellulose anion-exchange column and Sephadex G-100 gel-permeation column. Their chemical and physical characteristics were determined by chemical methods, gas chromatography, IR spectrophotometer and gel-permeation chromatography (GPC) with a model 410 refractive index detector. Antioxidant activities of these fractions were investigated using various *in vitro* assay systems. Results indicated that LFP1 was composed of arabinose, ribose, galactose and glucose, while LFP2 comprised arabinose, rhamnose and glucose. LFP3 was a heteropolysaccharide bounded with protein (2.6%) and composed of arabinose, rhamnose, galactose and glucose. The molecular weights of LFP1, LFP2 and LFP3 were 11.6×10^4, 3.1×10^4 and 2.9×10^4 Da, respectively. LFP3 showed the highest antioxidant activities among the three fractions of polysaccharides, and could be explored as a novel potential antioxidant.

1. Introduction

Oxidation is essential to many organisms for the production of energy to fuel biological processes. However, many human diseases (such as cancer, rheumatoid arthritis, and atherosclerosis, etc.) are due to the uncontrolled production of oxygen-derived free radicals (Mau, Lin, & Song, 2002). Since synthetic antioxidants have been suspected of being responsible for liver damage and carcinogenesis (Grice, 1988; Qi et al., 2005), it is essential to develop and utilize effective and naturally derived 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). In the past two decades, many reports have indicated that plant polysaccharides have strong antioxidant activities and can be explored as novel potential antioxidants (Hu, Xu, & Hu, 2003; Jiang, Jiang, Wang, & Hu, 2005; Qi et al., 2006; Ramarahnam, Osawa, Ochi, & Kawaishi, 1995). Previous studies indicate that polysacchrides antioxidant activity may be closely related to chemical properties and structural charateristics of these substances (Chen, Zhang, Qu, & Xie, 2008; Wang & Luo, 2007).

Litchi (*Litchi chinensis* Sonn.), a tropical to subtropical fruit originated from China is cultivated all over the world in warm climates (Gontier et al., 2000). Litchi fruits have been used to produce various types of healthy products and foods, e.g., medicinal beverages, drinks or soup (Chyau, Ko, Chang, & Mau, 2003; Jiang, 2000; Jiang & Li, 2003). It may merit investigation as a potential antioxidant for clinical use. Polysaccharide and major flavonoids from litchi pericarp tissues has been purified and found to exhibit beneficial antioxidant activities (Yang et al., 2006). However, no investigation has been carried out on polysacchrides of litchi pulp to better effectively exploit the structural and functional properties of these substances. More detailed work is required to fully elucidate the antioxidant activities of active polysaccharides.

In this study, the major polysaccharides of litchi fruit pulp were extracted and purified using a DEAE-52 cellulose column chromatography and a Sephadex G-100 column chromatography. Their chemical and physical characteristics were determined by chemical methods, gas chromatography, IR spectrophotometer and gel-permeation chromatography (GPC) with a model 410 refractive index detector. In addition, the structure properties and antioxidant activities of these major polysaccharides were also investigated.

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2. Materials and methods

2.1. Materials and chemicals

Fresh fruits of litchi (*Litchi chinesis* Sonn.) cv. Heiye at the commercially mature stage were offered by the Institute of Fruit Tree Research, Guangdong Academy of Agricultural Sciences in Guangzhou, China. Fruits with uniform shape and color were selected as materials.

DEAE-52 cellulose and Sephadex G-100 were perchased from Pharmacia Co. (Stockholm, Sweden). D-Arabinose, L-rhamnose, D-ribose and D-galactose, D-glucose, Nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), dihydronicotineamidadenine dinucleotide (NADH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of analytical grade.

2.2. Extraction of polysaccharides

Litchi fruit polysaccharide (LFP) was extracted and isolated from litchi pulp according to the methods of Miyazaki and Nishijima (1981) and Olafsdottir et al. (1999) with minor modifications. 1 kg litchi fruits pulp were refluxed three times to remove monosaccharides and saccharoses with 80% EtOH. Then it was comminuted and extracted twice with 1 l portions of distilled water for 4 h at 80 °C. The water extracts were filtered through Whatman No. 1 paper. The filtrates were combined and concentrated to 250 ml by a rotary evaporator at 65 °C. The proteins in the extracts were removed using the Sevag reagent, and then the polysaccharides were precipitated with four volumes of ethanol for overnight at 4 °C. The precipitate was collected by centrifugation, washed successively with ethanol and ether, and dried at reduced pressure, giving LFP as a crude polysaccharide.

2.3. Separation and purification of the polysaccharides

A DEAE-52 cellulose column (2.6 cm × 50 cm) was used to isolate negatively charged polysaccharides from non-negatively charged polysaccharides. After loading with sample, the column was eluted with distilled water for 300 ml at 0.5 ml/min, followed stepwise by NaCl aqueous solution (0–1 M) for 300 ml, respectively, at 0.5 ml/min. The major polysaccharide fractions were collected with a fraction collector and dialyzed against tap water and distilled water for 48 h, respectively, and concentrated using a rotary evaporator at 65 °C. A 0.5-ml sample of three highest peaks of polysaccharide material was loaded into a Sephadex G-100 gel column (1.6 cm × 60 cm) and eluted with distilled water at a flow rate of 0.3 ml/min. The major fractions with the highest polysaccharide content were collected and then freeze dried.

2.4. Determination of the polysaccharides purification

The samples were dissolved in distilled water, centrifuged, and then the filtrate was applied to a Sephadex G-100 column (1.6 cm × 80 cm), which was eluted with distilled water at 0.2 ml/min. In addition, the polysaccharides purification was also identified by cellulose acetate pellicle electrophoresis (borax–sodium hydroxide buffer, pH 10) at 40 V for 50 min with detection using Toluidine Blue.

2.5. Monosaccharide compositions and properties

The carbohydrate contents of the samples were determined by the phenol–sulfuric acid method, using glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The protein contents in the polysaccharide fractions were measured according to Bradford's method, using bovine serum albumin (BSA) as the standard (Bradford, 1976).

Gas chromatography (GC) was used for identication and quantification of monosaccharides in litchi polysaccharide fractions. The polysaccharide (10 mg) was hydrolyzed with 10 ml of 2 M trifluoroacetic acid (TFA) at 120 °C for 6 h (Erbing, Jansson, Widmalm, & Nimmich, 1995). Derivation was then carried out using the trimethylsilylation reagent according to the method of Guentas et al. (2001). The trimethylsilylated derivatives were loaded into a HP5 capillary gas chromatography (GC) column equipped with a flame-ionization detector (FID), using inositol as the internal standard. The operation was performed using the following conditions: H_2: 16 ml/min; air: 150 ml/min; N_2: 20 ml/min; injection temperature: 230 °C; detector temperature: 230 °C; column temperature programmed from 130 to 180 °C at 5 °C/min, holding for 2 min at 180 °C, then increasing to 220 °C at 5 °C/min and finally holding for 3 min at 220 °C.

2.6. Molecular weight determination

The molecular weight of the polysaccharides puried from litchi pulp was determined by gel-permeation chromatography (GPC) by the method of Yamamoto, Nunome, Yamauchi, Kato, and Sone (1995), in combination with a high-performance liquid chromatography instrument (Waters5215, USA) equipped with an Ultrahydrogel column, a model 410 refractive index detector and a Millennium 32 Workstation. The column was eluted with distilled water at a flow rate of 0.6 ml/min. Dextran standards (4400, 9900, 21,400, 43,500, 124,000, 196,000, 277,000 and 845,000 Da) were used for the calibration curve.

2.7. Infrared spectral analysis of the polysaccharides

The IR spectrum of the polysaccharides was determined using a Fourier transform infrared spectrophotometer (FTIR, Bruker, Germany) equippment. 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).

2.8. Determination of antioxidant activity

2.8.1. Superoxide anion-scavenging activity

The superoxide anion-scavenging activity was measured following the method of Qi et al. (2006). Briefly, 1 ml of various concentrations of sample was mixed with 1 ml of 0.1 M phosphate buffer (pH 7.4) containing 150 μM NBT, 60 μM PMS and 468 μM NADH. The reaction mixture was incubated at room temperature for 5 min and the absorbance was read at 560 nm. The capability of scavenging superoxide radical was calculated using the following equation: scavenging activity $(\%) = (1 - A_{\text{sample 560 nm}}/A_{\text{control 560 nm}}) \times 100$.

2.8.2. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was measured by consulting the method of Wang et al. (2003) with minor modification. The reaction mixture, total volume 4.0 ml, containing the sample solution with various concentration, EDTA-Fe^{2+} (220 μM), safranine O (0.23 μM), and H_2O_2 (60 μM) in potassium phosphate buffer (150 mM, pH 7.4), was incubated for 30 min at 37 °C and the absorbance was read at 520 nm against a blank. The control contained phosphate buffer instead of the H_2O_2 while blanks contained distilled water instead of sample solution. The capability of scavenging hydroxyl radical was calculated using the following equation: scavenging activity

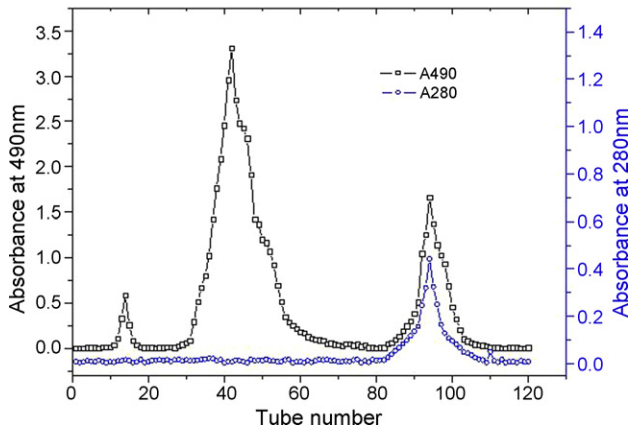

Fig. 1. DEAE-52 chromatogram of the crude polysaccharides extracted from litchi fruit.

$$(\%) = [(A_{\text{sample 520 nm}} - A_{\text{blank 520 nm}})/(A_{\text{control 520 nm}} - A_{\text{blank 520 nm}})] \times 100.$$

2.8.3. Reducing power

The reducing power was determined according to the method of Duan, Zhang, Li, and Wang (2006) with some modifications. Briefly, 1 ml of various concentrations of sample solution was mixed with 2.5 ml of 0.2 mM sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was then incubated at 50 °C for 20 min. After 2.5 ml of 10% trichloroacetic acid (w/v) was added, the mixture was centrifuged at 3000 rpm for 10 min. A 2.5 ml aliquot of the upper layer was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride, and the absorbance at 700 nm was measured. Reducing power was expressed as a percentage of the activity shown by a 1 mM solution of Vitamin C.

3. Results and discussion

3.1. Isolation and purification of polysaccharides

An aqueous extract of crude polysaccharides from litchi pulp tissue was chromatographed on a DEAE-52 anion-exchange column (2.6 cm × 50 cm) to yield three peaks (Fig. 1), and the yields of the three fractions were LFP1 2.37%, LFP2 63.92%, LFP3 20.00%. The polysaccharide absorption peak showed similar time and shape as the protein absorption peak, suggesting that LFP3 is a likely a glycoprotein. This finding was similar to previous report that polysaccharides existing as glycoprotein in grape berries (Saulnier & Brillouct, 1989). Three fractions were purified by gel chromatography on Sephadex G-100 column (1.6 cm × 60 cm), respectively (Fig. 2).

Table 1
Components of monosaccharide and properties of polysaccharides from fruits of litchi.

	LFP1	LFP2	LFP3
Protein (wt%)	nd[a]	nd	2.6
Carbohydrate (wt%)	99.4	99.6	97.4
Sugar components (mol%)			
D-Arabinose	1.67	1.00	2.42
L-Rhamnose	nd	1.37	3.44
D-Ribose	2.15	nd	nd
D-Galactose	1.54	nd	1.00
D-Glucose	1.00	1.21	1.65

[a] nd: Not detected.

To determine the purification of polysaccharides, three fractions were loaded into Sephadex G-100 column (1.6 cm × 80 cm). Three fractions were showed both only one symmetrical peak from gel-filtration chromatography, indicating that no other polysaccharide was present in the sample. In addtion, only a single spot on cellulose acetate pellicle electrophoresis was observed using Toluidine Blue. Based on above results, these three fractions have been purified to be homogeneous.

3.2. Chemical composition of polysaccharides

The polysaccharide content, protein content, and sugar compositions of LFP1, LFP2 and LFP3 were determined and given in Table 1. No proteins existed in the LFP1 and LFP2, while LFP3 had a low protein content of 2.6%. Monose compositions of three fractions were determined by the triuoroacetic acid hydrolysis method. GC analysis of the trimethylsilyl derivatives showed difference of these three fractions, with the presence of arabinose, ribose, galactose and glucose in the molar ratio of 1.67:2.15:1.54:1.00 for LFP1 fraction; and arabinose, rhamnose and glucose in the molar ratio of 1.00:1.37:1.21 for LFP2 fraction, also arabinose, rhamnose, galactose and glucose in the molar ratio of 2.42:3.44:1.00:1.65 for LFP3 fraction.

3.3. Molecular weight determination

The molecular weights of LFP1, LFP2 and LFP3 were determined by GPC (Yamamoto et al., 1995). The equation of the standard curve was drawn as $\log wt = 27.215 - 6.052t + 0.5446t^2 - 0.0167t^3$ (where wt represents molecular weight, while t represents elution time) using the Millennium32 software. The molecular weights of LFP1, LFP2 and LFP3 were 116440, 31424 and 28181 Da, respectively. The

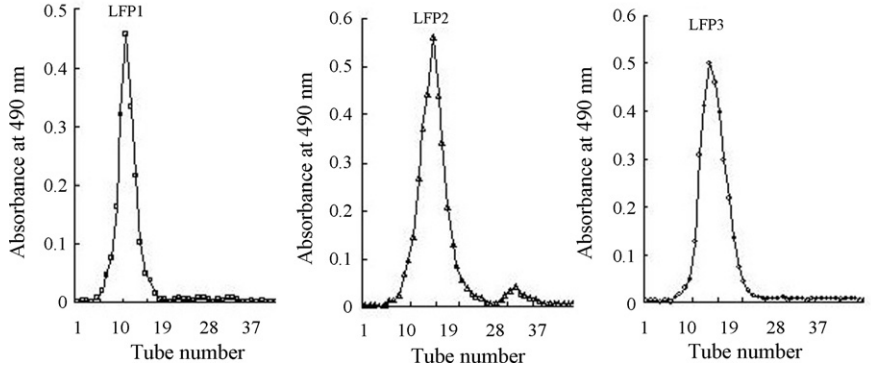

Fig. 2. Sephadex G-100 gel chromatogram of the polysaccharides. A 0.5-ml sample of three highest peaks of polysaccharide from DEAE-52 column was loaded into a Sephadex G-100 gel column (1.6 cm × 60 cm) for further purification.

Table 2
Molecular weight (MW) and molecular size (MN) of litchi polysaccharides.

Sample	MW	MN	MW/MN
LFP1	116440	94119	1.24
LFP2	31424	35982	1.15
LFP3	28181	61040	4.87

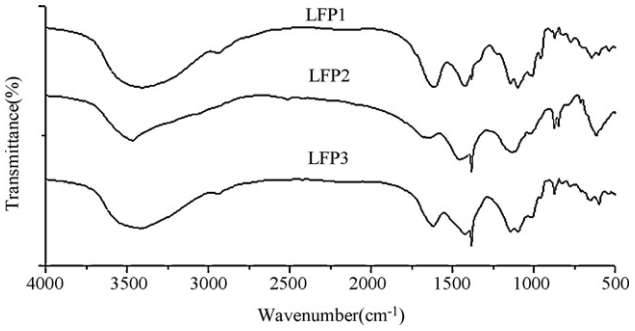

Fig. 3. IR spectra of LFP1, LFP2 and LFP3 in the frequency range 4000–500 cm^{-1}.

MW/MN values of LFP1 and LFP2 were closer to 1 than that of LFP3, indicating that LFP1 and LFP2 were more homogeneous (Table 2).

3.4. IR spectra of puried LFP fractions

As shown in Fig. 3, the IR spectra of the three LFP fractions displayed a broad stretching intense characteristic peak at around 3419 cm^{-1} for the hydroxyl group, and a weak C–H stretching band at around 2937 cm^{-1}(Santhiya, Subramanian, & Natarajan, 2002). The two peaks towards 1620 and 1420 cm^{-1} in the IR spectra of the three LFP fractions, resulted from the presence of the COO^- deprotonated carboxylic group (Manrique & Lajolo, 2002). Two stretching peaks, at 1144 and 1011 cm^{-1} in the IR spectra of LFP1, at 1137 and 1022 cm^{-1} in the IR spectra of LFP2, at 1143 and 1012 cm^{-1} in the IR spectra of LFP3, suggested the presence of C–O bonds (Kacuráková, Capek, Sasinková, Wellner, & Ebringerová, 2000).

3.5. Scavenging activity of superoxide radical

The three polysaccharide fractions were found to have the ability to scavenge superoxide radical at concentrations between 50 and 500 μg/ml (Fig. 4). The scavenging effects of litchi polysaccharide increased with increasing concentration. The IC_{50} values of LFP1, LFP2 and LFP3 for superoxide radical were 960.86 μg/ml, 423.54 μg/ml and 113 μg/ml, respectively. LFP3 exhibited the highest scavenging effects. Yang et al. (2006) reported that scavenging

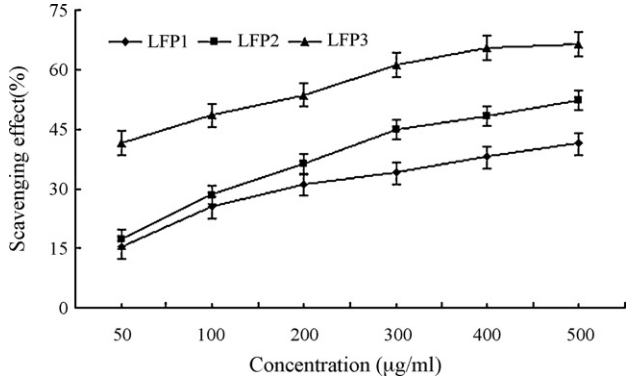

Fig. 4. Scavenging effect of LFPs on superoxide radicals. Each value is presented as mean ± standard error of three parallel measurements for each concentration.

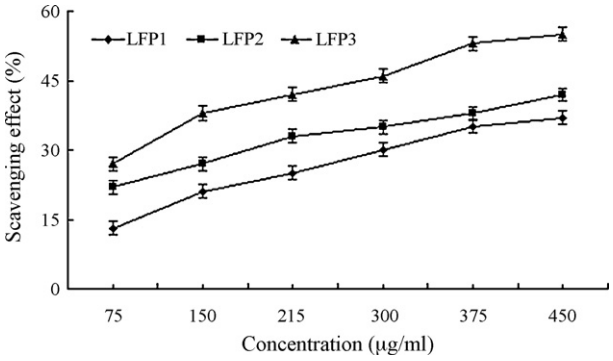

Fig. 5. Scavenging effect of LFPs on hydroxyl radicals. Data represent the mean ± standard deviation ($n = 3$).

activity of the polysaccharide fraction (F01) from pericarp tissues of litchi was about 50.7% and 62.5% at 50 μg/ml and 100 μg/ml. In the present study, LFP3 from litchi pulp exhibits a little lower scavenging activity than those from litchi pericarp by values of 41.62% and 48.53% at 50 μg/ml and 100 μg/ml, respectively. Qi et al. (2006) reported that scavenging activity of Vitamin C for superoxide radical was about from 30% at 500 μg/ml. Compared to this result, LFP1, LFP2 and LFP3 had stronger scavenging activity for superoxide radical than Vitamin C with values of 41.48%, 52.28% and 66.45% at 500 μg/ml respectively (Fig. 4). Our data on the activities of scavenging superoxides of different contents suggested that it was likely to contribute towards the observed antioxidant effect.

3.6. Scavenging activity of hydroxyl radical

As shown in Fig. 5, the three polysaccharide fractions were found to exhibit the ability to scavenge hydroxyl radicals at amounts between 75 and 450 μg. The scavenging effects increased with increasing concentration. Scavenging effects of three fractions were 37.23%, 42.54% and 55.67% at amount of 450 μg/ml, respectively. Previous report found that scavenging activity of the polysaccharide fraction from pericarp tissues of litchi was about 50.3% at 100 μg/ml (Yang et al., 2006). This value of scavenging activity for hydroxyl radical from litchi pericarp was higher than three polysaccharide fractions from litchi pulp (Fig. 5). It was reported that scavenging effect on hydroxyl radical of Vitamin C was about 20% at 1000 μg/ml (Qi et al., 2006). These results proved that polysaccharides from litchi (pulp and pericarp) had significant effect on scavenging hydroxyl radical, and some fractions was more pronounced than that of Vitamin C.

3.7. Reducing power

Reducing power of different polysaccharide fractions extracted and purified from litchi pulp was investigated and showed in Fig. 6. Values of reducing power of LFP1, LFP2 and LFP3 were 24.09%, 32.55% and 45.61% at concentration of 500 μg/ml, respectively. The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Gordon, 1990). Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. The antioxidant activity was concomitant with the reducing power (Pin-Der-Duh, Pin-Chan-Du, & Gow-Chin-Yen, 1999; Tanaka, Kuie, Nagashima, & Taguchi, 1988). Our data on reducing power of three different fractions from litchi pulp indicates that it may likely play a role in the antioxidation observed.

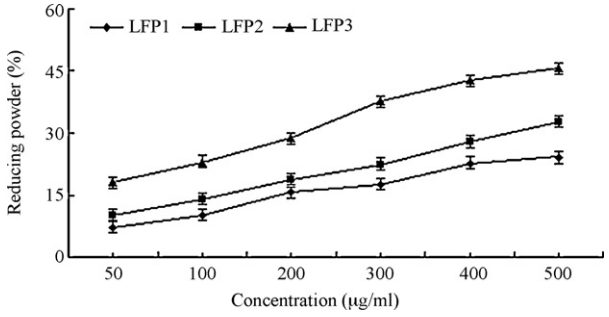

Fig. 6. Reducing power of LFPs. Each sample was assayed in triplicate for each concentration. Each value is presented as mean ± standard error ($n = 3$).

4. Conclusion

According to the results above, it was concluded that the water extracting crude polysaccharide of litchi pulp contained predominantly three polysaccharide fractions (LFP1, LFP2 and LFP3) purifed by DEAE-52 and Sephadex G-100 column chromatography, and purifcation polysaccharide prepared are confirmed of high purity. Antioxidation test *in vitro* shows that LFP3 possesses the strongest scavenging effect of superoxide radical, hydroxyl radical and reducing power. This evaluation may shed the light on a better understanding on the litchi pulp polysaccharide as a potential functional antioxidant for its high antioxidant activity.

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