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Isolation, chemical characteristics and antioxidant properties of the polysaccharides from marine fungus *Penicillium* sp. F23-2

Hai-Hong Sun, Wen-Jun Mao *, Yin Chen, Shou-Dong Guo, Hong-Yan Li, Xiao-Hui Qi, Yan-Li Chen, Jian Xu

Institute of Marine Drug and Food, Ocean University of China, Qingdao, Shandong 266003, China

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ABSTRACT

Three polysaccharides PS1-1, PS1-2 and PS2-1 were successfully isolated from marine fungus *Penicillium* sp. F23-2 and the chemical characteristics were determined. Antioxidant properties of the three polysaccharides were evaluated by assays of various antioxidant *in vitro* systems. The results showed that PS1-1, PS1-2 and PS2-1 were primarily consisted of mannose with variable amounts of glucose and galactose, whereas their glucuronic acid contents, molecular weights and glycosidic linkage pattern were different. The three polysaccharides possessed good antioxidant properties, especially scavenging abilities on superoxide radicals and hydroxyl radicals. Moreover, PS2-1 showed higher antioxidant properties than PS1-1 and PS1-2 and should be explored as a potential antioxidant.

1. Introduction

Reactive oxygen species are generated by normal metabolic process or from exogenous factors and agents. Furthermore, these reactive oxygen species and free radical-initiated reactions are known to induce a wide variety of pathological effects, such as carcinogenesis, atherosclerosis and DNA damage as well as in degenerative processes associated with aging (Blander, Oliveira, Conboy, Haigis, & Guarente, 2003; Harman, 1993; Liu, Ooi, & Chang, 1997; Mau, Lin, & Chen, 2002). In order to reduce the damage to the human body, antioxidants are commonly used in processed foods. Antioxidants could alleviate the oxidative damage of a tissue indirectly by increasing cells' natural defenses and directly by scavenging the free radical species (Liu & Ng, 2000; Schinella, Tournier, Prieto, Mordujovich de Buschiazzo, & Ríos, 2002). However, most of antioxidants used are synthetic and 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 natural antioxidants so that they can protect the human body from free radicals and retard the progress of many chronic diseases (Luo, 2008; Nandita & Rajini, 2004).

Marine microorganisms are now considered as efficient producers of biologically active and/or chemically novel compounds. And no 'supply issue' will appear in the process of industrializations of the microbial products since scaled-up productions can be achieved through bioreactors of any capacity that can be designed nowadays (Trischman, Jensen, & Fenical, 1994). However, few reports dealing with the isolation and function of polysaccharides from marine microbial origins could be found although secondary metabolites of microbes from oceans have been well studied. In recent years, there has been a growing interest in polysaccharides obtained from marine microorganism that may have wide diversity of chemical structures and biological activities, and may be one of the most promising groups of antioxidant compounds. EPS2, a polysaccharide produced by a marine filamentous fungus *Keissleriella* sp. YS 4108 exhibited profound free radical-scavenging activities (Sun, Shan, Gao, & Tan, 2005; Sun, Wang, Fang, Gao, & Tan 2004). The polysaccharide from *Pantoea agglomerans* KFS-9 isolated from mangrove forest, had a high ability to quench hydroxyl radicals and superoxide radicals at low amounts, the EC_{50} values of scavenging abilities on hydroxyl radicals and superoxide radicals were 0.07 and 0.15 mg/ml, respectively (Wang, Jiang, Mu, Liang, & Guan, 2007). With today's interest in new renewable sources of chemicals and polymers, the polysaccharides isolated from marine microorganisms represent a potential source to be explored. Further work on the polysaccharides isolated from various marine microorganisms will aid in the development of new drugs and health foods.

In this study, three polysaccharides PS1-1, PS1-2 and PS2-1 were isolated from marine fungus *Penicillium* sp. F23 and their chemical characteristics and antioxidant properties were investigated.

* Corresponding author. Tel.: +86 532 8203 1560; fax: +86 532 8203 3054.
E-mail address: wenjunmqd@hotmail.com (W.-J. Mao).

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2. Experimental

2.1. Materials

Marine fungus *Penicillium* sp. F23-2 was kindly provided by Professor Gu Qianqun and Professor Zhu Weiming, Ocean University of China. Trifluoroacetic acid (TFA) and standard monosaccharides (glucose, rhamnose, xylose, arabinose, mannose, fucose, galactose, glucuronic acid, galacturonic acid, mannuronic acid and *N*-acetyl-β-D-glucosamine), 1,1-diphenyl-2-picrylhydrazyl (DPPH), thiobarbituric acid (TBA), trichloroacetic acid (TCA), butylated hydroxytoluene (BHT), ascorbic acid and ferrozine were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ethylenediamine tetraacetic acid (EDTA) was purchased from Fluka Chemie (Buchs, Switzerland). Dialysis membranes were from Lvniao (China, molecular weight cutoff 3500). Q Sepharose Fast Flow and Sephacryl S-300 HR were from the Pharmacia Co. (Sweden). All other reagents used were analytical grade.

2.2. Microbial strain and culture conditions

The marine fungus *Penicillium* sp. F23-2 (E148°44.8′, N19°24.1′, 5080 m) was initially grown on PDA medium (fresh potato 20%, dextrose 2% and agar 2%) in a Petri dish at 28 °C for 7 days and then inoculated to 500 ml Erlenmeyer flasks containing 150 ml of seed culture medium including potato 20%, maltose 2%, mannitol 2%, glucose 1%, monosodium glutamate 0.5%, peptone 0.5% on a rotary shaker at 160 rpm at 28 °C for 7 days. The cultivation experiments was performed in 1000 ml flasks containing 200 ml of the above medium and was inoculated with 5% of the seed medium culture at room temperature, and then was stored for 45 days. Finally, a total of 100 l broth was collected. The cultivated broth was separated from the filtrate using centrifugation at 4500 rpm for 10 min to separate mycelia, and the aqueous fermentation liquid was obtained.

2.3. Isolation and purification of the polysaccharides

The aqueous fermentation liquid was concentrated under reduced pressure at 40 °C, and precipitated by adding fourfold of the volume of 95% ethanol (v/v) and was kept at 4 °C for overnight. The precipitate was collected by centrifugation, was washed three times with 95% ethanol, anhydrous ethanol and acetone, respectively. It was then dissolved in distilled water and dialyzed in cellulose membrane tubing (molecular weight cutoff 3500) against distilled water at room temperature for three successive days. The retained fraction was recovered, concentrated under reduced pressure and lyophilized to obtain the crude polysaccharide. The crude polysaccharide (500 mg) was dissolved in 5 ml distilled water, centrifuged, and then the supernatant was applied to a column of anion exchange chromatography on Q Sepharose Fast Flow (2.6 cm × 50 cm) with the AKTA FPLC eluting with a step gradient of 0–2 mol/l NaCl with a flow rate of 60 ml/h. Total sugar content of the eluate was determined by the phenol-sulfuric acid method. The fractions eluted with distilled water and 0.25 mol/l NaCl were pooled, desalted and further purified by a Sephacryl S-300 HR column (1.6 cm × 100 cm) eluting with 0.2 mol/l sodium acetate at a flow rate of 12 ml/h. By the process, three purified polysaccharides were obtained and named as PS1-1, PS1-2 and PS2-1, respectively.

2.4. Composition analysis

Total sugar content was measured by the phenol-sulfuric acid method using mannose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Sulfate content was determined after hydrolysis with trifluoroacetic acid according to the methods of Therho and Hartiala (1971). Protein content was assayed using the method of Lowry (Bensadoun & Weinstein, 1976). Uronic acid content was determined by the carbazole-sulfuric acid method using glucuronic acid as standard (Bitter & Muir, 1962).

2.5. Monosaccharide composition analysis

2.5.1. Gas chromatography

The neutral monosaccharide compositions were determined and quantified by converting them into acetylated aldononitrile derivatives and detected by gas chromatography with a flame-ionization detector (Amornrut et al., 1999). Briefly, 10 mg polysaccharide was hydrolyzed with 10 ml of 2 mol/l trifluoroacetic acid at 105 °C for 6 h and the hydrolyzed products were precipitated by ethanol addition and dried. Derivation was carried out using the hydroxylammonium reagent according to the method of Zhang et al. (2008) with some modifications. The above dried product was dissolved with 0.1 ml pyridine adding 3 mg hydroxylammonium and 1 mg inositol (as internal reference) at 90 °C for 30 min. The mixture was cooled to room temperature. Acetic anhydride (0.1 ml) was then added to the mixture and the solution was incubated at 90 °C for another 30 min. Gas chromatography was performed on a HP6890 instrument with a SE-54 fused silica capillary column (320 μm i.d. × 50 m) (Agilent Technologies Co., Ltd., USA) equipped with flame-ionization detector (FID). The operation was performed using the following conditions: H_2: 1.5 ml/min; air: 200 ml/min; N_2: 1.5 ml/min; injection temperature: 250 °C; detector temperature: 250 °C; column temperature: 212 °C. Sugar identification was done by comparison with reference sugars (rhamnose, fucose, arabinose, xylose, mannose and galactose and glucose).

2.5.2. High performance liquid chromatography

The method for quantitative determination of carbohydrates by reversed-phase, high performance liquid chromatography after pre-column derivatization and UV detection has been used. Each dry polysaccharide hydrolate was derivatised with 1-phenyl-3-methyl-5-pyrazolone (Zhang, Xu, Zhang, Zhang, & Zhang, 2003). Briefly, 1 mg dry hydrolysate was dissolved in 100 μl of 0.3 mol/l NaOH, and then added to 120 μl of 0.5 mol/l methanol solution of 1-phenyl-3-methyl-5-pyrazolone and incubated at 70 °C for 1 h. Finally, the mixture was added 100 μl of 0.3 mol/l HC1 solution. The mixture was vigorously shaken and centrifuged for 5 min. The supernatant, containing the labeled carbohydrates, was finally filtered through 0.22 μm nylon membranes (MSI, Westborough, MA, USA) and 10 μl of the resulting solution was injected into the C18 column. The mobile phase was a mixture of 0.1 mol/l KH_2PO_4 (pH 10)-acetonitrile (83:17). The flow-rate was 1.0 ml/min and column temperature was 30 °C. Sugar identification was done by comparison with reference sugars (rhamnose, fucose, arabinose, xylose, mannose, galactose, glucose, galacturonic acid, glucuronic acid, mannuronic acid and *N*-acetyl-β-D-glucosamine).

2.6. Determination of molecular weight

Molecular weights of polysaccharides were estimated by high performance gel permeation chromatography (Yamamoto, Nunome, Yamauchi, Kato, & Sone, 1995). The column (A Shodex OH-pak KB-804 column, 0.8 cm × 30 cm, Japan) was maintained at 35 °C and the mobile phase was 0.1 mol/l Na_2SO_4 at a flow rate of 0.5 ml/min. The samples were dissolved in 0.1 mol/l Na_2SO_4 to reach a final concentration of 5 mg/ml and the sample solution was filtered through 0.45 μm filter membrane before injection. The eluate was monitored by a refractive index detector (Agilent 1100 Series). The column calibration was performed with various

standard dextrans (M_w: 5.9, 11.6, 22.8, 47.3, 112.0, 212.0, 404.0 and 788.0 kDa, respectively, purchased from Fluker). Calculation of the molecular weights of samples was carried out using the Angilent GPC software (USA).

2.7. Methylation analysis

Each sample was treated according to the method of Hakomori (1964) with some modification. Each sample (2 mg) was dissolved in DMSO (2 ml) and anhydrous NaH (100–200 mg) were then added. The mixture was stirred at room temperature for 1.5 h. CH_3I then was added to the mixture and stirred for a further 1.5 h. After the reaction was terminated with addition of water, the residue was extracted with $CHCl_3$. The extract was washed with distilled water and evaporated to dryness. The completion of methylation was confirmed by IR spectroscopy as the disappearance of OH bands. Methylated samples were hydrolyzed with 2 mol/l trifluoroacetic acid at 105 °C for 6 h. The methylated products were converted into their corresponding alditols by reduction with $NaBH_4$ and acetylated. The products were analyzed by gas chromatography–mass spectrometric (GC–MS) on DB 225 using a temperature gradient: first 100–240 °C with a rate of 5 °C/min; then keeping at 240 °C for 15 min. The peaks on the chromatogram were identified from their retention times. GC–MS was performed on an HP6890II instrument (Mao et al., 2008).

2.8. IR spectroscopy analysis

For IR spectroscopy, the polysaccharide was mixed with KBr powder, ground and then pressed into a 1 mm pellets for Fourier transform infrared (FT-IR) measurement in the frequency range of 4000–500 cm^{-1}. FT-IR spectra of the polysaccharides were measured on a Nicolet Nexus 470 spectrometer.

2.9. Assay of antioxidant properties

2.9.1. Scavenging ability on 1,1-diphenyl-2-picrylhydrazyl radicals

The scavenging activity on DPPH radicals was measured according to the method described by Braca et al. (2001). Briefly, 1 ml of sample solution at different concentrations (0, 0.8, 1.6, 2.4, 3.2, 4.0, 4.8, 5.6 and 6.4 mg/ml) was added to 4 ml of 0.004% methanol solution of DPPH, the reaction mixture was shaken vigorously and incubated for 30 min in the dark at room temperature, and the absorbance of the resulting solution was measured at 517 nm. The ability of scavenging the DPPH radicals was calculated using the following equation: scavenging ability (%) = $(1 - A_{sample}/A_{control}) \times 100$, where $A_{control}$ is the absorbance of control without the tested samples, and A_{sample} is the absorbance in the presence of the tested samples. The EC_{50} value (mg/ml) is the effective concentration at which the DPPH radicals are scavenged by 50%. BHT and ascorbic acid were used for comparison because they are standard antioxidants.

2.9.2. Scavenging ability on hydroxyl radicals

Hydroxyl radical-scavenging activity was determined according to the method of Smirnoff and Cumbes (1989) with a few modifications. 0.3 ml of 5 mmol/l orthophenanthroline, 0.8 ml of 0.75 mol/l phosphate buffer (pH 7.4) and 0.3 ml of 7.5 mmol/l $FeSO_4$ were added to 0.5 ml of sample solution at different concentrations (0, 0.8, 1.6, 2.4, 3.2, 4.0, 4.8, 5.6 and 6.4 mg/ml). Finally, 0.2 ml of 1% H_2O_2 was added, and the reaction mixture was then incubated at 37 °C for 60 min. The absorbance of the resulting solution was measured spectrophotometrically at 532 nm. The scavenging ability of hydroxyl radicals was calculated according to the equation: scavenging ability (%) = $(1 - A_{sample}/A_{control}) \times 100$, where $A_{control}$ is the absorbance of control without the tested samples, and A_{sample} is the absorbance in the presence of the tested samples. The EC_{50} value (mg/ml) is the effective concentration at which the hydroxyl radicals are scavenged by 50%. BHT and ascorbic acid were used for comparison.

2.9.3. Scavenging ability on superoxide radicals

The scavenging effect on superoxide radicals was assessed according to the method of Marklund and Marklund (1974) with a minor modification. Briefly, 3 ml of 0.05 mol/l Tris–HCl buffer (pH 8.2) and 1 ml of samples at different concentrations (0, 0.8, 1.6, 2.4, 3.2, 4.0, 4.8, 5.6 and 6.4 mg/ml) were incubated at 25 °C for 10 min, then 200 μl of pyrogallol at the same temperature were added to the mixture and the reaction was proceed at 25 °C for 4 min. Finally, the reaction system was terminated by the addition of 0.5 ml of HCl. The absorbance of the mixture was measured at 320 nm against the blank, The percentage inhibition of superoxide anion radicals scavenging was calculated using the following formula: scavenging ability (%) = $(1 - A_{sample}/A_{control}) \times 100$, where $A_{control}$ is the absorbance of control without the tested samples, and A_{sample} is the absorbance in the presence of the tested samples. The EC_{50} value (mg/ml) is the effective concentration at which the superoxide radicals are scavenged by 50%. BHT and ascorbic acid were used for comparison.

2.9.4. Lipid peroxidation inhibition

The inhibition of lipid peroxidation was determined by quantification of the lipid peroxide decomposition product malondialdehyde (MDA) based on reaction to thiobarbituric acid using egg yolk as oxydable substrate (Zhang & Yu, 1997). Briefly, 0.8 ml egg homogenate ($V_{egg\ yolk}$:V_{PBS} = 1:25, 0.1 mol/l phosphate buffer, pH 7.45) and 0.5 ml of sample solution at different concentrations (0, 0.8, 1.6, 2.4, 3.2, 4.0, 4.8, 5.6 and 6.4 mg/ml) were mixed, then 0.4 ml of 25 mmol/l $FeSO_4$ was added to initiate lipid peroxidation. After incubation at 37 °C for 60 min, 1.0 ml of 20% (w/v) trichloroacetic acid and 1.0 ml of 0.8% (w/v) thiobarbituric acid were added to quench the reaction, the resulting mixture was shaken and heated at 100 °C for 15 min, and then centrifuged at 6000 rpm for 10 min. The absorbance of the upper layer was measured at 532 nm. The inhibition of lipid peroxidation was calculated by the following equation: inhibition effect (%) = $(1 - A_{sample}/A_{control}) \times 100$, where $A_{control}$ is the absorbance of control without the tested samples, and A_{sample} is the absorbance in the presence of the tested samples. The EC_{50} value (mg/ml) is the effective concentration at which the lipid peroxidation is inhibited by 50%. BHT and ascorbic acid were used for comparison.

2.9.5. Reducing power

Reducing power was determined referring to the method (Dorman & Hiltunen, 2004) with some modifications. 0.5 ml of sample solution at different concentrations (0, 0.8, 1.6, 2.4, 3.2, 4.0, 4.8, 5.6 and 6.4 mg/ml) was mixed with 2.5 ml of 0.2 mol/l phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. 2.5 ml of 10% trichloroacetic acid was added to the mixture which was then centrifuged at 6000 rpm for 10 min. 2.5 ml of upper layer solution was mixed with 2.5 ml of water and 1 ml of 0.1% $FeCl_3$, and the absorbance was measured at 700 nm. BHT and ascorbic acid were used for comparison. Higher absorbance of the reaction mixture indicated greater reductive potential.

2.10. Statistical analysis

All bioassay results were expressed as means ± standard deviation (*SD*). The experimental data were subjected to an analysis of variance (ANOVA) for a completely random design. For each

polysaccharide, three samples were prepared for assays of every antioxidant attribute.

3. Results and discussion

3.1. Isolation and purification of the polysaccharides

Crude polysaccharide was extracted from marine fungus *Penicillium* sp. F23-2 and then was purified by Q Sepharose Fast Flow column, and two fractions were obtained (Fig. 1A). The fractions eluted with water and 0.25 mol/l NaCl contained abundant total sugar. The polysaccharides fractions were, respectively, pooled, dialyzed, lyophilized and were further fractionated on a Sephacryl S-300 HR column eluting with 0.2 mol/l sodium acetate buffer (Fig. 1B and C). By the process, three polysaccharides were obtained and named as PS1-1, PS1-2 and PS2-1, respectively. The three polysaccharides appeared as only a single and symmetrical sharp peak in high performance gel permeation chromatography with Shodex OHpak SB-804 column.

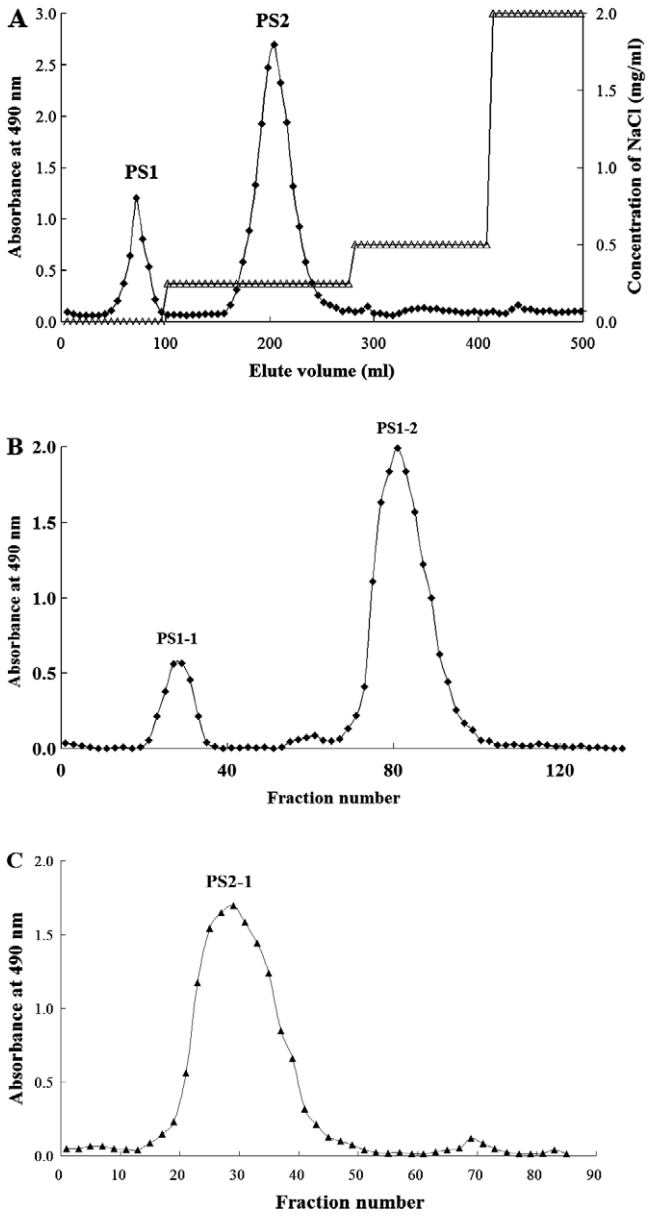

Fig. 1. Purification of the polysaccharides obtained from marine fungus *Penicillium* sp. F23-2. (A) A mixture of polysaccharides obtained from marine fungus *Penicillium* sp. F23-2 was applied to a Q Sepharose Fast Flow and eluted as described in Section 2. The fractions containing the polysaccharides were pooled and named as PS1 and PS2, respectively. (B) PS1 obtained on Q Sepharose Fast Flow was applied to a Sephacryl S-300 HR and eluted as described in Section 2. The fractions containing the polysaccharides were pooled and named as PS1-1 and PS1-2, respectively. (C) PS2 obtained on Q Sepharose Fast Flow was applied to a Sephacryl S-300 HR and eluted as described in Section 2. The fractions containing the polysaccharides were pooled and named as PS2-1.

3.2. Chemical compositions of the polysaccharides

The chemical compositions of the three polysaccharides isolated from marine fungus *Penicillium* sp. F23-2 were given in Table 1. The results showed that PS1-1, PS1-2 and PS2-1 contained minor amounts of protein (1.67–3.85%) and did not have any sulfate ester. PS2-1 contained 6.08% uronic acid, whereas the uronic acid contents of PS1-1 and PS1-2 were below detective limit. From gas chromatography, the three polysaccharides were found to contain mainly mannose with variable content of glucose and galactose. The molar ratios of the neutral monosaccharide were different for the three polysaccharides. The results from high performance liquid chromatography were similar to that of gas chromatography, except that PS2-1 contained glucuronic acid, which was consistent with the results of carbazole-sulfuric acid reaction. On the other hand, the molecular weight of PS2-1 was lower than that of PS1-1 and PS1-2 as determined by high performance gel permeation chromatography.

3.3. Methylation analysis

After successive permethylation of polysaccharide materials by the modified Hakomori method, completely methylated products were obtained. The completion of methylation was confirmed by IR spectroscopy as the disappearance of the hydroxyl group bands. The methylated products was depolymerized and converted into partially methylated alditol acetates for the GC–MS analysis. A major peak of 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-D-mannitol, originated from a 1,2-linked D-mannose residue, was found in PS1-1 (Table 2). 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-mannitol and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol were also detected in PS1-1 and indicated the presence of a 1,6-linked D-mannose residue and 1,3-linked D-glucose residue. The three kinds of glycosidic linkages may constitute the consecutive repeating units of PS1-1. A larger peak of 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-D-manitol, originated from a 1,2-linked D-mannose residure, was detected in PS1-2. 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-mannitol, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-mannitol and 1,2,5-tri-*O*-acetyl-

Table 1
Chemical compositions and molecular weights of PS1-1, PS1-2 and PS2-1 isolated from marine fungus *Penicillium* sp. F23-2.

Component	Polysaccharide		
	PS1-1	PS1-2	PS2-1
Protein content (%)	1.67	1.86	3.85
Uronic acid content (%)	nd[a]	nd[a]	6.08
Sulfate ester content (%)	nd[a]	nd[a]	nd[a]
Molecular weight (kDa)	67.4	15.4	12.1
Monosaccharide content (molar ratio)			
Mannose	28.0	18.9	22.2
Glucose	2.3	1.0	11.8
Galactose	1.0	6.7	7.1
Glucuronic acid	nd[a]	nd[a]	1.0
Galacturonic acid	nd[a]	nd[a]	nd[a]
Mannuronic acid	nd[a]	nd[a]	nd[a]

[a] Below detection limit (0.001).

Table 2
Methylation analysis of PS1-1, PS1-2 and PS2-1 isolated from marine fungus *Penicillium* sp. F23-2.

Retention time (min)	Methylation product	Deduced linkage pattern	Molar ratio		
			PS1-1	PS1-2	PS2-1
21.60	1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-mannitol	Man(1→	1.56	1.92	–
21.64	1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol	Glc(1→	–	–	2.06
21.70	1,5-di-*O*-acetyl-1-deuterio-2,3,4,6-tetra-*O*-methyl-D-galactitol	Gal(l→	–	–	1.00
24.27	1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-D-mannitol	→2)Man(1→	6.98	3.08	8.21
24.33	1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-D-glucitol	→2)Gal(1→	–	1.00	–
24.56	1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol	→3)Glc(1→	1.00	–	–
24.98	1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-mannitol	→4)Man(1→	–	2.97	2.12
25.09	1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-mannitol	→6)Man(1→	3.38	1.05	–
25.12	1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol	→6)Glc(1→	–	–	4.15
25.19	1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-galacitol	→6)Gal(1→	–	–	1.21
27.99	1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methyl-D-galacitol	→2,4)Gal(1→	–	1.97	2.08

3,4,6-tri-*O*-methyl-D-galacitol were also found in PS1-2 and indicated the presence of a 1,4-linked D-mannose residue, 1,6-linked D-mannose residue and 1,2-linked D-galacose residue, respectively. The results showed that PS1-2 may be composed of the four kinds of glycosidic linkages repeating units. In addition, PS1-2 should have a partially branched structure because it had branching point as a 2,4-linked D-galactose residue. A peak of 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-D-mannitol, originated from a 1,2-linked D-mannose residure, appeared most abundantly in PS2-1. 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-mannitol, and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-galacitol were also detected in PS2-1 and indicated the presence of a 1,6-linked D-glucose residue, 1,4-linked D-mannose residue and 1,6-linked D-galacose residue, respectively. The four kinds of glycosidic linkages may constitute the consecutive repeating units of PS2-1. In addition, PS2-1 should have a partially branched structure because it had branching point as a 2,4-linked D-galactose residue.

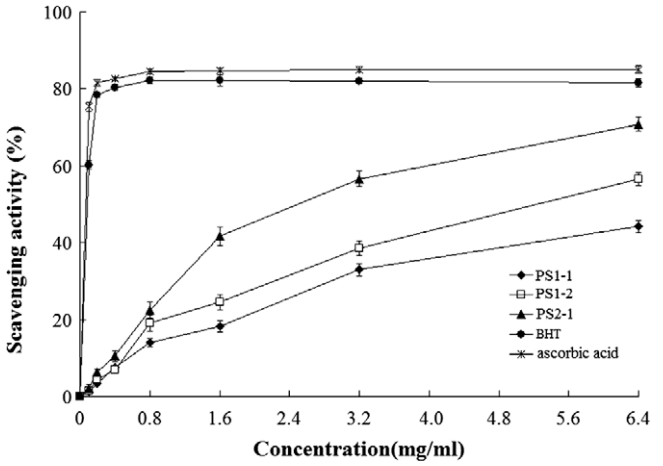

Fig. 2. Scavenging ability of PS1-1, PS1-2 and PS2-1 isolated from marine fungus *Penicillium* sp. F23-2 on 1,1-diphenyl-2-picrylhydrazyl radicals. Results were representative of three separated experiments.

3.4. IR spectroscopy

FT-IR spectra of PS1-1, PS1-2 and PS2-1 showed the signals at 3380–3419 cm^{-1} were from the stretch vibration of O–H existed in the hydrogen bond of the molecules; Signals at 2929–2936 cm^{-1} were from the stretch vibration of –CH; 1054–1059 cm^{-1}, the stretch vibration of C–O and change angle vibration of O–H. Any signals corresponding to sulfated ester were not found in IR spectra of the three polysaccharides. IR spectra of the three polysaccharides exhibited the obvious characteristic absorption at 812 cm^{-1} corresponding to the existence of mannose, and showed the absorption at around 880 cm^{-1}, typical for β configuration. In addition, FT-IR spectra of PS2-1 showed the signals at 1649 cm^{-1} were attributed to the asymmetric stretch vibration of COO^-; 1397 cm^{-1} were due to the symmetric stretch vibration of COO^- and the stretch vibration of C–O within –COOH.

3.5. Antioxidant properties

3.5.1. Scavenging ability on DPPH radicals

The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical-scavenging activities of materials (Nagai, Inoue, Inoue, & Suzuki, 2003; Soares, Dins, Cunha, & Ameida, 1997; Naik et al., 2003). The method is based on the reduction of the absorbance of methanolic DPPH solution at 517 nm in the presence of a proton-donating substance, due to the formation of the diamagnetic molecular by accepting an electron or hydrogen radicals. Scavenging abilities of the three polysaccharides isolated from marine fungus *Penicillium* sp. F23-2 on DPPH radicals were listed in Fig. 2. At 0.8 mg/ml, the three polysaccharides showed scavenging abilities of 13.98–22.46% on DPPH radicals. At 6.4 mg/ml, the scavenging effects increased to 44.2–70.9%. Scavenging abilities of the polysaccharides on DPPH radicals were in a concentration-dependent manner. Moreover, PS2-1 had higher scavenging ability than PS1-1 and PS1-2 in the same concentration. The differences of the scavenging ability of the three polysaccharides on DPPH radicals may be due to their chemical features discrepancy. PS2-1 could be better advantageous than PS1-1 and PS1-2 for reacting with DPPH radicals to convert them to more stable products and thereby terminate radical chain reactions. Tsiapali et al. (2001) also stated that the free radical-scavenging activity was partially related to monosaccharide constituents. In the study, BHT and ascorbic acid were used as positive controls. At 0.1–6.4 mg/ml, the scavenging abilities of BHT and ascorbic acid on DPPH radicals were in the range of 81.58–82.29% and 84.58–85.04%, respectively.

3.5.2. Scavenging ability on hydroxyl radicals

Hydroxyl radical is considered to be a highly potent oxidant, which can react with most biomacromolecules functioning in living cells and induce severe damage to the adjacent biomolecules. Thus, removing hydroxyl radical is important for antioxidant defense in cell or food systems (Aruoma, 1998). Scavenging abilities of the three polysaccharides isolated from marine fungus *Penicillium* sp. F23-2 on hydroxyl radicals were listed in Fig. 3. The three polysaccharides isolated from marine fungus *Penicillium* sp. F23-2 exhibited a strong scavenging activity on hydroxyl radicals. A significant increase of the scavenging activity was observed at the concentration range (0–0.8 mg/ml) of the polysaccharides. The

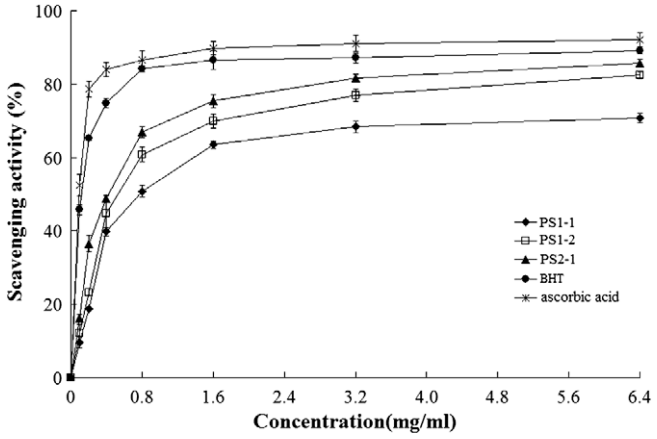

Fig. 3. Scavenging ability of PS1-1, PS1-2 and PS2-1 isolated from marine fungus *Penicillium* sp. F23-2 on hydroxyl radicals. Results were representative of three separated experiments.

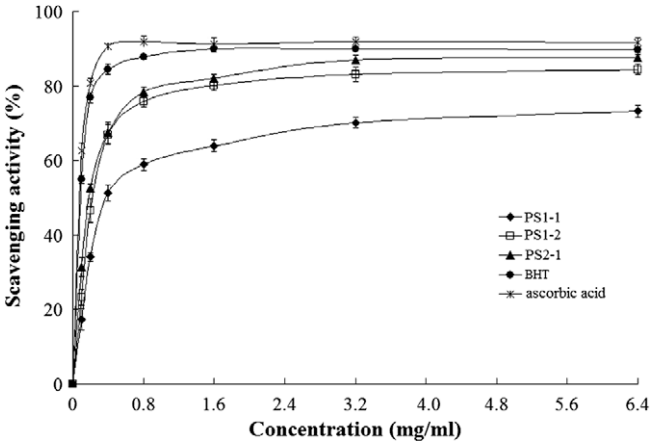

Fig. 4. Scavenging ability of PS1-1, PS1-2 and PS2-1 isolated from marine fungus *Penicillium* sp. F23-2 on superoxide radicals. Results were representative of three separated experiments.

scavenging ability of polysaccharides on hydroxyl radicals was in a concentration-dependent way. At 0.8 mg/ml, the abilities of PS1-1, PS1-2 and PS2-1 to scavenge hydroxyl radicals were 50.80%, 60.86% and 66.91%, respectively. At 6.4 mg/ml, the scavenging abilities of PS1-1, PS1-2 and PS2-1 were 70.83%, 82.46% and 85.67%, respectively. Moreover, PS2-1 was found to have higher scavenging activity on hydroxyl radicals than PS1-1 and PS1-2, and the scavenging ability of PS2-1 was close to that of BHT at 6.4 mg/ml. The present results proved that the polysaccharides isolated from marine fungus *Penicillium* sp. F23-2 were good scavengers for hydroxyl radicals. The polysaccharides minimized the concentration of Fe^{2+} in the Fenton reaction, and the scavenging abilities of the polysaccharides might be due to the active hydrogen donating ability of hydroxyl substitutions of polysaccharides. PS2-1 had better potency to donate hydrogen to reactive hydroxyl radicals than PS1-1 and PS1-2. However, the scavenging abilities of the three polysaccharides on hydroxyl radicals were all relatively lower than that of ascorbic acid at the same concentrations.

3.5.3. Scavenging ability on superoxide radicals

In cellular oxidation reactions, superoxide radicals are normally formed first, and its effects can be magnified because it produces other types of cell-damaging free radicals and oxidizing agents (Liu & Ng, 2000). Superoxide radical can be generated by pyrogallol autooxidation and it can produce a colored compound. Resulting from a color change from purple to yellow, the absorbance at 320 nm increased when the superoxide anion was scavenged by an antioxidant, which can represent the content of superoxide radicals and indicate the antioxidant activity of the sample (Chen, Xie, Nie, Li, & Wang, 2008). Scavenging abilities of the three polysaccharides isolated from marine fungus *Penicillium* sp. F23-2 on superoxide radicals were listed in Fig. 4. A significant increase of the scavenging activity was observed at the concentration range (0–0.8 mg/ml) of the polysaccharides. At 0.8 mg/ml, the abilities of PS1-1, PS1-2 and PS2-1 to scavenge superoxide radicals were 58.87%, 75.88% and 67.47%, respectively. The three polysaccharides had a noticeable effect on scavenging superoxide radicals at high concentration. At 6.4 mg/ml, the scavenging abilities of PS1-1, PS1-2 and PS2-1 were 73.27%, 84.48% and 87.66%, respectively. PS2-1 had significantly higher scavenging activities on superoxide radicals than PS1-1 and PS1-2. Moreover, the scavenging ability of PS2-1 was similar to that of BHT at 6.4 mg/ml. These results suggested that PS2-1 was an effective scavenger for superoxide radicals, and it might be advantageous for preventing injury induced by superoxide radicals in pathological conditions. At 0.1–6.4 mg/ml, scavenging abilities of ascorbic acid on superoxide radicals were in the range of 62.52–91.88%.

3.5.4. Lipid peroxidation inhibition

Lipid peroxidation is a typical free radical oxidation and proceeds via a cyclic chain reaction. Moreover, it is likely involved in numerous pathological events, including inflammation, metabolic disorders and cellular aging (Wiseman & Halliwell, 1996). In this study, a yolk suspension was used as the model system to evaluate sample against the inhibitory activities of lipid peroxidation. Egg yolk lipids undergo rapid non-enzymatic peroxidation when incubated in the presence of ferrous sulphate. Lipid peroxidation inhibitions of the three polysaccharides isolated from marine fungus *Penicillium* sp. F23-2 were given in Fig. 5. At 0.8 mg/ml, inhibition rates of PS1-1, PS1-2 and PS2-1 were 42.32%, 53.58% and 64.70%, respectively. The inhibiting effects of the three polysaccharides on the lipid peroxidation were concentration dependent. At 6.4 mg/ml, the inhibition rate of PS1-1, PS1-2 and PS2-1 increased to 65.90%, 68.99% and 79.19%, respectively. The present results suggested that the three polysaccharides had strong inhibition effects of lipid peroxidation, especially PS2-1. However, at 0.8–6.4 mg/ml, inhibition rates of BHT and ascorbic acid were in the range of 81.32–85.75% and 88.74–91.12%, respectively.

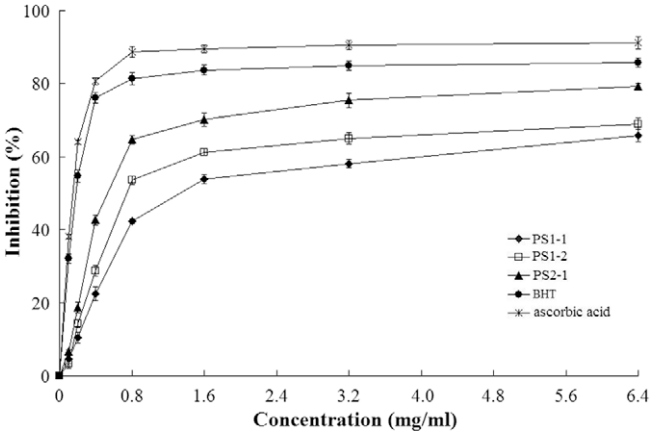

Fig. 5. Inhibition of lipid peroxidation of PS1-1, PS1-2 and PS2-1 isolated from marine fungus *Penicillium* sp. F23-2. Results were representative of three separated experiments.

3.5.5. Reducing power

The reducing capacity of a compound may serve as an indicator of its potential antioxidant activity. In the reducing power assay, the yellow color of test solution changes into various shades of green and blue colors depending on the reducing power of antioxidant samples. The reducing power of the three polysaccharides isolated from marine fungus *Penicillium* sp. F23-2 was shown in Fig. 6. At 0.8 mg/ml, PS1-1, PS1-2 and PS2-1 showed a reducing power of 0.59, 0.64 and 0.75, respectively. Like the other antioxidant attributes, the reducing powers of the three polysaccharides were correlated with its concentrations. At 6.4 mg/ml, the reducing power of PS1-1, PS1-2 and PS2-1 were 0.86, 1.07 and 1.18, respectively. Moreover, PS2-1 showed higher reducing power than PS1-1 and PS1-2. At the 6.4 mg/ml, reducing power of PS2-1 was close to that of BHT. The present results revealed that the polysaccharides isolated from marine fungus *Penicillium* sp. F23-2 were effective in reducing power.

3.5.6. EC_{50} in antioxidant properties

The antioxidant properties are summarized in Table 3. Effectiveness in antioxidant properties is inversely correlated with EC_{50} values. The three polysaccharides isolated from marine fungus *Penicillium* sp. F23-2 showed strong scavenging abilities on superoxide radicals and hydroxyl radicals as evidenced by their low EC_{50} value (<1.2 mg/ml), especially PS2-1 and PS1-2 (<0.5 mg/ml). With regard to reducing power, the EC_{50} value of the polysaccharides was less than 2.0 mg/ml. All EC_{50} values of lipid peroxidation inhibition were in range of 1.39–3.12 mg/ml, indicating that the three polysaccharides were good effective as antioxidants. With regard to scavenging abilities on superoxide radicals and hydroxyl radicals, the effectiveness was in the decreasing order of PS2-1 > PS1-2 > PS1-1. In addition, all EC_{50} values of scavenging ability on DPPH radicals were in the range of 2.53–6.81 mg/ml. Thus, the polysaccharides showed scavenging abilities on DPPH radicals, but were moderate effect in the antioxidant attribute. The present investigation suggested that the polysaccharides isolated from marine fungus *Penicillium* sp. F23-2, especially PS2-1, could be beneficial to help human body alleviate oxidative damages in cell induced by oxygen radicals and decelerate the progress of many chronic diseases, and effectively used as potential antioxidants, as a possible food supplement or ingredient in the pharmaceutical industry.

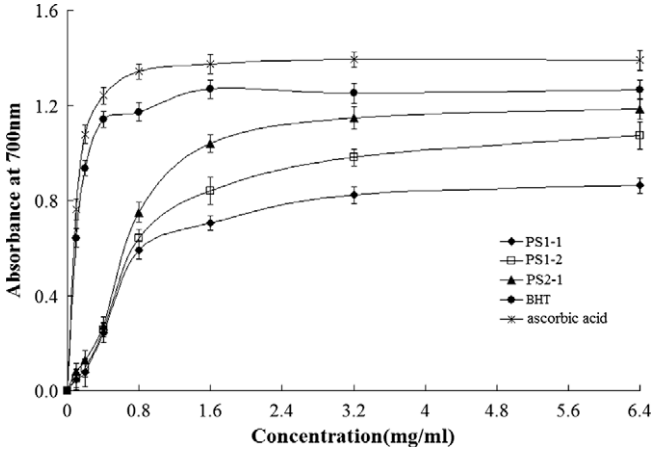

Fig. 6. Reducing power of PS1-1, PS1-2 and PS2-1 isolated from marine fungus *Penicillium* sp. F23-2. Results were representative of three separated experiments.

Table 3
EC_{50} values of PS1-1, PS1-2 and PS2-1 isolated from marine fungus *Penicillium* sp. F23-2 in antioxidant properties.

Antioxidant property	EC_{50}[a] value (mg/ml)		
	PS1-1	PS1-2	PS2-1
Scavenging ability on DPPH radicals	6.81 ± 0.07	4.56 ± 0.04	2.53 ± 0.03
Scavenging ability on hydroxyl radicals	1.14 ± 0.04	0.48 ± 0.03	0.36 ± 0.02
Scavenging ability on superoxide radicals	1.13 ± 0.03	0.26 ± 0.02	0.18 ± 0.01
Lipid peroxidation inhibition	3.12 ± 0.04	2.38 ± 0.03	1.39 ± 0.02
Reducing power	1.47 ± 0.03	1.81 ± 0.02	1.92 ± 0.03

[a] EC_{50} value, the effective concentration at which 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, hydroxyl radicals and superoxide radicals were scavenged by 50%; lipid peroxidation was inhibited by 50% and the absorbance was 0.5 at 700 nm for reducing power, respectively. EC_{50} value was obtained by interpolation from linear regression analysis.

4. Conclusion

Three polysaccharides PS1-1, PS1-2 and PS2-1 isolated from marine fungus *Penicillium* sp. F23-2 were mainly consisted of mannose with small amounts of glucose and galactose. However, molar ratios of the neutral monosaccharide, molecular sizes, uronic acid contents and glycosidic linkage pattern of the three polysaccharides were different. The polysaccharides exhibited strong antioxidant properties, especially scavenging ability on superoxide radicals and hydroxyl radicals. The antioxidant activity of the polysaccharides may be related to monosaccharide component, molecular size, structure and conformation. These monosaccharides in the polysaccharides are potent reductive agents as they can supply hydrogen, which when combined with radical forms a stable radical to terminate the radical reaction. PS2-1 had the highest antioxidant activity also suggested that a relatively low molecular weight and higher glucuronic acid might have increased antioxidant activity. The differences of the antioxidant abilities of the polysaccharides were directly due to their chemical features discrepancy. To elucidate the mechanism for this antioxidant behavior further investigations of structure characteristics of the polysaccharides isolated from marine fungus *Penicillium* sp. F23-2 are in progress.

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