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Correlation evaluation of antioxidant properties on the monosaccharide components and glycosyl linkages of polysaccharide with different measuring methods

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ABSTRACT

The relationships of antioxidant properties (AOPs), measured by four conventional *in vitro* methods, with monosaccharides and glycosyl linkages in the polysaccharide, were evaluated using multiple linear regression analysis with minor modifications. Polysaccharides extracted from culture broth filtrates of *Lentinula edodes* were used as model samples for evaluation. Results indicate that the composition of monosaccharides and the type of glycosyl linkage modulates the AOPs of the polysaccharides. The AOPs of the polysaccharides were dependent on the ratios of different monosaccharides in the composition. Among the monosaccharides, rhamnose was the most significant determinant factor associated with AOPs. The glycosyl linkages of the monosaccharides also affected the anti-oxidation characteristics of the polysaccharides. Specifically, the arabinose 1 → 4 and mannose 1 → 2 linkages of the side-chain were significantly related to the reducing power, whereas the glucose 1 → 6 linkage and arabinose 1 → 4 linkages were related to the scavenging on $DPPH^-$ radicals.

1. Introduction

Superoxide and hydroxyl radicals are potent oxidants that can react with all biological molecules, such as DNA, proteins, lipids, and carbohydrates; meanwhile, induced oxidative stress can mediate a wide variety of pathological effects (Chattopadhyay, Ghosh, Sinha, Chattopadhyay, Karmakar, & Ray, 2010; Kardošová & Machová, 2006; Tsai, Song, Shih, & Yen, 2007). Normally, living cells have the ability of self-protection against oxidative damage through several defense mechanisms, such as the enzymatic conversion of reactive oxygen species (ROS) into less toxic substances and detoxification by reaction with antioxidants (Tsai et al., 2007). The discovery of polysaccharides, a new antioxidant active agent that can be extracted from plants or fungi, have pushed many researchers to explore many clinically serviceable dietary supplements, as they offer advantages in the prevention of human diseases (Li, Zhou, & Han, 2006; Li, Zhang, Zeng, Huang, & Wang, 2006; Tsai et al., 2007; Tseng, Yang, & Mau, 2008).

Previously, polysaccharides extracted from mushrooms have been shown to be effective and non-toxic, and offers the ability to scavenge free radicals (Liu, Ooi, & Chang, 1997). Among various mushrooms, the shiitake mushroom, *Lentinula edodes*, which is widely cultivated in Korea, Russia, Taiwan, China, and Japan (Campbell & Slee, 1987; Fox, Burden, Chang, & Peberdy, 1994), have been intensively studied for their immune-modulating, antimicrobial, antioxidant, and anti-atherogenic activities (Hobbs, 2000). Liu et al. (1997) reported that proteins in polysaccharide extracts contribute directly in free radical scavenging activities. Particularly, the sulfated, acetylated, and phosphorylated derivatives, as well as the water extract from polysaccharides, could exhibit significant antioxidant properties (AOPs) in *in vitro* experiments (Kardošová & Machová, 2006; Zhang, Yu, Li, Zhang, Xu, & Li, 2003). Recently, Chen, Xie, Nie, Li, and Wang (2008) reported that a water-soluble protein-bound polysaccharide extracted from the fruiting bodies of *Ganoderma atrum* containing mannose (Man), galactose (Gal), and glucose (Glu) in a molar ratio of 1:1.28:4.91, with an average molecular weight about 1013 kDa, exhibits strong AOP. Li and Zhou (Li, Li, & Zhou, 2007) also showed the multiple AOPs of polysaccharides extracted from *Lycium barbarum* fruits (e.g., rhamnose (Rha), xylose (Xyl), arabinose (Ara), fucose (Fuc), Glu, and Gal). Bučková, Labuda, Šandula, Križková, Štěpánek, and Ďuračková (2002) found that AOP and protection against DNA damage by chelation of transition met-

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als of polysaccharides was in the order of mannan (*Candida krusei*), extracellular glucomannan (*Candida utilis*), mannan (*Candida albicans*), and glucomannan (*C. utilis*).

In general, the AOPs of polysaccharides are influenced by chemical characteristics like molecular weight, degree of branching (DB), types of monosaccharides, intermolecular associations of polysaccharides, glycosidic branching, and modification of polysaccharides. However, the detailed mechanisms of antioxidant effects reflected by monosaccharide composition and glycosyl linkage in polysaccharides remain largely unexplored, even if the vital roles of glucans and glycans have been well-established (Liu et al., 1997).

In previous papers, the monosaccharide composition, molecular weight, and structural linkage of polysaccharides extracted from 10 regional *L. edodes* were investigated and compared for genetic and structural similarities and differences (Lo, Kang, Wang, & Chang, 2007; Lo, Jiang, Chao, & Chang, 2007). All polysaccharides isolated from these mushrooms exhibited similar molecular weight ranging between 1×10^4 Da and 3×10^6 Da. A major form of polysaccharide linkage with a backbone of $(1 \rightarrow 4)$-glucan and side chains of $(1 \rightarrow 6)$-glucan was identified. Ara $(1 \rightarrow 4)$ and Man $(1 \rightarrow 2)$ linkages also existed in all polysaccharides. Based on composition analysis, the heterogeneous monosaccharides contained Ara, Xyl, Man, Gal, Glu, Fuc, and Rha. Structural analysis did not indicate presence of proteins and absence of uronic acids. The only differences in these linkages manifested in their monosaccharide compositions, leading to different degrees of backbone and branch formations.

Most of the extracts exhibited significant AOPs. All of the previously mentioned studies clearly demonstrate the AOPs of polysaccharides in fungi. In the present work, the AOPs of the extracted polysaccharides were assayed by using conventional methods; that is, by measuring conjugated diene, reducing power ability, scavenging ability on 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals, and chelating ability on ferrous ions.

Numerous methods have recently applied mathematics and statistics in the classification, summary, and/or simplification of complex data, which consequently have assisted in the interpretation of the relationships between structural characteristics and bio-functions of polysaccharides (Xu & Hagler, 2002). Meanwhile, regression analyses have been broadly used to predict sources or structure–function relationships in biological sciences (Lo, Jiang, et al., 2007; Pytelaa & Klimešova, 2011).

The aim of this study is to utilize this method to evaluate the relationship between *in vitro* AOP and chemical characteristics (i.e., monosaccharide ratio and glycosyl linkage) in polysaccharides by using conventional measurement methods. This study is the first to conduct research on the relationship between AOPs and monosaccharide components (i.e., Ara, Xyl, Man, Gal, Glu, Fuc, and Rha) of the L15 polysaccharide.

2. Experimental

2.1. Chemicals and reagents

Linoleic acid, ascorbic acid, potassium ferricyanide, trichloroacetic acid, ferric chloride, butylated hydroxylanisole (BHA), DPPH, ferrozine, ethylenediamine-tetraacetic acid (EDTA), Ara, Xyl, Man, Gal, Glu, Fuc, Rha, dextrose, and cellulose were purchased from Sigma (St. Louis, MO, USA). Sodium phosphate buffer and citric acid were purchased from Merck (Darmstadt, Germany). Methanol (ACS) was purchased from Echo (Hsinchu, Taiwan). The methanolic solution containing 0.2 mM DPPH radicals was obtained from Fluka (Buchs, Switzerland).

2.2. L. edodes strains, polysaccharide isolation, and structure characterization

Ten isolates of *L. edodes* were used in this study. These include Tainung No. 1 “white cap” (L1) and “red cap” (L4) from Taiwan; Japanese 271 (L11, L15), Jongxing 5 (L6), Jongxing 8 (L10), Hey-King-Gang (L21), and Jong-Wen 600 (L23) from Japan; and No. 135 (L24) and No. 939 (L25) from China. These following 10 isolates of *L. edodes* were grouped into three distinct clusters by amplified fragment length polymorphism (AFLP) analysis: (1) L24 and L25 isolates from China; (2) L1 and L4 isolates from Taiwan; (3) L6, L10, L11, L15, L21, and L23 isolates from Japan (Lo, Kang, et al., 2007). The culture broth of the mycelia of the *L. edodes* was submerged and fermented in a medium (pH 4.5) containing 2% oat, 0.5% yeast extract, 0.1% KH_2PO_4, 0.05% $MgSO_4$, and 0.15% $CaCO_3$, with reciprocating shaking (150 rpm min^{-1}) for 14 days at 26 °C.

The polysaccharides were isolated from culture broth filtrates (CBF) of different *L. edodes*. The CBF was added onto three volumes of 95% ethanol and stored at 4 °C overnight. The precipitate was collected by centrifugation, followed by washing with 75% ethanol, and then freeze-dried to obtain a culture precipitate (L15 polysaccharide). The L15 polysaccharide was treated with boiling water (100 °C) prior the preparation of sample solutions (0.5, 1, 1.5, 3, 4.5, 6, and 7.5 mg/ml). After filtration, a 0.22-μm filter (Milli-pore) was used for the AOP assay.

Analysis of monosaccharide composition and glycosyl linkage in the different polysaccharides had been described previously in detail (Lo, Kang, et al., 2007; Lo, Jiang, et al., 2007). Briefly, the compositions of monosaccharide of polysaccharides from the CBF (2 mg) were determined by methods described by Blakeney, Harris, Henry, and Stone (1983). The amounts of neutral monosaccharides in the polysaccharides were analyzed as alditol acetates by gas chromatography (GC, Varian 3800), with myo-inositol used as internal standard. To analyze glycosyl linkage, a sample containing 1.0 mg of each polysaccharide was methylated at 25 °C using the modified NaOH–DMSO (dimethyl sulfoxide) method (Bao, Wang, Dong, Fand, & Xiao, 2002; Capek & Hribalova, 2004; Komaniecka & Choma, 2003). The methylated product was then hydrolyzed, reduced, acetylated, and analyzed by Gas Chromatography–Mass Spectrometry (GC–MS) *(HP 6890/MSD 5973)* (Lo, Kang, et al., 2007).

2.3. Conjugated diene method

The antioxidant activities (AOAs) of the polysaccharide were evaluated according to the conjugated diene method, as described in literature (Lingnert, Vallentin, & Eriksson, 1979). In the method, each polysaccharide sample (1.5 mg/mL, 1 ml) was mixed with 2 ml of 10 mM linoleic acid emulsion stabilized with Tween-20 in 0.2 M sodium phosphate buffer (pH 6.5). These were put onto a test tube and placed in darkness at 37 °C in order to achieve oxidation. After incubation for 15 h, 8 ml of 80% methanol in de-ionized (DI) water was added onto each tube and mixed thoroughly. The absorbance of the mixture at 234 nm was re-measured against a blank. AOA was calculated as

$$\text{AOA}\ \ (\%) = \left\{1 - \left[\frac{A_{234}\ \text{nm of}\ \ (\text{sample})}{A_{234}\ \text{of}\ \ (\text{control})}\right]\right\} \times 100\%.$$

The blank comprises DI water and the control, which consisted of DI water and reagent solution without the polysaccharide extract. The strongest AOA value is denoted by 100%. Ascorbic acid (1.5 mg/ml) and BHA (1.5 mg/ml) were used as the positive controls for comparison.

2.4. Reducing power ability

The reducing power ability of polysaccharide was determined according to the method described in literature (Oyaizu, 1986; Tseng et al., 2008). Each polysaccharide sample (1.5 mg/ml, 2.5 ml) was mixed with 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. Then, 2.5 ml of 10% trichloroacetic acid was added and thoroughly mixed. Then, the mixture was centrifuged at 3000 rpm for 10 min. The upper layer of solution (5 ml) was collected and mixed with 5 ml of DI water and 1 ml of 0.1% ferric chloride. The mixture was allowed to stand for 10 min. The absorbance at 700 nm was measured against a blank. A higher absorbance in the reaction mixture indicates greater reducing power ability. For comparison, ascorbic acid (1.5 mg/ml), BHA (1.5 mg/ml), and citric acid (1.5 mg/ml) were used as positive controls.

2.5. Scavenging ability on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals

Each polysaccharide sample (1.5 mg/ml, 1 ml) was mixed with 1 ml of methanolic solution containing 0.2 mM DPPH radicals. The mixture was shaken vigorously and left to stand for 30 min in darkness. Next, absorbance at 517 nm was measured against a blank. A lower absorbance in the reaction mixture indicates higher free radical scavenging activity. Scavenging DPPH radical ability was calculated as

$$\text{Scavenging ability}\,(\%) = \left[\frac{A_{517}\ \text{of control} - A_{517}\ \text{of sample}}{A_{517}\ \text{of control}}\right] \times 100\%.$$

Ascorbic acid (1.5 mg/ml), BHA (1.5 mg/ml), and citric acid (1.5 mg/ml) were used as positive controls.

2.6. Chelating ability on ferrous ions

The chelating ability of the polysaccharide was determined according to the method prescribed in literature (Dinis, Madeira, & Almeida, 1994; Li, Zhang, et al., 2006). Each polysaccharide sample (1.5 mg/ml, 1 ml) was mixed with 3.7 ml of methanol and 0.1 ml of 2 mM ferrous chloride. Then, the mixture was allowed to stand for 30 s. Reaction was initiated by adding 5 mM ferrozine (0.2 ml). The mixture was allowed to stand at room temperature for 10 min. Finally, the absorbance at 562 nm of mixture solution was determined against a blank. A lower absorbance indicates higher chelating power. The percentage of chelating ability was calculated as

$$\text{Chelating ability}\ \ \% = \left[\frac{A_{562}\ \text{of control} - A_{562}\ \text{of sample}}{A_{562}\ \text{of control}}\right] \times 100\%.$$

For comparison, BHA (2 mg/ml) and EDTA (2 mg/ml) were used as positive controls.

2.7. Multiple linear regression analysis (MLRA)

MLRA is the method of statistics in regression that used to analyze the relationship between single response variable (dependent variable) with two or more controlled variables (independent variables) (Chen, 2004; Ghani & Ahmad, 2011). MLRA was conducted following a previous report but with minor modifications (Guisan, Edwards, & Hastie, 2002). In this research, AOPs value (AOA, reducing power ability, scavenging ability or chelating ability) was used as response variable, while monosaccharide components (Ara, Xyl, Man, Gal, Glu, Fuc, and Rha) or glycosyl linkage types (glucose (1 → 4), glucose (1 → 6), glucose (1 → 4, 6), Ara (1 → 4) and Man (1 → 2) linkages) were taken as variables. The backward elimination method was selected for stepwise-type procedures (Bakker, Busscher, Zanten, Vries, Klijnstra, & Mei, 2004). This method starts with all the variables in the model and variables are excluded on the basis of their non-significance (Chen, 2004). The backward eliminations are often a very good variable selection procedure (Ghani & Ahmad, 2011).

The relationship of AOPs and monosaccharide composition or glycosyl linkage value was built-up by generalizing the straight-line equation,

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \cdots + \beta_p X_p + e \quad (1)$$

where Y is the response variable (AOPs value); β_0 is the constant (intercept); X_1, X_2, …, X_p denote vectors of p predictor variables (monosaccharide composition or glycosyl linkage value) (see Table 1, Supplementary Table S1); and β_1, β_2, …, β_p denote vectors of p regression coefficients. Each predictor variable had its own coefficient. The outcome variable was predicted by combining all the variables multiplied by their respective coefficients plus an error term. The relationship was determined using the least square fitting technique. A residual analysis was used to check on model fit (Faraji, Crowe, Besant, Sokhansanj, & Wood, 2004). The residual is defined as follows:

$$\text{Residual} = \text{observed value} - \text{predicted value} \quad (2)$$

MLRA was performed using SPSS and S-PLUS. The normal P–P plot of the regression residuals in the SPSS was obtained in order to assess whether the normality assumption was violated. The plot supporting the normality assumption was constructed for the cumulative proportions of the residuals against the cumulative proportions of the normal distribution.

2.8. Comparison of the AOPs of L15 polysaccharide and its monosaccharide components

The AOPs of the polysaccharide and its monosaccharide components were determined and compared by measuring conjugated diene, reducing power ability, scavenging ability, and chelating ability at the dose of 1.5 mg/ml. Different concentrations (0.5–7.5 mg/ml) of L15 polysaccharide were also tested individually by using antioxidant evaluation methods.

3. Results

The relationships of AOPs, as measured by the four conventional *in vitro* methods (i.e., conjugated diene, reducing power, DPPH· radical-scavenging, and ferrous ions chelating activity methods) and structural characteristics of polysaccharides (i.e., monosaccharides and glycosyl linkages in the composition) were evaluated individually using MLRA.

3.1. Conjugated diene method

The conjugated diene method is widely used for monitoring lipid oxidation *in vitro*. In the method, the oxidation of linoleic acid was measured as an increase in 234-nm absorbance due to conjugated diene formation (Esterbauer, Striegl, Puhl, & Rotheneder, 1989; Esterbauer, Rotheneder, Waeg, Striegl, & Juergens, 1990). Antioxidants can inhibit conjugated diene formation. The polysaccharides of the 10 *L. edodes* strains were used to test for AOA to compare for ascorbic acid and citric acid. The results in Fig. 1 show that the AOAs of the polysaccharides (1.5 mg/ml) ranged from 14.56% to 58.27%. The strains with increasing AOA (% reduction) were in the following order: L21 > L25 > L4 > L24 > L11 > L23 > L1 > L15 > L6 > L10. The AOA of ascorbic acid and BHA at 1.5 mg/ml were 50.29% and 87.41%, respectively.

Table 1
Raw data of main monosaccharide compositions and glycosyl linkage abundances in different *Lentinula edodes* strains.

Structure/*L. edodes*	L1	L4	L6	L10	L11	L15	L21	L23	L24	L25
Monosaccharide (molar ratio)										
Arabinose	0.46	0.79	0.49	0.57	0.59	0.59	0.50	0.54	0.27	0.42
Xylose	0.37	0.75	0.35	0.41	0.46	0.42	0.35	0.39	0.14	0.29
Mannose	2.45	3.46	1.89	1.98	3.19	2.17	1.99	1.88	0.89	2.00
Galactose	0.11	0.36	0.15	0.26	0.38	0.15	0.22	0.22	0.00	0.12
Glucose	4.20	1.62	3.03	4.13	4.14	7.47	2.81	3.96	10.95	3.59
Rhamnose	0.00	0.02	0.01	0.00	0.01	0.03	0.03	0.02	0.06	0.03
Fucose	0.00	0.04	0.01	0.00	0.02	0.01	0.03	0.01	0.02	0.02
Methylated monosaccharide (abundance $\times 10^{-6}$)										
2,3,6-Me_3-Glcp[a]	3.35	3.10	3.90	5.70	4.90	5.80	4.40	4.60	7.70	3.20
2,3,4-Me_3-Glcp[b]	0.90	1.70	1.40	1.70	1.70	1.50	1.40	2.20	0.70	1.05
2,3-Me_2-Glcp[c]	0.90	0.90	1.70	1.90	1.70	2.40	1.40	1.40	3.60	1.10
3,4,6-Me_3-Manp[d]	1.40	3.50	1.70	2.20	2.50	1.70	1.70	1.30	0.10	0.10
2,3-Me_2-Arap[e]	2.60	0.50	0.90	0.50	1.20	2.90	1.10	0.70	0.30	0.70

[a] 1,4,5-Tri-O-acetyl-1-deuterio-2,3,6-tri-O-methyl-D-glucitol.
[b] 1,5,6-Tri-O-acetyl-1-deuterio-2,3,4-tri-O-methyl-D-glucitol.
[c] 1,4,5,6-Tetra-O-acetyl-1-deuterio-2,3-di-O-methyl-glucitol.
[d] 1,2,5-Tri-O-acetyl-1-deuterio-3,4,6-tri-O-methyl-mannitol.
[e] 1,4,5-Tri-O-acetyl-1-deuterio-2,3-di-O-methyl-arabinitol.

Table 2
The *P*-values and model fits (R^2) of multiple linear regression backward analysis for monosaccharide-AOP and glycosyl linkage-AOP relationship.

Structure/antioxidant activities	Antioxidation	Reducing power	Scavenging ability	Chelating effect
Arabinose	0.042	>0.100	0.013	0.079
Xylose	>0.100	>0.100	>0.100	0.091
Mannose	0.023	0.079	0.092	>0.100
Galactose	>0.100	>0.100	>0.100	>0.100
Glucose	0.060	0.083	0.081	0.070
Rhamnose	0.004	0.044	>0.100	0.015
Fucose	>0.100	0.037	>0.100	>0.100
Model fit (R^2)	0.867	0.647	0.704	0.821
2,3,6-Me_3-Glcp	>0.100	>0.100	0.031	>0.100
2,3,4-Me_3-Glcp	>0.100	>0.100	>0.100	>0.100
2,3-Me_2-Glcp	>0.100	>0.100	0.065	>0.100
3,4,6-Me_3-Manp	>0.100	0.045	>0.100	>0.100
2,3-Me_2-Arap	>0.100	0.011	>0.100	>0.100
Model fit (R^2)	–	0.709	0.558	–

–, model is not fit.

As proven by MLRA, there are differences in the monosaccharide composition and glycosyl linkage for the 10 strains of polysaccharides. The analytical results could also predict the characteristic components in each polysaccharide strain associated with the AOA. By employing a backward analysis on multiple linear regression, the *P*-values for each monosaccharide-AOA relationship (Supplementary Table S1 and Table 2) were 0.042, 0.023, 0.060, and 0.004 for Ara, Man, Glu, and Rha, respectively, and >0.1 for the other monosaccharides. A *P*-value <0.1 was considered statistically significant. The coefficient of determination (R^2) was 0.867.

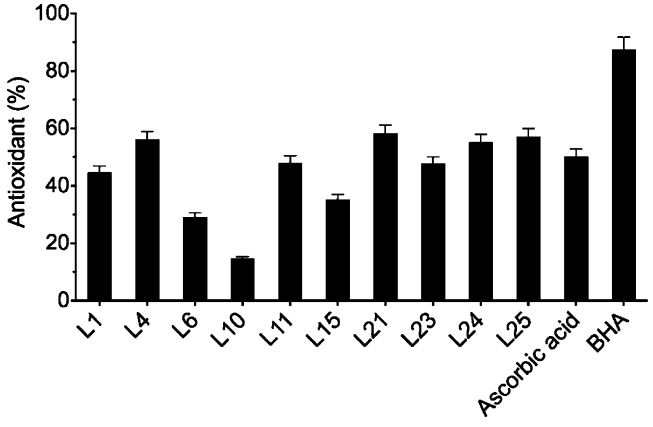

Fig. 1. Antioxidant activity (%) using polysaccharide of all strains.

Predicting the relationship of monosaccharide ratio with AOA (Supplementary Table S1) can be derived by

$$Y(\%\,\mathrm{AOA}) = 0.652 - 0.897X_{\mathrm{Ara}} + 0.207X_{\mathrm{Man}} - 3.25 \times 10^{-2}X_{\mathrm{Glu}} + 9.099X_{\mathrm{Rha}} \quad (3)$$

where X is the concentration of monosaccharide measured in the extract. In Eq. (3), the positive coefficient for X_{Man} and X_{Rha} indicates that AOA increases with increasing X_{Man} and X_{Rha}, whereas the negative coefficients for X_{Ara} and X_{Glu} indicate that the AOA decreases with corresponding increases in X_{Ara} and X_{Glu}. The positive coefficient of X_{Rha} had a stronger effect on AOA than on X_{Man}.

The *P*-values for testing linear relationship between the glycosyl linkage and AOA were all >0.1, and the value for R^2 showed that the model was not fit (Table 2). These indicate that no correlation exists between glycosyl linkage and AOA.

3.2. Reducing power ability

Generally, the reducing characteristics of chemicals are associated with their ability to break the chain reaction of free radicals by incorporating a hydrogen atom. Results in Fig. 2 show that the L15 polysaccharide had the highest reducing capacity compared with the other polysaccharide strains. The reducing power levels of the

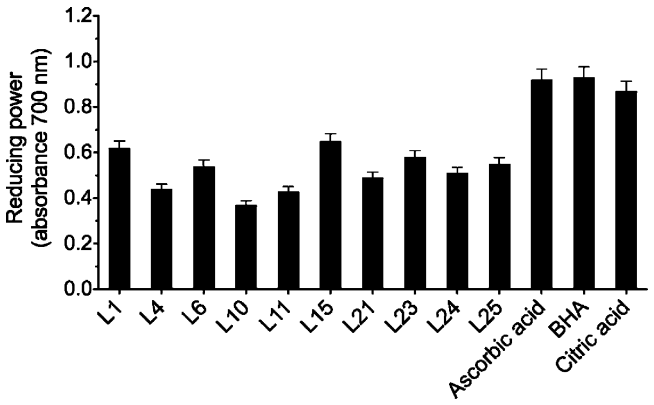

Fig. 2. Reducing power assay using polysaccharide of all strains.

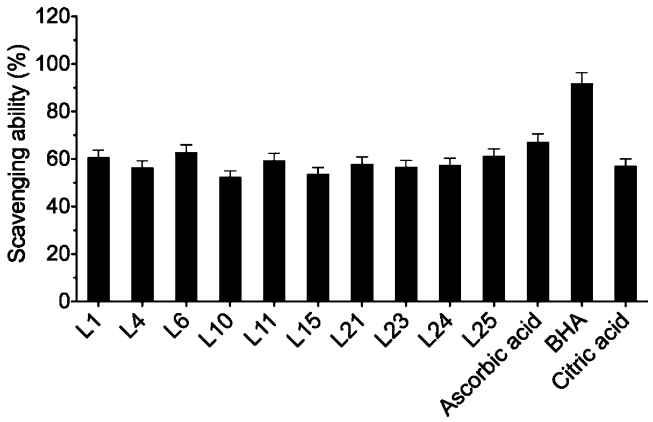

Fig. 3. Scavenging ability (%) on 1,1-diphenyl-2-picrylhydrazyl radicals using polysaccharide of all strains.

polysaccharides were in the range of 0.37–0.65, which were significantly lower than that of 1.5 mg/ml of ascorbic acid (0.92), BHA (0.93), and citric acid (0.87).

From the MLRA, the *P*-values were 0.079, 0.083, 0.044, and 0.037 for Man, Glu, Rha, and Fuc, respectively (Table 2). These reveal that the four monosaccharides influenced greatly the reducing power ability. Based on the MLRA, an equation for predicting the reducing power ability (Y) of each polysaccharide could be derived by

$$Y = 0.546 + 0.186X_{\text{Man}} - 5.44 \times 10^{-2}X_{\text{Glu}} + 16.853X_{\text{Rha}} - 19.389X_{\text{Fuc}} \quad (4)$$

The R^2 was 0.647, which suggests a weak relationship between monosaccharide compositions and reducing power ability (Table 2).

The type of glycosyl linkage and reducing power ability (Y) were positively correlated at $R^2 = 0.709$.

$$Y = 0.739 - 4.28 \times 10^{-2}X_{\text{Ara1}\rightarrow 4\ \text{linkage}} + 6.903 \times 10^{-2}X_{\text{Man1}\rightarrow 2\ \text{linkage}} \quad (5)$$

Li et al. (2007) reported that hydroxyl groups of polysaccharides can act as electron donors. They react with free radicals and convert them into more stable products, thereby terminating radical chain reaction. Thus, based on the findings, we can safely assume that the glycosyl linkage of the side chain structure has contributed greatly in the reducing reaction.

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

DPPHs are widely used as substrates for the evaluation of the free radical scavenging ability of antioxidants. The method is based on the reduction of methanolic $DPPH^-$ solution into non-radical form DPPH-H in the presence of a hydrogen-donating antioxidant (Li et al., 2007). In the results of the present study, all 10 polysaccharides (1.5 mg/ml) showed strong scavenging abilities ranging from 52.41% to 62.84% (Fig. 3). These were comparable to that of 1.5 mg/ml of ascorbic acid (67.25%), BHA (91.76%), and citric acid (57.18%).

As shown by the *P*-values obtained from the MLRA, there was a significant relationship between monosaccharide composition and scavenging ability. The *P*-values were 0.013, 0.092, and 0.081 for Ara, Man, and Glu, respectively, and >0.1 for other monosaccharides. These findings indicate that the levels of Ara, Man, and Glu in the polysaccharides are important facets of the scavenging ability. To predict the relationship between monosaccharide ratio and scavenging ability (Y), the present work employed

$$Y = 0.845 - 0.360X_{\text{Ara}} + 3.937 \times 10^{-2}X_{\text{Man}} - 7.63 \times 10^{-3}X_{\text{Glu}} \quad (6)$$

The R^2 was 0.704 (Table 2), suggesting a direct relationship between monosaccharide composition and scavenging ability. The MLRA revealed a weak relationship between glycosyl linkage and scavenging ability. The R^2 was 0.558 (Table 2). An equation correlating the glycosyl linkage and scavenging ability (Y) was derived by

$$Y = 0.823 - 5.17 \times 10^{-2}X_{\text{Glu1}\rightarrow 4\ \text{linkage}} + 7.430 \times 10^{-2}X_{\text{Glu1}\rightarrow 4,6\ \text{linkage}} \quad (7)$$

The scavenging ability of antioxidants was based on the reduction of methanolic $DPPH^-$, manifested by the hydrogen-donating role and the reaction from $DPPH^-$ to the non-radical of DPPH-H. Tseng et al. (2008) and Mau, Tsai, Tseng, and Huang (2005) found that the scavenging abilities of polysaccharides (5 mg/ml) from the filtrate were 36.4% and 50.0%. Results in this study showed that the monosaccharide composition on Ara, Man, and Glu in the polysaccharides were markedly observable for the free radical scavenging ability.

The water-soluble exopolysaccharide (WSEPS) from *Pantoea agglomerans* strain KFS-9, which is composed of Ara, Glu, Gal, and glucuronic acid, may be associated with strong reactive oxygen species-scavenging activity (Dong & Fang, 2001; Wang, Jiang, Mu, Liang, & Guan, 2007). The scavenging ability of polysaccharides may be due to the presence of hydrogen from the specific monosaccharide compositions and their side chain linkages. Polysaccharides are known to bind radicals and radical ions, and consequently, terminate radical chain reactions (Chen et al., 2008).

3.4. Chelating ability on ferrous ions

Metal chelating activity is widely known as one of the mechanisms of AOP. Ferrozine forms complexes with Fe^{2+} quantitatively. In the presence of antioxidants, the complex formation is disrupted, resulting in the decrease of red color of the complex (Li et al., 2007). The chelating ability of the compounds with Fe^{2+} was determined based on whether the Fe^{2+}-chelation could inhibit metal-catalyzed lipid oxidation. From the test results (Fig. 4), the chelating ability of polysaccharides ranged between 35.72% (L10) and 56.41% (L21). BHA (1.5 mg/ml), as a control, showed a chelating ability of 51.38%. For the EDTA (1.5 mg/ml), a well-known chelating agent for ferrous ions, the chelating ability was 97.46%.

The *P*-values for the linear relationship between the levels of monosaccharide and chelating ability are listed in Table 2. The *P*-values were 0.079, 0.091, 0.070, and 0.010 for Ara, Xyl, Glu, and Rha, respectively. For other monosaccharides, the *P*-values were >0.1. The results of the MLRA indicate that a high correlation existed between monosaccharide compositions and chelating ability (Y) on

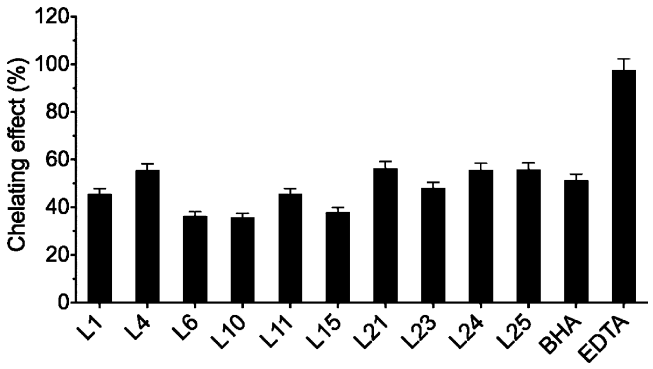

Fig. 4. Chelating effect (%) using polysaccharide of all strains.

ferrous ions ($R^2 = 0.821$). This was derived by

$$Y = 0.926 - 1.173X_{Ara} + 0.981X_{Xyl} - 2.14 \times 10^{-2}X_{Glu} + 4.498X_{Rha} \quad (8)$$

Eq. (8) shows that the level of Rha in the polysaccharide was the dominant component in modulating the *Y* response variable (chelating ability).

Upon examination of the relationship between glycosyl linkage and chelating ability, all *P*-values were >0.1 (Table 2), indicating the structure of glycosyl linkage was not related to chelating ability.

3.5. Comparison of the AOPs of the L15 polysaccharide and its monosaccharide components

The AOPs of the L15 polysaccharide and its monosaccharides (e.g., dextrose, cellulose, Ara, Xyl, Man, Gal, Glu, Fuc, and Rha) were tested for comparison with BHA, which was used as positive control (Table 3). Results indicate that the AOA (%) of the examined components in 1.5 mg/ml was in the order of Xyl (40.19%), cellulose (39.65%), Gal (39.83%), Ara (39.8%), dextrose (39.65%), Rha (39.34%), Fuc (38.59%), Glu (38.47%), Man (38.4%), and L15 (35.24%). Results also showed that the AOA of the L15 polysaccharide increased with concentration and ranged from 22.82% to 44.54% for concentrations of 0.5–7.5 mg/ml (Fig. 5A). BHA showed markedly higher AOA (87.41%) at 1.5 mg/ml, as opposed to the other examined species. These results suggest that the concentration of the L15 polysaccharide at over 6 mg/ml may be with more molecular structure hydrogen atoms; these neutralized the free radicals in this assay system.

Ara, Xyl, Man, Gal, Glu, Fuc, Rha, Dex, and cellulose showed limited reducing power ability at 1.5 mg/ml (Table 3). However, results also showed that the reducing power ability of L15 polysaccharides ranged from 0.34 to 0.95 for the concentration of 0.5–7.5 mg/ml (Fig. 5B). There was no significant difference in the reducing power ability between L15 polysaccharide at 3 mg/ml concentration (0.96) and BHA at 1.5 mg/ml concentration (0.93), as evidenced by the statistical analyses. Thus, the L15 polysaccharide over 1.5 mg/ml concentration could act as a strong electron donor and a radical chain reaction terminator.

As shown in Table 3, the L15 polysaccharide scavenging abilities of 53.71% on DPPH was highest at 1.5 mg/ml. The scavenging abilities of Ara, Xyl, Man, Gal, Glc, Fuc, Rha, Dex, and Cel showed negative abilities (Table 3). The L15 polysaccharide exhibited concentration-dependent antiradical activity by inhibiting the DPPH-radical (Fig. 5C). BHA showed a higher degree of scavenging ability (91.76%) than that of the L15 polysaccharide at each concentration.

The 1.5 mg/ml samples with increasing chelating ability were in the following order (Table 3): L15 (38%) > cellulose (36.09%) > Fuc (24.41%) > Ara (23.86%) > Gal (21.24%) > Xyl (20.94%) > Glu (19.7%) > Rha (18.81%) > dextrose (10.57%). The result depicted that the L15 polysaccharide, as a polymer structure, demonstrated higher effective capacity for iron binding compared with monosaccharide. The metal chelating ability of the L15 polysaccharide (51.76–51.26%) at the concentration range of 3–7.5 mg/ml and BHA (51.38%) at 1.5 mg/ml is almost similar (Fig. 5D).

4. Discussion

To the best of our knowledge, there have only been a few reports using objective statistical methods to conduct a comprehensive analysis of experimental data as a means of deducing polysaccharide structure–function relationships, particularly because data related to these are limited (Lo, Jiang, et al., 2007). The MLRA adopted in the current research allowed for the detection of differences in the major characteristic compositions (i.e., polysaccharide antioxidant competence) in each of the 10 strains of *L. edodes*. Specifically, the present study showed that the AOPs, using the four conventional methods (for different antioxidant reaction mechanisms) on multiple linear regression, could investigate and discern the relationships between AOPs and polysaccharide structural characteristics. Rha and Man generally show positive coefficients for the four different MLRA models (Eqs. (3), (4), (6) and (8)). Thus, the monosaccharide property (i.e., Rha and Man) and linkage type of side chain (Eqs. (5) and (7)) were important factors in the antioxidant effect. This further indicates that the Ara 1 → 4 and Man 1 → 2 linkages of the side chain in the polysaccharides were significantly related to reducing power ability. Meanwhile, the Glu 1 → 6 and Ara 1 → 4 linkages were related to the scavenging effect on $DPPH^-$ radicals. The results of this present work clearly show that the side chain structure in the monosaccharide (mainly, Rha) of the polysaccharide plays an important role in the inhibition of conjugated diene

Table 3
Comparison of antioxidant properties of monomers and polymers.

	Antioxidant (%)	Reducing power	Scavenging (%)	Chelating (%)
Monomers				
Ara	39.80	0.30	−0.41	23.86
Xyl	40.19	0.25	−0.22	20.94
Man	38.40	0.27	−3.25	18.29
Gal	39.83	0.25	−1.67	21.24
Glu	38.47	0.17	−0.88	19.70
Fuc	38.59	0.15	−2.84	24.41
Rha	39.34	0.26	−1.20	18.81
Dextrose	39.65	0.15	−2.87	10.57
Polymers				
Cellulose	40.13	0.13	−5.90	36.09
L15 polysaccharide	35.24	0.65	53.71	38.00
Control				
BHA	87.41	0.93	91.76	51.38

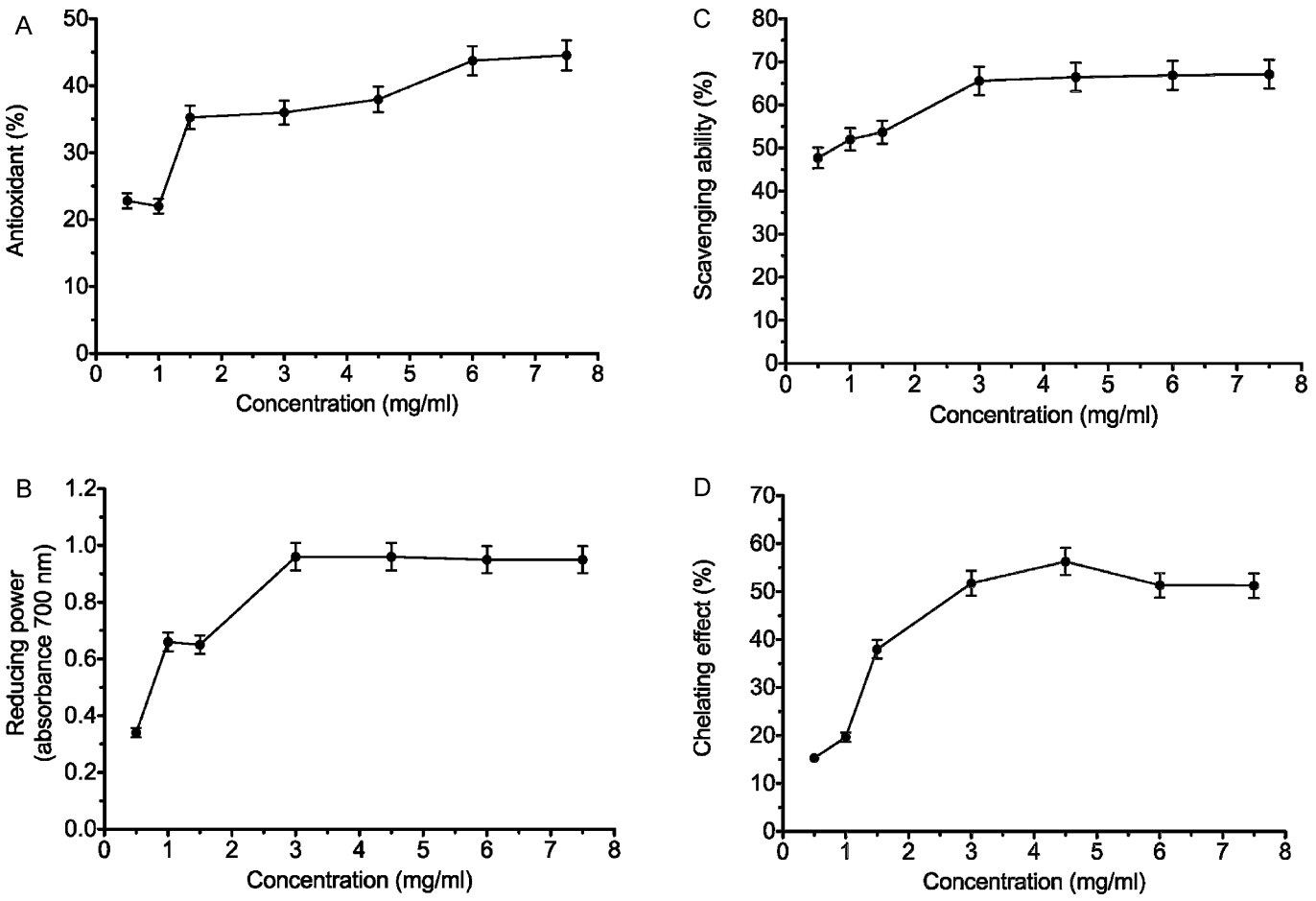

Fig. 5. (A) Antioxidant activity (%) using difference concentration of L15 polysaccharide. (B) Reducing power assay using difference concentration of L15 polysaccharide. (C) Scavenging activity (%) using difference concentration of L15 polysaccharide. (D) Chelating effect (%) using difference concentration of L15 polysaccharide.

formation, inclusion of a hydrogen atom, and in chelating metal ions.

Interestingly, Glu, which has been widely presented in all polysaccharide strains and is assumed as the backbone of polysaccharide structures, obtained low negative coefficient (Eqs. (3), (4), (6) and (8)), as opposed to all other AOPs. Therefore, the length of the backbone may not be an important factor in modulating AOP. Meanwhile, the effect of molecular mass on AOP was not obvious, as both poly- and oligosaccharides have similar levels of AOP (Kardošová & Machová, 2006).

The results further demonstrate that the AOPs of polymers and monomers, including Ara, Xyl, Man, Gal, Glu, Fuc, Rha, and dextrose, have scarce and weak scavenging ability, reducing power ability, and chelating effect at 1.5 mg/ml. Cellulose is a simple homopolymer of β-D-glucopyranose units linked by β-(1-4)-glycosidic bond in the formula $(C_6H_{10}O_5)_n$, where n ranges at 500–5000, depending on the source of the polymer. Particularly, except for the chelating effect, the polymeric structure cellulose and the monomeric structure Glu and dextrose were similar to the AOA, reducing power and scavenging ability. Trombino, Cassano, Bloise, Muzzalupo, Tavano, and Picci (2009) reported that the cellulose hydrogels containing ferulic moieties could be viewed as the best antioxidants against lipid peroxidation. Chen et al. (2008) mentioned that crude polysaccharide has better antioxidant effects than the purified components of the polysaccharide. Our results also show that the L15 polysaccharide exhibited better AOPs than each of its monosaccharide components (i.e., Ara, Xyl, Man, Gal, Glu, Fuc, and Rha). These results strongly suggest that special compositions, such as functional groups or monosaccharide combinations of the side chain in polymers, may induce and increase free radical scavenging ability.

5. Conclusion

The *in vitro* AOPs of polysaccharides extracted from the CBF of *L. edodes* are dependent on the composition and ratio of monosaccharide and the type of glycosidic side chain. This mechanism underscores the four different AOPs, which have been modulated by different characteristics of the polysaccharide structure (monosaccharide composition and glycosyl linkage). This study not only contributes to the understanding of the relationships between structures and AOPs, but also simplifies the problems encountered in research works on glyco-antioxidant molecular mechanisms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2011.04.056.

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