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Purification and antioxidant activities of intracellular zinc polysaccharides from *Pleurotus cornucopiae* SS-03

Jianjun Zhang [a], Zhao Ma [a], Lan Zheng [a], Guoyin Zhai [a], Liqin Wang [a], Mengshi Jia [b], Le Jia [a,*]

[a] College of Life Science, Shandong Agricultural University, Taian, Shandong 271018, PR China
[b] The Second High School of Taian, Taian, Shandong 271000, PR China

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ABSTRACT

Intracellular zinc polysaccharides (IZPS) from *Pleurotus cornucopiae* SS-03 were extracted and purified, and three subfractions (IZPS-1, IZPS-2, and IZPS-3) were separated by DEAE-52 cellulose anion-exchange column chromatography. They showed certain scavenging effects on superoxide anion ($O_2^{\bullet -}$) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, and positive rising of reducing power *in vitro*. All the subfractions were found upregulated the enzyme activities of superoxide dismutase (SOD), GSH peroxide (GSH-Px) and catalase (CAT) significantly, decreased the contents of malondialdehyde (MDA) and lipid peroxidation (LPO) remarkably *in vivo*. In addition, five monosaccharides components, including rhamnose, xylose, mannose, glucose, and galactose with the molar ratio of 1.65:0.05:1:1.50:1.07 and contents of 29.25%, 0.78%, 19.45%, 29.84%, and 20.67%, were investigated by gas chromatography.

1. Introduction

Pleurotus cornucopiae, which is classified in the genus *Pleurotus* of the family *Pleurotaceae* and found in Korea, Japan, China, Siberia, Turkey, Europe, North America, has been appreciated as an edible mushroom (Jang et al., 2001). It has high levels of proteins, carbohydrates, minerals (calcium, phosphorus, iron) and vitamins (thiamin, riboflavin and niacin) as well as low fat (Manzi, Gambelli, Marconi, Vivanti, & Pizzoferrato, 1999). Recently, it also has been used for medicine purposes due to its diverse physiological activities such as scavenging the free radicals, antitumour, immunoregulation and fatigue resistance (Chance, Sies, & Boveris, 1979; Petr, Vendula, Věra, & Jiří, 2005).

Zinc is an essential trace element in the human body and it participates in various pathways of metabolism (Jia et al., 2006). It has a recognized action on more than 300 enzymes by participating in their structure or in their catalytic and regulatory actions (Tsunenobu & Robert, 1996). It is a structural ion of biological membranes and closely related to protein synthesis. Zinc also interacts with important hormones involved in bone growth such as somatomedin-c, osteocalcin, testosterone, thyroid hormones, and insulin (Ortega, Quintas, & Andreś, 1999). After submerged fermentation with zinc-compound (zinc acetate), *P. cornucopiae* can significantly enrich zinc with polysaccharides, known as zinc-polysaccharides (Petr et al., 2005). To date, there has been no report on the antioxidant of intracellular zinc polysaccharides (IZPS) *in vitro* and *in vivo*.

In this work, IZPS from *P. cornucopiae* SS-03 was purified. Biological antioxidant activities of the subfractions were investigated, using free radicals scavenging abilities *in vitro*, enzyme activities and anti-lipid peroxide *in vivo* as antioxidant indicators, respectively.

2. Materials and methods

2.1. Microorganism and culture conditions

A strain of *P. cornucopiae* SS-03 was provided by our laboratory and used in this experiment. All experiments of liquid cultivation were carried out in the basal liquid medium composition (potato 200 g/l, glucose 20 g/l, KH_2PO_4 1.5 g/l and $MgSO_4 \cdot 7H_2O$ 1 g/l, with natural pH), and each 250-ml flask contained 100 ml basal medium and 3 ml zinc acetate (3 g/l). The mycelium was dried at 60 °C, shattered, and storied at 4 °C until used.

2.2. Separation and purification of IZPS

The powered of mycelium was mixed with proper water for 4 h (60 °C), then centrifugation (3600 r/min, 15 min), the supernatant liquid was mixed with 3 volumes of 95% ethanol (v/v), stirred

* Corresponding author. Tel.: +86 0538 8249958; fax: +86 0538 8248696.
E-mail address: ourlaboratory@163.com (L. Jia).

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vigorously, and kept at 4 °C for 24 h. After centrifugation, the precipitated IZPS was washed 3 times by 85% ethanol. Protein was removed by the method of Sevag until no macroscopic denatured-protein could be seen at the layer between the water and the chloroform–amyl alcohol. The purified IZPS was lyophilized.

The lyophilized powered of IZPS (0.5 g) was dissolved in distilled water (50 ml), and fractionated on DEAE-52 cellulose anion-exchange column (1.6 cm × 20 cm, DEAE–52 cellulose was purchased from Pharmacia Co. (New Jersey, USA)). The column was eluted with distilled water, and then with gradient solutions (0.1 M, 0.3 M, 0.5 M, and 1 M NaCl), at a flow rate of 5 ml/tube. Twenty-five test tubes were collected in each gradient (including gradient of water). The polysaccharide subfractions were collected with a fraction collector, detected at 490 nm by a spectrophotometer, until the polysaccharides were negative. The major fraction was collected and then freeze dried with vacuum freezing drying apparatus (Christ, Osterode, Germany). The dried subfractions of IZPS were dissolved in distilled water, and the concentration of IZPS was determined by the phenol–sulfuric acid method, using glucose as the standard (Chaplin & Kennedy, 1994).

2.3. Animal selection and experimental design

This study was approved by the animal ethics guidelines of Institutional Animal Ethics Committee. Twenty-five male mice (Kunming strain, 8-week-old, 20 ± 2 g) were obtained from Taibang Biologic Products Inc. and housed in cages under controlled conditions (temperature 22 ± 2 °C, humidity 60–65%, and 12 h light/dark cycles). All mice were aging induced by injecting D-galactose (0.2 ml). Diets and tap water were available *ad libitum*. The mice were randomly divided into 5 groups (5 in each group) including the normal control group (NCG), vitamin C positive group (CPG), IZPS-1 group, IZPS-2 group, and IZPS-3 group. The four dose groups (CPG, IZPS-1, IZPS-2 and IZPS-3) received doses of 150 mg/kg body weight of mice by gastric gavage (0.01 ml/g body weight) with a syringe. The control group (NCG) received the same volume of physiological saline solution (0.9%, w/v). All mice were fed every other day for 30 consecutive days. After overnight fasting following the last drug administration, the mice were sacrificed by cervical dislocation. Blood samples, from orbital sinus, were centrifuged at 4000 r/min at 4 °C for 10 min to afford the serums required. The heart, liver, spleen, and kidney were excised, weighed and homogenized in phosphate buffer solutions (PBS, 0.2 M, pH 7.4) based on a proportion of 1:9 (1 g wet organ to 9 ml PBS). The suspensions were centrifuged (4000 r/min, 4 °C, 10 min) and collected for the next antioxidant experiments *in vivo*.

2.4. Determinations of antioxidant activities of IZPS in vitro

2.4.1. Superoxide anion ($O_2^{\bullet -}$)

The scavenging ability on super anion was determined according to the method of Zhang, Zhang, Hong, and Dai (2003) with slight modification. Each sample (1 ml) was mixed with 4.5 ml Tris–HCl buffer (pH 8.2, 50 mM), and 3.2 ml deionized water. After keeping warm at the temperature of 25 °C for 20 min, 0.3 ml pyrogallol (keeping warm 20 min, 25 °C) was added immediately. The mixtures were reacted in the bath for 20 min (25 °C), using 1 ml vitamin C (5%, w/v) to end the reaction. The absorbance was measured at 420 nm against deionized water as a blank. The scavenging ability was calculated as follows:

$$\text{Scavenging ability } (\%) = \frac{A_0 - A}{A_0} \times 100\% \quad (1)$$

where A_0 is the absorbance of isometric water, and A is the absorbance of samples.

2.4.2. 1,1-Diphenyl-2-picrylhydrazyl radicals (DPPH)

The scavenging ability on DPPH radical was determined according to the method of He, Yue, and Tang (2009) with slight modification. Each sample (1 ml) was mixed with 4.0 ml DPPH (0.2 mM, obtained from Sigma Chemicals Co. (St. Louis, USA)) or ethanol to a final volume of 5 ml, using deionized water (1 ml) as contrast of samples. The mixtures were shaken vigorously and left still for 30 min in the dark, and the absorbance was measured at 517 nm against ethanol as a blank. The scavenging ability was calculated as follows:

$$\text{Scavenging ability } (\%) = \left[1 - \frac{A_i - A_j}{A_0}\right] \times 100\% \quad (2)$$

where A_0 is the absorbance of mixture contained DPPH and ethanol, A_i is the absorbance of mixture contained samples and DPPH, and A_j is the absorbance of mixture contained samples and ethanol.

2.4.3. Reducing power

Reducing power was carried out according to the method established by Oyaizu (1986) with slight modification. Each sample (1 ml) were mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6), and 2.5 ml potassium hexacyanoferrate solution (1%, w/v). The mixtures were incubated 20 min at 50 °C. After the reaction was cooled in flowing water, 2.5 ml trichloroacetic acid (10%, w/v), 2.5 ml deionized water and 2.5 ml ferric chloride (0.1%, w/v) were added successively. Immediately, the absorbance of the reaction mixture was measured by using spectrophotometer at 700 nm. Higher absorbance at this wavelength indicated stronger reducing power.

2.5. Determinations of antioxidant activities of IZPS in vivo

2.5.1. Superoxide dismutase (SOD)

The activity of SOD was measured according to the method of Bayer and Fridovich (1987) with slight modification. The mixture, including 0.05 ml samples, 1.5 ml potassium phosphate buffer (0.05 M, pH 7.8), 0.3 ml methionine (130 mM), 0.3 ml nitroblue tetrazolium (NBT, 750 μM), 0.3 ml EDTA (100 μM) and 0.3 ml riboflavin (20 μM), was reacted for 20 min under the illumination (4000 lx). The absorbance was measured at 560 nm against the mixture in dark as a blank. The SOD activity was expressed in relative units per milligram protein or micromoles per minute per liter of blood. The unit of SOD activity was expressed as U (50% inhibition of photochemical reduction of NBT as 1 U).

2.5.2. GSH peroxide (GSH-Px)

The activity of GSH-Px was measured by the method of Flohé and Günzler (1984). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM EDTA, 1 mM NaN_3, 0.15 mM NADPH, 1 mM GSH, and 2.4 U/ml of glutathione reductase (GR). The reaction was initiated by adding 0.15 mM H_2O_2. The rate of NADPH consumption was recorded at 340 nm. The activity of GSH-Px was expressed as mM of NADPH oxidized per minute per milligram of tissue or micromoles per minute per liter of blood.

2.5.3. Catalase (CAT)

The CAT activity was measured by the method of ammonium molybdate colorimetry (Kong, 2011) with slight modification. The substrate solution, mixed with sodium-potassium phosphate buffer (pH 7.4, 60 mM) and H_2O_2 (65 μM) (v/v = 1:1, pre-incubated for 5 min at 30 °C), and the buffer solution of sodium-potassium phosphate (pH 7.4, 60 mM), were pre-prepared. Then the reaction system, the measuring tubes (A_1, including 0.5 ml samples and 2.0 ml substrate solution), the standard tubes (A_0, including 2.0 ml substrate solution and 2.0 ml buffer solution), and the control tubes (A_2, including 0.5 ml samples, 2.0 ml buffer solution, and

2.0 ml substrate solution), was reacted at 30 °C for 60 s respectively. Immediately, the reactions were ended by adding 2.0 ml ammonium molybdate (32.4 mM). After stable for 10 min, all tubes were absorbed at 405 nm. And the ability of CAT was calculated by the following formula:

$$\text{CAT (U)} = \frac{A_1 - A_2}{A_0} \times \frac{60 \times 2}{0.5} \quad (3)$$

2.5.4. Malondialdehyde (MDA)

The content of MDA was measured by the method of Zhao, Shi, and Dong (2002) with slight modification. The mixture, including 0.2 ml sample and 2 ml thiobarbituric acid (TBA, 0.6%, w/v), was heated in boiling water for 15 min. After cooling rapidly, the mixture was centrifuged (3000 r/min, 10 min), and the supernatant was used for the determination of MDA content. The absorbance was measured at 532 nm, 600 nm and 450 nm. The content of MDA was calculated by the following formula:

$$\text{Content (mM)} = 6.45 \times (\text{OD}_{532\,\text{nm}} - \text{OD}_{600\,\text{nm}}) - 0.56 \times \text{OD}_{450\,\text{nm}} \quad (4)$$

2.5.5. Lipid peroxide (LPO)

The content of LPO was measured by the method of Zuo (2009) with slight modification. The mixture, containing 0.05 ml sample and 2.5 ml TBA (2.68 g/l), boiled 30 min. The reaction was cooled to termination immediately, and then 7.5 ml n-butyl alcohol was added. After standing for 5 min, the absorbance was measured at 532 nm, using deionized water for blank. And the positive control was performed with 1,1,3,3-tetraethoxypropane (TEP, 40 nM) instead of samples. The content of LPO was calculated by the following formula:

$$\text{Content (nM)} = \frac{A_i}{A_j} \times 40 \quad (5)$$

where A_i is the absorbance of samples, and A_j is the absorbance of TEP.

2.6. Monosaccharide composition analysis of IZPS

Monosaccharide composition of IZPS was analyzed by gas chromatography (GC) as described by Sheng et al. with some slight modifications (Sheng et al., 2007). Briefly, 0.4 g of IZPS was hydrolyzed with 4 ml trifluoroacetic acid (TFA) at 120 °C for 4 h. After removing the residual TFA with methanol and cooling to the room temperature, the sample (1 ml), including ammonium hydroxide (12 M, 0.6 ml) and $NaBH_4$ (dissolved in ammonium hydroxide, 0.4 ml), was incubated at 45 °C for 1 h, and ending the reaction at pH of 6–7 adjusting by glacial acetic acid. Afterwards, the sample (0.4 ml) was acetylated by adding 1-methylimidazole (0.6 ml) and acetic anhydride (4 ml). Eight standard monosaccharides, including rhamnose (Rha, Mw = 164), D-ribose (D-Rib, Mw = 150), arabinose (Ara, Mw = 150), xylose (Xyl, Mw = 150), inositol (Ino, Mw = 180), mannose (Man, Mw = 180), glucose (Glu, 180), and galactose (Gal, Mw = 180), were converted to their acetylated derivatives according to the above-mentioned method. GC was performed by a Shimadzu GC-2010 equipped with a capillary column of Rtx-1 (30 m × 0.25 mm × 0.25 μm). The temperature program was: 190 °C for 20 min, then to 200 °C at 10 °C/min holding for 25 min. The injector temperature was 260 °C, and samples (1.0 μl) were injected in the split model (1:20). N_2 was used as the carrier gas at 0.8 ml/min of cavity flow and 19.8 ml/min of total flow. The relative molar ratios of monosaccharides were calculated by the area normalization method according to the chromatogram (Deng, Liu, Li, & Yu, 2000)

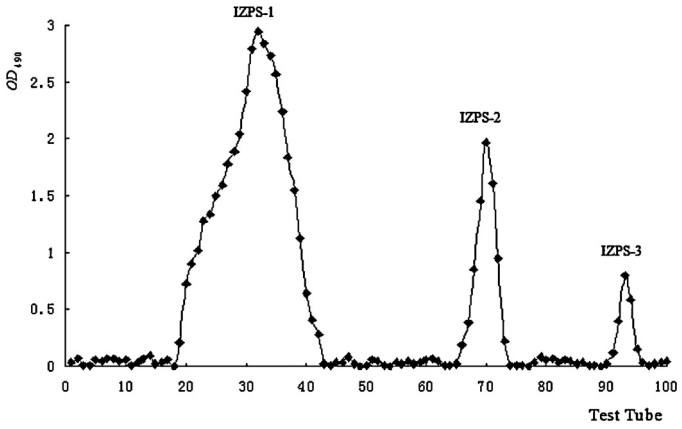

Fig. 1. DEAE-52 cellulose anion-exchange column chromatogram of the IZPS. The subfractions of IZPS (IZPS-1, -2, and -3) were obtained from distilled water, and NaCl gradient of 0.1, 0.3, 0.5, and 1.0 M.

2.7. Statistical analysis

Data were statistically analyzed by one-way ANOVA and paired-sample *T* test (SPSS 16.0 software package, USA). Differences were considered to be statistically significant if $P < 0.05$.

3. Results and discussions

3.1. Purification of IZPS subfractions

The chromatography results of IZPS were shown in Fig. 1. Three peaks (named as IZPS-1, IZPS-2, and IZPS-3) were obtained with five solvents (distilled water and NaCl gradients of 0.1 M, 0.3 M, 0.5 M, and 1.0 M). As expressed by Wu, Liu, Dou, and Zhao (2008), DEAE-52 was an anion exchanger. It could combine with electriferous-polysaccharides when crude polysaccharides were poured into the column. Then the polysaccharides could be eluted by increasing intensity of NaCl (or other saline solutions), and only strong enough ionic force could elute the fragments. Five solvents with only three peaks may be due to that no potential neutral-polysaccharides existed in the crude polysaccharides and NaCl gradient of 0.3 M was not strong enough to elute the polysaccharides. All the fragments were selected for the evaluation of antioxidant activities *in vitro* and *in vivo*.

3.2. Antioxidant activities of IZPS subfractions in vitro

A wide range of oxygen-free radicals and other reactive oxygen species (ROS), including free radicals such as superoxide anion radicals ($O_2^{\bullet -}$) and 1,1-diphenyl-2-picrylhydrazyl (DPPH), may form in the human body and in the food system. These radicals could cause oxidative damage by oxidizing biomolecules leading to cells death and tissues damage, such as atherosclerosis, cancer, emphysema, and cirrhosis (Kehrer, 1993).

Superoxide anion radicals were known to be very harmful to cellular components as precursors of ROS (Dong & Yao, 2008). So the scavenging ability of superoxide anion radicals was extremely important to antioxidation work. As shown in Fig. 2(A), all IZPS fragments exhibited dose-dependence on superoxide anion radical scavenging activities. From the concentration 0–1.4 mg/ml, IZPS-2 expressed higher scavenging activities than that of BHT, but lower effects were obtained by IZPS-1 and IPZS-3. At the concentration of 1.4 mg/ml, the scavenging ability of IZPS-2 was reached 55.68 ± 0.29%, which was about 45.61% higher than that of IZPS-1 (35.24 ± 0.43%, $P < 0.01$), 63.00% higher than that of IZPS-3 (34.16 ± 0.27%, $P < 0.01$), and only 10.28% higher than that of

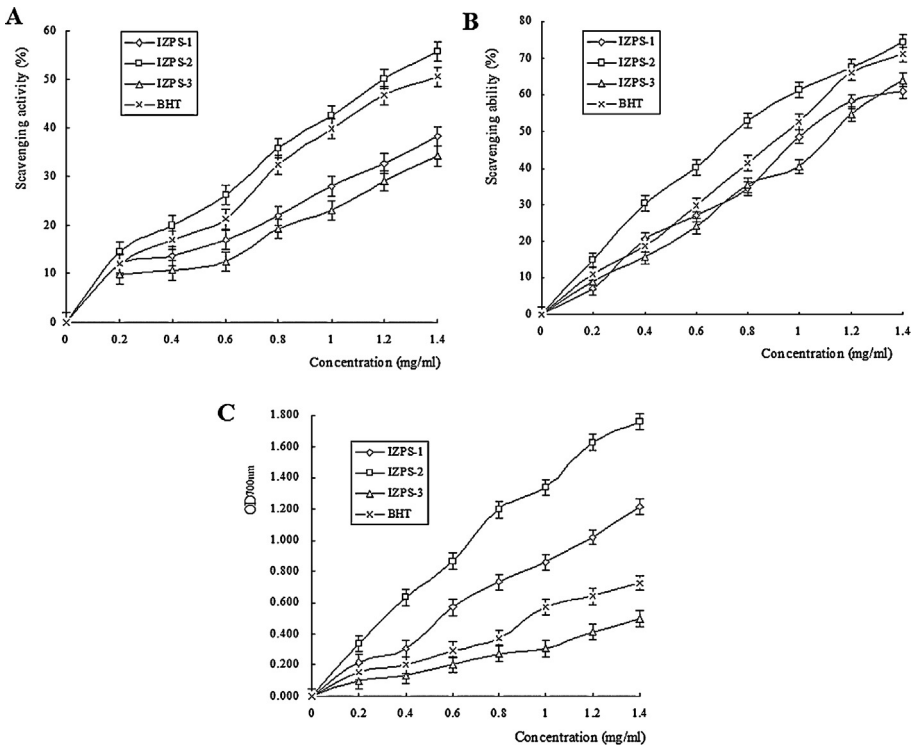

Fig. 2. Antioxidant activities of IZPS subfractions *in vitro*. (A) Scavenging abilities on superoxide radicals ($O_2^{\bullet -}$), (B) scavenging abilities on DPPH, and (C) reducing power.

BHT ($50.49 \pm 0.19\%$, $P > 0.05$), respectively. It was also higher than 24.6 of *Hypsizigus marmoreus* reported by Wang et al. (2006) and 46.3% of *Coriolus versicolor* reported by Zhou and Ma (2008), respectively. The results showed that the IZPS-2 of *P. cornucopiae* SS-03 significantly affects the scavenging of superoxide anion radicals.

The scavenging activity on DPPH, a stable free radical, was a widely index and a quick method to evaluate antioxidant activity (Ke et al., 2009). DPPH was a stable nitrogen-centered, lipophilic free radical that showed maximum absorption at 517 nm in methanol. When DPPH encountered a proton-donating substance, for example, an antioxidant, the radical would be scavenged and the absorbance was reduced. Based on this principle, the antioxidant activity of a substance could be expressed as its ability of scavenging the DPPH free radical. In the DPPH test, the antioxidants were able to reduce the stable DPPH radicals to the yellow-colored diphenylpicrylhydrazine (Wang et al., 2010). The dose-response curve for the scavenging activities of IZPS fragments were shown in Fig. 2(B). The scavenging abilities increased with the increasing concentrations of the three subfractions ($P < 0.01$). IZPS-2 had strong antioxidant activity than that of BHT, and the scavenging ability was $74.41 \pm 1.26\%$, which was higher than that of IZPS-1 ($60.97 \pm 0.94\%$, $P < 0.01$), IZPS-3 ($64.03 \pm 0.44\%$, $P < 0.05$), and BHT ($70.96 \pm 0.35\%$, $P > 0.05$) at the concentration of 1.4 mg/ml respectively. It also showed that the scavenging ability of IZPS-1 was better than that of IZPS-3 in low concentrations, but inversed in high concentrations (about 0.8 mg/ml as the demarcation point) in Fig. 2(B).

The reducing property indicated that these compounds were electron donors and could reduce the oxidized intermediates, and could act as primary and secondary antioxidants (Moktan, Saha, & Sarkar, 2008). In this paper, assay of reducing activity was based on the reduction of Fe^{3+}/ferricyanide complex to the ferrous form in the presence of antioxidant in the tested sample. The Fe^{2+} was then monitored by measuring the formation of Perl's Prussian blue at 700 nm (Oyaizu, 1986). As shown in Fig. 2(C), the reducing power of IZPS-2 was higher than that of other two subfractions and BHT ($P < 0.01$). At the concentration of 1.4 mg/ml, the *OD* values at 700 nm of IZPS-2 reached 1.758 ± 0.44, which was 44.8% higher than that of IZPS-1 (1.214 ± 0.61, $P < 0.05$), 253.0% higher than that of IZPS-3 (0.498 ± 0.21, $P > 0.05$), and 142.8% higher than that of BHT (0.724 ± 1.01, $P > 0.05$), respectively. In Fig. 2(C), we also could conclude that the antioxidant effect of IZPS-1 was better than that of BHT, but effect of IZPS-3 was still expressed the lowest effect in this work. (Li, Zhang, Fu & Liu 2008) reported that the reducing power of *H. marmoreus* was 0.080, significantly lower than that in our work. These results indicated that the IZPS subfractions of *P. cornucopiae* SS-03 had potential antioxidant capacities *in vitro*, and subfraction of IZPS-2 had the best capacity.

3.3. Activities of antioxidant enzymes of IZPS subfractions in vivo

Aging was a progressive deterioration of physiological function that it may impair the ability of an organism to maintain homeostasis and consequently increase the organism's susceptibility to disease and death (Nohl & Hegner, 1978). A vast number of evidences implicated that aging was associated with decrease of antioxidant enzymes activities (Hagihara, Nishigaki, Maseki, & Yagi, 1984). Reactive oxygen species (ROS) were free radicals and/or oxygen derivative produced during normal cellular functions (Mateś, 2000). Antioxidant enzymes, including SOD, GSH-Px and CAT, which could convert active oxygen molecules into nontoxic compounds, formed the first line of defense against ROS in the organism during oxidative stress (Liu et al., 2010; Abuja & Albertini, 2001). The mechanism may be that SOD could dismutate superoxide radicals to form hydrogen peroxide, which later could be decomposed to water and oxygen by GSH-Px and CAT, thereby the formation of ROS was prevented (Yao et al., 2005). Therefore, these enzymes acted cooperatively in the metabolic pathway of free radicals in different issues.

As the first enzyme which responded to oxygen radicals and offered the greatest response to oxidative stress, SOD could

Table 1
Effects of IZPS subfractions on the activities of SOD, GSH-Px, and CAT in heart, liver, spleen, kidney (U/mg protein) and blood (U/ml serums) in aging mice.[λ]

	NCG	CPG	IZPS-1	IZPS-2	IZPS-3
Heart					
SOD	37.1 ± 2.95	223.7 ± 14.40	247.9 ± 38.26[a]	266.3 ± 30.80[b]	209.5 ± 20.41[a]
GSH-Px	50.1 ± 2.98	177.6 ± 9.63	253.0 ± 22.40[a,c]	274.0 ± 12.48[a,c]	209.3 ± 19.43[a]
CAT	25.7 ± 1.11	233.0 ± 5.55	269.4 ± 15.02[a,c]	290.0 ± 12.21[a,c]	254.0 ± 1.06[a]
Liver					
SOD	41.2 ± 5.12	274.3 ± 32.53	211.3 ± 13.09[a,c]	229.4 ± 20.77[a]	192.3 ± 14.57[a]
GSH-Px	42.7 ± 2.18	197.3 ± 9.15	104.7 ± 6.31[a]	130.9 ± 15.05[a,d]	99.5 ± 6.51[a,d]
CAT	98.3 ± 2.16	230.4 ± 9.60	399.4 ± 11.36[a,c]	472.8 ± 17.36[a,c]	301.5 ± 8.23[a,d]
Spleen					
SOD	28.4 ± 2.91	256.9 ± 22.25	178.0 ± 16.36[a,c]	216.4 ± 19.80[a,d]	170.1 ± 19.20[a]
GSH-Px	28.7 ± 2.49	64.1 ± 2.67	73.0 ± 2.96[a,c]	85.0 ± 2.14[a,c]	69.2 ± 4.38[a]
CAT	30.2 ± 1.30	120.7 ± 2.11	176.8 ± 8.46[a,c]	199.9 ± 5.05[a,c]	161.0 ± 9.22[a,c]
Kidney					
SOD	14.6 ± 2.67	268.7 ± 27.53	137.9 ± 38.30[a,c]	154.0 ± 30.29[a,c]	105.4 ± 9.64[a,d]
GSH-Px	36.4 ± 2.07	137.1 ± 4.20	188.1 ± 4.95[a,c]	198.8 ± 7.86[a,c]	155.1 ± 9.20[a]
CAT	70.7 ± 2.77	274.2 ± 14.98	364.2 ± 23.37[a,c]	446.3 ± 16.29[a,c]	300.4 ± 16.11[a]
Blood					
SOD	80.0 ± 1.86	323.5 ± 12.66	260.6 ± 9.29[a,c]	344.6 ± 10.44[a,c]	299.0 ± 11.37[a,d]
GSH-Px	87.7 ± 2.08	148.3 ± 3.03	159.8 ± 10.37[a]	182.9 ± 6.02[a,c]	152.6 ± 5.22[a]
CAT	67.6 ± 2.54	245.8 ± 20.81	353.4 ± 15.22[a,c]	371.5 ± 18.46[a,c]	316.1 ± 19.20[a,c]

[a] Significant difference compare to NPG; $P < 0.01$.
[b] Significant difference compare to NPG; $P < 0.05$.
[c] Significant difference compare to CPG; $P < 0.01$.
[d] Significant difference compare to CPG; $P < 0.05$.
[λ] Values are expressed as mean ± SD (std. deviation, $n = 5$). Paired samples statistics (*T-T* test) are used for the significant difference compare to the NPG or CPG. Differences were considered significant at $P < 0.05$.

eliminate the superoxide anion radicals ($O_2^{\bullet -}$) to protect cells from damage (Zhang, Mu, Xu, Xu, & Liu, 2009). Experiments in animals demonstrated a correlation between SOD and tolerance on oxygen toxicity. As shown in Table 1, the SOD activities of IZPS-1 reached 247.9 ± 38.26 U/mg protein, 211.3 ± 13.09 U/mg protein, 178.0 ± 16.36 U/mg protein, 137.9 ± 38.30 U/mg protein, and 260.6 ± 9.29 U/ml serums; the activities of IZPS-2 reached 266.3 ± 30.80 U/mg protein, 229.4 ± 20.77 U/mg protein, 216.4 ± 19.80 U/mg protein, 154.0 ± 30.29 U/mg protein, and 344.6 ± 10.44 U/ml serums, and the activities of IZPS-3 reached 209.5 ± 20.41 U/mg protein, 192.3 ± 14.57 U/mg protein, 170.1 ± 19.20 U/mg protein, 105.4 ± 9.64 U/mg protein and 299.0 ± 11.37 U/ml serums, which increased by 568.0, 412.9, 526.8, 844.5, and 225.8% (all $P < 0.01$) of IZPS-1, 617.7 ($P < 0.05$), 456.8 ($P < 0.01$), 662.0 ($P < 0.01$), 954.8 ($P < 0.01$), and 330.8% ($P < 0.01$) of IZPS-2, and 521.3, 366.7, 500.0, 621.9, and 273.8% (all $P < 0.01$) of IZPS-3 (compared with the NCG) in heart, liver, spleen, kidney, and blood respectively. The results also indicated that IZPS-2 played an important role in increasing the activities of SOD due to the high activities among the three subfractions. The SOD activities of IZPS-1 were only higher than that of the CPG in heart with the increasing rate of 10.8% ($P > 0.05$), while the activities of IZPS-2 was higher in heart and blood with the increasing rate of 19.0 ($P < 0.01$) and 2.27% ($P < 0.01$) respectively. But no higher SOD activities of IZPS-3 could be seen in the detected tissues (Table 1). As Cu–Zn SOD was most abundant in the SOD families, and it existed in all eukaryotic cells (Asada, 1994), the existence of zinc had potential ability to ascend the activities of SOD in aging mice.

GSH was readily oxidized to glutathione disulphide (GSSG) upon reaction with GSH-Px, reducing the concentration of hydrogen peroxide, to protect the organ from oxidative stress (Cantin et al., 2007; Ting et al., 2011). As shown in Table 1, the GSH-Px activities of IZPS-1 reached 253.0 ± 22.40 U/mg protein, 104.7 ± 6.31 U/mg protein, 73.0 ± 2.96 U/mg protein, 188.1 ± 4.95 U/mg protein, and 159.8 ± 10.37 U/ml serums, the activities of IZPS-2 reached 274.0 ± 12.48 U/mg protein, 130.9 ± 15.05 U/mg protein, 85.0 ± 2.14 U/mg protein, 198.8 ± 7.86 U/mg protein, and 182.9 ± 6.02 U/ml serums, and the activities of IZPS-3 reached 209.3 ± 19.43 U/mg protein, 99.5 ± 6.51 U/mg protein, 69.2 ± 4.38 U/mg protein, 155.1 ± 9.20 U/mg protein, and 152.6 ± 5.22 U/ml serums, which increased by 405.0%, 145.2%, 154.4%, 416.8%, and 82.8% (all $P < 0.01$) of IZPS-1, 446.9%, 206.6%, 196.2%, 446.2%, and 108.6% (all $P < 0.01$) of IZPS-2, and 317.8%, 133.0%, 141.1%, 326.1%, and 74.0% (all $P < 0.01$) of IZPS-3 in heart, liver, spleen, kidney, and blood respectively (compare with NCG mice). In comparison to CPG mice, all GSH-Px activities of IZPS-1, IZPS-2 and IZPS-3 were higher than that of CPG mice except in liver (Table 1).

CAT was an enzyme present in nearly all animals and plants, and it combined rapidly with hydrogen peroxide to catalyze H_2O_2, breakdown into oxygen and water (Zhang et al., 2009). Chance et al. (1979) documented that the physiological variation of CAT concentration in different organs and tissues leaded to different steady-state levels of hydrogen peroxide concentration for the same rate of hydrogen peroxide generation. So CAT was the most important enzyme to provide a homeostasis for hydrogen peroxide. As shown in Table 1, all IZPS subfractions produced significant increase compared with NCG mice (both $P < 0.01$) in heart, liver, spleen, kidney, and blood. The CAT activities of IZPS-1 reached 269.4 ± 15.02 U/mg protein, 399.4 ± 11.36 U/mg protein, 176.8 ± 8.46 U/mg protein, 364.2 ± 23.37 U/mg protein, and 353.4 ± 15.22 U/ml serums, which were significant increase by 648.2%, 306.3%, 485.4%, 415.1%, and 422.9% (all $P < 0.01$), CAT activities of IZPS-2 reached 290.0 ± 12.21 U/mg protein, 472.8 ± 17.36 U/mg protein, 199.9 ± 5.05 U/mg protein, 446.3 ± 16.29 U/mg protein, and 371.5 ± 18.46 U/ml serums which also had significant increase by 728.4%, 381.0%, 562.0%, 531.3%, and 449.6% (all $P < 0.01$), and CAT activities of IZPS-3 reached 254.0 ± 1.06 U/mg protein, 301.5 ± 8.23 U/mg protein, 161.0 ± 9.22 U/mg protein, 300.4 ± 16.11 U/mg protein and 316.1 ± 19.20 U/ml serums with the significant increasing by 888.3%, 206.7%, 63.8%, 324.9%, and 367.6% (all $P < 0.01$) in these issues orderly mentioned above, respectively. In addition, the activities of GSH-Px of all IZPS subfractions were also higher than that of CPG mice, and also had a significant increase by 15.6%, 73.4%, 46.0%, 32.8%, and 43.8% of IZPS-1 (all $P < 0.01$); 24.6%, 105.2%, 65.6%, 62.7%, and 51.1% of IZPS-2, and 9.0%, 30.9%, 33.4%, 148.9%, and 28.6% of IZPS-3 (all $P < 0.01$) in heart, liver, spleen, kidney,

Table 2
Effects of IZPS subfractions on the contents of MDA and LPO in heart, liver, spleen, kidney, and blood in aging mice.[λ]

	NPG	CPG	IZPS-1	IZPS-2	IZPS-3
Heart					
MDA[e]	11.69 ± 0.87	0.93 ± 0.15	3.92 ± 0.19[a,c]	3.60 ± 0.11[a,c]	4.11 ± 0.37[a,d]
LPO[f]	7.93 ± 0.63	2.16 ± 0.31	4.40 ± 0.35[a,c]	2.93 ± 0.67[a,d]	4.74 ± 0.91[a]
Liver					
MDA	8.19 ± 0.18	1.78 ± 0.21	3.06 ± 0.15[a,c]	2.29 ± 0.42[a]	3.55 ± 0.28[a]
LPO	4.81 ± 0.44	3.04 ± 0.27	1.28 ± 0.42[a,c]	0.97 ± 0.28[a,c]	1.20 ± 0.19[a,d]
Spleen					
MDA	19.16 ± 2.13	8.21 ± 0.88	11.94 ± 1.25[a,c]	9.37 ± 0.74[a]	9.69 ± 0.76[a,d]
LPO	6.01 ± 0.36	2.29 ± 0.31	3.57 ± 0.36[a,c]	3.14 ± 0.29[a,c]	4.60 ± 0.14[a]
Kidney					
MDA	18.89 ± 2.20	8.14 ± 1.33	12.08 ± 1.79[a,d]	7.16 ± 1.18[a]	9.48 ± 0.33[a]
LPO	6.08 ± 0.27	3.75 ± 0.27	3.66 ± 0.36[a]	3.01 ± 0.21[a,c]	4.90 ± 1.01[a]
Blood					
MDA	16.90 ± 1.08	5.26 ± 0.49	6.93 ± 1.17[a,d]	6.90 ± 0.98[a,d]	7.09 ± 0.61[a,c]
LPO	3.16 ± 0.36	0.72 ± 0.22	1.10 ± 0.22[a,d]	0.81 ± 0.19[a]	2.20 ± 0.34[a,d]

[a] Significant difference compare to NPG; $P < 0.01$.
[b] Significant difference compare to NPG; $P < 0.05$.
[c] Significant difference compare to CPG; $P < 0.01$.
[d] Significant difference compare to CPG; $P < 0.05$.
[e] The unit of MDA was μmol/mg protein in heart, liver, spleen, kidney, and μmol/ml serums in blood.
[f] The unit of MDA was nmol/mg protein in heart, liver, spleen, kidney, and nmol/ml serums in blood.
[λ] Values are expressed as mean ± SD (std. deviation, $n = 5$). Paired samples statistics (*T-T* test) are used for the significant difference compare to the NGP or CGP. Differences were considered significant at $P < 0.05$.

and blood respectively. Due to the higher values of antioxidant enzyme activities, IZPS-2 could be considered as the most bioactive subfraction in the detected tissues.

3.4. Effects of the IZPS subfractions on the contents of MDA and LPO in vivo

Lipid peroxidation was the process that involved the chain reactions of free radicals with PUFA (polyunsaturated fatty acid). These reactions leaded to hydroperoxide generation and lipid breakdown to yield products like MDA (Thabrew, Joice, & Rajatissa, 1987). Therefore, inhibition of lipid peroxidation was of great importance in disease processes involving free radicals (Abuja & Albertini, 2001).

As shown in Table 2, the MDA contents of IZPS-1 reached 3.92 ± 0.19 μmol/mg protein, 3.06 ± 0.15 μmol/mg protein, 11.94 ± 1.25 μmol/mg protein, 12.08 ± 1.79 μmol/mg protein, 6.93 ± 1.17 μmol/ml serums; contents of IZPS-2 reached 3.60 ± 0.11 μmol/mg protein, 2.29 ± 0.42 μmol/mg protein, 9.37 ± 0.74 μmol/mg protein, 7.16 ± 1.18 μmol/mg protein, 6.90 ± 0.98 μmol/ml serums, and contents of IZPS-3 reached 4.11 ± 0.37 U/mg protein, 3.55 ± 0.28 U/mg/protein, 9.69 ± 0.76 U/mg protein, 9.48 ± 0.33 U/mg protein, 7.09 ± 0.61 U/ml serums, which had significant decrease by 66.4%, 62.6%, 37.7%, 36.1%, and 59.0% (all $P < 0.01$) of IZPS-1, 69.2%, 72.0%, 51.1%, 62.1%, and 59.2% (all $P < 0.01$) of IZPS-2, and 64.8%, 56.6%, 49.4%, 49.8%, and 58.0% (all $P < 0.01$) of IZPS-3 in heart, liver, spleen, kidney, and blood compared to the NCG mice respectively. However, the MDA contents of mice induced by IZPS subfractions were higher than that induced by vitamin C in all studied issues, which indicated that IZPS had lower effects on decreasing MDA contents.

LPO (lipid peroxidation) was the product of the reaction of oxygen radicals and PUFA, and the concentration was low in body at normal (Vande, Sedman, & Russin, 2001). The reaction of LPO played an important role in the metabolism. When the LPO was in a higher level, it would destroy the normal function of the cells which could lead the protein degeneration, and the enzyme inactivation. Furthermore, it may lead to some disease (Hessel, Haber, & Mulle, 2000). As shown in Table 2, the LPO contents reached 4.40 ± 0.35 nmol/mg protein, 1.28 ± 0.42 nmol/mg protein, 3.57 ± 0.36 nmol/mg protein, 3.66 ± 0.36 nmol/mg protein, 1.10 ± 0.22 nmol/ml serums of IZPS-1, the contents reached 2.93 ± 0.67 nmol/mg protein, 0.97 ± 0.28 nmol/mg protein, 3.14 ± 0.29 nmol/mg protein, 3.01 ± 0.21 nmol/mg protein, 0.81 ± 0.19 nmol/ml

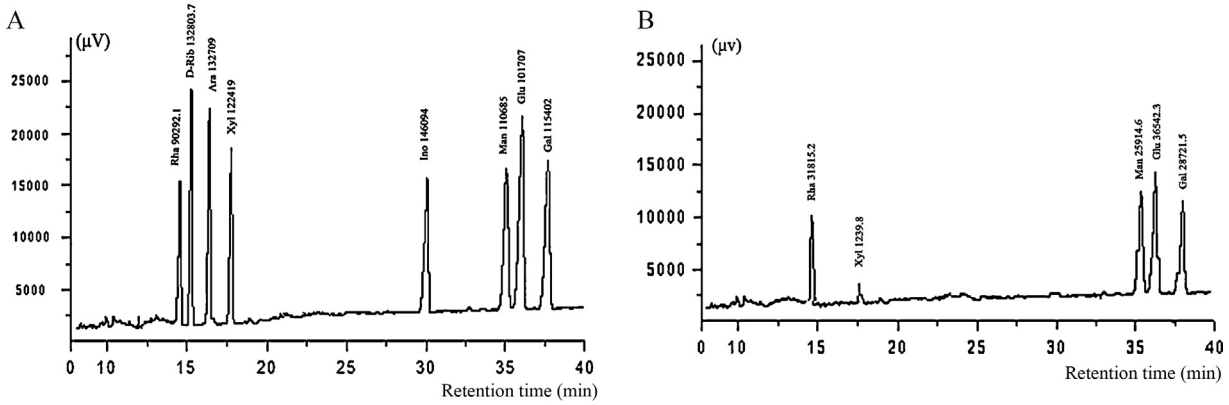

Fig. 3. Gas chromatography of monosaccharides. (A) Monosaccharides standards and (B) IZPS.

serums of IZPS-2, and the contents reached 4.74 ± 0.91 U/mg protein, 1.20 ± 0.19 U/mg protein, 4.60 ± 0.14 U/mg protein, 4.90 ± 1.01 U/mg protein, 2.20 ± 0.34 U/ml serums of IZPS-3, which had significant decrease by 44.5%, 73.4%, 40.6%, 39.8%, and 65.2% of IZPS-1 (all $P < 0.01$), 63.5%, 79.8%, 47.8%, 50.5%, and 74.4% of IZPS-2 (all $P < 0.01$), and 40.2%, 75.1%, 23.5%, 18.5%, and 30.4% of IZPS-3 (all $P < 0.01$) in heart, liver, spleen, kidney, and blood compared to the NCG mice, respectively. From Table 2, we also could see that liver played positive roles in the suppression of lipid peroxidation due to the low contents of LPO induced by IZPS subfractions.

3.5. Monosaccharide compositions

According to the retention time of the standard substances and samples, the monosaccharide types could be determined. The monosaccharide compositions were shown in Fig. 3. The content of monosaccharide is calculated by each peak area. As can be noted in Fig. 3(B), monosaccharides presented in IZPS were five kinds. And the contents were 29.25%, 0.78%, 19.45%, 29.84%, and 20.67% corresponding to Rha, Xyl, Man, Glu, and Gal with the molar ratio of 1.65:0.05:1:1.50:1.07. These data suggested that Rha and Glu were the predominant monosaccharides, but the content of Xyl was the lowest. Comparing with other species of *Pleurotus*, Dong and Lu (2004) found five sugar components in *P. ferulae*, including Rha, Xyl, Glu, Gal, and Man, and the Gal and Glu were the predominant monosaccharides (54.74% and 37.05%, respectively). Wu, Hu, Huang, and Jiang (2013) found three sugar components in *Pleurotus tuber-regium* (glucuronic acid, Gal, and Glu), and the content of Glu was the highest (44.232%).

4. Conclusions

The IZPS subfractions, IZPS-1, IZPS-2 and IZPS-3, showed better antioxidant activities *in vitro* and in *vivo*, suggesting that microbial sources could be used as a potential mean of producing natural antioxidants in various fermented products produced by *P. cornucopiae* SS-03. Five monosaccharides were also found in IZPS. Scarcely paper of the relationship between zinc and polysaccharides of *P. cornucopiae* had been published; however, the relationship between antioxidant and monosaccharides components, their structure–activity relationships, and the possible mechanisms were the areas for future studies.

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