PRODUCTION AND ANTIOXIDANT ACTIVITY OF INTRACELLULAR POLYSACCHARIDE BY *HYPSIZIGUS MARMOREUS* SK-01

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The extraction parameters of intracellular polysaccharide (IPS) from *Hypsizigus marmoreus* SK-01 mycelia were optimized, and the antioxidant activities of IPS were investigated. The optimum conditions of IPS extraction were predicted to be: an ultrasonic treatment time of 618.07 s, an extraction temperature of 84.53 °C, a pH of 7.57, and an IPS yield of *ca.* 6.84%. The *in vitro* inhibition effects of IPS on hydroxyl, superoxide anion, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals were 52.63 ± 3.19%, 68.21 ± 5.09%, and 63.43 ± 5.27%, respectively, and the EC_{50} values of IPS were 0.85 ± 0.05 g/L, 0.47 ± 0.03 g/L, and 0.62 ± 0.04 g/L, respectively. The activities of glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT) in mice blood were 2.52 ± 0.02 U/mL, 453.19 ± 38.43 U/mL, and 118.59 ± 9.64 U/mL, respectively. The results provide a reference for large-scale extraction of IPS by *H. marmoreus* SK-01 in industrial fermentation, suggesting that IPS can be used as a potential antioxidant that enhances adaptive immune responses.

Keywords: Hypsizigus marmoreus SK-01; Intracellular polysaccharide; Central composite design; Antioxidant activity; In vitro; In vivo

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INTRODUCTION

Hypsizigus marmoreus (Peck) Bigelow, an edible and nutrient mushroom with high medicinal and economic values, is widely distributed and has been artificially cultivated in China for the past few years. It contains many biological active materials, such as carbohydrates, protein, trace elements, essential amino acids, lectins, terpenoids, tocopherols, ascorbic acid, enzymes, dietary fiber, vitamins, *etc.* (Lee *et al*. 2009; Chang *et al*. 2009). The polysaccharides from *H. marmoreus* fruiting bodies or fermentation broths have potential antioxidant, antitumor, antivirus, and immunomodulating properties (Zhang *et al*. 2012).

Many reports on polysaccharide content of mushrooms have focused on the exopolysaccharide (EPS) of fermentation broth and polysaccharides of fruiting bodies by *Cordyceps sinensis* (Yan *et al*. 2009), *Shiraia bambusicola* (Cai *et al*. 2010), *Pleurotus sajor-caju* (Telles *et al*. 2011), *Agaricus brasiliensis* (Lima *et al*. 2008), *Gomphidius rutilus* (Gao *et al*. 2012), *Lentinus edodes* (Feng *et al*. 2010), *A. bisporus* (Tian *et al*.

2012), *Tricholoma lobayense* (Wang *et al*. 2012), *Ganoderma lucidum* (Zhao *et al*. 2012) and *Dictyophora indusiata* (Hua *et al*. 2012). Moreover, the intracellular polysaccharide (IPS) extracted from mycelia of *P. nebrodensis* (Liu *et al*. 2010), *G. applanatum* (Li and Wu 2009), *Phellinus igniarius* (Huang *et al*. 2010), *Antrodia camphorate* (Zhang *et al*. 2008) and *Boletus edulis* (Chen *et al*. 2012) have been reported. However, there are fewer reports about the extraction parameters of IPS from *H. marmoreus* mycelia in submerged culture and its antioxidant activities.

In this work, factors that affect the extraction of IPS from *H. marmoreus* SK-01 mycelia were analyzed by Plackett–Burman (PB) tests, and three significant variables (ultrasonic treatment time, extraction temperature, and pH) were chosen to optimize the extraction conditions using central composite design (CCD). In addition, the *in vitro* and *in vivo* experiments were performed to investigate the antioxidant activities of IPS.

EXPERIMENTAL

Chemicals

Butylated hydroxytoluene (BHT), nitroblue tetrazolium (NBT), methionine (MET), riboflavin (RF), and 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) were obtained from Sigma Chemicals Co. (St. Louis, USA). All other chemicals used in this study were analytical reagent grade and purchased from local chemical suppliers in China.

Strain and Liquid Culture

A strain of *H. marmoreus* SK-01 used in this experiment was from our laboratory and maintained on potato dextrose agar (PDA) slant. The culture was incubated at 25 °C for 7 days, stored at 4 °C, and subcultured every two months.

Cultivation in liquid media was carried out in 250-mL Erlenmeyer flasks containing 100 mL of (g/L): potato, 200; glucose, 20; KH_2PO_4, 1.5; and $MgSO_4 \cdot 7H_2O$, with natural pH. Each flask was inoculated with a 0.5-cm^2 mycelial block of *H. marmoreus* SK-01 from the solid media, incubated at 25 °C without shaking for 24 h, and then shaken on a rotary shaker (Anting, Shanghai, China) at 140 rpm for 7 days.

Measurement and Preparation of IPS

The mycelia precipitates of *H. marmoreus* SK-01 were obtained by filtration and dried to a constant weight at 60 °C for 24 h. The mycelia power was dissolved in distilled water for 3 h and then fragmented with an ultrasonic processor (Sonics & Materials, USA) at 600 W for 15 min. The supernatant was mixed with 3 volumes of ethanol (95%, v/v), stirred vigorously, and kept at 4 °C for 24 h. After centrifugation (3,000 g, 15 min), the precipitated IPS was dissolved in distilled water (60 °C), and the IPS content was determined by the phenol–sulfuric acid method, using glucose as the standard (Chaplin and Kennedy 1994).

IPS powder was obtained by quick prefreezing at -35 °C for 1 h and then by vacuum freeze drying (Labconco, USA) for 8 h, and applied to evaluate the *in vitro* and *in vivo* antioxidant activities. The extraction rate of IPS was expressed as a percentage of IPS to dried mycelia (w/w).

PB Experiments for IPS Extraction

Ten variables, including water multiple, pH, ultrasonic power, ultrasonic treatment time, extraction temperature, extraction time, ethanol concentration, ethanol multiple, precipitation temperature, and precipitation time, were initially screened by PB design as reported by Plackett and Burman (1946). In addition, five center points were added for the variables that could be assigned numerical values. The experimental design with the name and symbol code is shown in Table 1.

Table 1. Levels and Codes of Variables for Plackett–Burman Design

Variables	Symbol Code	Coded Levels		
		-1	0	1
Water multiple	A_1	20	30	40
pH	A_2	5	7	9
Ultrasonic power (W)	A_3	400	600	800
Ultrasonic treatment time (s)	A_4	400	600	800
Extraction temperature (°C)	A_5	60	75	90
Extraction time (h)	A_6	1	2	3
Ethanol concentration (%)	A_7	75	85	95
Ethanol multiple	A_8	2	3	4
Precipitation temperature (°C)	A_9	-4	0	4
Precipitation time (h)	A_{10}	12	24	36

CCD Optimization for IPS Extraction

Three parameters that significantly affect IPS extraction of *H. marmoreus* SK-01, ultrasonic treatment time, extraction temperature, and pH, were selected by PB tests and taken into consideration for optimization of IPS extraction by CCD design. The experimental design with name and symbol code is shown in Table 2. The test factors were coded according to the following equation,

$$x_i = (X_i - X_0) / \Delta X_i \qquad i = 1, 2, 3, \ldots, k \tag{1}$$

where x_i is the coded value of an independent variable, X_i is the real value of an independent variable, X_0 is the real value of the independent variable at the center point, and ΔX_i is the step change value. To correlate the response variable to the independent variable, the following quadratic polynomial equation was applied to fit the response variable to a quadratic model,

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \tag{2}$$

where Y is the predicted response value, β_0 is the intercept term, β_i is the linear term, β_{ii} is the squared term, β_{ij} is the interaction term, and X_i and X_j are the coded level of independent variables.

Table 2. Levels and Codes of Variables for Central Composite Design

Variables	Symbol		Coded Levels		
	Uncoded	Coded	-1	0	1
pH	X_1	x_1	5	7	9
Ultrasonic treatment time (s)	X_2	x_2	400	600	800
Extraction temperature (°C)	X_3	x_3	60	75	90

Antioxidant Capacity of IPS *in vitro*

The hydroxyl radical-scavenging assay was measured according to the method of Winterbourn and Sutton (1984) with slight modification. The reaction mixture (10 mL) contained 4 mL of phosphate buffer saline (0.2 M, pH 7.4), 1.5 mL of 1,10-phenanthroline (7.5 mM), 1 mL of $FeSO_4$ (0.75 mM), 1 mL of H_2O_2 (1%, v/v), and 2.5 mL of the IPS (0.1 to 1 g/L). After incubating at 37 °C for 30 min, the absorbance of the IPS was measured at 560 nm, using BHT as a positive control. The EC_{50} value (g/L) of IPS is the effective concentration at which the hydroxyl radicals were scavenged by 50%. The hydroxyl radical scavenging activity was expressed as,

$$\text{Scavenging rate (\%)} = [(A_0 - A_1)/A_0] \times 100 \quad (3)$$

where A_0 is the absorbance of the blank and A_1 is the absorbance of IPS or BHT.

The superoxide anion scavenging activity of IPS was determined according to the method of Stewar and Beewley (1980) with slight modification. The reaction mixture contained 0.2 M phosphate buffer saline (pH 7.8), 10 mM riboflavin (RF), 13 mM methionine, 0.25 mL of 0.51 mM NBT, and the IPS (0.1 to 1 g/L). After illuminating the reaction mixture with a fluorescent lamp at 25 °C for 20 min, the absorbance of the IPS was measured at 560 nm, using BHT for a positive control. The EC_{50} value (g/L) of IPS is the effective concentration at which the superoxide anion radicals were scavenged by 50%. The scavenging rate was calculated using the following formula,

$$\text{Scavenging rate (\%)} = [(A_0 - A_1)/A_0] \times 100 \quad (4)$$

where A_0 is the absorbance of the blank and A_1 is the absorbance of the IPS or BHT.

The DPPH scavenging activity of IPS was measured according to the method of Shimada *et al*. (1992). The reaction mixture contained 2 mL of 95% ethanol, 0.1 μM DPPH, and 2 mL of the IPS (0.1 to 1 g/L). The solution was incubated at 25 °C for 30 min, and the absorbance of IPS was determined at 517 nm. The EC_{50} value (g/L) of IPS is the effective concentration at which the DPPH radicals were scavenged by 50%. The antioxidant activity of IPS was evaluated according to the following formula,

$$\text{Scavenging rate (\%)} = [(A_0 - A_1)/A_0)] \times 100 \quad (5)$$

where A_0 was the absorbance of the DPPH solution and A_1 was absorbance of IPS or BHT. The reducing power of IPS was evaluated according to the method of Oyaizu (1980) with slight modification. The reaction mixtures contained 2.5 mL phosphate buffer (pH 6.6, 0.2 M), 2.5 mL potassium ferricyanide (1%, w/v), and the IPS (0.1 to 1 g/L). After incubating at 50 °C for 20 min, 2.5 mL of trichloroacetic acid (10%, w/v) was added to the mixture for terminating the reaction, and then centrifuged at 1,200 g for 10 min.

An aliquot of 2.5 mL supernatant was collected and mixed with 2.5 mL deionized water and 0.5 mL $FeCl_3$ (0.1%, w/v). After incubating at 25 °C for 15 min, the absorbance of the IPS was measured at 700 nm. The EC_{50} value (g/L) of IPS is the effective concentration at which the absorbance at 700 nm was 0.5.

Animal Experiments

Sixty male mice (Kunming strain), weighing 20 ± 2 g, were purchased from Taibang Biological Products Ltd. Co. (Taian, China) and housed in stainless steel cages under controlled conditions (temperature 22 ± 1°C, humidity 60 to 65%, lights on 12 h every day) with free access to standard food. After a 3-day acclimatization period, all animals were randomly divided into six groups (10 in each group). The five experiment groups received doses of 25, 50, 100, 200, 400 mg/kg body weight of mice by filling the stomach (0.01 mL/g body weight) with a syringe. The control group received the same volume of saline. The mice were allowed to have free access to water and food for 28 days. Experiments were performed in accordance with the institutional ethical guidelines and under guidance of Shandong Agricultural University Committee. At the end of the experiment, all animals were sacrificed under ether anesthesia, and blood samples were taken from the retrobulbar vein with a vacutainer and anticoagulated by heparin (stored at -80 °C). The hearts, livers, spleens, and kidneys were rapidly removed, weighed, and homogenized (1:9, w/v) immediately in 0.2 M phosphate buffer (4 °C, pH 7.4), respectively. The homogenates were centrifuged (6,000 g) at 4 °C for 20 min and the supernatants were stored at -20 °C for further biochemical analysis.

Measurement of Biochemical Parameters *in vivo*

The activity of GSH-Px was measured as described by Rong *et al*. (1994). 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 milliliter of blood.

The superoxide dismutase (SOD) activities were measured according to the method of Bayer and Fridovich (1987). Fifty microliters of the samples were mixed with 1 mL of physiological saline, centrifuged at 2,500 g for 3 min, and the pellet was used for the enzyme reaction. Ice-cold water (0.2 mL), 0.2 mL of 95% ethanol, and 0.1 mL of trichloromethane were added to the pellet and mixed thoroughly with a homogenizer (Bagmixer 400, France). The mixture presented three layers after centrifugation (3,500 *g*, 5 min): SOD extract (top layer), hemoglobin precipitation (middle layer), and trichloromethane (bottom layer). The top layer was removed by micropipettor, and the mixture, including 0.05 mL of SOD extract, 50 mM potassium phosphate buffer (pH 7.0), 13 mM methionine, 0.075 mM NBT, 0.01 mM EDTA, and 0.002 mM riboflavin, was reacted for 20 min. The enzyme activity was expressed as relative units per milligram protein or micromoles per minute per milliliter of blood. The unit of SOD activity was expressed as U (50% inhibition of photochemical reduction of NBT as 1 U).

The activity of catalase (CAT) was determined by the method of Schlorff *et al*. (1999) with a slight modification. The mixture, including 2 mL sodium-potassium phosphate, 65 μM of H_2O_2, and 0.5 mL of blood or tissue homogenate, was reacted at 37 °C for 10 min. Ammonium molybdate (2 mL, 32.4 mM) was added for termination reaction at 25 °C. The activity of CAT was expressed as 1 μM of H_2O_2 degraded per minute per milligram of tissue or per minute per milliliter of blood.

The content of malondialdehyde (MDA) was measured by the method of Zhao *et al*. (2002) with a slight modification. The mixture, including 0.2 mL sample and 2 mL of 0.6% thiobarbituric acid (TBA, w/v), was heated in boiling water for 15 min. After

cooling rapidly, the mixture was centrifuged at 3,000 g for 10 min, and the supernatant was used for the determination of MDA level.

Statistical Analysis

All the data obtained were expressed as means ± SD (standard deviation) of three replicated measurements. Design Expert software (Version 7.1.3, Stat-Ease. Inc., Minneapolis, USA) including ANOVA was applied for Statistical analysis, and $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Determination of Parameters of IPS Extraction

As showed in Table 3, the maximum yield of IPS extraction reached 5.56 ± 0.15%, while the optimal conditions were: water multiple 20, pH 9; ultrasonic power, 400 W; ultrasonic treatment time, 800 s; extraction temperature, 90 °C; extraction time, 1 h; ethanol concentration, 95%; ethanol multiple, 4; precipitation temperature, 4 °C; and precipitation time, 12 h . Ultrasonic treatment time, extraction temperature, and pH had a highly significant influence on IPS extraction at the 1% level, and the influence of other parameters was at the 5% level or not significant ($P > 0.05$) (Table 1). Therefore, these three important variables were chosen to optimize the process of IPS extraction using CCD.

Table 3. Results of Plackett–Burman for IPS Extraction by *H. marmoreus* SK-01

Runs	A_1	A_2	A_3	A_4	A_5	A_6	A_7	A_8	A_9	A_{10}	IPS yield (%)
1	1	1	-1	1	1	1	-1	-1	-1	1	4.35 ± 0.17
2	-1	1	1	-1	1	1	1	-1	-1	-1	3.86 ± 0.09
3	1	1	-1	1	-1	1	1	1	-1	-1	2.01 ± 0.08
4	-1	1	-1	1	1	-1	1	1	1	-1	5.56 ± 0.15
5	-1	-1	1	-1	1	1	-1	1	1	1	2.51 ± 0.07
6	-1	-1	-1	1	-1	1	1	-1	1	1	2.29 ± 0.06
7	1	-1	-1	-1	1	-1	1	1	-1	1	1.83 ± 0.05
8	1	1	-1	-1	-1	1	-1	1	1	-1	2.54 ± 0.11
9	1	1	1	-1	-1	-1	1	-1	1	1	2.37 ± 0.13
10	-1	1	1	1	-1	-1	-1	1	-1	1	4.02 ± 0.16
11	1	-1	1	1	1	-1	-1	-1	1	-1	4.14 ± 0.18
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	1.03 ± 0.04
13	0	0	0	0	0	0	0	0	0	0	3.55 ± 0.12
14	0	0	0	0	0	0	0	0	0	0	3.43 ± 0.18
15	0	0	0	0	0	0	0	0	0	0	3.32 ± 0.15
16	0	0	0	0	0	0	0	0	0	0	3.63 ± 0.11
17	0	0	0	0	0	0	0	0	0	0	3.65 ± 0.17
	*	**		**	**				*	*	

* Significant at 5 % level.
** Significant at 1 % level.

CCD Optimization of IPS Extraction

The CCD matrix together with the experimental and predicted IPS data is shown in Table 4, while adequacy and fitness were evaluated by ANOVA (Table 5). By using

multiple regression analysis, the polynomial model for an empirical relationship between the extraction rate of IPS and test variables in coded units was expressed by Eq. (6),

$$Y_{IPS} = 6.44 + 1.29x_1 + 0.5x_2 + 0.3x_3 - 0.035x_1x_2 + 0.085x_1x_3 + 0.29x_2x_3 - 1.2x_1^2 - 0.43x_2^2 - 0.33x_3^2 \quad (6)$$

where Y_{IPS} is the predicted response for IPS yield (%), and x_1, x_2, and x_3 are the coded test variables for pH, ultrasonic treatment time (s), and extraction temperature (°C), respectively.

Table 4. Experimental and Predicted Values of IPS Based on Central Composite Design

Runs	x_1	x_2	x_3	IPS yield (%)	
				Experimental	Predicted
1	-1	-1	0	3.01 ± 0.16	2.99
2	1	-1	0	5.64 ± 0.21	5.64
3	-1	1	0	4.06 ± 0.19	4.06
4	1	1	0	6.55 ± 0.22	6.57
5	-1	0	-1	3.40 ± 0.13	3.41
6	1	0	-1	5.83 ± 0.18	5.82
7	-1	0	1	3.82 ± 0.11	3.83
8	1	0	1	6.59 ± 0.23	6.58
9	0	-1	-1	5.16 ± 0.20	5.17
10	0	1	-1	5.61 ± 0.27	5.60
11	0	-1	1	5.18 ± 0.19	5.19
12	0	1	1	6.78 ± 0.28	6.77
13	0	0	0	6.39 ± 0.24	6.44
14	0	0	0	6.41 ± 0.22	6.44
15	0	0	0	6.48 ± 0.23	6.44
16	0	0	0	6.44 ± 0.21	6.44
17	0	0	0	6.50 ± 0.25	6.44

Table 5. ANOVA for the Evaluation of the Quadratic Model

Source	Coefficients	S.E.	Sum of Squares	Mean Square	*F*-value	*P*
Model	–	–	24.24	2.69	1822.48	<0.0001**
Intercept	6.44	0.017	–	–	–	–
x_1	1.29	0.014	13.31	13.31	9008.18	<0.0001**
x_2	0.50	0.014	2.01	2.01	1360.09	<0.0001**
x_3	0.30	0.014	0.70	0.70	475.09	<0.0001**
x_1x_2	-0.035	0.019	0.0049	0.0049	3.32	0.1114
x_1x_3	0.085	0.019	0.029	0.029	19.56	0.0031**
x_2x_3	0.29	0.019	0.33	0.33	223.72	<0.0001**
x_1^2	-1.20	0.019	6.07	6.07	4107.80	<0.0001**
x_2^2	-0.43	0.019	0.77	0.77	522.51	<0.0001**
x_3^2	-0.33	0.019	0.47	0.47	316.41	<0.0001**
Lack-of-fit			0.18	0.006	0.29	0.8344

R^2 = 0.9996. R = 0.9998. * Significant at 5% level. ** Significant at 1% level.
adj-R^2 = 0.9990.

It can be seen from Table 5 that the linear term regression coefficients (x_1, x_2, x_3) , quadratic coefficients (x_1^2, x_2^2, x_3^2), and the interaction coefficient (x_1x_3, x_2x_3) were significant at the 1% level, indicating that the precipitation time, ultrasonic treatment time, and pH are all significantly correlated with the yield of IPS extraction. The model was also significant ($P < 0.0001$) with a very high *F*-value (1822.48). The value of correlation coefficient (R = 0.9998) indicated good agreement between the experimental and predicted values of IPS, and R^2 (coefficient of determination) was 0.9996, showing a good agreement between experimental and predicted values which can explain 99.96% variability of the responses. The value of adjusted determinant coefficient (adj-R^2) was 0.9990, suggesting that the total variation of 99.9% for IPS can be attributed to the independent variables and only 0.1% of the total variation cannot be explained by the model. The *F*-value (0.29) and *P*-value (0.8344) of lack-of-fit implied that it was not significant relative to the pure error, which indicated that the model equation was appropriate to predict the yield of IPS extraction under any combination of values.

By solving the inverse matrix (from Eq. (6)), the optimal values of the variables affecting IPS yield were ultrasonic treatment time 618.07 s, extraction temperature 84.53 °C, and pH 7.57. Under these optimal conditions, the model gave the maximum predicted values of IPS extraction (6.84%). In view of the operating convenience, the optimal extraction parameters were determined to be ultrasonic treatment time 620 s, extraction temperature 85 °C, and pH 7.6, while the predicted value of IPS extraction was 6.85%.

Triplicate experiments were performed under the determined conditions and the value of IPS extraction (6.93 ± 0.22%) in agreement with the predicted value (6.84%) was obtained, indicating that the model was adequate for the process of IPS extraction.

Antioxidant Capacities of IPS *in vitro*

Antioxidant activities also have been attributed to various reactions and mechanisms, such as radical scavenging, reductive capacity, prevention of chain initiation, binding of transition metal ion catalysts, *etc.* (Frankel and Meyer 2000). In this experiment, the *in vitro* antioxidative capacities of IPS from *H. marmoreus* SK-01 were evaluated using different biochemical methods of hydroxyl, superoxide anion, and DPPH radical scavenging assay and reducing power analysis.

Hydroxyl radicals are main reactive oxygen free radicals in living organisms, which are the important reasons for causing the general processes of aging and tissue damage, and could influence the evolution of many degenerative diseases (In *et al*. 2002). As shown in Fig. 1A, the scavenging rate of IPS of *H. marmoreus* SK-01 reached 52.63 ± 3.19% ($P < 0.01$) at a dosage of 1 g/L, which was about 27% higher than that of BHT (41.51 ± 2.86%, $P < 0.05$). It was higher than 44.1% of *P. nebrodensis* (Li *et al*. 2003), 28.3% of *C. militaris* (Zhu *et al*. 2011), 47.3% of *Pholiota adiposa* (Yang *et al*. 2009), 39.8% of *G. applanatum* (Li *et al*. 2010), 35.9% of *G. lucidum* (Yi *et al*. 2010), 38.1% of *T. matsutake* (Wang *et al*. 2009), 20.9% of *C. sinensis* (Li *et al*. 2009), and 51.2% of *Inonotus obliquus* (Xu *et al*. 2011), respectively. The EC_{50} value of IPS (0.85 ± 0.05 g/L, $P < 0.01$) was lower than 1.6 g/L of *P. nebrodensis* (Li *et al*. 2003), 2.7 g/L of *C. militaris* (Zhu *et al*. 2011), 1.2 g/L of *P. adiposa* (Yang *et al*. 2009), 1.8 g/L of *G. applanatum* (Li *et al*. 2010), 1.3 g/L of *G. lucidum* (Yi *et al*. 2010), and 7.6 g/L of *T. matsutake* (Wang *et al*. 2009), respectively. The results showed that the IPS of *H. marmoreus* SK-01 significantly affects the scavenging of hydroxyl radical.

Superoxide anion is one of the precursors of the singlet oxygen and hydroxyl radicals; therefore, it indirectly initiates lipid peroxidation. Apart from that, the presence

of superoxide anion can magnify cellular damage because it produces other kinds of free radicals and oxidizing agents (Athukorala *et al*. 2006). The results of superoxide anion radical scavenging assay are shown in Fig. 1B, and the inhibition activity of IPS was concentration-dependent at a dosage range of 0.1 to 1 g/L. The scavenging rate of IPS of *H. marmoreus* SK-01 at 1 g/L was 68.21 ± 5.09% ($P < 0.01$), about 34% higher than that of BHT (50.81 ± 4.35%, $P < 0.01$). It was also higher than 21.4% of *C. militaris* (Zhu *et al*. 2011), 26.6% of *P. adiposa* (Yang *et al*. 2009), 56.6% of *Morchella esculenta* (Yang *et al*. 2010), 36.4% of *G. applanatum* (Li et al. 2010), and 43.8% of *C. sinensis* (Li *et al.* 2009), respectively. The EC_{50} value of IPS was 0.47 ± 0.03 g/L ($P < 0.01$), which was about 106% lower than that of BHT (0.97 ± 0.05 g/L, $P < 0.05$), indicating that the IPS significantly affects the scavenging of superoxide anion radical.

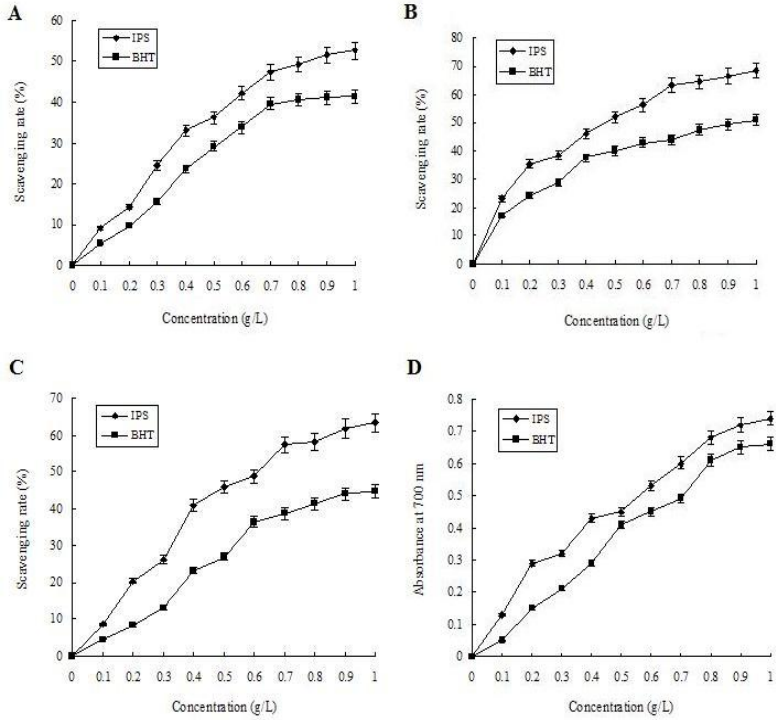

Fig. 1. Antioxidant activities of IPS *in vitro*. (A) Scavenging effect of IPS on hydroxyl radical, (B) Scavenging effect of IPS on superoxide anion radical, (C) Scavenging effect of IPS on DPPH, and (D) Reducing power of IPS

DPPH is a stable free radical that shows maximum absorbance at 517 nm in ethanol. When DPPH encounters a proton-donating substance such as an antioxidant, the radical would be scavenged and the absorbance is reduced (Shimada *et al*. 1992). It can be seen from Fig. 1C that the DPPH scavenging ability of IPS at 1 g/L reached 63.43 ± 5.27% ($P < 0.01$), which was higher than 44.79 ± 3.82% of BHT ($P < 0.05$), 30.8% of *C. militaris* (Zhu *et al*. 2011), 27.3% of *P. baumii* (Xie *et al*. 2011), 45.3% of *G. applanatum* (Li *et al*. 2010), 17.8% of *C. sinensis* (Li *et al*. 2009), and 22.4% of *Lentinula edodes* (Turło *et al*. 2010), respectively. The EC_{50} value of IPS was 0.62 ± 0.04 g/L ($P < 0.05$), which was much lower than 2.1 g/L of *C. militaris* (Zhu *et al.*, 2011), 1.8 g/L of *P. baumii* (Xie *et al*. 2011), 1.2 g/L of *G. applanatum* (Li *et al*. 2010), and 2.7 g/L of *I. obliquus* (Xu *et al*. 2011), respectively.

Figure 1D shows that the reducing power of IPS at 1 g/L was 0.74 ± 0.05 (P < 0.01), which was not only 12.12 ± 1.07% higher than that of BHT (0.66 ± 0.04, P < 0.05), but also higher than 0.1 of *P. baumii* (Xie *et al*. 2011), 0.4 of *G. applanatum* (Li *et al*. 2010), and 0.5 of *L. edodes* (Turło *et al.* 2010), respectively. The EC50 value of IPS (0.54 ± 0.02 g/L, P < 0.01) was about 31% lower than that of BHT (0.71 ± 0.05 g/L, P < 0.05). These results indicated that the IPS of *H. marmoreus* SK-01 in this study has potential antioxidant capacities *in vitro*.

Antioxidant Activity of IPS *in vivo*

GSH-Px catalyzes the reduction of hydrogen peroxide that derived from oxidative metabolism as well as peroxides from oxidation of lipids and is considered to be the most effective enzyme against lipid peroxidation (Zhang *et al*. 2009). As shown in Fig. 2A, the GSH-Px activities of 400 mg/kg group in blood, heart, liver, spleen, and kidney of mice were 2.52 ± 0.02 U/mL, 1.04 ± 0.01 U/mg, 1.81 ± 0.02 U/mg, 0.52 ± 0.01 U/mg, and 0.65 ± 0.03 U/mg, respectively, which were 51.81 ± 3.64%, 40.54 ± 3.29%, 110.47 ± 9.05%, 79.31 ± 6.17%, and 66.67 ± 5.64% higher than that of the control, respectively. The ANOVA showed that GSH-Px activities of blood, heart, liver, spleen, and kidney of mice treated with IPS were significantly higher (P < 0.01). These data showed that the IPS tested has a noticeable effect on improving GSH-Px activity.

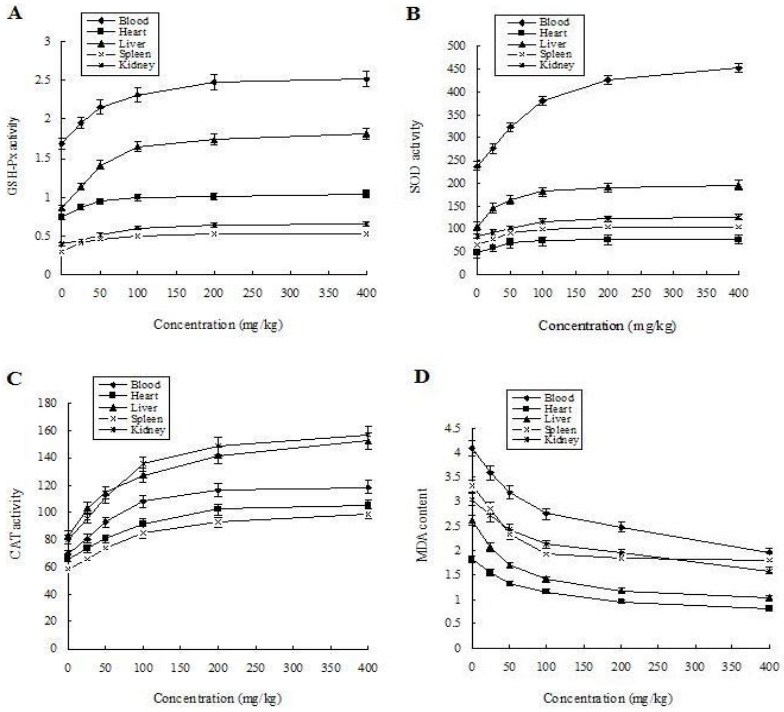

Fig. 2. Effects of IPS on biochemical parameters in blood, heart, liver, spleen, and kidney of mice. (A) GSH-Px activity, (B) SOD activity, (C) CAT activity, and (D) MDA content. The units of GSH-Px, SOD, and CAT activity are expressed as U/mg protein in heart, liver, spleen, and kidney, and U/mL in blood. The unit of MDA content is expressed as nmoL/mg protein in heart, liver, spleen, and kidney, and nmoL/mL in blood

SOD catalyzes the dismutation of superoxide radicals into oxygen and hydrogen peroxide plus, thus participating with other antioxidant enzymes in the enzymatic defense against oxidative injury (Luo *et al*. 2010). It can be seen from Fig. 2B that the SOD

activities of 400 mg/kg group in blood, heart, liver, spleen, and kidney of mice reached 453.19 ± 38.43 U/mL, 76.47 ± 5.62 U/mg, 195.83 ± 17.05 U/mg, 104.21 ± 9.25 U/mg, and 125.87 ± 11.48 U/mg, respectively, which were about 91%, 62%, 92%, 57%, and 47% higher than that of the control, respectively. The ANOVA showed that the activity of SOD at a dosage of 400 mg/kg was significant compared to that of the control group ($P < 0.01$).

CAT is the most important enzyme to provide a homeostasis for hydrogen peroxide. Chance *et al.* (1979) reported that the physiological variation of CAT concentration in different organs and tissues leads to different steady-state levels of hydrogen peroxide concentration for the same rate of hydrogen peroxide generation. Figure 2C showed that the CAT activities of 400 mg/kg group in blood, heart, liver, spleen, and kidney of mice reached 118.59 ± 9.64 U/mL, 105.01 ± 8.67 U/mg, 152.15 ± 13.47 U/mg, 99.05 ± 8.25 U/mg, and 156.73 ± 12.08 U/mg, respectively, which were about 71%, 58%, 83%, 69%, and 96% higher than that of the control, respectively.

MDA, a secondary product of lipid peroxidation, is used as an indicator of oxidative stress, and the value of MDA can effectively reflect the content of free radical produced by lipid peroxidation (Jayakumar *et al*. 2011). As shown in Fig. 2D, the MDA contents at 400 mg/kg group in blood, heart, liver, spleen, and kidney of mice were 1.96 ± 0.02 nmoL/mL, 0.82 ± 0.01 nmoL/mg, 1.03 ± 0.01 nmoL/mg, 1.81 ± 0.02 nmoL/mg, and 1.59 ± 0.02 nmoL/mg, respectively, which were about 109%, 122%, 154%, 83%, and 91% lower than that of control, respectively.

These data indicated that the IPS of *H. marmoreus* SK-01 can increase antioxidant abilities by improving SOD, GSH-Px, and CAT activities, and reducing MDA content in blood, heart, liver, spleen, and kidney of mice. The reason for the strong ability of antioxidation may be due to the molecular biochemical composition of IPS and the higher extraction rate of IPS. The antioxidant mechanism of IPS is an area for future studies.

CONCLUSIONS

1. A three-factor-three-level central composite design was a successful tool for extraction optimization of intracellular polysaccharide (IPS) produced by *H. marmoreus* SK-01 in submerged culture.
2. The IPS showed antioxidant activities *in vitro* and *in vivo*.
3. The results provide a reference for large-scale extraction of IPS by *H. marmoreus* SK-01 in industrial fermentation, suggesting that IPS can be used as a potential antioxidant which enhances adaptive immune responses.

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