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Extraction optimization and antioxidant activity of intracellular selenium polysaccharide by *Cordyceps sinensis* SU-02

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ABSTRACT

Central composite design (CCD) was used to optimize the extraction parameters of intracellular selenium polysaccharide (ISPS) from *Cordyceps sinensis* SU-02 mycelium in submerged culture. The optimum conditions for ISPS extraction were predicted to be, ultrasonic treatment for 15.11 min, extraction temperature at 59.26 °C and pH 7.17, and ISPS production was estimated at 11.52%. The *in vivo* antioxidant results of ISPS showed that the activities of glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) of mice blood were 2.36 ± 0.17 U and 477.92 ± 35.14 U, respectively, and malonaldehyde (MDA) level was 0.65 ± 0.04 μM/l. The *in vitro* inhibition effects of ISPS on superoxide anion radical and hydroxyl radical were 38.53 ± 3.19% and 43.22 ± 3.29%, respectively, and the reducing power of ISPS was 0.61 ± 0.04. The results suggested that the ISPS could be used as a potential antioxidant which enhances adaptive immune responses.

1. Introduction

Selenium (Se) is an essential trace element of glutathione peroxidase (GSH-Px) (Ursini et al., 1995), Type I, II and III iodothyronine deiodinase (Berry et al., 1991; Pallud et al., 1997; Ramauge et al., 1996), and thioredoxin reductase in human and animal bodies (Tamura & Stadtman, 1996). Se also participates in synthesis of enzymes and protects the structure and function of biomembrane from overoxidation and damage. Se-deficiency can cause about 40 diseases (Yang, Zhang, Wang, & Huang, 2003). There are 700 million people with lacking or lower selenium in China (Ge & Chang, 1999).

Cordyceps sinensis is widely used as a traditional medicine in China for treatment of a wide variety of diseases (Yu, Wang, Huang, & Du, 2006). It contains many biological active materials, such as cordycepin, protein, fat, trace elements, ash, fiber, and carbohydrates (Leung, Zhang, & Wu, 2006). Polysaccharides from *C. sinensis* fruiting bodies or fermentation broths have potential antioxidation, antitumor, antivirus, and immunomodulating properties (Chen, Zhang, Shen, & Wang, 2010; Cheung et al., 2009; Ohta et al., 2007).

Since extraction parameters for intracellular selenium polysaccharide (ISPS) from *C. sinensis* mycelium have not been studied, statistical designs were applied to optimize extraction conditions. Factors affecting the extraction of ISPS 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 by central composite design (CCD). In addition, the *in vivo* and *in vitro* experiments were performed to explore the antioxidant activities of ISPS.

2. Materials and methods

2.1. Strain and liquid culture

A strain of *C. sinensis* SU-02 was provided by our laboratory and maintained on synthetic potato dextrose agar (PDA). The cultures were incubated for 7 days at 25 °C, stored at 4 °C and subcultured every 2 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, $MgSO_4 \cdot 7H_2O$, 1 and Na_2SeO_3 0.04 g with natural pH. Flasks were inoculated with a 0.5-cm^2 mycelial block of *C. sinensis* SU-02 from the solid media, incubated at 25 °C for 24 h without shaking, and then shaken on a rotary shaker (Anting, China) at 160 rpm for 5 days.

2.2. Measurement and preparation of ISPS

The mycelium precipitate of *C. sinensis* SU-02 was obtained by centrifugation (3000 × *g*, 10 min), and dried to a constant weight at

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60 °C for 24 h. The mycelia powder (8 g) was dissolved in distilled water for 2 h, and then was fragmented with ultrasonic processor (Sonics & Materials, USA) for 15 min. The supernatant was mixed with 3 volumes of 95% ethanol (v/v), stirred vigorously, and kept at 4 °C for 18 h. After centrifugation (3000 × *g*, 15 min), the precipitated ISPS was dissolved in distilled water (60 °C), and the ISPS content was determined by the phenol–sulfuric acid method, using glucose as the standard (Chaplin & Kennedy, 1994). ISPS 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 detect the antioxidant activities *in vivo* and *in vitro*. The extraction rate of ISPS was expressed as a percentage of ISPS to mycelium (w/w). The intracellular polysaccharide (IPS) powder of *C. sinensis* SU-02, a positive comparison, was obtained according to the former method of ISPS preparation.

2.3. PB design for ISPS extraction

Initial screening of the most significant parameters affecting ISPS extraction by *C. sinensis* SU-02 was performed by PB design as reported by Plackett and Burman (1946). Eight variables including mesh number, water multiple, ultrasonic treatment time, ultrasonic power, concentration multiple, extraction time, extraction temperature, and pH were studied in this experiment. In addition, 5 center points were added for the variables that could be assigned numerical values. The experimental design with the name, symbol code, and actual level of the variables is shown in Tables 1 and 2.

2.4. CCD for ISPS extraction

Based on the results of the PB tests, ultrasonic treatment time, extraction temperature, and pH were chosen for optimization of ISPS extraction by CCD. The experimental design with name, symbol code, and actual level of the variables is shown in Tables 3 and 4. The test factors were coded according to the following equation:

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad i = 1, 2, 3, \ldots, k \tag{1}$$

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 variables, the following quadratic polynomial equation was applied to fit the response variable to a quadratic model:

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

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, x_i and x_j are the coded level of independent variables.

Table 1
Levels and codes of variables for Plackett–Burman design.

Variables	Symbol code	Coded levers		
		−1	0	1
Mesh number	A_1	40	60	80
Water multiple	A_2	10	20	30
Ultrasonic treatment time (min)	A_3	10	15	20
Ultrasonic power (W)	A_4	500	600	700
Concentration multiple	A_5	3	2	1
Extraction time (min)	A_6	30	60	90
Extraction temperature (°C)	A_7	40	60	80
pH	A_8	5	7	9

Table 2
Results of Plackett–Burman for extraction rate of ISPS.

Runs	A_1	A_2	A_3	A_4	A_5	A_6	A_7	A_8	ISPS (%)
1	1	1	−1	1	1	1	−1	−1	9.71
2	−1	1	1	−1	1	1	1	−1	10.70
3	1	−1	1	1	−1	1	1	1	11.59
4	−1	1	−1	1	1	−1	1	1	10.09
5	−1	−1	1	−1	1	1	−1	1	10.37
6	−1	−1	−1	1	−1	1	1	−1	8.67
7	1	−1	−1	−1	1	−1	1	1	10.28
8	1	1	−1	−1	−1	1	−1	1	10.14
9	1	1	1	−1	−1	−1	1	−1	10.98
10	−1	1	1	1	−1	−1	−1	1	10.36
11	1	−1	1	1	1	−1	−1	−1	9.55
12	−1	−1	−1	−1	−1	−1	−1	−1	7.59
13	0	0	0	0	0	0	0	0	11.13
14	0	0	0	0	0	0	0	0	11.01
15	0	0	0	0	0	0	0	0	10.86
16	0	0	0	0	0	0	0	0	11.68
17	0	0	0	0	0	0	0	0	10.81
F-value	57.829	44.843	144.211	0.024	5.364	15.671	61.187	92.243	
P	0.061	0.072	0.001**	0.086	0.104	0.029*	0.004**	0.002**	

** $P < 0.01$.
* $P < 0.05$.

Table 3
Levels and codes of variables for central composite design.

Variables	Symbols		Coded levels				
	Uncoded	Coded	−2	−1	0	1	2
Ultrasonic treatment time (min)	X_1	x_1	5	10	15	20	25
Extraction temperature (°C)	X_1	x_2	20	40	60	80	100
pH	X_3	x_3	3	5	7	9	11

Table 4
Experimental and predicted values of ISPS based on central composite design.

Runs	x_1	x_2	x_3	ISPS (%)	
				Experimental	Predicted
1	−1	−1	−1	7.48	7.63
2	1	−1	−1	7.44	7.85
3	−1	1	−1	7.27	7.58
4	1	1	−1	6.99	7.36
5	−1	−1	1	6.78	7.36
6	1	−1	1	7.14	7.78
7	−1	1	1	6.97	7.51
8	1	1	1	6.69	7.49
9	−2	0	0	6.66	6.35
10	2	0	0	7.19	6.55
11	0	−2	0	7.04	6.62
12	0	2	0	6.81	6.28
13	0	0	−2	6.29	6.15
14	0	0	2	6.83	6.02
15	0	0	0	11.28	11.30
16	0	0	0	11.25	11.30
17	0	0	0	11.24	11.30
18	0	0	0	11.17	11.30
19	0	0	0	12.49	11.30
20	0	0	0	11.33	11.30

2.5. Antioxidant activity of ISPS in vivo

Sixty-three male mice (Kunming strain), weighing 20 ± 2 g, were obtained from Missile Co., Ltd. (Taian, China) and housed in stainless steel cages under controlled conditions (temperature 22 ± 1 °C, humidity 60–65%, lights on 12 h every day). After a 3-day acclimatization period, all animals were randomly divided into 3 groups (21 in each group): (1) Group 1 (control group): treated with 0.4 ml saline; (2) Group 2 (ISP group): treated with 0.4 ml of ISP solution (0.3 mg/l) of *C. sinensis* SU-02; (3) Group 3 (ISPS): treated with 0.4 ml of ISPS solution (0.3 mg/l) of *C. sinensis* SU-02. The mice were allowed to have free access to water and food for 18 days. All the different doses of tested samples were rinsed into the stomach of mice by a drencher at intervals of 3 days, respectively. The experiments were performed under guidance of the Shandong Agricultural University Committee.

The animals were sacrificed under ether anesthesia, and retrobulbar venous blood specimens of mice were collected from three mice chosen randomly from each group on 0, 3, 6, 9, 12, 15, and 18 days, respectively. Blood samples were anticoagulated by heparin and stored at −80 °C for the evaluation of the activities of glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and the content of malonaldehyde (MDA).

Blood sample, 10 μl, diluted to 1 ml by distilled water, was placed for 4 h, and the activity of GSH-Px was measured by the method of Rong, Liu, and Bao (1994). One unit of GSH-Px activity was defined as the amount of enzyme that decreased 1 μM of GSH of blood (1 μl) within the reaction time (1 min) at 37 °C and pH 6.5, compared to that in the nonenzyme reaction.

The SOD activity was measured according to the method of Bayer and Fridovich (1987). Fifty microliters of blood sample was mixed with 1 ml of physiological saline, centrifuged for 3 min at 2500 rpm, and the pellet was used for the enzyme reaction. Ice-cold water (0.2 ml), 0.2 ml 95% ethanol, 0.1 ml trichloromethane were added to the pellet and mixed thoroughly with a homogenizer (Bagmixer 400, France). The mixture presented three layers after centrifugation (3500 × *g*, 5 min): SOD extract (top layer), haemoglobin precipitation (middle layer) and trichloromethane (bottom layer). The top layer was removed by micropipettor carefully and the mixture, including 0.05 ml of SOD extract, 50 mM potassium phosphate buffer (pH 7.0), 13 mM methionine, 0.075 mM nitroblue tetrazolium (NBT), 0.01 mM EDTA and 0.002 mM riboflavin, was reacted for 20 min. The enzyme activity was expressed in relative units per 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).

The content of malondialdehyde (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 of 0.6% thiobarbituric acid (TBA (w/v)), was heated in a boiling water for 15 min. After cooling rapidly, the mixture was centrifuged at 3000 × *g* for 10 min, and the supernatant was used for the determination of MDA level.

2.6. Antioxidant capacity of ISPS in vitro

The superoxide anion scavenging activity of ISPS was determined according to the method of Stewar and Beewley (1980) with slight modification. The reaction mixture (3 ml) contained 13 mM methionine, 10 mM riboflavin (RF), 75 μM NBT, 100 mM EDTA, 50 mM phosphate buffer (pH 7.8), and the ISPS (10–80 μg/ml). After illuminating the reaction mixture with a fluorescent lamp at 25 °C for 30 min, the absorbance of the ISPS was measured at 560 nm, using IPS for a positive control. The whole reaction was assembled in a box lined with aluminium foil. The inhibition percentage of superoxide anion generation was calculated using the following formula:

$$\text{Scavenging rate}\,(\%) = \frac{A_0 - A_1}{A_0} \times 100 \quad (3)$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the ISPS/IPS.

Hydroxyl radical scavenging activity was measured according to the method of Winterbourn and Sutton (1984). The reaction mixture contained 1 ml of 0.15 M phosphate buffer saline (pH 7.4), 1 ml of 40 μg/ml safranin, 1 ml of 0.945 mM EDTA–Fe (II), 1 ml H_2O_2 (3% (v/v)), and 0.5 ml of the ISPS (10–80 μg/ml). After incubating at 37 °C for 30 min, the absorbance of ISPS was measured at 560 nm, using IPS for a comparison. The hydroxyl radical scavenging activity was expressed as:

$$\text{Scavenging rate}\,(\%) = \frac{A_0 - A_1}{A_0} \times 100 \quad (4)$$

where A_0 is the absorbance of the control and A_1 is the absorbance of ISPS/IPS.

The reducing power of ISPS was evaluated according to the method of Oyaizu (1986) 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 ISPS (10–80 μg/ml). 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 1200 × *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 room temperature for 15 min, the absorbance of the ISPS was measured at 700 nm, using IPS for a comparison.

2.7. Statistical analysis

All experiments were carried out in triplicates. Data were processed and analyzed using Design Expert Software (version 7.1.3, Stat-Ease. Inc., Minneapolis, USA) including ANOVA.

3. Results and discussion

3.1. Determination of parameters of ISPS extraction

The maximum rate of ISPS extraction reached 11.68%, while the extraction parameters were: mesh number, 60; water multiple, 20; ultrasonic treatment time, 15 min; ultrasonic power, 600 W;

Table 5
ANOVA for the evaluation of the quadratic model.

Source	Coefficients	S.E.	Sum of squares	Mean square	*F*-value	*P*
Model	–	–	32,859.86	3651.10	17.51	<0.0001**
Intercept	226.05	5.76	–	–	–	–
x_1	1.01	3.61	16.24	16.24	0.08	0.0959
x_2	−1.70	3.61	46.44	46.44	0.22	0.0771
x_3	−0.67	3.61	7.21	7.21	0.03	0.0862
x_1x_2	−2.21	5.11	39.07	39.07	0.19	0.0243*
x_1x_3	0.98	5.11	7.64	7.64	0.04	0.0466*
x_2x_3	0.99	5.11	7.84	7.84	0.04	0.0351*
${x_1}^2$	−24.27	2.88	14,808.44	14,808.44	71.02	<0.0001**
${x_2}^2$	−24.26	2.88	14,796.24	14,796.24	70.96	<0.0001**
${x_3}^2$	−26.09	2.88	17,114.44	17,114.44	82.08	<0.0001**
Lack-of-fit			1568.21	313.64	3.03	0.1243

R^2 = 0.9403.
Adj-R^2 = 0.9676.
R = 0.9697.
** P < 0.01.
* P < 0.05.

concentration multiple, 2; extraction time, 60 min; extraction temperature, 60 °C; and pH, 7 (Table 2). ANOVA results showed that ultrasonic treatment time, extraction temperature and pH had a highly significant influence on ISPS extraction at the 1% level. The influence of other parameters was at the 5% level or not significant (Table 2). Therefore, ultrasonic treatment time, extraction temperature and pH were chosen for the optimization process of ISPS extraction by CCD.

3.2. CCD optimization for ISPS extraction

The experiments were planned to obtain a quadratic model consisting of 14 runs and 6 center points. The range and levels of three independent variables are shown in Table 3. The CCD matrix together with the experimental and predicted ISPS 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 ISPS and test variables in coded units is expressed by Eq. (5).

$$Y_{ISPS} = 226.05 + 1.01x_1 - 1.70x_2 - 0.67x_3 - 2.21x_1x_2 + 0.98x_1x_3 + 0.99x_2x_3 - 24.27x_1^2 - 24.26x_2^2 - 26.09x_3^2 \quad (5)$$

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

The F value and P value were used to check the significance of each coefficient, which also indicated the interaction strength between independent variables. The larger the magnitude of the F value and smaller the P value, the more significant is the corresponding coefficient (Elibol, 2004). Table 5 shows that the quadratic coefficients of ${x_1}^2$, ${x_2}^2$, and ${x_3}^2$ were significant at the 1% level, and that the cross product coefficients of x_1x_2, x_1x_3, and x_2x_3 were significant at the 5% level. These results indicated that the ultrasonic treatment time, extraction temperature, and pH all correlated with a direct relationship with the extraction rate of ISPS.

The ANOVA results for the effect of parameters on ISPS extraction (Table 5) demonstrated that the model was highly significant (P < 0.001). The value of correlation coefficient (R = 0.9697) indicated good agreement between the experimental and predicted values of ISPS. The lack-of-fit measured the failure of the model to represent data in the experimental domain at points which are not included in the regression. The value of determinations coefficient (R^2) was 0.9403, indicating a good agreement between experimental and predicted values which can explain 94.03% variability of the responses. The value of adjusted determinant coefficient (adj-R^2) was 0.9676, suggesting that the total variation of nearly 97% for ISPS is attributed to the independent variables and only about 3% of the total variation cannot be explained by the model. The tested model was statistically significant at the 1% level, and the non-significant lack of fit also indicated that the model was a good fit.

By solving the inverse matrix (from Eq. (5)), the optimal values of the variables affecting the extraction rate of ISPS given by the software were ultrasonic treatment time 15.11 min, extraction temperature 59.26 °C, and pH 7.17. In view of the operating convenience, the optimal extraction parameters were determined to be ultrasonic treatment time 15 min, extraction temperature 59 °C and pH 7.2, while the predicted value of ISPS extraction was 11.28%, slightly lower than that of the maximum predicted value (11.52%).

Triplicate experiments were performed under the determined conditions and the extraction rate of ISPS (11.91%) was in agreement with the predicted value (11.28%), indicating that the model was adequate for ISPS extraction process.

3.3. Antioxidant activities of ISPS in vivo

As main index of antioxidant activities *in vivo*, the values of GSH-Px, SOD, and MDA can effectively reflect the degree of cell injury and lipid hydroperoxide in organism (Fang & Zheng, 2002).

GSH-Px catalyzes the reduction of hydrogen peroxide that derived from oxidative metabolism as well as peroxides from oxidation of lipids and is considered the most effective enzyme against lipid peroxidation (Zhang et al., 2009). The GSH-Px activity of ISPS group in mice blood reached the maximum (2.36 ± 0.17 U, P < 0.01) on day 9, which was 6.41 ± 0.49% and 2.79 ± 0.16% higher than that of control group (2.22 ± 0.13 U, P < 0.01) and IPS group (2.30 ± 0.14 U, P < 0.05), respectively (Table 6). SOD can eliminate the superoxide anion radical to protect cells from damage. Experiments in animals demonstrate a correlation between SOD and tolerance to oxygen toxicity (Zhang et al., 2009). It can be seen from Table 6 that SOD activity of ISPS group reached its peak (477.92 ± 35.14 U; P < 0.01) on day 12, which was 23.78 ± 2.17% and 7.68 ± 0.53% higher than that of control group (386.09 ± 27.81 U; P > 0.05) and IPS group (443.83 ± 31.27 U; P < 0.05), respectively. MDA is considered as a secondary indicator formed by lipid peroxidation, reflecting the content of free radicals produced by lipid peroxidation (Niki, Yoshida, Saito, & Noguchi, 2005). As shown in Table 6, the MDA level of ISPS group diminished and reached the minimum (0.65 ± 0.04 μM/l; P < 0.05) on day 18, which was

Table 6
Antioxidant activity of ISPS of *Cordyceps sinensis* SU-02 *in vivo*.

Index	Time (d)						
	0	3	6	9	12	15	18
GSH-Px (U)							
Control	1.99 ± 0.09	2.10 ± 0.11	2.13 ± 0.14	2.22 ± 0.13	2.20 ± 0.17	2.17 ± 0.15	2.11 ± 0.14
IPS	1.97 ± 0.11	2.23 ± 0.16	2.25 ± 0.15	2.30 ± 0.14	2.28 ± 0.15	2.26 ± 0.13	2.25 ± 0.12
ISPS	1.97 ± 0.11	2.25 ± 0.15	2.30 ± 0.17	2.36 ± 0.17	2.34 ± 0.16	2.33 ± 0.15	2.30 ± 0.17
SOD (U)							
Control	245.23 ± 19.01	260.56 ± 21.32	296.07 ± 23.09	321.28 ± 24.67	386.09 ± 27.81	367.25 ± 25.04	337.11 ± 24.18
IPS	239.03 ± 18.72	292.26 ± 22.19	351.92 ± 26.43	428.31 ± 29.38	443.83 ± 31.27	425.04 ± 29.04	401.01 ± 28.36
ISPS	237.56 ± 18.04	324.21 ± 22.15	387.03 ± 26.45	466.17 ± 34.27	477.92 ± 35.14	453.04 ± 33.27	420.15 ± 31.09
MDA (μM/l)							
Control	3.22 ± 0.16	3.02 ± 0.21	2.89 ± 0.15	2.68 ± 0.14	2.45 ± 0.12	2.33 ± 0.12	2.16 ± 0.11
IPS	3.22 ± 0.18	2.59 ± 0.14	1.78 ± 0.11	1.58 ± 0.11	1.36 ± 0.09	1.04 ± 0.08	0.73 ± 0.05
ISPS	3.25 ± 0.21	2.11 ± 0.18	1.53 ± 0.14	1.29 ± 0.11	0.96 ± 0.09	0.69 ± 0.05	0.65 ± 0.04

Table 7
Antioxidant activity of ISPS of *Cordyceps sinensis* SU-02 *in vitro*.

Concentration (mg/l)	Superoxide radical Scavenging rate (%)		Hydroxyl radical Scavenging rate (%)		Reducing Power	
	IPS	ISPS	IPS	ISPS	IPS	ISPS
0	0	0	0	0	0	0
10	5.23 ± 0.24	6.99 ± 0.41	9.08 ± 0.63	11.66 ± 0.91	0.04 ± 0.00	0.06 ± 0.00
20	8.96 ± 0.35	10.05 ± 0.72	12.81 ± 0.86	14.72 ± 1.11	0.09 ± 0.00	0.12 ± 0.00
30	15.38 ± 1.01	19.44 ± 1.25	19.23 ± 1.47	24.11 ± 1.49	0.15 ± 0.01	0.19 ± 0.01
40	22.19 ± 1.73	25.82 ± 1.97	26.04 ± 2.19	30.49 ± 2.26	0.22 ± 0.01	0.25 ± 0.01
50	25.56 ± 1.82	29.76 ± 2.38	35.41 ± 2.67	37.98 ± 2.31	0.31 ± 0.02	0.37 ± 0.02
60	31.28 ± 2.17	35.87 ± 2.81	35.59 ± 2.90	40.26 ± 3.28	0.43 ± 0.03	0.46 ± 0.02
70	33.47 ± 2.33	36.83 ± 3.07	36.52 ± 3.02	41.11 ± 3.42	0.45 ± 0.03	0.53 ± 0.04
80	34.82 ± 2.37	38.53 ± 3.19	36.89 ± 3.11	43.22 ± 3.29	0.52 ± 0.04	0.61 ± 0.04

285.71 ± 22.15% and 12.31 ± 1.06% lower than that of control group (2.16 ± 0.11 μM/l; $P < 0.05$) and IPS group (0.73 ± 0.05 μM/l; $P < 0.01$), respectively.

These data showed that the ISPS of *C. sinensis* SU-02 can increase antioxidant abilities by improving SOD and GSH-Px activities, and reducing the MDA content in mice blood. The mechanism by which the activity of these enzymes is increased and by which MDA production is inhibited could be direct activation of the enzymes or binding of metal ions which are necessary for organisms to produce free radical (Volpi & Tarugi, 1999).

3.4. Antioxidant capacities of ISPS 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 & Meyer, 2000). In this experiment, the *in vitro* antioxidative capacities of ISPS from *C. sinensis* SU-02 were evaluated using different biochemical methods of superoxide anion radical assay, hydroxyl radical scavenging assay and reducing power analysis.

The superoxide anion radical scavenging ability of ISPS was concentration-dependent at the dosage of 10–80 μg/ml (Table 7). The inhibition percentage of ISPS at 80 μg/ml was 38.53 ± 3.19% ($P < 0.01$), which was 10.65 ± 0.81% higher than that of IPS (34.82 ± 2.37%, $P < 0.01$). Some reports showed that the scavenging rates of IPS of *Marasmius androsaceus* (Wang et al., 2006), *Coprinus comatus* (Wu, Xie, Xie, & Lin, 2007), *Coriolus versicolor* (Zhou & Ma, 2008) and *Hypsizigus marmoreus* (Li, Zhang, Fu, & Liu, 2008) at 80 μg/ml were 14.4%, 9.2%, 8.5%, and 15.3%, respectively, indicating that the ISPS of *C. sinensis* SU-02 significantly affects the scavenging of superoxide anion radical.

The results of hydroxyl radical scavenging assay are described in Table 7. The inhibition rate of ISPS at 80 μg/ml reached 43.22 ± 3.29% ($P < 0.01$), which was not only higher than that of IPS (36.89 ± 3.11%, $P < 0.05$) of *C. sinensis* SU-02, but also much higher than that of IPS of *M. androsaceus* (12.6%) (Wang et al., 2006), *C. comatus* (9.1%) (Wu et al., 2007), *C. versicolor* (7.4%) (Zhou & Ma, 2008), and *H. marmoreus* (10.3%) (Li et al., 2008), respectively.

As shown in Table 7, the reducing power (absorbance at 700 nm) of ISPS at 80 μg/ml was 0.61 ± 0.04 ($P < 0.01$), which was 17.31 ± 1.35% higher than that of IPS. Wu et al. (2007) and Li et al. (2008) reported that the scavenging rates of IPS of *C. comatus* and *H. marmoreus* were 0.16 and 0.08, respectively, remarkably lower than that in this experiment.

4. Conclusion

A three-factor-five-level central composite design was a successful tool for optimization of extraction of ISPS produced by *C. sinensis* SU-02 in submerged culture. The ISPS showed antioxidative activities *in vivo* and *in vitro*. The results suggested that the ISPS could be used as a potential antioxidant which enhances adaptive immune responses.

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