Carbohydrate Polymers 95 (2013) 644–648

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Optimizing conditions of polysaccharide extraction from *Shiitake* mushroom using response surface methodology and its regulating lipid metabolism

Min Zhu [a], Ping Nie [a], YongKang Liang [b], Bing Wang [b,*]

[a] *Department of Oral & Cranio-maxillofacial Science, Shanghai Ninth People's Hospital, College of Stomatology, Shanghai Jiao Tong University School of Medicine, Shanghai Key Laboratory of Stomatology, Shanghai 200011, China*
[b] *Department of General Surgery, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200011, China*

ARTICLE INFO

Article history:
Received 8 January 2013
Received in revised form 5 March 2013
Accepted 7 March 2013
Available online 15 March 2013

Keywords:
Shiitake mushroom polysaccharides
RSM
Extraction
Antioxidant
Lipid metabolism

ABSTRACT

Process conditions (extraction time, extraction temperature, water/solid ratio and incubation time) of *Shiitake* mushroom polysaccharides (SMP) were optimized by conducting experiments at three different levels using the response surface method (RSM). A second-order polynomial response surface equation was developed indicating the effect of variables on polysaccharides yield. Contour maps generated using the response surface equation showed that all the experimental variables significantly affected the yield. The effect of SMP on oxidative damage in mice fed by high cholesterol diet (HCD) was done in vivo. Results showed that SMP can decreased serum total cholesterol (TC), triglyceride (TG), low density lipoprotein (LDL-c) levels, and increased high density lipoprotein (HDL-c) levels in HCD mice. Treatment with SMP reduced blood, liver lipid peroxidation level and increased antioxidant enzymes activities. Thus it can be concluded that SMP can improve lipid metabolism and decreased oxidative damage in HCD mice.

1. Introduction

Edible mushrooms have long been used in folk medicines and health foods. The xylotropic basidiomycete *Lentinus edodes*, commonly known as the *Shiitake* mushroom, is one of the most popular edible mushrooms on the global market (Hatvani & Mećs, 2001). Various biocompounds extracted from *L. edodes* show antifungal activity, and inhibit the infectivity and the cytopathic effect of the human immunodeficiency virus (HIV). Recent research on this mushroom has also revealed the presence of many bioactive compounds that function as host defense potentiators, antibacterials, antitumor agents, and anti-HIV agents (Ngai & Ng, 2001; Song, Jeon, Yang, Ra, & Kim, 1998; Zhang, Li, Xu, & Zeng, 2005). The consumption of *shiitake* mushrooms has been shown to significantly lower blood cholesterol levels significantly and is reported to lower high blood pressure in laboratory animals (Hirasawa, Shouji, Neta, Fukushima, & Takada, 1999; Kabir, 1987). *Shiitake* contains all eight essential amino acids in better proportions than soy beans, meat, milk, or eggs as well as a good blend of vitamins and minerals including vitamins A, B, B12, C, D and Niacin (Brodziak, 1984; Murakami, 1997).

Cholesterol feeding has often been used to elevate serum or tissue cholesterol levels to study the etiology of hypercholesterolemia-related metabolic disturbances (Bocan, 1998; Stehbens, 1986). Rabbits are susceptible to the development of atherosclerosis, whereas rats are considered to be resistant to the induction of hypercholesterolemia by cholesterol feeding (Bocan, 1998; Stehbens, 1986). Oxidative stress is currently suggested as a mechanism underlying hypercholesterolemia. Free radicals are continually produced in the body as the result of normal metabolic processes and interaction with environmental stimuli. Oxidative stress results from imbalance between radical-generating and radical scavenging systems, i.e. increased free radical production or reduced activity of antioxidant defenses or the both.

In the present study, we examine the effect of *Shiitake* mushroom polysaccharides on oxidative damage of HCD mice.

2. Methods and materials

2.1. Preparation of aqueous extract of Shiitake mushroom polysaccharides

100 g of dried *Shiitake* mushroom was immersed in distilled water and boiled for 3 h under reflux. The filtrate was freeze-dried, and the resulting powder was used as the crude aqueous extract of the *Shiitake* mushroom.

* Corresponding author. Tel.: +86 13166107798; fax: +86 13166107798.
E-mail address: wangbsjges@sina.com (B. Wang).

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http://dx.doi.org/10.1016/j.carbpol.2013.03.035

Table 1
Box–Behnken design for three independent variables used in this study.

Run	X1 (extraction time, min)	X2 (extraction number)	X3 (liquid/solid)	*R* (extraction yield, %)
1	−1.00 (140)	−1.00 (5)	0.00 (1/7)	3.68
2	1.00 (200)	−1.00	0.00	4.62
3	−1.00	1.00 (7)	0.00	4.59
4	1.00	1.00	0.00	5.31
5	−1.00	0.00 (6)	−1.00 (1/8)	3.86
6	1.00	0.00	−1.00	4.95
7	−1.00	0.00	1.00 (1/6)	3.97
8	1.00	0.00	1.00	5.08
9	0.00 (170)	−1.00	−1.00	3.99
10	0.00	1.00	−1.00	5.13
11	0.00	−1.00	1.00	4.58
12	0.00	1.00	1.00	5.12
13	0.00	0.00	0.00	4.77
14	0.00	0.00	0.00	4.71
15	0.00	0.00	0.00	4.73
16	0.00	0.00	0.00	4.75
17	0.00	0.00	0.00	4.72

2.2. Optimization experimental design

The experimental design and statistical analysis were performed using design expert software. A three-level three-factor Box–Behnken design was chosen to evaluate the combined effect of three independent variables extraction time, number and solid–liquid ratio, coded as X1, X2 and X3, respectively. The minimum and maximum values for extraction time were set at 140 min and 200 min, extraction number between 5 and 7 and solid–liquid ratio 1:6 and 1:8 (Table 1). The response values were extraction yield. The complete design consisted of 17 combinations including five replicates of the center point (Table 1). The responses function (*R*) was partitioned into linear, quadratic and interactive components.

The analysis of variance (ANOVA) tables were generated and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. The significances of all terms in the polynomial were judged statistically by computing the *F*-value at a probability (*P*) of 0.001, 0.01 or 0.05. The regression coefficients were then used to make statistical calculations to generate contour maps from the regression models (Table 2 and Fig. 1).

2.3. Animal experimental design

Kunming mice ($n = 60$) weighing 22 ± 2 g were used in this study. Mice were divided into 4 group ($n = 15$): normal control (group 1), HCD group (group 2) and low, high dose of *Shiitake* mushroom polysaccharides-treated groups: Mice in group 1 was feed standard diet for 30 days. Mice in group 2 were fed with 1% cholesterol-enriched diet for 30 days. Mice in group 3 and 4 were fed with 1% cholesterol-enriched diet and *Shiitake* mushroom polysaccharides (100 mg or 200 mg/kg) for 30 days. Diets and tap water were freely available. Animals were kept in wire bottom cages at a temperature of 24 °C and relative humidity of 60%, and lights were automatically turned on from 7:00 AM to 7:00 PM. The General Guidelines on the Use of Living Animals in Scientific Investigations were followed, and the protocol and use of animals were approved by the institutional committee on animal care and use.

2.4. TC, TG, LDL-c and HDL-c levels

TC, TG, LDL-c and HDL-c levels were measured with commercially available kits.

2.5. Total GSH (reduced) and MDA content

The reduced glutathione (GSH) level was determined by the method of Ellman (1959).

MDA levels were determined by the fluorometric method described by Wasowich, Neve, and Peretz (1993) based on thiobarbituric acid (TBA) reactivity.

2.6. Antioxidant defense system assays

Catalase (CAT) activity was determined according to Lartillot, Kedziora, and Athias (1988) with little modification as described by Bergmeyer (1974). The enzyme activity was expressed as U/mg protein. Superoxide dismutase (SOD) activity was estimated according to the method of Beauchamp and Fridovich (1971). The developed blue color in the reaction was measured at 560 nm. Units of SOD activity were expressed as the amount of enzyme required to inhibit the reduction of NBT by 50% and the activity was expressed as U/mg protein. Glutathione peroxidase (GSH-Px) activity was measured according to the method of Shati, Elsaid, and Hafez (2011). The enzyme activity was expressed as U/mg protein.

2.7. Statistical analysis

All data are presented as mean ± SD. Statistical significance was examined through one-way analysis of variance and Duncan's multiple range tests. *P* values of <0.05 were assumed to be statistically significant.

3. Results

The results obtained from the design experiments were evaluated for the extraction of polysaccharides. An empirical relationship between the response and the independent variables has been expressed by the following quadratic model (1).

$$R = +4.74 + 0.48 \times A + 0.41 \times B + 0.10 \times C - 0.055 \times A \times B + 5.000E - 003 \times A \times C - 0.15 \times B \times C - 0.21 \times A^2 + 0.027 \times B^2 - 0.058 \times C^2 \quad (1)$$

The results of second-order response surface model in the form of analysis of variance (ANOVA) are shown in Table 2. The statistical significance of the model equation was evaluated by the *F*-test ANOVA. The significance of each coefficient was determined by *F* values and *P*-values. The *F*-value (51.91) with a low probability value ($P < 0.0001$) demonstrates a high significance for the regression model. The goodness of fit of the model was also checked by the multiple correlation coefficient (R^2). The value of predicted multiple correlation coefficient (pred. $R^2 = 0.7730$) is in reasonable

Table 2
Analysis of variance (ANOVA) of the response surface quadratic model for polysaccharides extraction.

Source	Sum of squares	df	Mean square	*F* value	*P*-value prob. > *F*	
Model	3.61	9	0.40	51.91	<0.0001	Significant
A–A	1.86	1	1.86	241.34	<0.0001	
B–B	1.34	1	1.34	174.26	<0.0001	
C–C	0.084	1	0.084	10.89	0.0131	
AB	0.012	1	0.012	1.57	0.2507	
AC	1.000E−004	1	1.000E−004	0.013	0.9126	
BC	0.090	1	0.090	11.66	0.0112	
A2	0.19	1	0.19	24.75	0.0016	
B2	3.069E−003	1	3.069E−003	0.40	0.5483	
C2	0.014	1	0.014	1.84	0.2176	
Residual	0.054	7	7.717E−003			
Lack of fit	0.052	3	0.017	29.71	0.0034	Significant
Pure error	2.320E−003	4	5.800E−004			
Cor. total	3.66	16				
Std. dev.		0.088		R^2	0.9852	
Mean		4.62		Adj. R^2	0.9663	
C.V. %		1.90		Pred. R^2	0.7730	
Press		0.83		Adeq. precision	26.493	

Table 3
Effect of *Shiitake* mushroom polysaccharides on serum TC, TG, LDL-c and HDL-c levels.

Group	TC (mmol/L)	TG (mmol/L)	LDL-c (mmol/L)	HDL-c (mmol/L)
1	3.04 ± 0.43	0.94 ± 0.08	0.69 ± 0.06	1.64 ± 0.18
2	7.12 ± 0.68[a]	1.81 ± 0.17[a]	1.57 ± 0.14[a]	0.91 ± 0.11[a]
3	5.39 ± 0.55[c]	1.45 ± 0.16[c]	1.09 ± 0.12[c]	1.28 ± 0.13[b]
4	4.27 ± 0.49[b]	1.19 ± 0.13[b]	0.88 ± 0.09[b]	1.57 ± 0.17[b]

[a] $P < 0.01$, compared with group 1.
[b] $P < 0.01$, compared with group 2.
[c] $P < 0.05$.

Table 4
Effect of *Shiitake* mushroom polysaccharides treatment on serum and liver MDA and GSH levels.

Group	MDA		GSH	
	Serum	Liver	Serum	Liver
1	3.07 ± 0.25	2.96 ± 0.24	159.28 ± 12.07	173.08 ± 15.83
2	7.28 ± 0.52[a]	6.44 ± 0.73[a]	111.05 ± 10.62[a]	122.15 ± 11.42[a]
3	5.02 ± 0.36[b]	4.61 ± 0.42[b]	144.82 ± 13.29[b]	156.03 ± 14.28[b]
4	4.26 ± 0.39[b]	3.38 ± 0.35[b]	155.37 ± 12.47[b]	170.29 ± 13.72[b]

[a] $P < 0.01$, compared with group 1.
[b] $P < 0.01$, compared with group 2.

agreement with the value of the adjusted multiple correlation coefficient (adj. $R^2 = 0.9663$).

The three-dimensional response surface curve for RSM was a rising ridge with a potential stationary point outside the experimental region (Fig. 1). The stationary point is the point at which the slope of the response surface is zero when taken in all directions. The response surface had a nearly stationary region. A near stationary region is defined as a region where the surface slopes (or gradient along the variable axis) are small compared to the estimate of experimental error. The response surface sharply increased as extraction was increased from 140 min to 200 min, became nearly stationary between 5.1% and 5.3% and then gradually started decreasing. Three-dimensional ridge shaped surface rose gradually along the extraction number axis, however the rise was small indicating that extraction number played a small role in increasing polysaccharides extraction yield. Maximum extraction yield was achieved at extraction time 200 min and extraction number 6.

Table 3 shows the effect of administration of *Shiitake* mushroom polysaccharides on serum TC, TG, LDL-c and HDL-c levels in different groups of mice. There was a significant ($P < 0.01$) increase in serum TC, TG, LDL-c and decrease in HDL-c levels in group 2 mice compared with group 1 mice. Treatment of mice with *Shiitake* mushroom polysaccharides (groups 3 and 4) resulted in a marked increase in serum HDL-c, and decrease in serum TC, TG, LDL-c levels compared to group 2 mice.

As shown in Table 4, serum and liver MDA levels were significantly ($P < 0.01$) increased and GSH levels were decreased in group 2 mice compared with group 1 mice. *Shiitake* mushroom polysaccharides treatment significantly increased GSH level and decreased ($P < 0.01$) MDA levels in groups 3 and 4 compared to group 2 mice.

Tables 5 and 6 showed that SOD, CAT and GSH-Px activities in serum and liver tissue of group 2 mice were significantly ($P < 0.01$) decreased compared to group 1 animals. A significant increase ($P < 0.01$) of the SOD, CAT and GSH-Px activities in serum and liver tissue of groups 3 and 4 was detected compared to group 2 animals.

Table 5
Effect of *Shiitake* mushroom polysaccharides treatment on serum SOD, CAT and GSH-Px activities.

Group	SOD (U/ml)	CAT (U/ml)	GSH-Px (U/ml)
1	241.07 ± 28.05	66.04 ± 5.09	50.27 ± 4.63
2	121.39 ± 14.52[a]	31.27 ± 3.51[a]	24.08 ± 2.99[a]
3	198.32 ± 18.05[b]	51.25 ± 4.89[b]	44.05 ± 4.72[b]
4	237.48 ± 26.13[b]	63.17 ± 5.71[b]	53.11 ± 4.94[b]

[a] $P < 0.01$, compared with group 1.
[b] $P < 0.01$, compared with group 2.

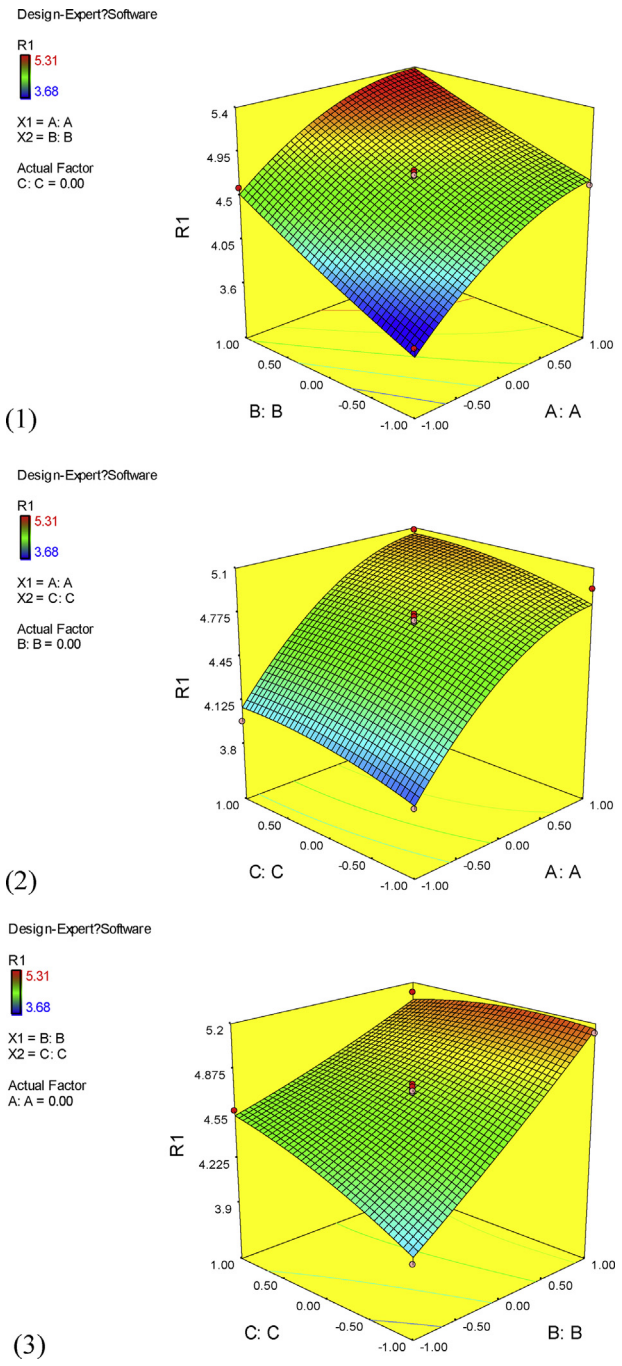

Fig. 1. Response surface plots for the yield of the *Shiitake* mushroom polysaccharides extract.

Table 6
Effect of *Shiitake* mushroom polysaccharides treatment on liver SOD, CAT and GSH-Px activities.

Group	SOD (U/mg)	CAT (U/mg)	GSH-Px (U/mg)
1	249.31 ± 21.05	56.28 ± 4.89	49.04 ± 4.82
2	150.72 ± 16.66[a]	20.59 ± 2.61[a]	15.38 ± 1.77[a]
3	203.19 ± 20.22[b]	43.05 ± 3.99[b]	34.49 ± 3.88[b]
4	238.28 ± 25.37[b]	52.42 ± 4.87[b]	47.63 ± 3.94[b]

[a] $P < 0.01$, compared with group 1.
[b] $P < 0.01$, compared with group 2.

4. Discussion

In the present study, we examined whether the *Shiitake* mushroom polysaccharides treatment might improve the lipid profile and oxidative damage resulting from a high-cholesterol diet in mice.

Our work shows that rats fed with a high-cholesterol diet had a higher concentration of serum TC. LDL-c and TG level, and lower concentration of serum HDL-c compared to mice maintained on a standard diet. However, there was an obvious reduction in serum TC, LDL-c and TG levels, increase in serum HDL-c level in mice fed with the high-cholesterol diet supplemented with *Shiitake* mushroom polysaccharides. It is also widely accepted that an elevated plasma level of LDL-C is a major risk factor for CHD (Berliner & Heinecke, 1996). The levels of HDL-C are thought to reflect the rate of removal of excess peripheral cholesterol, and raised levels are thereby associated with reduced risk of atherosclerosis. This indicated that *Shiitake* mushroom polysaccharides treatment can decrease blood lipid level in mice fed with high-cholesterol diet.

It had been reported that the high-fat diet would induce free radical production, followed by hypercholesterolemia and oxidative stress (Devi et al., 2000; Ohara, Peterson, & Harrison, 1993). Therefore, it is important not only to value the scavenging effects of free radicals in vitro, but also the activities of antioxidant enzymes in vivo. On the other hand, blood is the primary substance affected by oxidative stress when the cholesterol ingested in excess and assay of blood biochemical markers is of great clinical importance in the diagnosis of malfunctioning of body organs.

Several investigators found that HC diet had an increasing effect on lipid peroxidation in plasma and tissues in rabbits (Balkan et al., 2002; Del Boccio et al., 1990; Mahfouz & Kummerow, 2000). In contrast to this, there were conflicting findings on lipid peroxidation in rats fed an HC diet. It has been found that plasma and liver lipid peroxide levels increased (Tsai, 1975; Uysal, Kutalp, & Seçkin, 1988), remained unchanged (Bobek, Ozdin, & Hromadava, 1997; Bulur et al., 1995; Mahfouz & Kummerow, 2000), or even decreased (Lu & Chiang, 2001) after HC diet in rats. Tests frequently used to evaluate products of lipid peroxidation in serum and tissues include determinations of MDA, lipid hydroperoxides, and DC (Sattler, Malle, & Kostner, 1998). The MDA assay is the most frequently used method because of its simplicity, but several substances have interfering effects on this assay (Sattler et al., 1998). SOD, which dismutates O_2^- to H_2O_2, and CAT, which decomposes H_2O_2 to H_2O and O_2, were chosen as main antioxidant enzyme activities. GST is a detoxicant enzyme capable of conjugating GSH with electrophiles and is also related to antioxidant defense (Hayes & Strange, 1995).

Thus, in this study, SOD, CAT, GSH-Px and MDA in serum were selected to determine the antioxidant effects in vivo. In the present study, markedly increased MDA level, decreased SOD, CAT and GSH-Px activities were detected in blood and liver in HCD mice. This confirms that abnormal blood lipid and oxidative damage have occurred in HCD mice. In the livers of HCD-fed mice, a net decrease of all these enzyme activities was found, indicating that HCD impaired antioxidant defense. By contrast, *Shiitake* mushroom polysaccharides can significantly decreased blood lipid level, increase antioxidant enzymes activities in HCD mice. This indicated that *Shiitake* mushroom polysaccharides are useful for decreasing body damage caused by long-term HCD consumption.

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