Original paper

Optimization of Enzyme-assisted Extraction of Antitumor Polysaccharides from *Hericium erinaceus* mycelia

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In this study, an efficient complex enzyme-assisted extraction technology was developed and optimized to extract polysaccharides from *Hericium erinaceus* mycelia (HEP) using the Box-Behnken design based on the results of single factor experiments. As a result, the optimal protocol was as follows: an extraction time of 79 min, an extraction temperature of 50°C, a pH of 5.7 and a ratio of water to raw materials of 33.4 mL/g. These optimal extraction conditions achieved the highest yield ($13.9 \pm 0.3\%$) of HEP. The purified fraction (HEP-2) was composed of glucose, galactose, mannose, fucose, arabinose, xylose and rhamnose. HEP-2 manifested an obvious inhibitory effect on the growth of HeLa cells. The overall findings implied that enzyme-assisted procedure is a superior method and that HEP-2 might be useful for developing natural and safe antitumor drugs.

Keywords: *Hericium erinaceus* mycelia, enzyme-assisted extraction, polysaccharides, antitumor activity

Introduction

Hericium erinaceus, which belongs to the *Aphylllophorales*, *Hydnaeae* and *Hericiaceae* families (Li *et al.*, 2014), is a well-known edible and medicinal mushroom in East-Asia. It is widely used for treating gastric ulcers, chronic gastritis and other digestive tract-related diseases (Xu *et al.*, 1985; Jia *et al.*, 2004). Polysaccharides, the key active compounds in *H. erinaceus* (HEP), are reputed to possess various pharmacological activities, including antioxidant activity, antitumor activity, hypoglycemic activity, anti-bacterial activity, anti-inflammatory properties, hepatoprotective and anti-aging properties and so on (Kim *et al.*, 2012; Abdulla *et al.*, 2011; Wang *et al.*, 2005; Elisashvili, 2012).

Artificial cultivation of *H. erinaceus* is difficult and requires a long training period whereas liquid fermentation is a fast and efficient way to acquire desired products. Hot water extraction, enzyme-assisted extraction, ultrasonic-assisted extraction and microwave-assisted extraction are the commonly used methods to extract polysaccharides. In contrast, enzyme-assisted extraction

technology offers many advantages such as high efficiency, low investment cost, simplified manipulation and environmental compatibility (Puri *et al.*, 2012; Nagendra *et al.*, 2013).

As far as we know, there are no reports available in the literature regarding the optimization of enzyme-assisted extraction of polysaccharides from *H. erinaceus* mycelia. In this study, Polysaccharide extraction was optimized from *H. erinaceus* mycelia using response surface methodology (RSM). HEP was then purified and the antitumor activities of the purified polysaccharide was evaluated *in vitro* for further application in pharmaceutical industries.

Materials and Methods

Materials *H. erinaceus* mycelia were obtained according to our previous methods (Zhang *et al.*, 2012). The mycelia were washed by distilled water, dried at 40°C, and ground for further use. DEAE cellulose-52, trifluoroacetic acid (TFA), 5-fluorouracil (5-Fu) and standard monosaccharides (fucose, rhamnose,

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mannose, xylose, glucose, galactose, fructose and arabinose) were all purchased from Sigma-Aldrich. HeLa cells were provided by the Cell Bank of Institute of Biochemistry and Cell Biology, Chinese Academy of Science (Shanghai, China). Papain (200 U/mg), cellulase (50 U/mg) and pectolyase (5 U/mg) were obtained from Aoji Chemical Reagent Co., Ltd (Hangzhou, China) and all other reagents were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

Extraction procedure The mushroom powder (1g) was extracted with compound enzymes solutions (the proportion of cellulase: pectinase: papain was 2:1:1) at a given concentration with designed parameters (temperature, time and pH). The mixture was occasionally shaken during extraction. Water soluble crude polysaccharides were obtained by precipitation of the concentrated supernatant using 3 volumes of dehydrated alcohol, and then recovered by centrifugation. The polysaccharide content was measured by the phenol-sulfuric acid method (Dubosi *et al.*, 1956). The yield of polysaccharides of *H. erinaceus* was calculated by the following equation:

$$\text{Polysaccharide yield \%} = \frac{\text{polysaccharide weight}}{\text{mushroom weight}} \times 100 \quad \dots\dots \text{Eq. 1}$$

Box-Behnken design and statistical analysis The influences of extraction time(A), extraction temperature(B), ratio of water to raw material(C) and pH(D) were carried out by a Box-Behnken experimental design (BBD) on the basis of the preliminary value from the single-factor test. All experiments were performed in triplicate and the average yield of polysaccharide was taken as the responses of the design.

Design Expert software was used for the experimental design, as well as the variance and regression analysis of the experimental data. The regression coefficients were then used to make statistical calculations and generate contour maps from the regression models.

Purification of HEP HEP (0.5 g) was dissolved in 10 mL water and separated on DEAE cellulose-52 anion-exchange column (2.6 cm × 40 cm). Briefly, the crude polysaccharide solutions (2 mL) were applied to the column which was eluted by the step-wise addition of NaCl solutions of different concentrations (0, 0.1, 0.2 and 0.4 M NaCl) at a flow rate of 1.0 mL/min. Eluate was collected automatically (5 mL/tube) and the polysaccharides were detected by the phenol-sulfuric acid method (Dubois *et al.*, 1956). The main fraction (with high absorbance values) was collected, dialyzed against tap water for 48 h (cutoff MW 3000 Da), and freeze-dried for further use.

Analytical methods of purified polysaccharide Monosaccharide composition analysis was conducted according to the methods of Yang *et al.* (2005). The polysaccharide (2 mg) was hydrolyzed with 2 M TFA at 110°C for 2 h, and the monosaccharides composition was determined by high-performance anion-exchange chromatography (HPAEC), using a Dionex LC30 equipped with a

CarboPac™ PA20 column (3 mm×150 mm). The column was eluted with 2 mM NaOH (0.45 mL/min) and the monosaccharides were monitored using a pulsed amperometric detector (Dionex).

The average molecular weight (MW) was determined using a gel permeation chromatography method (Fu *et al.*, 2007). Briefly, the polysaccharides (2 mg/mL) were applied to an HPLC system of Agilent 1100 (America) equipped a gel-filtration chromatographic column, eluted with the deionised water and detected by a SEDEX 75 evaporation light scatter detector. T-series Dextran standards were used for estimating the molecular weight of HEP-2.

Assay for antitumor activity in vitro The HeLa cancer cell line was maintained with DMEM medium supplemented with 10% fetal bovine serum (FBS) in a humidified 5% CO₂ incubator (Thermo Electron Corporation, MA, USA) at 37°C.

The MTT assay was used to determine the inhibition effects of HEP-2 on HeLa cells (Gan *et al.*, 2011). Briefly, cells were incubated in 96-well plates at 1×10⁵ cells per well containing 100 µL culture medium and permitted to adhere for 24 h. One hundred microlitres of HEP-2 solutions at different concentrations (0.1, 0.2, 0.4, 0.8, 1.2, 1.6 mg/mL) and 5-Fu (0.03 mg/mL) were added. After incubation for 72 h, the HEP-2/medium was removed and MTT (0.5 mg/mL, 100 µL) was added. After further incubation for 4 h, the supernatant was aspirated and 150 µL of DMSO was added. Absorbance was measured at 570 nm by a 96-well microplate reader. The growth inhibition rate was calculated as a percentage as follows:

$$\text{Growth inhibition rate (\%)} = (1 - A_{\text{experiment}}/A_{\text{control}}) \times 100\% \quad \dots\dots \text{Eq. 2}$$

where A_{control} is the absorbance of the blank control, and A_{experiment} is the absorbance of the sample or 5-Fu.

Statistical analysis All the experiments were carried out in triplicate and data were expressed as mean± standard deviation. Stat-Ease Design-Expert 8.0.5 (Trial version, Stat-Ease Inc., Minneanopolis, MN, USA) was used for the experimental design and the regression analysis of experimental data. Statistical analysis was performed with ANOVA followed by the Student's t-test. A level of *P* < 0.05 was taken as statistically significant.

Results and Discussion

Effect of compound enzyme concentration on extraction yield of polysaccharides Enzymes such as cellulase, pectinase and papain are widely used to extract the bioactive components. Cellulase can degrade cellulose into glucose, cellobiose and higher glucose polymers. Pectinase has the ability to disintegrate pectic compounds. Papain is used to hydrolyze proteins and accelerate polysaccharides dissolution (Liang *et al.*, 2012; Nagendra Chari *et al.*, 2013). In the present study, the enzyme concentrations of 2.0%, 2.5%, 3.0%, 3.5% 4.0% and 4.5% (weight of raw material) were chosen to examine the influence of enzyme concentration on the extraction HEP when the other reaction conditions were fixed (extraction temperature of 50°C, extraction time of 1h, ratio of

Fig. 1. Effects of different (a) compound enzyme concentration; (b) extraction time; (c) extraction pH; (d) extraction temperature; and (e) ratio of water to raw material on the extraction yield of polysaccharides from *Hericium erinaceus* mycelia.

water to raw material of 30:1 and pH of 5.0). The yield of HEP increased from 9.7% to 13.3% when the enzyme concentration increased from 1.5% to 3.0%. There were no significant differences when the enzyme concentration exceeded 3.0%, thus a concentration of 3.0% was selected for this study (Fig. 1a).

Effect of extraction time on extraction yield of polysaccharides
The effect of extraction time on HEP yield is shown in Fig. 1b, when the other three factors (extraction temperature, ratio of water to raw materials and pH) were fixed at 50°C, 30:1, and 5.0,

respectively. The HEP yield increased with increasing time and the HEP yield reached a maximum value (13.8%) when the sample was extracted for 80 min.

Effect of different pH on extraction yield of polysaccharides
The pH value can strongly affect the conformation and activity of enzymes (Yin *et al.*, 2011), therefore, it was important to investigate the optimal pH of the tested enzyme. The extraction procedure was performed at different pH conditions with the following fixed extraction variables: extraction time of 80 min,

Table 1. Box-Behnken design and the response values for the yields of polysaccharide from the *Hericium erinaceus* mycelia with the enzyme-assisted extraction.

Runs	Extractiontime (min)	Extraction temperature (°C)	pH	Ratio of water to raw material	Yield ^a (%)
1	−1 (60)	−1 (40)	0 (5)	0 (30)	7.9
2	−1 (60)	1 (60)	0 (5)	0 (30)	12.7
3	1 (100)	−1 (40)	0 (5)	0 (30)	8.8
4	1 (100)	1 (60)	0 (5)	0 (30)	13.4
5	0 (80)	0 (50)	−1 (4)	−1 (20)	9.1
6	0 (80)	0 (50)	−1 (4)	1 (40)	10.8
7	0 (80)	0 (50)	1 (6)	−1 (20)	11.7
8	0 (80)	0 (50)	1 (6)	1 (40)	12.5
9	−1 (60)	0 (50)	0 (5)	−1 (20)	9.9
10	−1 (60)	0 (50)	0 (5)	1 (40)	12.4
11	1 (100)	0 (50)	0 (5)	−1 (20)	10.5
12	1 (100)	0 (50)	0 (5)	1 (40)	11.4
13	0 (80)	−1 (40)	−1 (4)	0 (30)	7.5
14	0 (80)	−1 (40)	1 (6)	0 (30)	9.9
15	0 (80)	1 (60)	−1 (4)	0 (30)	12.9
16	0 (80)	1 (60)	1 (6)	0 (30)	14.0
17	−1 (60)	0 (50)	−1 (4)	0 (30)	11.4
18	−1 (60)	0 (50)	1 (6)	0 (30)	12.3
19	1 (100)	0 (50)	−1 (4)	0 (30)	11.3
20	1 (100)	0 (50)	1 (6)	0 (30)	12.5
21	0 (80)	−1 (40)	0 (5)	−1 (20)	8.3
22	0 (80)	−1 (40)	0 (5)	1 (40)	9.0
23	0 (80)	1 (60)	0 (5)	−1 (20)	12.6
24	0 (80)	1 (60)	0 (5)	1 (40)	13.6
25	0 (80)	0 (50)	0 (5)	0 (30)	12.2
26	0 (80)	0 (50)	0 (5)	0 (30)	12.4
27	0 (80)	0 (50)	0 (5)	0 (30)	12.2
28	0 (80)	0 (50)	0 (5)	0 (30)	12.5
29	0 (80)	0 (50)	0 (5)	0 (30)	12.1

^aMean values (n=3)

extraction temperature of 50°C and ratio to water to raw material of 30:1. As shown in Fig. 1c, the HEP yield increased with elevated pH levels and reached a critical value (13.3%) at pH 5.0 which was in agreement with previous works (Wang *et al.*, 2015; Zhao *et al.*, 2016). However, further increase in pH resulted in decreasing HEP yield, which may be due to the decrease of enzyme activities at higher pH value (Wang *et al.*, 2013).

Effect of temperature on extraction yield of polysaccharide
The effect of different temperatures from 30 to 70°C on HEP yield is shown in Fig. 1d, when other factors were as follows: extraction time of 80 min, pH of 5.0, and ratio of water to raw material of 30:1. The yield of HEP increased with extraction temperature and reached a highest value 13.6% at an extraction temperature of 50°C, then began to decrease due to lower enzyme activity at higher temperatures (Zhang *et al.*, 2011).

Effect of ratio of water to raw material on extraction yield of polysaccharide
It can be seen from Fig. 1e, the highest extraction yield of polysaccharide was 13.8% at a ratio of water to raw

materials of 30:1. The yield increases with a ratio of 10:1 to 30:1, the increase of the driving force for the mass transfer of the polysaccharide may be the main reason (Bendahou *et al.*, 2007). However, when the ratio is higher than 30, the polysaccharide yield was slightly decreased. This might be due to the loss of HEP induced by excessively dissolving in lower concentration solvent (Yin & Dang, 2013). Based on these initial experiments, the following conditions were used for RSM experiments: a pH of 4.0 to 6.0, an extraction temperature of 40 – 60°C, a ratio of water to raw material of 20:1–40:1, an extraction time of 60 – 100 min.

Optimization of extraction conditions by BBD
A total of 29 runs were used to optimize the extraction of HEP in the current BBD. The values for the responses (yield of polysaccharides) at different experimental combinations of variables were recorded in Table 1. The HEP yield ranged from 7.5 to 14.0%. The response variable and the test variables were related by the following second-order polynomial equation by applying multiple regression analysis on the experimental data:

Table 2. ANOVA for response surface quadratic model.

Variables	Sum of squares	DF	Mean square	F value	p-value
Model	87.92	14	6.28	32.04	<0.0001
A	0.16	1	0.16	0.80	0.3868
B	64.59	1	64.59	329.47	<0.0001
C	8.27	1	8.27	12.17	<0.0001
D	4.83	1	4.83	24.62	0.0002
AB	0.016	1	0.016	0.08	0.7818
AC	0.022	1	0.022	0.11	0.7398
AD	0.64	1	0.64	3.26	0.0923
BC	0.43	1	0.43	2.19	0.1612
BD	100	1	100	0.051	0.8246
CD	0.17	1	0.17	0.88	0.3645
A ²	1.41	1	7.19	7.19	0.0179
B ²	5.88	1	30.00	30.00	<0.0001
C ²	0.59	1	3.02	3.02	0.1040
D ²	4.27	1	21.76	21.76	0.0004
Residual	2.74	14	0.20	0.20	
Lack of Fit	2.63	10	0.26	8.9	0.0247
Pure Error	0.12	4	0.03		
Cor Total	90.67	28			
R ²	0.9697				
Adj R ²	0.9395				
Pred R ²	0.8311				
Adeq precision	12.92				
CV%	3.91				

A:extractin time, B :extraction temperature, C: pH, D: ratio of water to raw material

$$Y=12.36+0.11A+2.32B+0.83C+0.63D-0.06AB+0.08AC-0.44AD-0.33BC+0.05BD+0.21CD-0.47A^2-0.95B^2-0.3C^2-0.81D^2 \quad \dots\dots Eq. 3$$

where Y is the polysaccharide yield and A, B, C, and D are the coded values for extraction time, extraction temperature, pH, and ratio of water to raw material, respectively.

The ANOVA results for the response surface quadratic polynomial model are summarized in Table 2. A low *p*-value (*p* < 0.0001) indicated that the fitness of this model was highly significant. The high value of the determination coefficient (*R*²=0.9697) represented the ratio of the explained variation to the total variation. The value of the adjusted determination coefficient (*R*²=0.9395) indicated that a good relevance of the dependent variables in the model. A low value for the coefficient of the variation (CV=3.9%) was indicative of a high reliability of the experiments. The coefficient estimated for the parameter optimization suggested that the independent variable (B, C, and D) significantly affected the polysaccharide yield (*p* < 0.05). The extraction temperature (B) was the most significant single parameter which influenced the polysaccharide yield.

The 3D response surface is the graphical representations of the regression equation. It provides a method to visualize the relationship between responses and each variable, as well as the types of interactions between two test variables (Chen *et al.*, 2014).

Results from Fig. 2 further confirmed the ANOVA findings. During HEP extraction, extraction temperature, pH and ratio of water to raw material were significantly correlated with the yield of polysaccharides. Among the four factors, extraction temperature was the most significant variable that affected the yield of polysaccharide, followed by pH, ratio of water to raw material and extraction time. Therefore, the optimal values for the tested variables for extraction of HEP that were obtained by the prediction of computing program: an extraction time of 79.4 min, an extraction temperature of 50°C, a pH of 5.7 and a ratio of water to raw materials of 33.4 mL/g. The maximum predicted extraction yield of HEP was 14.0%, which corresponded well with the actual yield (13.9 ± 0.3%, *n*=3). These results suggested that the model designed in this study was valid.

Purification of HEP and preliminary characterization of the purified polysaccharide HEP was fractionated by DEAE-52 cellulose column and obtained three fractions. The second fraction (HEP-2) was the main fraction, representing 66.5% of HEP. This fraction was collected, dialyzed and freeze-dried. HPAEC analysis showed that HEP-2 was composed of glucose, galactose, mannose, fucose, arabinose, xylose and rhamnose at molar ratios of 42.05: 21.31: 13.07: 12.47: 0.96: 8.76:1.38. Glucose and galactose were the predominant monosaccharides. Monosaccharide composition of polysaccharides play an important role in evaluating the quality

Fig. 2. 3D response surface plots (a-f) showing the effects of extraction time, extraction temperature, pH and ratio of water to raw materials on the yield of polysaccharides from the *Hericium erinaceus* mycelia.

Table 3. The molecular weight and monosaccharide compositions of HEP-2

Sample	Molecular weight (kDa)	Sugar components (mol%)						
		Glucose	Galactose	Mannose	Fucose	Arabinose	Xylose	Rhamnose
HEP-2	38.76	42.05	21.31	13.07	12.47	0.96	8.76	1.38

Fig. 3. Inhibitory effect of the purified polysaccharide (HEP-2) from the *Hericium erinaceus* mycelia on HeLa cells treated for 72 h.

control and basic information on the polysaccharides (Guan & Li, 2010). The monosaccharide composition analysis was helpful to elucidate the physicochemical properties, structure and structure-activity relationship of polysaccharide. In comparison with other polysaccharides from *H. Erinaceus* mycelia (Lee *et al.*, 2009; Zhang *et al.*, 2012), the monosaccharide composition was different which may be due to the difference of the extraction and separation conditions. The average MW of HEP-2 was about 38.76 kDa calibrated using dextran standards of varying molecular weights (Table 3).

Antitumor activity of HEP-2 HeLa cells are widely used to investigate the antitumor activities of many extracts or compounds (Chen *et al.*, 2013). In this study, the *in vitro* anti-proliferative activities of HEP-2 on HeLa cells were investigated and 5-Fu (a well-known anticancer agent) was used for comparison. As shown in Fig. 3, HEP-2 exerted a concentration-dependent inhibition effect on HeLa cells. At concentrations between 0.1 and 1.6 mg/mL, the inhibition rates of HEP-2 were 6.9–55.6%. The % inhibition of HEP-2 on HeLa cell was 19.6 at 0.2 mg/mL. It was reported that, at this concentration, the inhibition of a polysaccharide from *Gynostemma pentaphyllum* Makino was only 6.2% (Chen *et al.*, 2011). Zhao *et al.* (2012) reported that the inhibition rate of a polysaccharide extracted from *Asparagus officinalis* was 67.86% at 10 mg/mL. As expected, the inhibition rate of 5-Fu reached 74.3% at a concentration of 0.03 mg/mL. However, its high toxicity to normal cells need to be considered (Chen *et al.*, 2011). These results implied that HEP-2 had an

efficient anti-proliferation effect on HeLa cells.

Extraction conditions effected the yield, molecular weight distribution, monosaccharide composition and biological activities of polysaccharides (Jia *et al.*, 2013; Li & Wang, 2016; Zhu *et al.*, 2016). In the past decade, numerous reports of their antitumor activity have pushed research on polysaccharides extracted from mushrooms to the modern scientific frontier (Brander *et al.*, 1958; Miyazaki & Nishijima, 1981; Zhang *et al.*, 2015). It is widely shared that the antitumor activities of mushroom polysaccharides depend on their molecular weight, chemical composition, expanded chain, and specific conformation (Cui *et al.*, 2007; Jin *et al.*, 2003; Ren *et al.*, 2013). Several proposed mechanisms were put forward such as inducing apoptosis of tumor cells, recognition with receptor on cell surface, the cell cycle arrest and necrosis anti-angiogenesis and so on (Park *et al.*, 2009; Song *et al.*, 2013; Yang *et al.*, 2012; Ou Yang *et al.*, 2013). Immunomodulation may be the most important one (Zhou *et al.*, 2004). However, the mode of action and pharmacokinetics are still unclear for these polysaccharides, thus the anti-proliferation mechanisms of HEP-2 in HeLa cells require further investigation.

Conclusion

BBD was employed to optimize an efficient complex enzyme-assisted extraction technology from *Hericium erinaceus* mycelia based on the results of single factor experiments. The effects of extraction time, extraction temperature, pH and ratio of water to raw materials on HEP yield were studied. The optimized conditions

were as follows: an extraction time of 79 min, an extraction temperature of 50°C, a pH of 5.7 and a ratio of water to raw materials of 33.4 mL/g. The optimal extraction conditions resulted in the highest yield ($13.9 \pm 0.3\%$). The main purified polysaccharide (HEP-2) from HEP was composed of glucose, galactose, mannose, fucose, arabinose, xylose and rhamnose. HEP-2 manifested obvious inhibitory effect on the growth of HeLa cells. As it turned out, enzyme-assisted procedure is a superior method and HEP-2 might be useful for developing natural safe antitumor drugs.

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