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Optimization of ultrasonic extraction of *Flammulina velutipes* polysaccharides and evaluation of its acetylcholinesterase inhibitory activity

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ABSTRACT

Polysaccharide was testified to be the main component of *Flammulina velutipes* for inhibiting AChE activity in our preliminary study. Therefore, response surface methodology, based on Box–Behnken design, was used to optimize the ultrasonic extraction conditions of *F. velutipes* polysaccharides (FVP). Four independent variables (ratio of water to raw material, ultrasonic power, ultrasonic time, and ultrasonic temperature) were taken into consideration. A quadratic model, adequate for reasonably predicting the yield of FVP, was constructed between ultrasonic conditions and yield of FVP. A yield of FVP of 8.33% was obtained under a modified condition (ratio of water to material of 25 ml/g, ultrasonic power of 620 W, ultrasonic time of 20 min, and ultrasonic temperature of 45 °C). Subsequently, acetylcholinesterase (AChE) inhibitory activity and 1,1-diphenyl-2-picryl hydrazine (DPPH) scavenging activity of FVP were determined. AChE inhibitory rate of 18.51% and DPPH scavenging rate of 61.24% were obtained at 0.6 mg/ml of FVP, indicating a good potential of FVP to enhance learning and cognitive ability.

1. Introduction

Nowadays, edible mushrooms are distinguished as important natural resources of immunomodulating and anticancer agents and have been cultured on a large scale in Asia (Wasser, 2002). *Flammulina velutipes*, one of the most popular edible mushrooms, has attracted considerable attention in the fields of biochemistry and pharmacology due to its biological activities. Polysaccharides, as one of the important active components of *F. velutipes*, have been proved to be beneficial in immunomodulating antitumor and anti-inflammatory activities (Leung, Fung & Choy, 1997). Therefore, much attention has been paid to the studies of *F. velutipes* polysaccharides (FVP).

Although polysaccharides have been well known for their various pharmacological functions, their extraction is still mainly performed with conventional techniques, which are based on proper solvents, prolonging extraction time, heating process, and agitation to increase extraction yield (Wang, Cheng, Mao, Fan & Wu, 2009). In these methods, the extraction process usually consumes a long time and a lot of energy, but the extraction efficiency is very low. Therefore, it is essential and desirable to find out an economical and highly efficient extraction method. Ultrasonic has been used to increase extraction yield of bioactive substances from natural products, which is mainly attributed to disruption of cell walls, particle-size reduction, and enhanced mass transfer to the cell contents as a result of cavitation bubble collapse (Li, Pordesimo, & Weiss, 2004; Vinatoru et al., 1997; Wang et al., 2009). However, there is hardly any report that ultrasonic is applied to separate FVP. Therefore, ultrasonic was employed for the extraction of FVP in our study. Especially, temperature was controlled during the extraction process to prevent overheating-induced oxidation and degradation of polysaccharides.

The worldwide population ageing has increased the incidence of cognitive deficits, such as the age-associated memory impairment and senile dementias and Alzheimer's disease (Hornick et al., 2008). Extensive evidence supports the view that cholinergic mechanisms modulate learning and memory formation. Neuropathological occurrences of cognitive deficits are associated with the cholinergic deficiency (Gold, 2003; Roberson & Harrell, 1997). Inhibitors of acetylcholinesterase (AChE) have been extensively used to increase the effectiveness of cholinergic transmissions and endogenous acetylcholine levels and thus overcome cognitive deficits (Hornick et al., 2008; Silman & Sussman, 2005). *F. velutipes* is beneficial to human memory. FVP has been proven to improve learning and memory ability of scopolamine hydrobromid-induced model mice and rats using step-through test and Morris water test (Zou, Liao, Wu & Liu, 2010). However, the effect of FVP on AChE activity has not been studied. In our preliminary study, crude polysaccharide solution was testified to be the main fraction in *F. velutipes* for inhibiting AChE activity. In view of the above, it is necessary to research the AChE inhibitory activity of FVP. In addition, it is suggested that polysaccharides induced cognitive improvement owing to their antioxidant activity (Fan et al., 2005; Zhang, Zhang, Wang & Mao, 2008), so antioxidant activity of FVP was also investigated.

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The objective of this study was to optimize the ultrasonic-assisted extraction conditions of FVP using response surface methodology. Effects of ratio of water to raw material, ultrasonic power, ultrasonic time, and ultrasonic temperature on the extraction yield of FVP were fully examined. Moreover, AChE inhibitory activity of FVP was investigated to study its potential to improve memory impairment and cognitive deficit. On account of the relationship between oxidative stress and cognitive deficit, 1,1-diphenyl-2-picryl hydrazine (DPPH) radicals scavenging assay was also conducted to evaluate the antioxidant ability of FVP.

2. Materials and methods

2.1. Materials and chemicals

F. velutipes was purchased from local market (Nanjing, China) and then dried at 60 °C and ground to pass through 80 mesh screen, the powder was stored at 4 °C until used. Glucose, phenol, and sulfuric acid were obtained from Shanghai Chemical Co. (Shanghai, China). 1,1-Diphenyl-2-picryl hydrazine (DPPH), 5,5′-dithio-*bis*-(2-nitrobenzoic) acid, acetylthiocholine iodide, ascorbic acid, acetylcholinesterase (AChE, type VI-S, EC 3.1.1.7), and galanthamine were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All other chemicals used in experiments were of analytical grade.

2.2. AChE inhibitory activity of Flammulina velutipes extracts

10 g *F. velutipes* powder was extracted with 200 ml of different solvents (deionized water, ethanol, petroleum ether, and ethyl acetate), respectively, and AChE inhibitory activities of the extracts were compared. Subsequently, the water extract was mixed with quadruplicate anhydrous ethanol and then centrifuged. The precipitate, crude polysaccharides, was lyophilized and redissolved in water as crude polysaccharide solution (CPS). The supernatant was concentrated under reduced pressure, lyophilized, and redissolved in water as water–ethanol solution (WES). The AChE inhibitory activities of CPS and WES were further investigated.

2.3. Ultrasonic extraction and determination of polysaccharides

F. velutipes powder was weighed accurately (10.0 g) and extracted with distilled water in ultrasonic cell disintegrator ((DCTZ-2000, Beijing Hongxianglong Biotechnology Development Co. Ltd). Subsequently, the treated mixture was air cooled to room temperature and centrifuged (10,000 rpm/min, 15 min). The supernatant was concentrated under reduced pressure at 65 °C. The polysaccharides extracts obtained above were then mixed with 4-fold volume anhydrous ethanol (ethanol final concentration, 80%) and kept at 4 °C for 24 h. After centrifugation at 5000 rpm/min for 15 min, the precipitate was washed three times with anhydrous ethanol and then dialyzed and lyophilized to yield FVP sample. The percentage polysaccharides yield (%) is calculated as follows:

$$\text{Yield of polysaccharide (\%)} = \frac{\text{weight of dried crude FVP (g)}}{\text{weight of Flammulina velutipes powder (g)}} \times 100$$

2.4. Experimental design

A three-level-four-factor, Box–Behnken factorial design (BBD) was employed in this optimization study. Ratio of water to raw material (X_1), ultrasonic power (X_2), ultrasonic time (X_3), and ultrasonic temperature (X_4) were chosen for independent variables to be optimized for the extraction of FVP. Yield of polysaccharides (Y) was taken as the response of the design experiments. Twenty-nine experiments were carried out in BBD (Table 1). Five replicates at the center point were used for estimation of a pure error sum of squares. Triplicate determinations were performed at all design points in randomized order.

Table 1
Experiment of ultrasonic extraction of polysaccharides from *Flammulina velutipes*. (Data presented are the mean of triplicate determinations.)

Run	X_1-ratio (ml/g)	X_2-ultrasonic power (W)	X_3-ultrasonic time (min)	X_4-ultrasonic temperature (°C)	Yield of FVP (%)	
					Actual value	Predicted value
1	20	600	15	50	8.21	8.08
2	30	400	15	50	6.88	7.05
3	20	600	15	50	7.99	8.08
4	20	400	15	65	6.63	6.76
5	20	800	15	65	6.46	6.38
6	20	800	25	50	6.64	6.82
7	20	600	5	35	7.57	7.30
8	20	400	5	50	6.45	6.25
9	10	800	15	50	6.21	6.16
10	10	600	5	50	6.33	6.57
11	10	400	15	50	6.18	6.14
12	20	600	5	65	6.89	7.09
13	20	400	25	50	7.59	7.56
14	30	600	15	65	7.79	7.48
15	20	600	15	50	8.17	8.08
16	30	800	15	50	7.46	7.61
17	30	600	5	50	7.55	7.55
18	20	600	25	65	6.58	6.96
19	30	600	25	50	8.39	8.03
20	20	600	15	50	8.29	8.08
21	10	600	15	65	6.28	5.94
22	20	800	15	35	7.91	7.66
23	20	400	15	35	6.75	6.70
24	20	600	25	35	8.06	7.98
25	10	600	15	35	6.63	6.92
26	10	600	25	50	6.77	6.64
27	20	600	15	50	7.78	8.08
28	30	600	15	35	7.42	7.74
29	20	800	5	50	7.57	7.58
Optimum conditions	24.81	618.98	18.64	44.73	–	8.32
Modified conditions	25	620	20	45	8.33	8.30

A quadratic polynomial model was fitted to correlate the response variable (yield of polysaccharide) to the independent variables. The general form of quadratic polynomial equation is as follows:

$$Y = \beta 0 + \sum_{i=1}^{4} \beta iXi + \sum_{i=1}^{4} \beta iiXi^2 + \sum_{i=1} \sum_{j=i+1}^{4} \beta ijXiXj$$

where Y is the response variable, and β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients for intercept, linearity, square, and interaction, respectively, while X_i and X_j are the independent variables.

2.5. AChE inhibitory activity

The AChE inhibitory activity assay was performed according to the protocol described by Langjae, Bussarawit, Yuenyongsawad, Ingkaninan and Plubrukarn (2007) with slight modifications. Briefly, 125 μl of 3 mM 5,5′-dithio-*bis*-(2-nitrobenzoic) acid, 25 μl of 1.5 mM acetylthiocholine iodide, 50 μl of 50 μM Tris–HCl buffer (pH 8.0), 25 μl of sample, and 25 μl of 0.25 U/ml AChE were added consecutively into 96-well plate. Then the absorbance was measured immediately at 412 nm using an ELISA plate reader (TECAN Infinite F200, Switzerland). The potency of AChE inhibitory activity of FVP was expressed as the inhibition rate. Galanthamine was used as a positive control.