Cellulase-assisted extraction and anti-ultraviolet activity of polysaccharides from the root of *Flammulina velutipes* on *Caenorhabditis elegans*

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Abstract: We investigated the cellulase-assisted extraction and anti-ultraviolet activity of water-soluble polysaccharides from the root of *Flammulina velutipes* on *Caenorhabditis elegans*. A Box–Behnken design experiment with three factors and three levels, including enzymolysis temperature, microwave time, and microwave power, was designed on the basis of the results of single-factor experiments. For improving the polysaccharide yield of *F. velutipes* root, the following optimal extraction conditions were used: 52.67°C enzymolysis temperature, 80s microwave time, and 144 W microwave power. Under optimal conditions, the actual measured value of the yield was 2.01% (w/w) and the predicted value was 2.06% (w/w). One fraction (FRP-2) was isolated and purified, and its characteristics were analyzed. The average mean molecular weight of FRP-2 was measured to be 2.60×10^5 Da, and its monosaccharide composition is mainly glucose. The sugar units are present both in the α-configuration and β-configuration. Moreover, FRP-2 exhibited certain anti-ultraviolet activity to *C. elegans* when the polysaccharide concentration ranged between 0.05mg/mL and 0.20mg/mL.

Keywords: Anti-ultraviolet activity, *C. elegans*, cellulase-assisted extraction, *Flammulina velutipes* root, polysaccharides.

INTRODUCTION

Flammulina velutipes, which is also called the golden needle mushroom, is classified as *Basidiomycotina*, *Hymenomycetes*, *Holobasidiomycetidae*, *Agaricel*, *Tricholomataceae*, and *Flammulina* (Leifa *et al.*, 2001). *F. velutipes* is the most popular edible mushroom in the world because of its attractive taste (Cai *et al.*, 2013). The consumption of *F. velutipes* has increased rapidly in recent years. *F. velutipes* is a rich source of protein, fiber, polysaccharides, vitamins, and essential amino acids and is low in fat (Fukushima *et al.*, 2001). Furthermore, *F. velutipes* prevents metabolic disorders, such as hypercholesterolemia, hypertension, and cancer (Leung *et al.*, 1997; Pang *et al.*, 2007). In recent years, *F. velutipes* has attracted considerable attention in the fields of pharmacology and biochemistry because of its antioxidant, anti-inflammatory, immunomodulatory, and antitumor activities (Leung *et al.*, 1997; Wu *et al.*, 2010). Polysaccharides, which constitute one of the most important active components in *F. velutipes*, have also been the subject of considerable research.

Most studies have focused on the body polysaccharides of *F. velutipes* in terms of its extraction and various pharmacological effects. However, *F. velutipes* root polysaccharides (FRP) have rarely been investigated. In this study, the cellulase-assisted extraction procedure of FRP was developed. The optimal extraction conditions were selected by using the response surface methodology (RSM), and the anti-ultraviolet activity of FRP to *Caenorhabditis elegans* was investigated.

MATERIALS AND METHODS

Materials

The root of *F. velutipes* was provided by a food factory in Shandong, China. Food-grade cellulase with 1,000U/g enzymatic activity was purchased from Tianjin Nuoao Technology Development Co. Ltd. (Tianjin, China).

FRP extraction

The roots of *F. velutipes* were dried at 60°C in a drying oven (DGG-101-2B, China) until a constant weight was obtained. Thereafter, the samples were stored in a dry container until use after being powdered and sifted through a 60-mesh sieve.

The samples were placed in distilled water to reach the ratio of 1/30(w/v). Varying amounts of cellulase were added to the suspension, which was immersed in a constant temperature bath. After cooling, the sample was placed in microwave, light wave and ultrasonic wave extraction instruments (SCIENTZ-IIDM, China) to extract polysaccharides. After microwave-assisted extraction, the extract solutions were centrifuged (3,000rpm, 10min), and the supernatant was collected.

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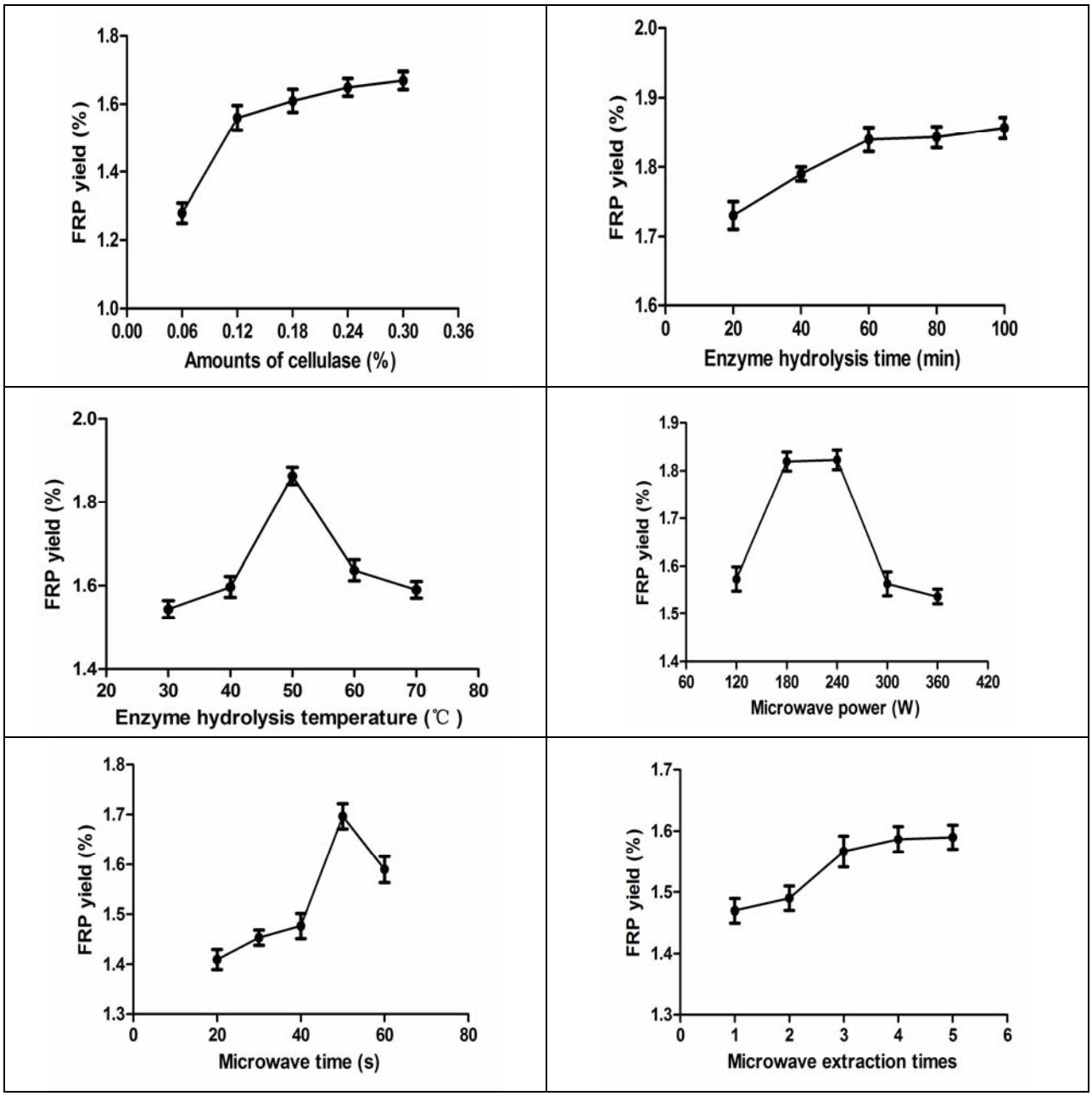

Fig. 1: Effect of different extraction parameters on yield of *F. velutipes* root polysaccharides (FRP). a. Amounts of cellulose (%, w/w); b. Enzyme hydrolysis time (min); c. Enzyme hydrolysis temperature (°C); d. Microwave power (W); e. microwave time (s); f, Microwave extraction times.

By adding the triploid of ethanol for 24h at 4°C, the supernatant fluids were precipitated. Thereafter, the sediments were collected by centrifugation (3,000rpm, 10 min) and freeze-dried. The proteins were removed following the Sevag method. With D-glucose as standard, FRP were measured by phenol–sulfuric acid method. The FRP yield (%) was measured using the following equation:

$$\text{FRP yield}(\%) = \frac{\text{Weight of dried crude FRP(g)}}{\text{Weight of sample(g)}} \times 100 \quad (1)$$

Single-factor experimental design

The samples were placed in distilled water to reach the ratio of 1/30(w/v). Varying amounts of cellulose (ratio of cellulose weight to material weight) were added to the suspension. The following enzyme hydrolysis times were selected: 20, 40, 60, 80, and 100min. Enzyme hydrolysis temperature was adjusted to 30°C, 40°C, 50°C, 60°C and 70°C, and the selected microwave times were 20, 30, 40, 50, and 60 s. The selected microwave power values were 120, 180, 240, 300 and 360W. Finally, microwave extractions were conducted five times. The single-factor

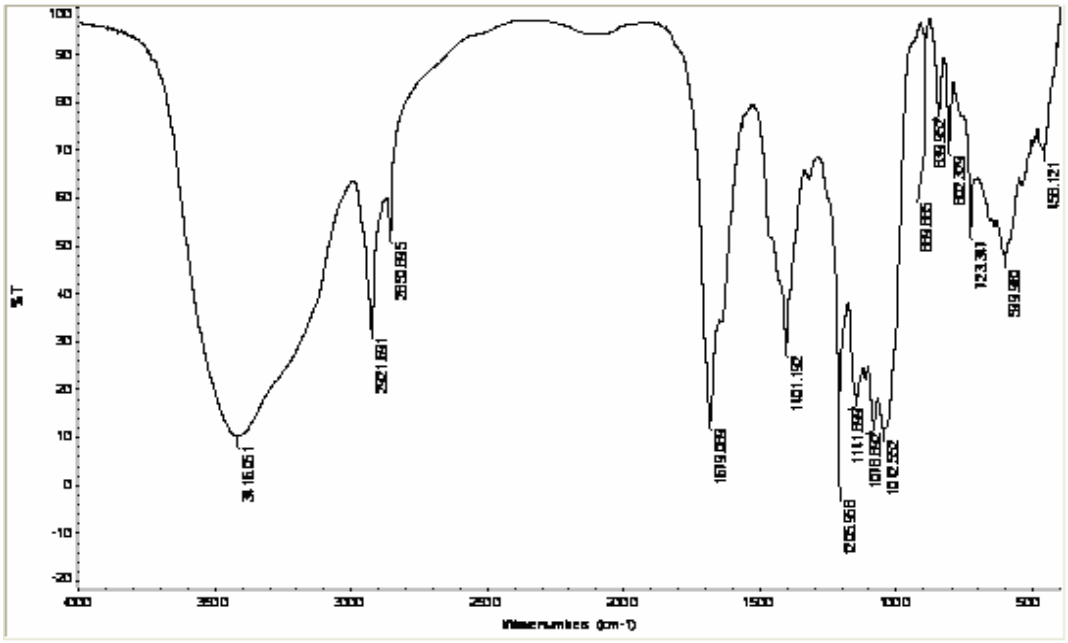

Fig. 2: The infrared spectral (IR) of FRP-2

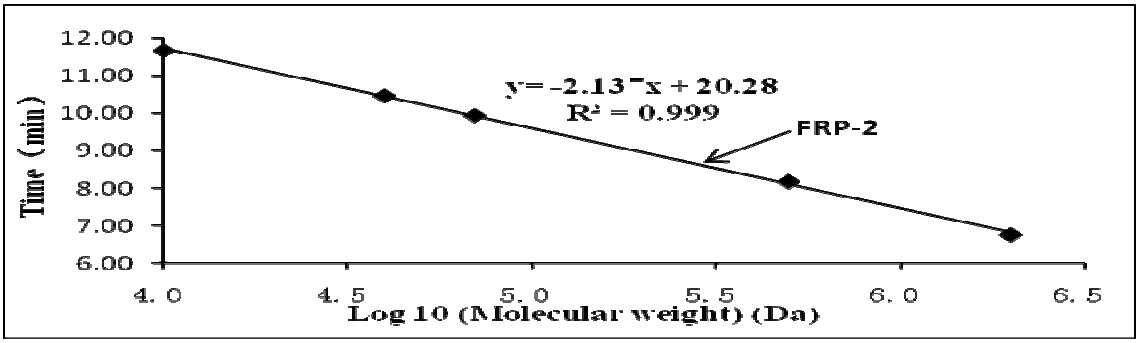

Fig. 3: The calibration curve of standard dextrans

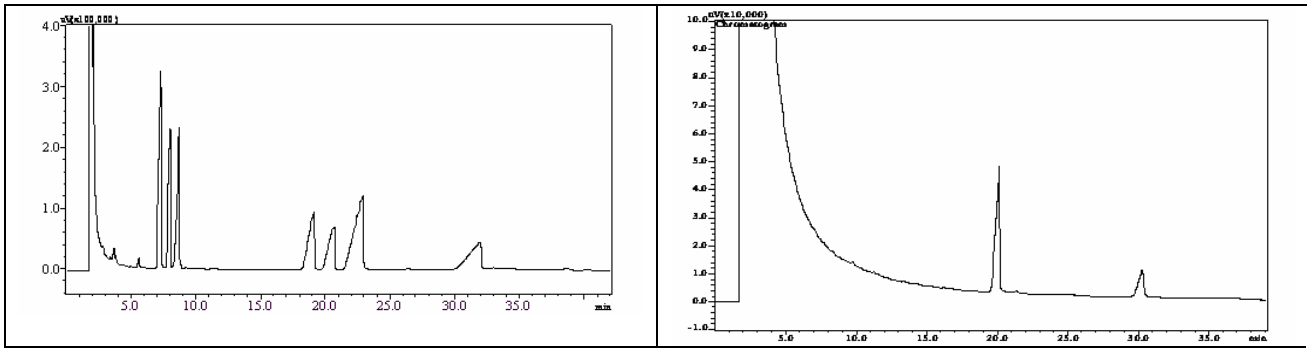

Fig. 4: GC chromatogram of monosaccharide composition of FRP-2 (The upper profile (a) represents the monosaccharide standard. The bottom profile (b) represents the FRP-2 tested)

experimental design was as follows: one factor was changed, and the other factors were kept constant in each experiment. From the single-factor experiments, a central composite experiment of three factors and three levels was used. Finally, enzymolysis temperature, microwave time and microwave power were designed.

Box–Behnken design (BBD)

A BBD was used to optimize the extraction conditions utilizing cellulase and for fitting a polynomial model, as follows:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{223} B^2 C, \quad (2)$$

where Y denotes the FRP yield, β_0 denotes the intercept term, β_1, β_2, and β_3 denote the linear coefficients, β_{12}, β_{13}, and β_{23} denote the interaction coefficients, β_{11} and β_{22} denote the squared coefficients, β_{223} denotes the cube coefficient, and A, B, and C are independent variables. Design-Expert software (version 8.05b, State-Ease Inc., Minneapolis, MN, USA) was used for the design, data analysis, and model building.

FRP separation and purification

FRP were dissolved in 20% ethanol solution (analytically pure; v/v) and centrifuged at 3,000rpm for 10min. Supernatants were added to absolute ethanol until the ethanol concentration reached 50%. Then, the supernatants were centrifuged. The precipitates were collected and freeze-dried to obtain purified white FRP-2.

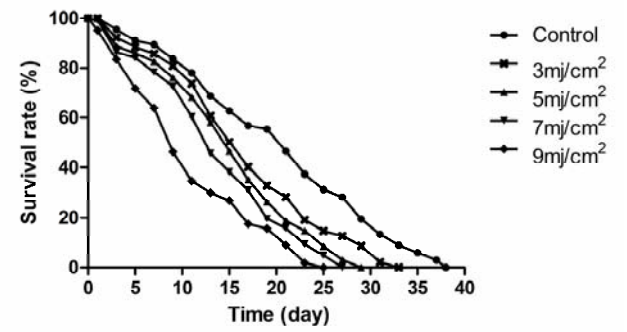

Fig. 5: The survival effect to *C. elegans* of different ultraviolet dosage

Infrared (IR) spectral analysis

The IR spectrum was tested by a spectrophotometer (Vector 22, China) as KBr pellets in the range of 4,000 cm^{-1} to 400cm^{-1}.

Estimation of molecular weight

The molecular weight of FRP-2 was determined by a high-performance liquid chromatography (HPLC) instrument (Agilent 1200, USA) equipped with a TSK gel 4,000 PWXL column and an Agilent refractive index detector (RID) at 35°C. The sample was eluted by distilled water at a flow rate of 0.6mL/min and detected by RID. Different molecular weights of standard dextrans (10^4, 4×10^4, 7×10^4, 5×10^5 and 2×10^6 Da) were used for the calibration curve. The standard curve was plotted according to the retention time and logarithm of the respective molecular weights. The molecular weight of FRP-2 was evaluated by comparison with the standard curve.

Identification of monosaccharide composition

FRP-2 was hydrolyzed with2 mol/L trifluoroacetic acid at 120°C for 3h. The excess acid was removed by using absolute methanol in a rotary evaporator three times (Susumu *et al.*, 1981). The residual acid was acetylated by acetic anhydride. Alditol acetates were analyzed by gas chromatography (GC) on a Shimadzu-G-9A instrument equipped with a capillary column of DB-17 (30m×0.32 mm×0.5m) and a flame ionization detector (FID). The temperature of the injector was 280°C, that of the capillary column was 190°C, and that of the FID was 280 °C. Moreover, nitrogen was used as the carrier gas at 1.0mL/min.

C. elegans preparation

C. elegans was cultivated at 20°C on nematode growth medium agar plates, as described previously (Brenner, 1974), and fed with OP_{50} bacteria. Age-synchronized animals were prepared by hypochlorite treatment. Young *C. elegans* were cultured in a liquid S-Medium for 48h and added to nematode growth medium agar plates. *C. elegans* grew to adulthood on nematode growth medium agar plates (Day 3 post-hatching) and were subsequently transferred and cultured in liquid S-Medium with or without different concentrations of FRP-2 for 18h. The test *C. elegans* was transferred onto new nematode growth medium agar and irradiated using a UV crosslinker (UVC 5000, USA) at the selected dose. Thereafter, the worms were counted and transferred to fresh nematode growth medium plates every day until the post-egg-laying period.

Effect of survival rate and head swing frequency on C. elegans

The survival rate of the test worms was counted at the ninth day, and the head swing frequency was measured for 1 min on the second day. Head swing from left to right or from right to left was counted once. All experiments were performed by using 20 adult worms and a minimum of 3 independent studies.

Activity determination of total superoxide dismutase (T-SOD) and catalase (CAT)

The irradiated worms were subjected to grinding by using an M9 buffer and were centrifuged at 1,500 rpm for 3 min. The supernatant was collected to determine the activity of T-SOD and CAT. The activity of T-SOD was detected by using a T-SOD kit (Nanjing Jiancheng, China) and the hydroxylamine method. The activity of CAT was detected by using UV colorimetry. The optical density value was 0.01 for one enzyme activity unit per milligram of tissue protein in 1 min. The tissue protein was detected by using Coomassie brilliant blue G-250 method.

STATISTICAL ANALYSIS

All data are presented as the means ±SD. SPSS Statistics version 17 was used to analyze the data. A multi-factor ANOVA with posterior multiple-range test was used for determining statistical significance.

RESULTS

Effect of different amounts of cellulase on FRP yield

The effect of cellulase amount on the polysaccharide extraction yield is shown in fig. 1a. Extraction was

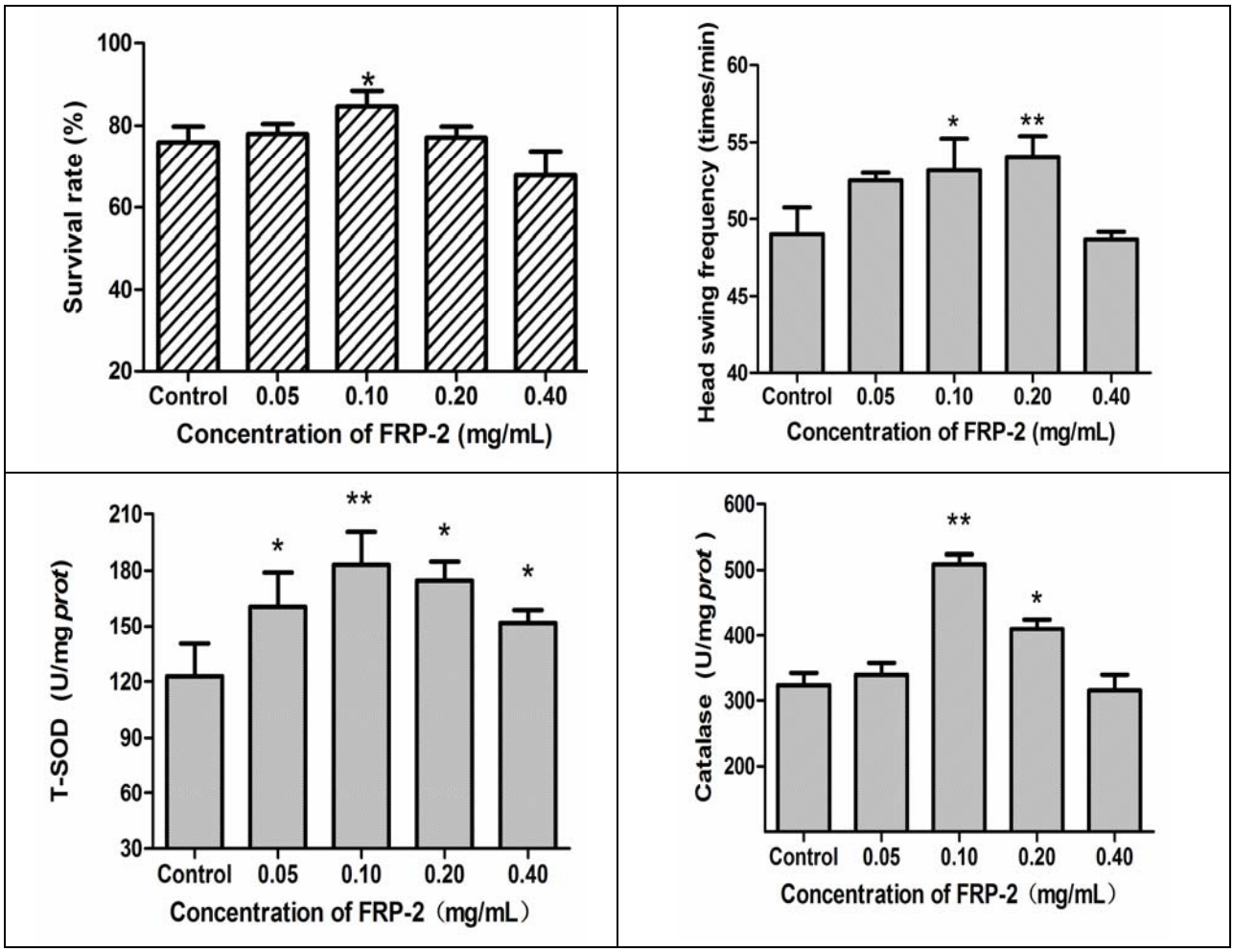

Fig. 6: The anti-ultraviolet activity on *C. elegans* of FRP-2. a. Survival rate at the 9th day (%); b. Head swing frequency (times/min); c. Activity of T-SOD (U/mg *prot*); d. Activity of CAT (U/mg *prot*). Bars represent the standard deviation. The values are presented as means ± S.D. (n = 3). Significant differences (**p<0.01 and *p <0.05) compared with the control group.

conducted with different cellulase amounts of 0.06%, 0.12%, 0.18%, 0.24%, and 0.3% (w/w). Other extraction parameters included 60min hydrolysis time, 45°C enzyme hydrolysis temperature, 120W microwave power, and 40s microwave time. Microwave extraction was conducted twice. The polysaccharide yield increased rapidly when the amounts of cellulase ranged from 0.06% to 0.18%. Thereafter, the polysaccharide yield increased slightly. Thus, 0.18% was selected as the amount of cellulase for producing polysaccharides.

Effect of enzyme hydrolysis time on FRP yield

The effect of enzyme hydrolysis time on the polysaccharide extraction yield is shown in fig. 1b. Extraction was conducted at different enzyme hydrolysis times (i.e., 20, 40, 60, 80, and 100min). Other extraction parameters included 0.18% (w/w) cellulase, 45°C enzyme hydrolysis temperature, 240W microwave power, and 40s microwave time. Microwave extraction was conducted twice. Polysaccharide yield increased rapidly when enzyme hydrolysis time ranged from 20min to 60min. After 60min, the polysaccharide yield increased only slightly. Thus, 60min was selected as the enzyme hydrolysis time.

Effect of enzyme hydrolysis temperature on FRP yield

The effect of enzyme hydrolysis temperature on polysaccharide extraction yield is shown in fig. 1c. Extraction was conducted at different enzyme hydrolysis times (30, 40, 50, 60, and 70min). Other extraction parameters included 0.18% (w/w) cellulase, 60min enzyme hydrolysis time, 240W microwave power, and 40 s microwave time. Microwave extraction was conducted twice. The polysaccharide yield increased rapidly when the enzyme hydrolysis temperature ranged from 20°C to 50°C and decreased after 50°C. Thus, 50°C was selected as the optimum enzyme hydrolysis temperature.

Effect of different microwave power values on FRP yield

The effect of microwave power on polysaccharide extraction yield is shown in fig. 1d. Extraction was processed at microwave power values of 120, 180, 240, 300, and 360W. Other extraction parameters included

0.18% (w/w) cellulase, 60min enzyme hydrolysis time, 50°C enzyme hydrolysis temperature, and 40s microwave time. Microwave extractions were conducted twice. The polysaccharide yield increased rapidly when the microwave power ranged from 120W to 240W and decreased thereafter. Thus, 240W was considered the optimum microwave power.

Table 1: The Box–Behnken Design for optimizing extraction conditions

Run	A	B	C	Yield (%)
1	50	240	50	1.82
2	40	240	40	1.55
3	60	180	50	1.57
4	50	180	60	1.68
5	60	300	50	1.44
6	50	300	60	1.72
7	40	240	60	1.54
8	50	240	50	1.85
9	50	180	40	1.53
10	50	240	50	1.79
11	60	240	40	1.73
12	50	300	40	1.49
13	50	240	50	1.8
14	40	180	50	1.32
15	40	300	50	1.39
16	50	240	50	1.84
17	60	240	60	1.65

A=enzymolysis temperature (°C), B= microwave power (W), C= microwave time (s)

Effect of different microwave times on FRP yield

The effect of microwave time on polysaccharide extraction yield is shown in fig. 1e. Extraction was conducted at different microwave times (i.e., 20, 40, 60, 80, and 100s). Other extraction parameters included 0.18% (w/w) cellulase, 60min enzyme hydrolysis time, 50°C enzyme hydrolysis temperature, and 240W microwave power. Microwave extraction was conducted twice. Polysaccharide yield increased rapidly when the microwave time ranged from 20s to 50s and decreased thereafter. Thus, 50s was considered the best microwave time.

Effect of microwave extraction times on FRP yield

The effect of microwave extraction times on polysaccharide extraction yield is shown in fig. 1f. Extraction was conducted at different microwave extraction times (i.e., one, two, three, four and five times) with the following extraction parameters: 0.18% (w/w) cellulase amount, 60min enzyme hydrolysis time, 50°C enzyme hydrolysis temperature, 240W microwave power, and 50s microwave time. Polysaccharide yield increased rapidly when the number of microwave extractions ranged from one time to three times. After three times, polysaccharide yield only increased slightly. Thus, the optimum microwave extraction time was three times.

Enzymolysis temperature, microwave time and microwave power are the three factors and three levels in the BBD experiment.

Optimization of FRP extraction

The optimum combination of the three variables, namely, enzymolysis temperature, microwave time, and microwave power, was investigated by using BBD. The design and results are shown in table 1. The regression model was as follows:

$$Y = 1.81 + 0.074A - 0.0075B - 0.023C - 0.05AB + 0.018AC + 0.02BC - 0.19A^2 - 0.2B^2 + 0.12B^2C,$$

(3) where Y is the FRP yield (%, w/w), A is the enzymolysis temperature (°C), B is the microwave time (s), and C is the microwave power (W). The statistical significance of Eq. (3) was verified through ANOVA for the RSM model, which indicated that the model was highly significant. The F value of the model is 88.24. Moreover, the value of the determination coefficient (R^2 = 0.9913) and the adjusted determination coefficient (R^2_{Adj}=0.9880) indicated that the response model could explain 99.13% of the total variations. The lack of fit was insignificant. Overall, the model was fit for navigating the design space.

The significance of each coefficient was verified by the p value. The coefficients of A, A^2, and B^2 were highly significant (p<0.001) and that of AB and B^2C were significant (p<0.01). By contrast, the coefficients of B, C, AC, and BC were insignificant (p>0.05) (Zheng, 2014).The interaction between enzymolysis temperature and microwave time was significant, which is different from that between enzymolysis temperature and microwave power and that between microwave time and microwave power (table 2).

From this model, the extraction conditions were optimized as follows: 52.67°C enzymolysis temperature, 80s microwave time, and 144W microwave power. The maximum FRP yield was 2.06% (w/w), which was consistent with the predicted value of 2.01% (w/w).

Purity of FRP-2

The total content of carbohydrate in FRP-2 was measured as 93.45% by the phenol–sulfuric acid method and the UV spectrum of FRP-2 showed that absorption peaks were not detected at 260 and 280nm, respectively, indicating the absence of proteins and nucleic acids in FRP-2 (Peng *et al.*, 2014).

IR analysis of FRP-2

The IR spectrum of FRP-2 is shown in fig. 2. The absorption bands were at 3,416, 2,921, 2,850, 1,679, 1,401, 1,205, 1,141, 1,078, 1,042, 889, and 839cm^{-1}.

Determining the molecular weight of FRP-2

The molecular weight of FRP-2 was estimated to be 2.60×10^5 Da in reference to standard dextran (fig. 3).

Table 2: Analysis of variance for the experimental results of the Box-Behnken Design

Factor	Degree of freedom	Sum of square	F value	P > F	Significance
Model	9	0.049	88.24	<0.0001	***
A	1	0.044	78.94	<0.0001	***
B	1	0.00045	0.82	0.3963	
C	1	0.0002025	3.67	0.0968	
A B	1	0.01	18.14	0.0037	**
AC	1	0.001225	2.22	0.1796	
BC	1	0.00016	2.90	0.1322	
A^2	1	0.15	274.99	<0.0001	***
B^2	1	0.17	312.47	<0.0001	***
B^2C	1	0.028	50.09	0.0002	**
Residual	7	0.0005512			
Lack of fit	3	0.0004195	0.65	0.6254	
Pure error	4	0.00065			
Cor total	16				

*Significant, p<0.05. **Very significant, p < 0.01. ***Highly significant, p<0.001. A = enzymolysis temperature (°C), B = microwave power (W), C= microwave time (s)

Monosaccharide composition of FRP-2

In terms of qualitative determination and monosaccharide quantity, GC was selected because of its sensitivity. The retention time of the alditol acetate derivatives in GC indicates that FRP-2 is mainly composed of glucose (fig. 4).

Anti-ultraviolet activity analysis of FRP-2 to C. elegans

The different ultraviolet doses were 3, 5, 7 and 9mJ/cm^2. The results of the ultraviolet effect on the survival of *C. elegans* are shown in fig. 5. Moreover, 7mJ/cm^2 was selected as the ultraviolet dose for this test. The survival rate of the test worms was checked on the ninth day.

The results of the anti-ultraviolet activity assay by using FRP-2 are shown in fig. 6. The *C. elegans* survival rates on the ninth day were 77.94%, 84.75%, 76.78% and 67.88% with the FRP-2 concentrations of 0.05, 0.10, 0.20 and 0.40mg/mL, respectively. The control survival rate was 75.93% (fig. 6a). The head swing frequencies at the second day were 52.50, 53.17, 54.04 and 48.69 times/min with the FRP-2 concentrations of 0.05, 0.10, 0.20 and 0.40mg/mL, respectively. The control was 49.02 times/min (fig. 6b). The activity of T-SOD was 160.55, 183.08, 174.48, and 151.86 U/mg *prot* with the FRP-2 concentrations of 0.05, 0.10, 0.20 and 0.40mg/mL, respectively. The control was 122.77 U/mg *prot* (fig. 6c). The activities of CAT were 339.18, 508.97, 410.30 and 315.59 U/mg *prot* with the FRP-2 concentrations of 0.05, 0.10, 0.20 and 0.40mg/mL, respectively. The control was 323.66 U/mg *prot* (fig. 6d).

DISCUSSION

The FRP was purified by ethanol precipitation and named the fraction FRP-2. The structural characterization of FRP-2 was carried out by IR spectrum, HPLC and GC. IR spectrum is a method that can be used to determine the types of functional groups and glycosides in polysaccharides (Ovsepyan *et al.*, 1977; Zhang *et al.*, 2005). The IR spectrum of FRP-2 exhibited an intense peak at approximately 3,416cm^{-1} in the hydroxyl group. A stretching band at 2,921cm^{-1} was ascribed to the CH_2 stretching vibration. The peak at 2,850cm^{-1} was assigned to the C–H stretching bands of CH_2. Two bands at 1,679 and 1,401cm^{-1} were ascribed to the deprotonated carboxylic group (COO–) (Manrique and Lajolo, 2002). The band at 1,205cm^{-1} was ascribed to the C-O stretching vibration. The bands at 1,141, 1,078 and 1,042cm^{-1} corresponded to the characteristic absorption peaks of the pyranose ring with the nonsymmetrical C-O-C glycosidic linkage (Zhao *et al.*, 2005). The characteristic absorption at 889cm^{-1} indicates that sugar units are present in the β-configuration and 839cm^{-1} can be inferred for the sugar units in the α-configuration (Sun *et al.*, 1996). No bands exist at 875 and 810cm^{-1}, indicating that FRP-2 does not contain mannose (Wang *et al.*, 2014). The results of HPLC and GC showed that the molecular weight of FRP-2 was 2.60×105 Da, and its monosaccharide composition is mainly glucose.

C. elegans, studied in great detail, is simple and cheap enough to be used as a model organism for biomedical research (Li *et al.*, 2015; Katiki *et al.*, 2011). And, *C. elegans* has been used as an excellent model to elucidate the DNA damage caused by exposure to ultraviolet radiation (Hartman *et al.*, 1989). In our present study, the *C. elegans* was taken as the ultraviolet radiation-induced damage model and the ultraviolet dosage was chosen 7mj/cm^2. The *C. elegans* was given different dose FRP-2 for 18 h. The research showed that the medium dose group (0.1mg/mL) significantly increased the survival rate and head swing frequency of *C. elegans* compared with the control group (p<0.05). Meanwhile, the activity of T-SOD and CAT significantly of *C. elegans* increased in 0.1mg/mL dose

group comparing to those in control group (p<0.01). Therefore, the FRP-2 has a certain anti-ultraviolet radiation activity, in part because it can improve the activity of T-SOD and CAT in *C. elegans.*

CONCLUSION

In our research, cellulase-assisted extraction of FRP was performed. The optimal conditions were determined as follows: 52.67°C enzymolysis temperature, 80s microwave time, 144 W microwave power, 0.18% (w/w) cellulase, 60min enzymolysis time, and three microwave extraction times. Under these conditions, the actual FRP yield was 2.06% (w/w) and the predicted value was 2.01% (w/w). The FRP-2 product samples were water-soluble and white. The FRP-2 samples contained 93.45% (w/w) total sugar and did not contain any protein or nucleic acid. The sugar units existed in the α-configuration and β-configuration. The average molecular weight was estimated to be 2.60×10^5 Da. The monosaccharide composition was mostly glucose. The anti-ultraviolet activity to *C. elegans* of FRP-2 was determined. The results indicate that FRP exhibits anti-ultraviolet potential for functional food and drug applications.

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