文章编号:1001-6880(2012)01-0105-05

海金沙草多糖的提取及抗氧化活性

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摘　要:用响应面优化技术研究了提取时间、固液比、提取温度等对海金沙草粗多糖提取的定量影响,获得了提取工艺的最优工艺参数:提取时间为 123.3 min,固液比(s/w)为 1∶20.9,提取温度为 49.9 ℃,二阶多项式曲线回归模型预测多糖产量为 12.466%,多糖提取验证试验结果(提取率)为 12.85 ± 0.18%(n = 3),比模型预测稍高。多糖经纯化并进行体外抗氧化活性研究(以维生素 C 为对照品),结果发现,多糖产物对超氧阴离子($O_2^{-\cdot}$)和羟基氧自由基(·OH)具有较好的清除作用。

关键词:海金沙草;多糖;超氧阴离子(SOAFR);羟基氧自由基(HOFR);氧自由基(OFRs);响应面法(RSM)

中图分类号:R284.2;R285.5　　**文献标识码**:A

Extraction and Antioxidation of Polysaccharides from *Lygodium japonicum* (Thumb.) Sw

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Abstract: The quantitative effects of extraction time (ETm), the ratio of sample to water (S/W), and extraction temperature (ETp) on polysaccharide extraction rate from *Lygodium japonicum* (Thumb.) Sw were investigated using response surface methodology (RSM), and the optimum extraction parameters were determined: ETm of 123.3 min, S/W of 1∶20.9 (w/v) and ETp of 49.9 ℃. The predicted PS extraction rate reached 12.446%, and the result of verification experiment is 12.85 ± 0.18% (n = 3). The antioxidant activities of the purified PS *in vitro* were evaluated and compared to standard antioxidant as Vitamin C. The results indicated that the purified PS showed strong antioxidant activities against $O_2^{-\cdot}$ and ·OH.

Key words: *Lygodium japonicum* (Thumb.) Sw; polysaccharide (PS); superoxide anion free radical; hydroxyl free oxygen free radicals (OFRs); response surface methodology (RSM)

Introduction

In recent years, some bioactive polysaccharide (PS) isolated from natural sources have attracted much attention in the field of biochemistry and pharmacology. They exhibit various biological activities[1], such as strong antioxidant properties, and can be explored as a novel potential antioxidant[2].

Fern is a sporous plant with fascicular, and now exists approximately 12,000 species around the globe. In China, there is about 61 families, 223 genus, 2600 species, there exist around 300 species may be selected for the purpose of medicine usage[3]. *Lygodium japonicum* (Thumb.) SW, one of the most valued traditional Chinese medicines (TCMs), can be found in hillside, forest, bosk, and lawn in the Yangtze River drainage region and several southern provinces in China. The whole plant and mature spore of this plant have been used for the treatment of pneumonia, acute gastroenteritis, dysentery, urinary tract infection, skin eczema, nephritis dropsy, urine calculus, and so on[4].

Currently, many works mainly centralized in its curative effects[5], but less on the extraction parameters optimization using response surface methodology (RSM) and the function mechanism for supporting its versatile bioactivity, such as antioxidant activity. In this study, a RSM based on a three-factor-three-level Box-Bohnken Design (BBD) was employed to identify and optimize the critical, and significant extraction conditions that

Received August 23, 2010; Accepted January 27, 2011

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will maximize the production of PS. And meanwhile, antioxidant activities *in vitro* against $O_2^{-\cdot}$ and ·OH of purified PS were also investigated.

Materials and Methods

Plant materials and chemicals

Aerial part of *L. japonicum* was collected from Jiulang Mountain located in Zhuzhou city, Hunan Province and identified by LI Lan who graduated from Hunan University of Chinese medicine. The collection was naturally dried, and ground into powder. Vitamin C was purchased from Nanjing Jianchen Ltd., Co.. And all other chemicals and reagents were of analytical grade.

Extraction and preparation of crude PS

L. japonicum was weighed (*approx*. 5.0 g) and dipped in proper amount of double distilled water, and extracted at a certain temperature for a period, and the resulting suspension was centrifuged (4000 g for 30 min), the resulting supernatant was then membrane-filtered (0.45 μm, Millipore), the filtered aqueous solutions were cooled, and then, vacuum freeze-drying at -14 ℃, giving a final volume of about 50 mL. Three volumes of anhydrous alcohol were added to this concentrate, the resulting mixture was placed in a refrigerator at -4 ℃ overnight. The resulting precipitate was separated by centrifugation, washed exhaustively using 96% alcohol, dissolved in deionized water, and prepared for further purification.

Purification of crude PS

Trichloroacetic acid (TCA) was added to the crude PS solution to denature and precipitate protein, and dialyzed using dialysis bag at -4 ℃ overnight. Trace pigment was absorbed using activated charcoal. One hundred milligrams of crude PS was dissolved in distilled water and the resulting solution was membrane-filtered (0.45 μm) and applied to a DEAE-52 cellulose column (1.6 × 40 cm). The column was eluted firstly with deionized water, and then successively with 0.5 mol/L KCl at a flow rate of 30 mL/h (data omitted). PS concentration in each fraction was monitored by the phenol-sulfuric acid method[6], and then, purified PS was gained and collected for further investigations.

Extraction parameters optimization of PS using RSM

The experimental design employed in the present study was a Box-Bohnken Design with three affected factors, i. e., extraction time (ETm), the ratio of sample to water (S/W) and extraction temperature (ETp). The experimental plan consisted of 17 trails and the value of the independent response was the mean of the triplicate. The second-order polynomial coefficients were calculated and analyzed using the 'Design expert' (Version 7.0.0, Stat-Ease Inc., Minneapolis, USA) statistical package. Statistical analysis of the fitted model was employed to evaluate the analysis of variance (ANOVA).

Determination of OFR scavenging activities in vitro

Sample solutions with different concentrations (0 ~ 300 μg/mL) were prepared for antioxidant appraisal.

Determination of $O_2^{-\cdot}$

The ability of the purified PS to scavenge $O_2^{-\cdot}$ was determined according to the method represented by Nishikimi [7]. The scavenging effect was calculated using the following equation.

Scavenging effect (%) = $[(A_{control}-A_{Sample})/A_{control}] \times 100\%$

Determination of ·OH

Measurement of ·OH scavenging activity of purified PS was investigated based on the approach described by Chung[8] with some modifications. The reaction mixture consisted of 0.1 mL of 10 mM $FeSO_4$, 0.1 mL of 10 mM EDTA, 0.5 mL of 10 mM α-deoxyribose, 0.9 mL of sodium phosphate buffer (pH7.4) and 1.0mL of various concentrations of PS sample and standard were thoroughly mixed in a tube separately. Hydrogen peroxide (0.2 mL, 10 mM) was then added and the reaction mixture was incubated at 37 ℃ for 1 h. One milliliter of 2.8% TCA and 1.0mL of 1.0% thiobarbituric acid (TBA) were added to the test tubes and boiled for 15 min. After cooling the mixture, the absorbance was measured at 532 nm. Sodium phosphate buffer (pH 7.4) instead of sample was used as blank. The scavenging activity was evaluated as the inhibition rate of α-deoxyribose oxidation by ·OH. The scavenging effect was determined using the formula described by Ren [9].

Statistics

All the data were expressed as mean ± standard deviation (SD) of the triplicate. The values were regarded to be significantly different when $P < 0.05$.

Results and Discussion

Optimization of extraction parameters

In RSM, a prior knowledge to understand performance of the process and process variables under investigation is necessary for achieving a more realistic model[10]. Preliminary trials [(Plackett - Burman design, PBD) and (steepest ascent design, SAD)] indicated that the range of ETm (90 ~ 150 min), S/W (1∶10 ~ 1∶30, w/v) and ETp (45 ℃ ~ 55 ℃) is beneficial for PS extraction. The range and levels of the three independent variables are presented in Table 1.

Table 1 Level and code of variables chosen for Box-Bohnken Design

Variables	Symbols		Coded levels		
	Uncoded	Coden	-1	0	1
ETm/min	X_1	x_1	90	120	150
S/W(w/v)	X_2	x_2	1∶10	1∶20	1∶30
ETp/℃	X_3	x_3	45	50	55

The design matrix of the variables in coded format is presented in Table 2. Each run was repeated in triplicate, and thus the values list in Table 2 were the average value of the triplicate, and meanwhile, the predicted values of the response were obtained from quadratic model fitting techniques and also be displayed in Table 2.

Table 2 Box-Bohnken Design matrix along with the experimental and predicted values

Run order	ETm (x_1)	S/W (x_2)	ETp (x_3)	PS extraction rate (%)	
				Experimental value	Predicted value
1	-1	0	-1	8.545	8.23
2	1	1	0	9.849	9.83
3	0	1	1	7.81	7.52
4	1	0	-1	9.12	9.16
5	1	-1	0	9.396	9.06
6	-1	1	0	8.682	9.01
7	0	-1	1	9.183	9.20
8	1	0	1	8.583	8.90
9	0	0	0	12.413	12.85
10	0	-1	-1	6.611	6.90
11	-1	-1	0	8.611	8.63
12	0	0	0	12.317	12.85
13	0	0	0	12.216	12.85
14	0	0	0	12.312	12.85
15	0	1	-1	9.743	9.73
16	-1	0	1	8.627	8.59
17	0	0	0	12.825	12.85

It can be seen from Table 2, there is a considerable variation for PS extraction rate depending on the extraction conditions. The replication at the central point conditions resulted in higher the PS extraction rate than at other levels. The predicted value (y) can be fitted and described as:

$$y = 12.85 + 0.31x_1 + 0.29x_2 + 0.023x_3 + 0.095x_1x_2 - 0.15x_1x_3 - 1.13x_2x_3 - 1.45x_1^2 - 1.83x_2^2 - 2.25x_3^2 \quad (1)$$

Where y is the predicted value, and x_1, x_2 and x_3 are the coded value of the tested variables.

The statistical significance of the fitted Eq. (1) was checked by F-test, and the ANOVA for quadratic model is summarized in Table 3.

Table 3 ANOVA for the fitted quadratic polynomial model

Source	Sum of squares	d_f	Mean square	F-value	Probability (p) > F
Model	55.87	9	6.21	52.96	<0.0001
Lack-of-fit	0.59	3	0.20	3.47	0.1305
Pure error	0.23	4	0.057		
Corrected total	56.69	16			

$R^2 = 0.9855$, *Adj.* $R^2 = 0.9669$, and R = 0.9943

The value of *Adj.* R^2 (0.9669) suggests that 96.69% of total variation is attributed to the independent variables and only 3.31% of the total variation can not be explained effectively by this model. The value of R (0.9943) indicates good agreement between the predicted and experimental value. The value of lack-of-fit for quadratic regression Eq. (1) is not significant (p = 0.1305), which indicates that the model equation was adequate for predicting the response under any combination of values of the variables. The coefficient estimates of Eq. (1), along with the corresponding p-value are presented in Table 4.

Table 4 Results of regression analysis of a full second-order polynomial model

Model term	Coefficient estimate	Standard error	Sum of Squares	Mean square	*F*-value	Probability(p) > F
Intercept	12.85	0.15				
x_1(ETm)	0.31	0.12	0.77	0.77	6.57	0.0373
x_2(S/W)	0.29	0.12	0.65	0.65	5.56	0.0505
x_3(ETp)	0.023	0.12	0.004	0.004	0.036	0.8547
x_1x_2	0.095	0.17	0.036	0.036	0.31	0.5943
x_1x_3	−0.15	0.17	0.096	0.096	0.82	0.3961
x_2x_3	−1.13	0.17	5.07	5.07	43.28	0.0003
x_1^2	−1.45	0.17	8.85	8.85	75.52	<0.0001
x_2^2	−1.83	0.17	14.13	14.13	120.56	<0.0001
x_3^2	−2.25	0.17	21.27	21.27	181.48	<0.0001

The p-values can be obtained from Table 4 that x_1 is significant($P < 0.05$), x_1^2, x_2^2, x_3^2 are extremely significant ($P < 0.01$), nevertheless, x_2, x_3, x_1x_2, x_1x_3 and x_2x_3 are not significant($P > 0.05$). The 3D surface plot of the graphical discription for the regression equation is presented in Fig. 1-A, 1-B and 1-C.

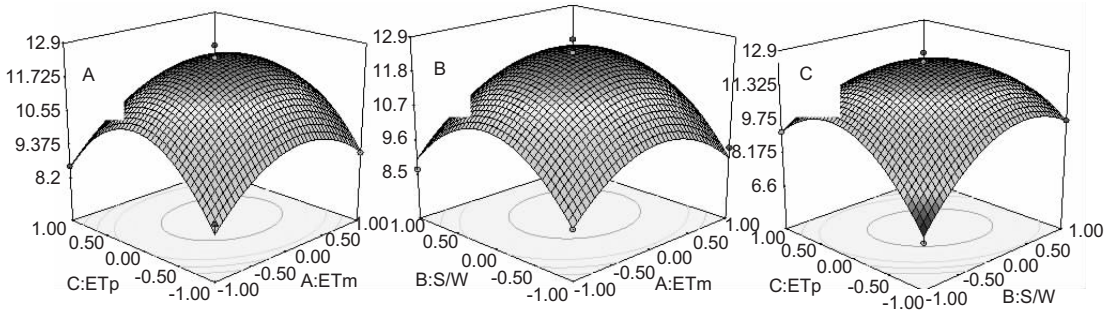

Fig. 1 Response surface plot of effects of ETp and ETm, S/W and ETm, and ETp and S/W on PS extraction rate

The main task of response surface is to hunt efficiently for the optimum values of the variables for maximizing the response. Elliptical contours are obtained when there is a perfect interaction between the independent variables [11]. The optimal predicted value was obtained by fitted model as approximately 12.446%. By solving the inverse matrix (from Eq. (1)), the optimum value of the tested variables in coded format are $x_1 = 0.11$, $x_2 = 0.09$ and $x_3 = -0.02$, in uncoded (natural) units are ETm of 123.3 min, S/W of 1∶20.9(w/v) and ETp of 49.9 ℃, respectively. Under these conditions, the crude PS extraction rate of verification experiment is 12.85% ± 0.18% ($n = 3$), slightly greater than that obtained from the plot analysis.

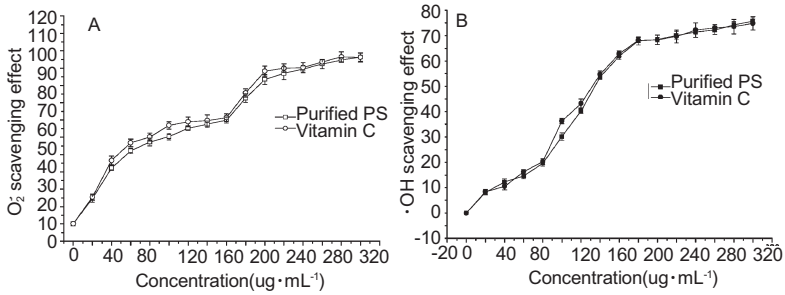

Fig. 2 $O_2^{\cdot-}$ and ·OH scavenging capacity of the purified PS and Vitamin C

Scavenging activity of the purified PS to $O_2^{-\cdot}$ and ·OH

The $O_2^{-\cdot}$ and ·OH scavenging capacities of the purified PS and standard vitamin C are shown in Fig. 2-A and Fig. 2-B, respectively.

Fig. 2-A illustrates that the purified PS was capable of scavenging $O_2^{-\cdot}$ in an concentration- dependent manner (linear regression equation is $y = 5.9838x + 0.081$, $R^2 = 0.9449$), which is similar with the results reported by Li X[3]. The 50% of $O_2^{-\cdot}$ inhibition concentration ($IC_{50\%}$-$O_2^{-\cdot}$) of purified PS and Vitamin C are 83.42 μg/mL and 62.06 μg/mL, respectively. The inhibition precentage of $O_2^{-\cdot}$ generation by 300 μg/mL doses of the purified PS and Vitamin C was found as 96.26% and 96.14%, respectively. The results demonstrated that the purified PS exhibits strong $O_2^{-\cdot}$ scavenging activity, and could bear comparison with that of Vitamin C. The ·OH scavenging activity of purified PS and Vitamin C are presented in Fig. 2-B, the inhibition percentage of different purified PS concentrations on ·OH scavenging can be formulated as a linear regression equation $y = 5.5912x - 1.2655$ ($R^2 = 0.9207$). Results illustrated that the purified PS possesses a strong ·OH scavenging capacity. 50% of ·OH inhibition concentration ($IC_{50\%}$-·OH) for PS and Vitamin C are 91.69 μg/mL and 66.16 μg/mL, respectively. The precentage inhibition of ·OH generation by 300 μg/mL doses of the purified PS and Vitamin C was found as 75.63% and 74.82%, respectively.

Conclusion

In this study, a RSM based on a Box-Bohnken Design was applied for optimization of crude PS extraction. The optimum conditions were determined as ETm of 123.3 min, S/W of 1∶20.9 (w/v) and ETp of 49.9 ℃, the predicted value and verification experimental value of PS extraction rate are 12.446 % and 12.850%, respectively. The antioxidant evaluation of purified PS *in vitro* revealed that PS possesses strong $O_2^{-\cdot}$ and ·OH scavenging activity, which may be comparable to vitamine C. Through our investigations, it may be rational to assure that PS from *L. japonicum* can be used as an effective OFRs scavenger and play its curative role in traditional medicine due to the mechanism of antioxidant ability in it.

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