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Extraction and antioxidant activity of polysaccharides from *Rana chensinensis* skin

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ABSTRACT

The extraction process of polysaccharides from *Rana chensinensis* skin was optimized by using a Box–Behnken design. The optimum extraction conditions were as follows: extraction time, 4.96 h; extraction temperature, 100 °C; ratio of water to raw material, 60; and extraction frequency, 1. Under these conditions, the experimental polysaccharide yield was 2.03 ± 0.14%, which agreed with the predicted yield. The purified polysaccharide RCSP II was successfully obtained by diethylaminoethanol–Sepharose and Sepharose CL-6B column chromatography. In vitro experiments showed that RCSP II exhibited a strong scavenging activity against superoxide anion and 1,1-diphenyl-2-picrylhydrazyl radicals but a weak scavenging activity against hydroxyl radicals. RCSP II also showed a strong reducing capacity. Thus, this polysaccharide can be used as a natural antioxidant in functional foods or medicines.

1. Introduction

The Chinese brown frog *Rana chensinensis* is widely distributed in North-Central China (Zhao, Sun, Li, & Su, 2011). The unisex health product "Linwayou" can be extracted from the fallopian tube of female *R. chensinensis*. The production of "Linwayou" from *R. chensinensis* yields a high amount of residues that are not well utilized. The skin of amphibians contains numerous bioactive substances with great application value and exploitation potential. Thus, many studies concentrated on the antimicrobial peptides of amphibian skin (Iwakoshi-Ukena et al., 2011; Rozek et al., 2000; Wang et al., 2012). Roseghini, Erspamer, Falconieri, and Cei (1986) analyzed the extracts prepared from dried or fresh skin of 140 American amphibian species to determine the presence and concentration of aromatic biogenic amines. Barra and Simmaco (1995) found that amphibian skin is a rich source of biologically active compounds with diverse physiological and defense functions. Amphibian skin secretions contain many biologically active compounds, such as biogenic amines, complex alkaloids, or peptides (Rinaldi, 2002; Simmaco, Mignogna, & Barra, 1998). The antibacterial peptide dermatoxin was successfully extracted from the skin of the tree frog *Phyllomedusa bicolor* in 2000. This peptide exhibits bactericidal effects on Gram-positive and Gram-negative eubacteria (Amiche, Seon, Wroblewski, & Nicolas, 2000). Six peptides that demonstrate antimicrobial activities were also isolated from an extract of freeze-dried skin of the Japanese mountain brown frog *Rana ornativentris* (Kim, Iwamuro, Knoop, & Conlon, 2001).

However, information on the extraction, purification, and functionality of polysaccharides from amphibian skin is lacking. In this study, the production process of polysaccharides from the skin of *R. chensinensis* (RCSP) was optimized by using a Box–Behnken design (BBD). The antioxidant activities of RSCP were evaluated for use in the food and pharmaceutical industries.

2. Materials and methods

2.1. Materials and chemicals

The residues of *R. chensinensis* were provided by Fushun East Star Institute of *R. chensinensis* competitive products (Fushun, Liaoning Province, China). Ascorbic acid, nitroblue tetrazolium, methionine, 1,1-diphenyl-2-picrylhydrazyl (DPPH), and riboflavin were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Unless otherwise stated, all chemicals used were of analytical grade.

2.2. RCSP extraction

Skin samples were collected from the residues of *R. chensinensis*. After cleaning with deionized water and drying, the samples were homogenized in a blender and then defatted by petroleum ether (boiling range 30–60 °C) at room temperature for 12 h. The

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Table 1
Experimental domain of BBD.

Variables	Levels		
	−1	0	1
Extraction time (X_1) (h)	3	4	5
Extraction temperature (X_2) (°C)	80	90	100
Ratio of water to raw material (X_3) (n)	40	50	60

defatted sample was centrifuged at 2000 × *g* for 15 min, and the deposit was vacuum dried at 60 °C for 16 h to obtain a constant weight. Each dried pretreated sample (10 g) was extracted with deionized water at a designated temperature, extraction time, ratio of water to raw material, and extraction frequency. The mixture was centrifuged at 2000 × *g* for 15 min, and the supernatant was separated from the insoluble residue. The extract was precipitated by the addition of ethanol to a final concentration of 75% (v/v) and then incubated overnight. The polysaccharide precipitate was collected by centrifugation at 2000 × *g* for 15 min and then deproteinated by a combination of proteinase and Sevag method (Staub, 1965). The supernatant was lyophilized to obtain RCSP. The polysaccharide content was measured by using the phenol–sulfuric acid method with D-glucose as a standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1958).

2.3. Experimental design and statistical analysis

The preliminary range of extraction variables was determined through a single-factor test. A BBD with three independent variables (X_1, extraction time; X_2, extraction temperature; and X_3, ratio of water to raw material) was adopted. For statistical calculations, the variables were coded as follows:

$$x_i = \frac{X_i - X_0}{\Delta X_i}, \quad (1)$$

where x_i is a coded value of the independent variable, X_i is the actual value of the independent variable, X_0 is the actual value of X_i at the centerpoint, and ΔX_i is the value of the step change. The ranges of independent variables and their levels, which were chosen based on the preliminary experimental results, are presented in Table 1.

The design comprised 17 experimental points, each of which was performed in triplicate in a randomized order. The response value of each trial was calculated as the average of the duplicates.

The BBD data were analyzed by multiple regressions to fit the following quadratic polynomial model:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta k_{ij} x_i x_j, \quad (2)$$

where Y is the predicted response (RCSP yield); β_0 is an intercept; β_i, β_{ii}, and β_{ij} are the coefficients of the linear, quadratic, and interactive terms, respectively; and x_i and x_j represent the coded independent variables.

The regression coefficients of the linear, quadratic, and interaction terms were determined according to the results of variable analysis and were used in statistical calculations to generate 3D surface and contour plots from the fitted polynomial equation. The second-order polynomial coefficients were calculated and analyzed using the Design Expert software (Version 8.0.6, Stat-Ease Inc., Minneapolis, USA). Statistical significance was considered at $P < 0.05$.

2.4. RCSP purification

RCSP was dissolved in distilled water, freeze thawed, and then centrifuged until all insoluble substances disappeared. The solutions were applied to a diethylaminoethyl (DEAE)–cellulose (Amersham Biosciences, Sweden) column (3 cm × 45 cm) equilibrated with a linear gradient of NaCl from 0 M to 1.0 M. After the adsorption of most pigments in the column, the eluted sample was collected and further purified by gel filtration chromatography on a Sepharose CL-6B (Amersham Biosciences, Sweden) column (2.5 cm × 90 cm) with distilled water. The different fractions were dialyzed against distilled water and then lyophilized.

2.5. Antioxidant activity test in vitro

2.5.1. Superoxide radical scavenging assay

Approximately 4.5 mL of 50 mM Tris–HCl buffer (pH 8.2) was mixed with 4.2 mL of deionized water. After incubation at 25 °C for 20 min, 1 mL of polysaccharide solution and 0.4 mL of pyrogallic acid were added to the mixture. The resulting mixture was rapidly shaken and then incubated at 25 °C for 5 min. Subsequently, 8 mM HCl was added to the mixture to terminate the reaction, and the absorbance of the mixture was measured at 320 nm with ascorbic acid as the positive control. The ability of polysaccharide to scavenge superoxide radicals was calculated as follows:

$$\text{Scavenging rate}\,(\%) = \frac{A_0 - A_i}{A_0} \times 100 \quad (3)$$

where A_0 is the absorbance of the blank and A_i is the absorbance of polysaccharide/ascorbic acid.

2.5.2. Hydroxyl radical scavenging assay

The scavenging activity of polysaccharide against hydroxyl radicals was measured as previously described (Winterbourn & Sutton, 1984). The reaction mixture contained 1 mL of 0.15 M phosphate buffer (pH 7.4), 1 mL of 40 μg/mL safranin, 1 mL of 0.945 mM ethylenediaminetetraacetic acid–Fe(II), 1 mL of 3% H_2O_2 (v/v), and 0.5 mL of polysaccharide. After incubation at 37 °C for 30 min, the absorbance of the solution was measured at 560 nm with ascorbic acid as the positive control. The EC_{50} value (mg/L) of polysaccharide or ascorbic acid was defined as the effective concentration at which 50% of the hydroxyl radicals were scavenged. The scavenging activity of polysaccharide against hydroxyl radicals was expressed as follows:

$$\text{Scavenging rate}\,(\%) = \frac{A_0 - A_i}{A_0} \times 100 \quad (4)$$

where A_0 is the absorbance of the blank and A_i is the absorbance of polysaccharide/ascorbic acid.

2.5.3. DPPH scavenging assay

The scavenging activity of polysaccharide against DPPH radicals was determined according to the method described by Shimada, Fujikawa, and Yahara (1992) with slight modifications. The reaction mixture comprised 2 mL of DPPH (0.1 μM in 95% ethanol) and 2 mL of polysaccharide. The mixture was incubated at 25 °C for 15 min, and the absorbance of the mixture was determined at 517 nm with ascorbic acid as the positive control. The EC_{50} value (mg/L) of polysaccharide was defined as the effective concentration at which 50% of the DPPH radicals were scavenged. The antioxidant activity of polysaccharide was evaluated as follows:

$$\text{Scavenging rate}\,(\%) = \frac{1 - A_i}{A_0} \times 100 \quad (5)$$

where A_i is the absorbance of polysaccharide/ascorbic acid and A_0 is the absorbance of the DPPH solution.

2.5.4. Determination of the reducing power of polysaccharide

The reducing power of polysaccharide was evaluated according to the method reported by Deng et al. (2011). The reaction mixture contained 2.5 mL of phosphate buffer (pH 6.6, 0.2 M), 2.5 mL of potassium ferricyanide (1%, w/v), and polysaccharide. After incubation at 50 °C for 20 min, 2.5 mL of trichloroacetic acid (10%, w/v)

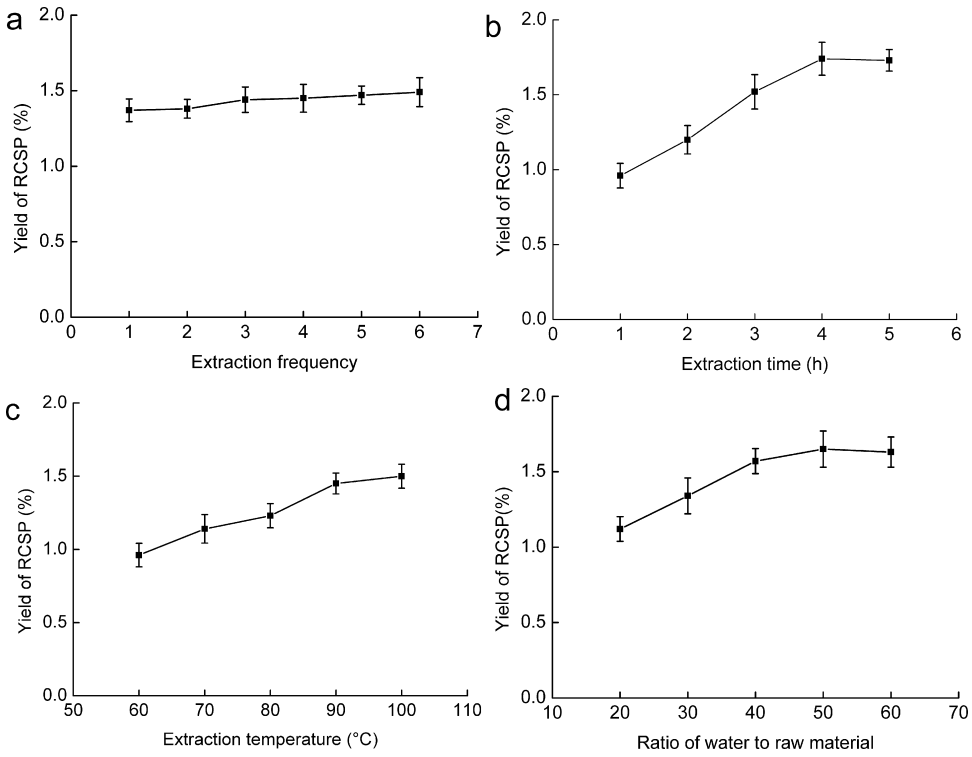

Fig. 1. Effect of different extraction parameters on the yield of RCSP: (a) extraction frequency; (b) extraction time; (c) extraction temperature; (d) ratio of water to raw material.

was added to the mixture to terminate the reaction. The mixture was centrifuged at 1200 × *g* for 10 min, and approximately 2.5 mL of the supernatant was collected and mixed with 2.5 mL of deionized water and 0.5 mL of $FeCl_3$ (0.1%, w/v). After incubation at room temperature for 15 min, the absorbance of the solution was measured at 700 nm with ascorbic acid as the positive control.

3. Results and discussion

3.1. Effect of extraction frequency on RCSP yield

The effect of extraction frequency on RCSP yield is shown in Fig. 1a. The extraction was conducted at different frequencies while the other extraction conditions were fixed as follows: temperature, 90 °C; time, 4 h; and ratio of water to raw material, 40. The variance of polysaccharide yield did not significantly increase when the extraction frequency varied from 1 to 6. Thus, the extraction frequency of 1 was selected to obtain the maximum RCSP.

3.2. Effect of extraction time on RCSP yield

The effect of extraction time on RCSP yield is illustrated in Fig. 1b. The extraction was conducted at different extraction times while the other extraction conditions were fixed as follows: frequency, 1; temperature, 90 °C; and ratio of water to raw material, 40. The extraction yield remarkably increased as the extraction time varied from 1 h to 4 h. The highest yield of 1.74 ± 0.11% was obtained at 4 h. The yield no longer significantly changed beyond 4 h. Thus, the extraction time of 4 h was selected to obtain the maximum RCSP yield.

Table 2
The levels of each variable and corresponding yield of RCSP obtained from BBD.

No.	Coded variable level			Real variable level			Yield of RCSP (%)	
	X_1	X_2	X_3	Extraction time (h)	Extraction temperature (°C)	Ratio of water to raw material	Predicted	Experimental
1	−1	−1	0	3	80	50	1.22	1.17
2	1	−1	0	5	80	50	1.50	1.42
3	−1	1	0	3	100	50	1.51	1.59
4	1	1	0	5	100	50	1.97	2.02
5	−1	0	−1	3	90	40	1.39	1.38
6	1	0	−1	5	90	40	1.83	1.85
7	−1	0	1	3	90	60	1.57	1.55
8	1	0	1	5	90	60	1.87	1.88
9	0	−1	−1	4	80	40	1.69	1.44
10	0	1	−1	4	100	40	1.93	1.86
11	0	−1	1	4	80	60	1.67	1.74
12	0	1	1	4	100	60	2.17	2.11
13	0	0	0	4	90	50	1.76	1.77
14	0	0	0	4	90	50	1.76	1.76
15	0	0	0	4	90	50	1.76	1.75
16	0	0	0	4	90	50	1.76	1.75
17	0	0	0	4	90	50	1.76	1.77

Table 3
ANOVA for the yield of RCSP according to response surface quadratic model.

Source	Statistical analysis				
	Sum of squares	df	Mean square	*F*-value	*P*-value
Model	0.9103	9	0.1011	29.3475	<0.0001
X_1	0.2738	1	0.2738	79.4446	<0.0001
X_2	0.4095	1	0.4095	118.8220	<0.0001
X_3	0.0703	1	0.0703	20.4016	0.0027
X_1X_2	0.0081	1	0.0081	2.3503	0.1691
X_1X_3	0.0049	1	0.0049	1.4217	0.2720
X_2X_3	0.0006	1	0.0006	0.1813	0.6830
X_1^2	0.1164	1	0.1164	33.7668	0.001
X_2^2	0.0081	1	0.0081	2.3384	0.1701
X_3^2	0.0214	1	0.0214	6.2021	0.0416
Residual	0.0241	7	0.0034		
Lack of fit	0.0237	3	0.0079	79.0833	0.0005
Pure error	0.0004	4	0.0001		
Cor total	0.9344	16			
	$R^2 = 0.9742$	$R^2_{adj} = 0.9410$	CV = 3.46		

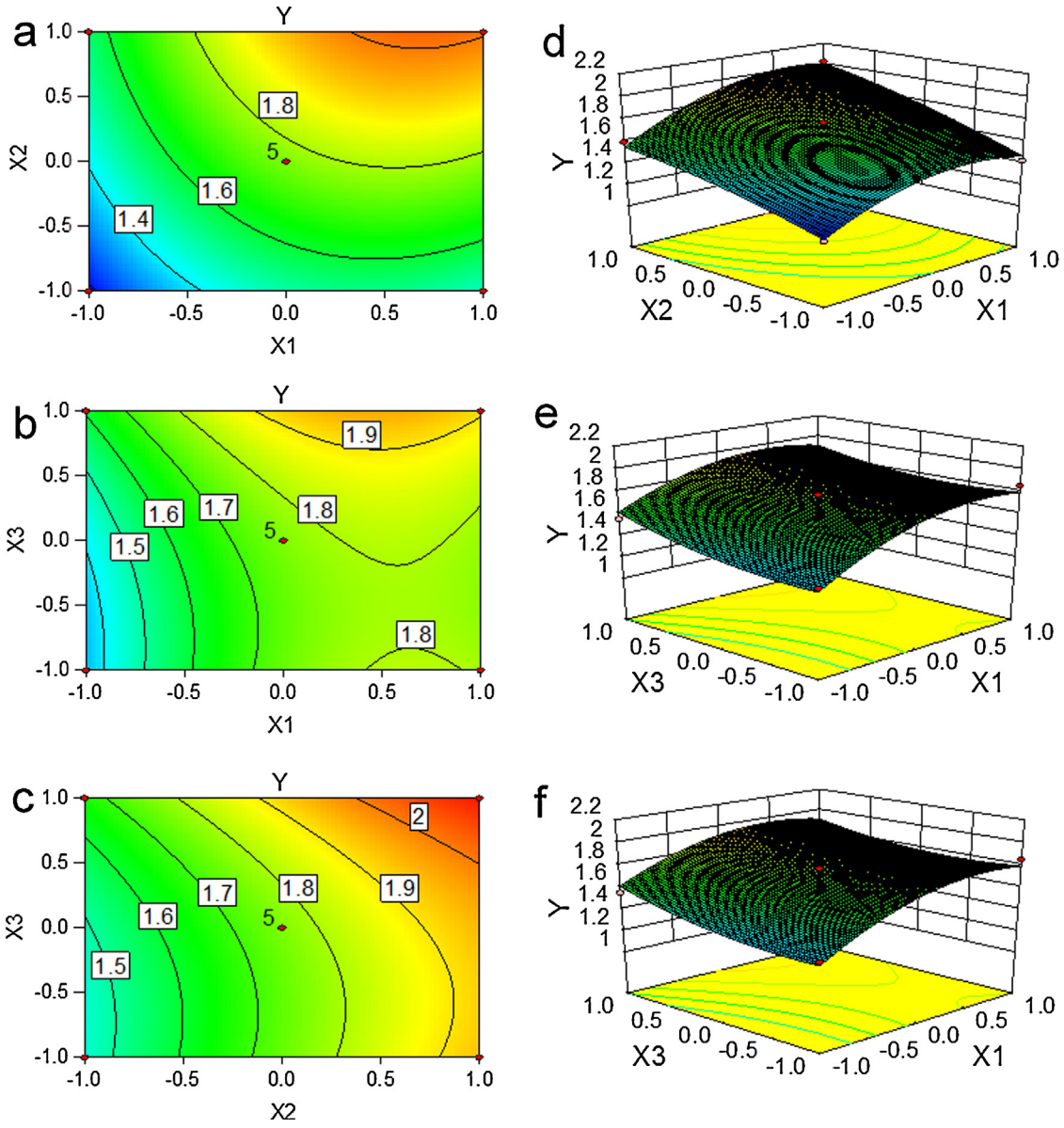

Fig. 2. Three-dimensional and plots response surface showing the effects of variables (X_1, extraction time; X_2, extraction temperature; X_3 ratio of water to raw material) on the response (yield of RCSP).

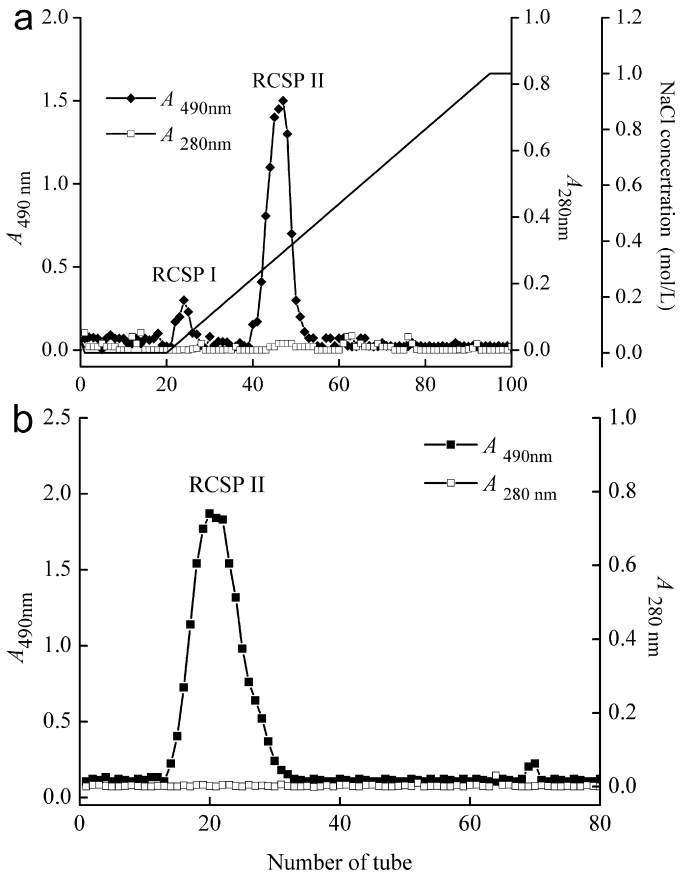

Fig. 3. Elution curve of RCSP on DEAE–cellulose DE-52 column (a) and elution curves of RCSP II on Sepharose CL-6B column (b).

3.3. Effect of extraction temperature on RCSP yield

The extraction coefficient increases with increasing temperature because of improved polysaccharide solubility (Braga, Moreschi, & Meireles, 2006). The effect of extraction temperature on RCSP yield is illustrated in Fig. 1c. The extraction was conducted at different temperatures while the other conditions were fixed as follows: frequency, 1; time, 4 h; and ratio of water to raw material, 40. The RCSP yield continuously increased as the extraction temperature was increased from 60 °C to 90 °C. The extraction yield no longer significantly increased beyond this temperature range.

3.4. Effect of ratio of water to raw material on RCSP yield

The effects of different ratios of water to raw material on RCSP yield are illustrated in Fig. 1d. The extraction was conducted at different ratios of water to raw material while the other conditions were fixed as follows: temperature, 90 °C; time, 4 h; and frequency, 1. The extraction yield increased with increasing water-to-raw material ratio. The highest yield of 1.65 ± 0.12% was obtained at 50. The yield no longer significantly changed beyond this ratio.

According to the above single-factor experiments, BBD experiments were conducted under the following extraction conditions: frequency, 1; time, 4–6 h; temperature, 80–100 °C; and water-to-raw material ratio, 40–60.

3.5. Statistical analysis and model fitting

The design matrix and corresponding results of the BBD were considered to determine the effects of the four independent variables, including X_1, X_2, and X_3 (Table 2). The experimental data were subjected to multiple regression analysis. The response and test

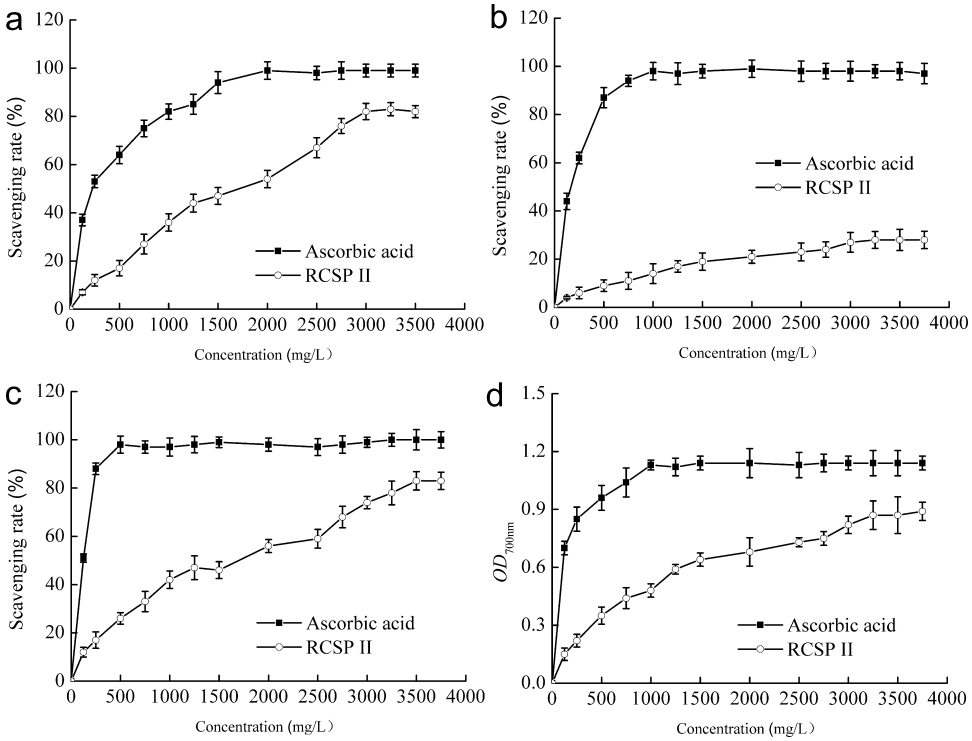

Fig. 4. Antioxidant activity assay of RCSP II and ascorbic acid. (a) Scavenging effects on superoxide anion radicals. (b) Scavenging effects on hydroxyl radicals. (c) Scavenging effects on DPPH. (d) Reducing power of RCSP II and ascorbic acid.

variables were related by the following second-order polynomial equation:

$$Y = 1.758 + 0.185X_1 + 0.1875X_2 + 0.055X_3 + 0.045X_1X_2 - 0.035X_1X_3 + 0.065X_2X_3 - 0.204X_1^2 - 0.004X_2^2 + 0.111X_3^2 \quad (6)$$

The fitting statistics of the yield Y for the selected quadratic predictive model is shown in Table 3. The model was used to predict responses within the range of the experimental variables. From ANOVA of the quadratic regression model, the determination coefficient is $R^2 = 0.9742$, which indicates that 2.58% of the total variations cannot be explained by the model. The adjusted determination coefficient ($R^2_{adj} = 0.9410$) also confirms that the model is highly significant. The low coefficient of variation (3.46) indicates high degrees of precision and reliability of the experimental values. As shown in the table, the linear coefficients (X_1, X_2, and X_3) and quadratic term coefficients ($X_1{}^2$ and $X_3{}^2$) were significant with low P values ($P < 0.05$). The other coefficients were not significant ($P > 0.05$).

3.6. Optimization of RCSP extraction conditions

The full model was created as a 3D response surface with contour plots to predict the relationships between independent and dependent variables. The RCSP yield was affected by extraction time (X_1), extraction temperature (X_2), and ratio of water to raw material (X_3), as shown in Fig. 2.

In the response surface and contour plots, the RCSP yield was obtained along with two continuous variables because the other variable was fixed at 0 (center value of the testing ranges). In the two figures, the maximum predicted value indicated by the surface was confined in the smallest ellipse in the contour diagram. Elliptical contours were obtained when perfect interaction existed between the independent variables. The independent variables and the maximum predicted values from the figures corresponded to the optimum values of the dependent variables (responses) obtained using Eq. (6).

The 3D surface and contour plots constructed based on the independent variables (X_1 and X_2) are shown in Fig. 1a and d. The independent variable X_3 was maintained at 0. The highest RCSP yield was achieved when X_1 and X_2 were 4.59 h and 100 °C, respectively. As shown in Fig. 2b and e, the RCSP yield varied with X_1 and X_3 when X_2 was maintained at 0. The highest RCSP yield was achieved when X_1 and X_3 were 4.59 h and 60, respectively. Fig. 2c and f show the effects of different X_2 and X_3 on the RCSP yield when X_1 was maintained at 0. The maximum RCSP yield was achieved when X_2 and X_3 were set at 100 °C and 60, respectively.

The following optimal extraction conditions for RCSP are shown in Fig. 2: frequency, 1; time, 4.59 h; temperature, 100 °C; and ratio of water to raw material, 60.

3.7. Verification of the predictive model

The suitability of the model equations for predicting the optimum response values was determined under optimized conditions. This set of conditions was considered optimum and was used to validate experimentally and predict the values of the responses by using the model equation. A mean value of 2.03 ± 0.14 ($n = 3$) obtained from the real experiments validated the RSM model compared with the predicted value (2.15), indicating that the model can be used for extraction.

3.8. Purification of RCSP

RCSP solution was loaded into a DEAE–cellulose column equilibrated with a linear gradient of NaCl from 0 M to 1.0 M. Two independent elution peaks (RCSPs I and II, Fig. 3a) detected by the phenol–sulfuric acid assay were obtained. The fraction content of RCSP I was lower than that of RCSP II and was not conducive to collection. Thus, only the RCSP II fraction was collected for the subsequent purification and antioxidant activity assays. The RCSP II fraction was collected, dialyzed, concentrated, and loaded into a Sepharose CL-6B column. The fraction produced a single elution peak (Fig. 3b).

3.9. Antioxidant activity assay

The scavenging rates of RCSP II and ascorbic acid against superoxide radicals were directly proportional to their concentrations (Fig. 4a). The scavenging rates of RCSP were lower than those of ascorbic acid within the test dosage range. The EC_{50} of RCSP (1709.4 mg/L) was considerably higher than that of ascorbic acid (232.3 mg/L).

The scavenging activity of ascorbic acid against hydroxyl radicals was remarkably higher than that of RCSP II within the tested dosage range (Fig. 4b). The scavenging effect of RCSP II was weak, and equilibrium was reached at 3250 mg/L concentration with a maximum scavenging rate of $28.2 \pm 0.8\%$. Within the test dosage range, the EC_{50} of RCSP II was unconfirmed, whereas that of ascorbic acid was 174.6 mg/L.

The scavenging effects of RCSP II on DPPH radicals were determined (Fig. 4c). The scavenging activity of RCSP II against DPPH radicals directly increased with increasing concentration within the test dosage range. The maximum scavenging rate of $83.1 \pm 3.8\%$ was obtained at a relatively high concentration range (3500 mg/L). Ascorbic acid elicited an evident scavenging activity against DPPH radicals at a relatively low concentration range and reached the maximum scavenging rate (98%) at a relatively low concentration (500 mg/L). RCSP II exhibited an EC_{50} of 1691 mg/L against DPPH radical, and this value was significantly higher than that of ascorbic acid (119.8 mg/L).

The reducing capacities of RCSP II and ascorbic acid were also investigated (Fig. 4d). The reducing capacity of RCSP was lower than that of ascorbic acid, but RCSP II had potential antioxidant properties.

4. Conclusion

The extraction process of polysaccharides from *R. chensinensis* skin was optimized using a BBD. The effect of independent variables (extraction time, extraction temperature, and ratio of water to raw material) on the process was determined. The optimal experimental RCSP yield of $2.03 \pm 0.14\%$ was obtained under the following extraction conditions: time, 4.96 h; temperature, 100 °C; ratio of water to raw material, 60; and frequency, 1. Under these optimized conditions, the experimental RCSP yield was consistent with the predicted yield (2.15%). The purified homogeneous polysaccharide RCSP II was successfully obtained by DEAE–Sepharose and Sepharose CL-6B column chromatography. RCSP II exhibited a strong radical scavenging activity against superoxide anion and DPPH radicals but a weak scavenging activity against hydroxyl radicals. RCSP II also showed a strong reducing capacity in vitro.

Further studies on polysaccharide properties, such as monosaccharide composition, structure, and splenocyte activation, are currently in progress. RCSP can be used as an antioxidant and immunostimulating agent in functional foods or medicines.

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