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PII: S0141-8130(18)30047-3
DOI: doi:10.1016/j.ijbiomac.2018.03.145
Reference: BIOMAC 9367

To appear in:

Received date: 4 January 2018
Revised date: 26 January 2018
Accepted date: 23 March 2018

Please cite this article as: Kunli Wang, Mo Li, Xin Wen, Xiaosong Chen, Zhengyu He, Yuanying Ni , Optimization of ultrasound-assisted extraction of okra (Abelmoschus esculentus (L.) Moench) polysaccharides based on response surface methodology and antioxidant activity. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Biomac(2017), doi:10.1016/j.ijbiomac.2018.03.145

Optimization of ultrasound-assisted extraction of okra (*Abelmoschus esculentus (L.) Moench*) polysaccharides based on response surface methodology and antioxidant activity

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Abstract

This study determined the optimal conditions for ultrasound-assisted extraction of a water-soluble polysaccharide, Raw Okra Polysaccharide, from the fruit of okra using response surface methodology. The optimal extraction temperature, extraction time and ultrasonic power were 59 °C, 30 min and 522 W, respectively, giving a yield of 10.35 ± 0.11 %. ROP was further isolated, lyophilized and purified using a DEAE-Sepharose Fast Flow column and Sepharose CL-6B column, revealing three elution peaks subsequently designated ROP-1, -2, and -3, respectively. Of these, ROP-2 showed the highest yield, and was therefore selected for physicochemical analysis and evaluation of antioxidant activity. Gas chromatography, fourier transform infrared spectroscopy, and high-performance liquid chromatography were used to characterize the primary

structural features and molecular weight, revealing that ROP-2 is composed of glucose, mannose, galactose, arabinose, xylose, fructose, and rhamnose (molar percentages: 28.8, 12.5, 13.1, 15.9, 9.2, 13.7, and 6.8 %, respectively) and has an average molecular weight of 1.92 × 105 Da. A superoxide radical scavenging assay and DPPH radical scavenging assay further revealed the significant *in vitro* antioxidant activity of ROP-2. These findings present an effective technique for extraction of the natural antioxidant ROP-2, warranting further analysis of its potential application in the food industry.

Keywords: Okra polysaccharides, ultrasonic-assisted extraction, response surface methodology, optimization, antioxidant activity

1. Introduction

Okra, *Abelmoschus esculentus (L.) Moench*, is an annual herb grown in tropical and Mediterranean climates such as Southeast Asia, Africa, southern states of the USA, and the Middle East; however, okra also has a wide growth potential in other countries [1, 2]. The okra pod is eaten as a vegetable, while its extract is used to thicken stews and soup [3, 4]. Water-soluble polysaccharides from okra are also used in ice-cream, potato chips, and baked goods, providing a healthy option and more stable shelf-life [5-7]. Interest in okra is therefore growing, with many of its components (flavonoids, polysaccharides and vitamins) possessing significant biological activities [8] such as antioxidant, hypoglycemic, antineoplastic, and hypolipidemic [9-12].

Of these compounds, polysaccharides are among the most important, having been found to possess immunomodulatory, antioxidant, and antiproliferative activities [13]. However, while the backbone, molecular weight, composition, and biological activity of okra polysaccharides have been examined [2], little is known about their extraction and antioxidant activities.

Extraction methods tend to influence the yield, chemical structure and biological activity of polysaccharides [14, 15]. Hot water extraction is traditionally used to isolate bioactive materials; however, it has disadvantages including a high extraction temperature, long extraction time, and high consumption rate of solvents and energy [16, 17]. Various new techniques for polysaccharide extraction have therefore been developed such as supercritical fluid, enzyme-assisted, microwave-assisted, and ultrasonic-assisted extraction [18], all of which have advantages and disadvantages. Ultrasound extraction technology has a moderate solvent requirement, relatively short extraction time, and reasonably high extraction yield, and has therefore been used widely to extract highly bioactive polysaccharides from plant materials with a minimal impact on biological

activity [19-22].

In this study, we chose the ultrasound extraction technique to optimize the extraction yield of okra polysaccharide (Raw Okra Polysaccharide, ROP). To do so, three extraction factors (temperature, time, and ultrasonic power) were examined based on single factor experiments. ROP was then deproteinized, lyophilized, and purified using a diethylaminoethyl (DEAE)-Sepharose Fast Flow column and Sepharose CL-6B column, and the purified fraction characterized along with evaluation of *in vitro* antioxidant activity.

2. Materials and methods

2.1 Reagents and materials

Fresh okra was purchased from Gansu Province, China. Bovine serum albumin (BVA), dextran T-series standards and monosaccharide standards were obtained from Sigma Chemical Co. (St. Louis, MO, USA). DEAE-Sepharose Fast Flow and Sepharose CL-6B were purchased from Pharmacia. Remaining chemicals were of analytical grade.

2.2 Extraction of ROP

2.2.1 Pretreatment of okra

The plant material was freeze-dried (-56 ℃, 36 h), pulverized using a mechanical disintegrator then screened through a 40-mesh sieve. The resulting powder was subjected to heat circumfluence extraction at 50 ℃ for 6 h to remove oligosaccharides, colored substances and lipids using 95 % ethanol–petroleum ether at 1:1 (v/v) as the refluxing solvent. After centrifugation, the residue was collected then dried to a constant weight at 40 ℃ for ROP extraction.

2.2.2 ROP extraction

Ultrasound-assisted extraction was carried out in an ultrasonic cleaning bath (KQ-500DE, Kunshan Ultrasonic Instrument Co. Ltd., Jiangsu, China) using distilled water to extract the pretreated sample (10 g). Water extraction solutions were obtained by centrifugation (CR21GIII, Hitachi Koki Instrument Development Co., Ltd.) at 8000 rpm for 8 min. Polysaccharides were measured based on the phenol-sulfuric acid method using D-glucose as a standard [23] and the protein content measured according to the Bradford method using BSA as a standard [24]. ROP extraction yield (%) was then calculated as follows:

$$\mathrm{Y}\ (\%) = \frac{C}{W} \times 100\%$$

Where C is the polysaccharide content and W is the sample dry weight.

2.2.3 Single-factor design

The initial range of the following three extraction factors was determined using a single factor design: extraction temperature (40 - 80 °C), extraction time (10 - 30 min), and ultrasonic power (360 - 720 W). The dependent variable was ROP extraction yield. Preliminary experiments revealed a high extraction yield under a liquid-solid ratio of 30:1, with no significant improvements thereafter. A liquid-solid ratio of 30:1 was therefore selected.

2.2.4 Optimization of ROP extraction

Using the results of the single factor experiments, Design Expert (Trial Version 8.0.6.1, Stat-Ease Inc., USA) was used for experimental design, data analysis and model building. Three independent variables (extraction temperature (A), extraction time (B), and ultrasonic power (C)) at three levels were then used to perform Box-Behnken design (BBD) analysis to determine the best combination for ROP extraction. Seventeen experimental points were determined using the design results and the level of each variable, all of which were included in a random order (Table

1). A computer-generated nonlinear quadratic model was then used to fit the data as follows:

$$Y = A_0 + \sum_{j-1}^{K} A_j X_j + \sum_{j-1}^{K} A_{jj} X_j^2 + \sum \sum_{i<j} A_{jj} X_i X_j$$

where Y is the ROP yield, A_0, A_j, A_{jj} and A_{ij} are regression coefficients of variables representing intercept, linear, quadratic and interaction terms, and X_i and X_j are the independent coded variables (i ≠ j).

2.3 Purification of ROP

Based on the results of optimal extraction, the water extraction solutions were combined then concentrated to 1/3 volume at 50 ℃ using a rotary evaporator (R-100, BUCHI) under reduced pressure. Sevag reagent (chloroform: butyl alcohol, 4:1 v/v) [25] was then used to remove proteins from the ROP fraction until there was no precipitate at the junction of the two phases. The solution was then concentrated and precipitated with the addition of ethanol (3 times the volume of aqueous extract) at 4 ℃ for 12 h then centrifuged at 8000 rpm for 20 min. The flocculent precipitates were then dialyzed in double-distilled water for three days followed by lyophilization to a constant weight. The resulting powder was dissolved in 50 ml Tris-HCl buffer (pH 7.4) at 5 mg / ml and filtered through a 0.45 μm membrane (Nucleopore). The solution was then loaded onto a DEAE-Sepharose Fast Flow column (2.6 × 20 cm) pre-equilibrated with Tris-HCl buffer (pH 7.4). After elution with 150 mL Tris-HCl buffer (pH 7.4), stepwise elution with increasing concentrations of sodium chloride solution (0 - 0.6 M) was carried out at a flow rate of 4 mL / min and 8 mL fractions collected using an automatic fraction collector (BSZ-100, Shanghai Huxi Instrument Factory, Shanghai, China). The total polysaccharide content in each fraction was then measured at 490 nm using the phenol-sulfuric acid method. Three large, overlapping elution peaks were subsequently revealed in the elution profiles, and designated ROP-1, ROP-2, and ROP-3,

respectively. Of these, ROP-2 showed the highest yield and was therefore selected for further analysis. It was collected, concentrated, dialyzed against distilled water then further purified using a Sepharose CL-6B column eluted with 0.15 M NaCl. A single elution peak was subsequently generated, and the responsible fraction dialyzed against distilled water and freeze-dried for further analysis.

2.4 Characterization

2.4.1 Homogeneity and molecular weight

The molecular weight of the purified ROP-2 fraction was determined by gel permeation chromatography using Shodex SB-805HQ with an injection volume of the sample (5 mg / mL) of 20 µL. The column was eluted using 0.1 M $NaNO_3$ at a flow rate of 0.8 mL / min The molecular weight was calculated by comparison with a standard curve established using T-series Dextran standards (1, 7, 10, 40, 500, and 1000 KD).

2.4.2 UV spectroscopy

For UV spectroscopy, 1.0 mg ROP-2 / mL distilled water was used. Ultraviolet absorption spectra were recorded within a wavelength range of 190–350 nm using a T6 UV spectrometer (Beijing Puxi General Instrument Co. Ltd., Beijing, China).

2.4.3 FT-IR spectroscopy

Fourier transform infrared (FT-IR) spectroscopy was performed using a Nicolet Nexus FT-IR spectrometer (Thermo Electron, Madison, WI, USA), and absorption spectra recorded at wavelengths of 4000 to 400 cm^{-1}. The ROP-2 was ground with KBr powder and pressed into pellets.

2.4.4 Starch, phenolic substance, and reducing sugar contents

Starch was estimated using the iodine - potassium iodide reaction [26], phenolic substances using the ferric chloride reaction [27], and reducing sugar using the Fehling's test [28].

2.4.5 Monosaccharide composition

To determine the monosaccharide composition, ROP-2 (10 mg) was dissolved in trifluoroacetic acid (TFA, 2 M, 2 mL) then hydrolyzed at 120 °C for 2 h. The resulting hydrolysate was co-concentrated with methanol repeatedly then successively dissolved in distilled water and freeze-dried. The freeze-dried hydrolysate of the above procedure was then dissolved in pyridine (0.3 mL), and after the addition of trimethylchlorosilane (0.4 ml) and hexamethyldisilazane (0.2 ml) shaken vigorously for 30s. Distilled water (1 ml) was then added and the mixture centrifuged for 5 min at 10,000 rpm to remove any white precipitates. The supernatant was then analyzed by gas chromatography – mass spectrometry (GC–MS) using a fused silica capillary column (DB-5MS, 30 m × 0.25 mm × 0.25 μm). The column oven was first cooled to 50 °C for 2 min then the temperature increased to 100 °C at 15 °C / min, 170 °C at 10 °C / min and 300 °C at 3 °C / min, and maintained for 3 min. The temperatures of the injection port, detector, and ion-source were 250, 280, and 200 °C, respectively [29].

2.5 *In vitro* antioxidant activity

2.5.1 Hydroxyl radical scavenging assay

The hydroxyl radical scavenging activity of ROP-2 was measured using the Fenton's reaction. Samples were prepared in a series of concentrations (0.157, 0.313, 0.625, 1.250, 2.500, 5.000 mg / mL) using distilled water. The reaction mixture contained 1 mL polysaccharide solution, 1 mL hydrogen peroxide (1 % v/v), 1 mL orthophenanthroline (6 mM in ethanol), and 1 mL iron sulfate ($FeSO_4$, 2 mM in water), and was incubated at 37 °C for 1 h. Absorbance was then measured at

526 nm. Vitamin C diluted in distilled water was used as a positive control. Hydroxyl radical scavenging activity (%) was then calculated as follows:

$$\text{Hydroxyl radical scavenging activity}(\%) = \left(1 - \frac{A_i - A_j}{A_c}\right) \times 100\%$$

where A_c is the absorbance of the control reaction in which distilled water was used, A_i is the absorbance of the reaction mixture using the polysaccharide sample, and A_j is the absorbance of the control reaction in which orthophenanthroline (in ethanol) was replaced with ethanol [30].

2.5.2 Superoxide radical scavenging assay

The superoxide radical scavenging capacity of ROP-2 was determined using a pyrogallic acid auto-oxidation system as described previously with slight modifications [31]. The ROP-2 sample was prepared in a series of concentrations (0.157, 0.313, 0.625, 1.250, 2.500, 5.000 mg / mL) using distilled water. Tris–HCl buffer (4.5 mL, 0.05 mol / L, pH 8.2) was incubated at 25 °C for 20 min then mixed with 1 mL of sample solution at each concentration and 25 mM pyrogallic acid (0.4 mL). After rapid shaking, the resulting mixture was incubated at 25 °C for 4 min. The reaction was terminated by adding 8M HCl and the absorbance measured at 299 nm. Vitamin C diluted in distilled water was used as a positive control. The superoxide radical scavenging percentage was calculated as follows:

$$\text{Superoxide radical scavenging activity}(\%) = \frac{A_c - A_i}{A_c} \times 100\%$$

where A_c is the absorbance of the control reaction in which deionized water was used and A_i is the absorbance of the reaction mixture containing the polysaccharide sample.

2.5.3 DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay

The ability of ROP-2 to scavenge DPPH free radicals was determined as described previously [32] with slight modifications. The ROP-2 sample was prepared in a series of concentrations (0.157,

0.313, 0.625, 1.250, 2.500, 5.000 mg/mL) using distilled water. Freshly prepared DPPH (2 ml, 0.2 mg / mL in ethanol), 1.5 mL of sample solution, and 0.5 mL of distilled water comprised the reaction mixture. After vigorous shaking, the mixture was incubated at 25 ℃ for 30 min in the dark then the absorbance determined at 517 nm. Vitamin C diluted in distilled water was used as a positive control. DPPH scavenging ability (%) was then calculated as follows:

$$\text{DPPH scavenging activity}(\%) = \left(1 - \frac{A_i - A_j}{A_c}\right) \times 100\%$$

where A_c is the absorbance of the control reaction in which deionized water was used A_i is the absorbance of the reaction mixture containing the polysaccharide sample, and A_j is the absorbance of the control reaction in which the DPPH (in ethanol) was replaced by ethanol.

2.6 Statistical analysis

All experiments were carried a minimum of three times. Data were analyzed using SPSS software (Version 20, SPSS Inc., IL, USA). Duncan's test was used to determine the statistical significance between groups. All values are expressed as the mean ± SD with $p < 0.05$ used to determine statistical significance.

3. Results and Discussion

3.1 Single factor experimental analysis

3.1.1 Effect of extraction temperature on ROP yield

Fig. 1A shows the effect of extraction temperature on the yield of ROP. As previously reported, extraction temperature directly affects the yield of polysaccharides [33]. Here, a significant increase in extraction yield was observed with increasing extraction temperature from 40 - 60 ℃ ($p < 0.05$) with a decrease thereafter. Increasing extraction temperature reduces the viscosity of the solvent and increases molecular movement, accelerating the mass transfer of intracellular

substances [34]. However, excessively high temperatures can also cause degradation of certain thermo-sensitive materials, resulting in a decrease in yield [35]. A temperature range of 55 – 65 ℃ was therefore selected as the optimal extraction temperature.

3.1.2 Effect of extraction time on ROP yield

Fig. 1B shows the effect of extraction time on the extraction yield of ROP. Yield increased with increasing extraction time, reaching a maximum at 25 min and decreasing slightly thereafter, albeit it in an insignificant manner. This slight decrease was possibly due to overexposure of ROP to the release medium, the liquid dissolving ROP and then ROP diffusing out from the raw materials [36]. These findings suggest that if the extraction time is too long degradation of the polysaccharides occur [37]. An optimal extraction time of 20 - 30 min was therefore selected.

3.1.3 Effect of ultrasonic power on ROP yield

Fig. 1C shows the effect of ultrasonic power on the extraction yield of ROP. Yield increased significantly with increasing power from 360 - 540 W decreasing slightly thereafter. This slight decrease was possibly due to acceleration in mass transfer of intracellular substances. Thus, as with the previous two variables, if the ultrasonic power is too high degradation of the polysaccharides can occur. An optimal ultrasonic power of 360 - 540W was therefore selected.

3.2 Model fitting and optimization of ROP extraction

3.2.1 Model fitting

Table 2 shows the design matrix and the results of analysis of adequacy, variance and fitness of the model obtained. Using the following second-order polynomial equation, multiple regression analysis of the experimental data was then used to correlate the test variables and response variable (extraction yield of ROP). In this statistical analysis section, ANOVA was carried out:

$Y = 10.21 - 0.029A + 0.19B - 0.18C - 0.25AB - 0.13AC - 0.16BC - 0.44A^2 + 0.086B^2 - 0.93C^2$

where Y represents yield, and A, B and C represent the extraction temperature, extraction time, and ultrasonic power, respectively.

Analysis of variance of the quadratic regression model (Table 2) revealed a determination coefficient (R^2) and adjusted determination coefficient (R^2_{Adj}) of 0.9497 and 0.8851, respectively. Thus, the total variance was explained by the model and the model was highly significant. In line with this, the P-value of the model was 0.0009, suggesting extremely significant fitness ($p < 0.001$), while the lack-of-fit value was 0.0776; in other words, insignificant ($p > 0.05$). The model was therefore proven to be adequate for prediction with the range of experimental variables determined here. As a result, the linear coefficients (B and C), cross coefficient (AB), and quadratic coefficients (A^2 and C^2) were all found to significantly affect yield of ROP ($p < 0.05$) using the coefficient estimates for optimization.

3.2.2 Optimization of ROP extraction

Fig. 2 shows the three-dimensional (3D) response surface of the model, giving an understanding of the interactions between test variables and confirming the optimal conditions for maximum yield [35]. As shown, the optimal test variables providing a ROP extraction yield of 10.56 % were: extraction temperature: 58.5 °C, extraction time: 30 min, and ultrasonic power: 525.6 W. However, considering actual production operation, modification of the optimal conditions was subsequently carried out as follows: extraction temperature: 59 °C, extraction time: 30min, and ultrasonic power: 522 W. A verification experiment was subsequently carried out under these optimal conditions to verify the rationality of the model equations. As a result, an experimental extraction yield of 10.35 ± 0.11 % (n = 3) was obtained, in strong agreement with the predicted value. This

finding suggests that the regression model was accurate and can be used to predict ROP extraction yield. The polysaccharide and protein contents of ROP were subsequently determined as 47.92± 1.27 % and 2.13 ± 0.09 %, respectively.

3.3. ROP separation and purification

After determining the optimal extraction conditions, the ROP sample was purified using a DEAE-Sepharose Fast Flow cellulose anion-exchange column and a Sepharose CL-6B column, resulting in three large elution peaks (Fig. 3A). The peaks were detected using the phenol-sulfuric acid method and subsequently named ROP-1, -2, and -3 accordingly. The resulting fractions were collected, concentrated, dialyzed and lyophilized. As a result, yields of ROP-1 and ROP-3 were found to be too low for further analysis; therefore, the ROP-2 fraction was collected for identification of physicochemical properties and antioxidant activity.

3.4 Characterization of ROP-2

3.4.1 Physicochemical properties of ROP-2

Fig. 3B shows the homogeneity and molecular weight of ROP-2 as determined by high-performance liquid chromatography (HPLC). ROP-2 had a single symmetrical sharp peak, indicating homogeneity. The regression equation was LogMw = -1.2827X + 20.136 with a correlation coefficient (R^2) of 0.9989. The average molecular weight was represented by Mw, and the elution volume was represented by X. According to the equation, the average molecular weight of ROP-2 was calculated as 1.92 × 105 Da. A maximum absorption peak around 200 nm was displayed in the UV–Vis spectrum of ROP-2, confirming that it is a polysaccharide. Moreover, there was no absorption peak between 250 and 350 nm, suggesting no protein or nucleic acid [38, 39].

Fig. 3C shows the FT-IR spectrum of the ROP-2 sample. An intense broad absorption peak around 3340 cm^{-1} was attributed to O-H stretching vibration, which is common to all polysaccharides [40], while the weak band near 2922 cm^{-1} was assigned to C-H asymmetric stretching vibration [41]. Both absorption bands are characteristic of polysaccharides [42]. There was also a characteristic weak peak at 1700–1750 cm^{-1}, suggesting the existence of uronic acids [43], while signals at 1373 and 1152-995 cm^{-1} represent araboxylan [44, 45]. The bands at approximately 890cm^{-1} suggest a β anomeric configuration [46]. Thus, ROP-2 does not contain proteins, nucleic acids, starch, phenolic substances, raw sugar, or reducing sugar.

3.4.2 Monosaccharide composition

Fig. 4 shows the monosaccharide composition of ROP-2. Peaks were identified by comparing retention times to those of monosaccharide standards. The results show that ROP-2 is composed of glucose, mannose, galactose, arabinose, xylose, fructose, and rhamnose with molar percentages of 28.8, 12.5, 13.1, 15.9, 9.2, 13.7, and 6.8c%, respectively.

3.5 Antioxidant activity of ROP-2

3.5.1 Hydroxyl radical scavenging activity

Hydroxyl radicals are considered the most harmful of all reactive oxygen species, since they can damage human biomolecules such as proteins, lipids, carbohydrates, and DNA [47, 48]. The removal of hydroxyl radicals is therefore thought to help protect the body against oxidization-related injury. As shown in Fig. 5A, the hydroxyl radical scavenging activity of ROP-2 was concentration dependent. At 5 mg / mL, the scavenging activity was 19.38 % with an IC_{50} of 11.99 mg / mL, Compared to the activity of ascorbic acid (100 %), these results suggest that ROP-2 has only a weak hydroxyl radical scavenging ability.

3.5.2 Superoxide radical scavenging activity

The superoxide anion is a weak free radical, generated by the mitochondrial electron transport system. However, importantly, it can create other strong free radicals that increase the risk of various types of disease [49-51]. As shown in Fig. 5B, the superoxide radical scavenging activity of ROP-2 was also concentration dependent. At 5 mg / mL, the scavenging rate was 61.18 % with an IC_{50} of 3.12 mg / mL. Compared with the scavenging rate of ascorbic acid (100 %), the superoxide radical scavenging ability of ROP-2 is relatively low.

3.5.3 DPPH radical scavenging activity

Although DPPH is a relatively stable free radical, antioxidants can be used to reduce levels by providing electrons or hydrogen atoms. The DPPH radical can therefore be used to evaluate antioxidant free radical scavenging activity [52, 53]. As shown in Fig. 5C, the DPPH radical scavenging activity of ROP-2 was also concentration dependent. At 5 mg / mL, the DPPH radical scavenging activity was 79.82 % with an IC_{50} value of 1.87 mg / mL. Compared with ascorbic acid (100%) these findings suggest that ROP-2 can provide hydrogen atoms or for DPPH radical scavenging.

4. Conclusions

Extraction methods have a significant impact on the quantity of the extract obtained. Here, ultrasound-assisted extraction was successfully applied to extraction of the oligosaccharide ROP from okra. Under optimal extraction conditions (extraction temperature: 59 °C, extraction time: 30 min, and ultrasonic power: 522W), the extraction yield of ROP was 10.35 ± 0.11 %, which strongly agreed with the predicted value. ROP was further purified using a DEAE-Sepharose Fast Flow column and a Sepharose CL-6B column, indicating three separate fractions. Of these, ROP-2

showed the highest yield, with an average molecular weight of 1.92×10^5 Da, and composed of glucose, mannose, galactose, arabinose, xylose, fructose, and rhamnose at molar percentages of 28.8, 12.5, 13.1, 15.9, 9.2, 13.7, and 6.8 %, respectively. The UV and FT-IR spectrum of ROP-2 were also evaluated, and the strong scavenging activities against superoxide radicals and DPPH and weak scavenging activity against hydroxyl radicals revealed. Overall, these findings suggest that ultrasound-assisted extraction technology can be successfully applied to polysaccharide extraction from okra. The obtained polysaccharide (ROP-2) can now be examined for its potential as a functional food ingredient. Accordingly, further experiments on the relationship between structure and bioactivity are now under way.

Acknowledgements

We would like to thank the native English speaking scientists of Elixigen Company (Huntington Beach, California) for editing our manuscript. This work was supported by the Special Fund for Agro-scientific Research in the Public Interest [grant number 201503142].

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Table 1

Box-Behnken experimental design and results of each set of variables on ROP extraction yield.

NO.	Variable			Yield (%)
	A: extraction temp. (°C)	B: extraction time (min)	C: ultrasonic power (W)	
1	-1(55)	1(30)	0(540)	10.13
2	0(60)	0(25)	0	10.24
3	1(65)	-1(20)	0	10.09
4	1	0	1(630)	8.28
5	0	0	0	10.31
6	-1	-1	0	9.37
7	1	1	0	9.83
8	-1	0	-1(450)	9.13
9	0	-1	-1	9.03
10	-1	0	1	8.87
11	1	0	-1	9.06
12	0	0	0	10.32
13	0	1	-1	9.88
14	0	-1	1	9.17
15	0	1	1	9.36
16	0	0	0	10.01
17	0	0	0	10.18

Table 2

ANOVA of the response surface quadratic model of ROP yield.

Source	SS[a]	df[b]	MS[c]	F-value	P-value	Significance
Model	5.67	9	0.63	14.69	0.0009	**
A	$6.962E^{-003}$	1	6.962E-003	0.16	0.6991	
B	0.30	1	0.30	6.91	0.0340	*
C	0.25	1	0.25	5.86	0.0461	*
AB	0.26	1	0.26	5.97	0.0446	*
AC	0.070	1	0.070	1.62	0.2432	
BC	0.11	1	0.11	2.51	0.1574	
A^2	0.82	1	0.82	19.21	0.0032	*
B^2	0.031	1	0.031	0.72	0.4228	
C^2	3.67	1	3.67	85.41	< 0.0001	**
Residual	0.30	7	0.043			
Lack of fit	0.24	3	0.079	4.97	0.0776	
Pure error	0.064	4	0.016			
Correlation	5.98	16				
R^2	0.9497		R^2_{Adj}	0.8851		

a Sum of Squares; b Degree of Freedom; c Mean Square; * Means significant ($p < 0.05$; ** extremely significant ($p < 0.01$)

Figure legends

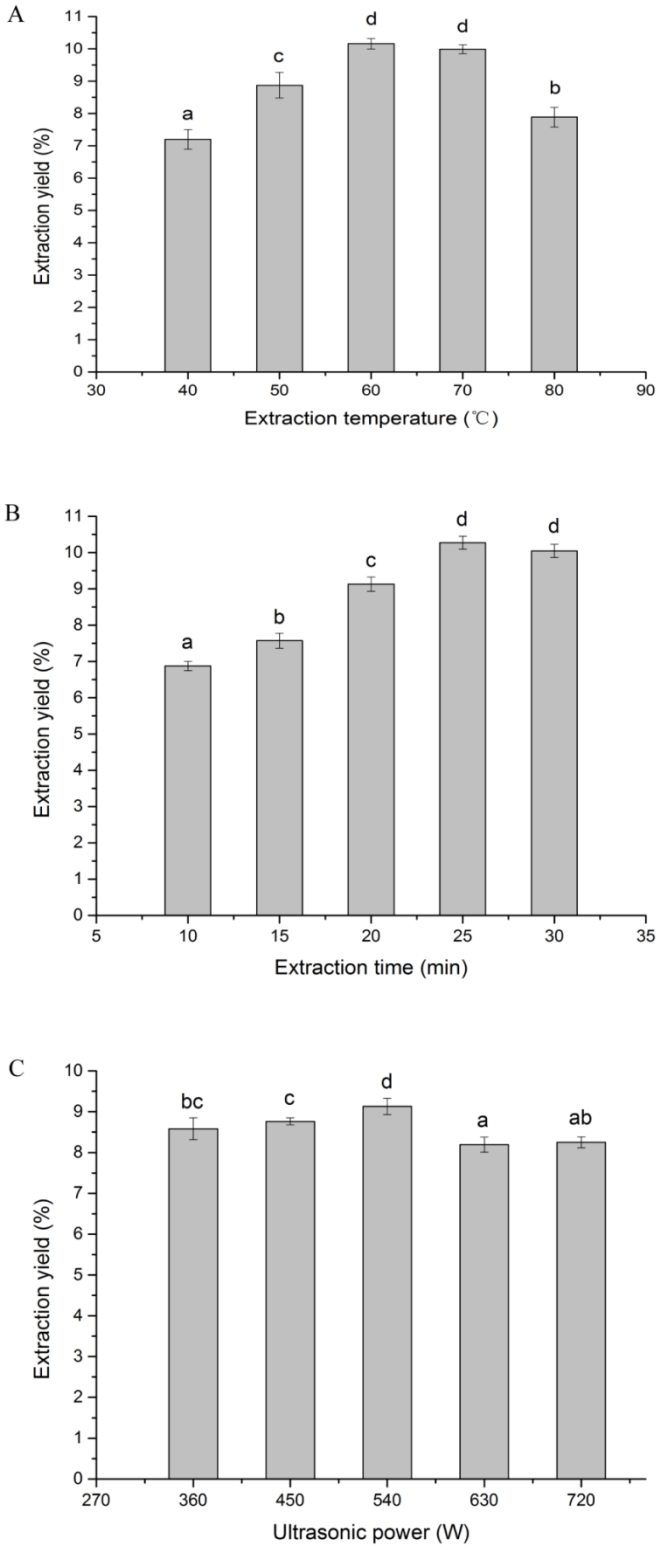

Fig. 1. Effect of extraction temperature (A), extraction time (B), and ultrasonic power (C) om ROP extraction yield according to single-factor experiments.

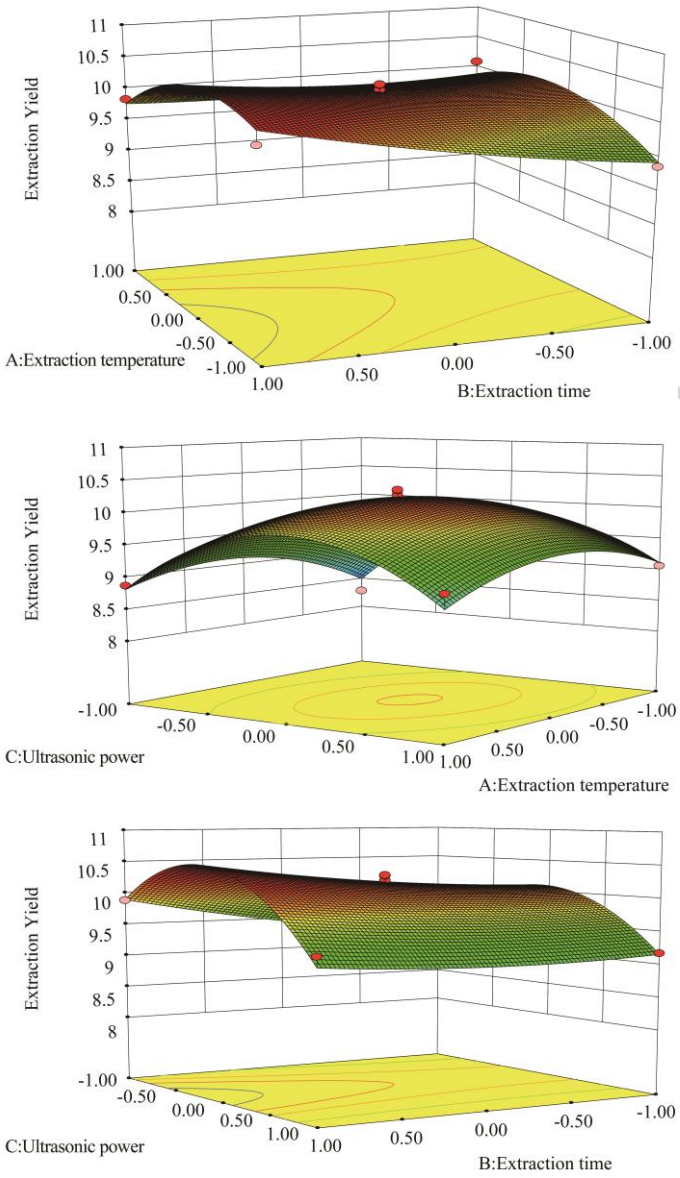

Fig. 2. Response surface plots showing the individual and mutual effects of extraction temperature (A), extraction time (B), and ultrasonic power (C) on the extraction yield of ROP.

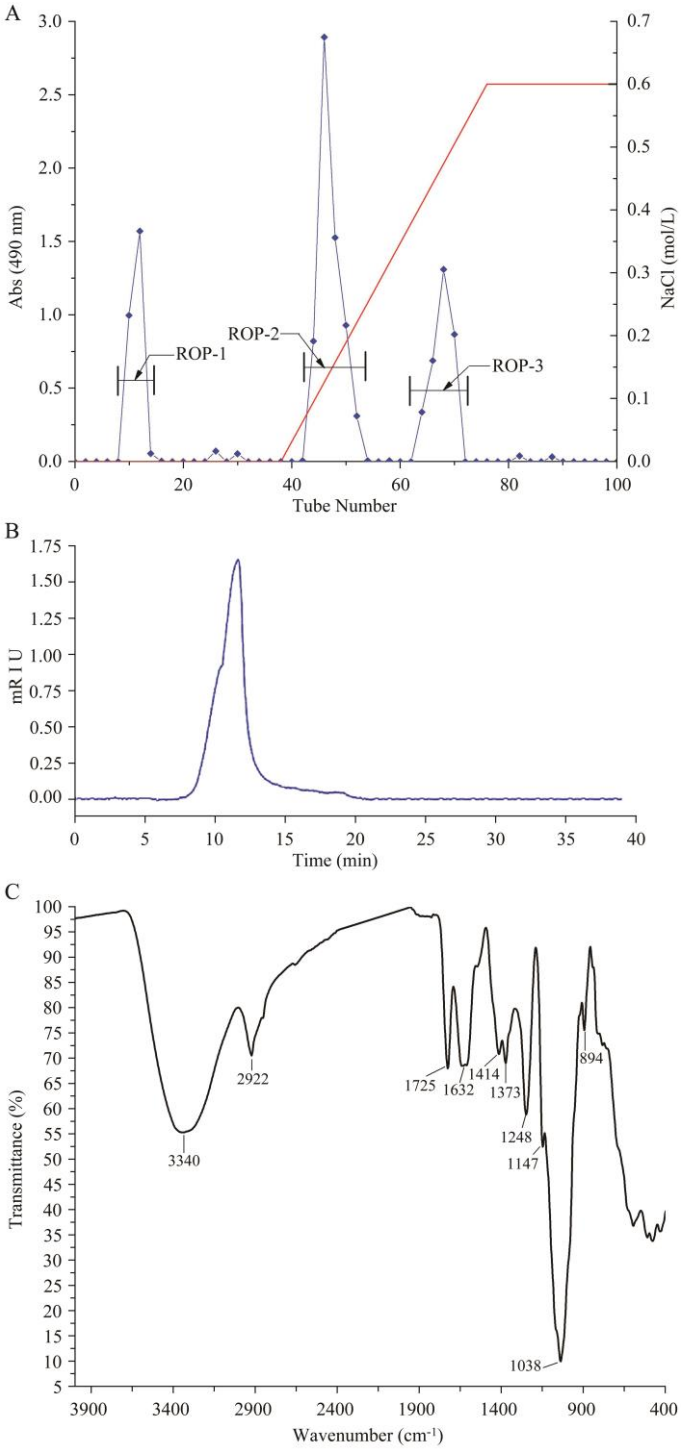

Fig. 3. (A) DEAE-Sepharose Fast Flow cellulose anion-exchange column elution profile of ROP. (B) HPSEC elution curve of ROP-2. (C) FT-IR spectrum of ROP-2.

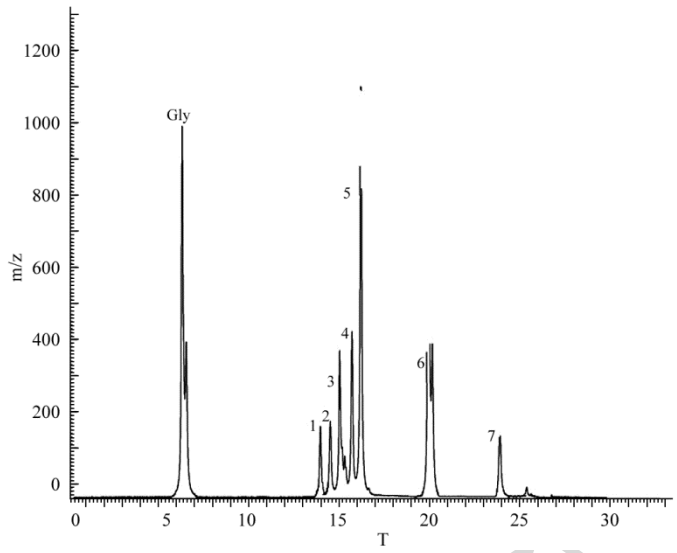

Fig. 4. Monosaccharide composition of ROP-2 1: glucose, 2: mannose, 3: galactose, 4: arabinose, 5: xylose, 6: fructose, and 7: rhamnose)

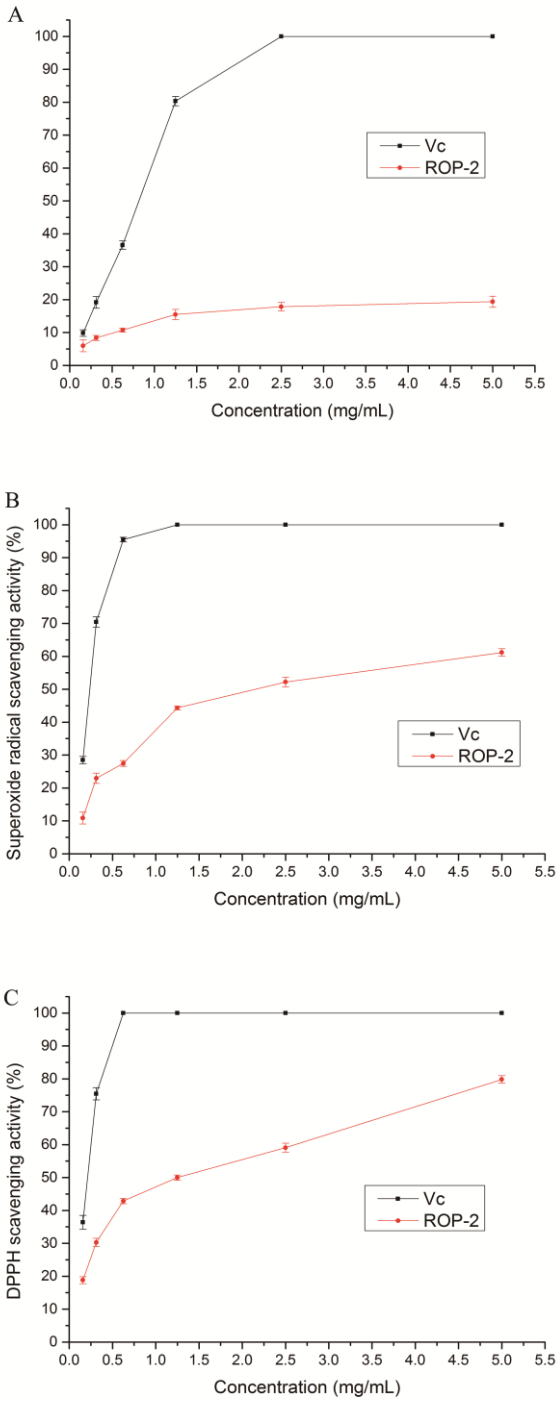

Fig. 5. (A) Hydroxyl radical scavenging activity, (B) superoxide radical scavenging activity, and (C) DPPH scavenging activity of ROP-2.