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A comparison study on microwave-assisted extraction of *Potentilla anserina* L. polysaccharides with conventional method: Molecule weight and antioxidant activities evaluation

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ABSTRACT

The conventional extraction methods for polysaccharides were time-consuming, laborious and energy-consuming. Microwave-assisted extraction (MAE) technique was employed for the extraction of *Potentilla anserina* polysaccharides (PAP_{MAE}), which is a traditional Chinese functional food. The extracting parameters of PAP_{MAE} were optimized by Box–Behnken design. The optimum conditions were ratio of water to raw material 14.5:1, microwave power 369 W, extraction temperature 63.3 °C and extraction time 76.8 min with an enhanced yield of 13.33%. In microwave heating process, a sharp decrease in M_w was detected in SEC-LLS measurement. A d_f value of 3.03 indicated that PAP_{MAE} exhibited a compact hard sphere conformation in an aqueous solution. Furthermore, it showed stronger antioxidant activities compared with hot water extraction by evaluating in superoxide radical ($\cdot O_2^-$), hydroxyl radical ($\cdot OH$), 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical, reducing power and metal chelating assay. The data obtained clearly showed that the molecular weights played a more important role in antioxidant activities.

1. Introduction

Hot water extraction (HWE) is the most common method used for the extraction of polysaccharides. Many papers aimed at investigating the influence of extraction parameters, such as particle size, ratio of solvent to raw material, extraction time, extraction temperature, pH value and number of extraction (Hou & Chen, 2008; Liang, 2008; Wang, Luo, & Cai, 2007; Wu, Cui, Tang, & Gu, 2007; Yin & Dang, 2008). It should be noted that HWE of polysaccharides is associated with long extraction time and high temperature. Recently, alternative extraction techniques such as ultrasonic assisted extraction (UAE) and membrane separation technology with lower temperature and enhanced yields had been also reported (Li, Ding, & Ding, 2007; Ye, Wang, Zhou, Liu, & Zeng, 2008).

Many reports have been published on the extraction of secondary metabolite from plants using microwave-assisted extraction (MAE) (Keyson et al., 2007). Microwaves are composed of electric and magnetic fields and thus represent electromagnetic energy. The spectral frequency of microwave ranges from 300 to 300,000 MHz (Mahesar et al., 2008). Microwave energy acts as a nonionizing radiation that causes rotation of the dipoles, but does not affect molecular structure. The highly localized temperature of MAE can cause selective migration of the target compounds from the material to the surroundings at a more rapid rate and with similar or better recoveries compared to the conventional extraction, with the main advantages of reducing both extraction time and solvent consumption. However, Tao and Xu reported that degradation occurred in the structure of polysaccharides during the microwave heating process (Tao & Xu, 2008). Marshall et al. also studied the possibility of minimize the disassembly of polysaccharides during MAE (Marshall, Chau, Cooke, Yadav, & Hotchkiss, 2009). Therefore, it is necessary to optimize the extraction conditions during MAE.

Oxidation is essential to many organisms for the production of energy in biological processes (Xu et al., 2009). Oxidative stress, induced by oxygen radicals, is believed to be a primary factor in various diseases such as cancer, rheumatoid arthritis and atherosclerosis as well as in degenerative processes of aging. Reactive oxygen species (ROS), in the forms of superoxide anion ($\cdot O_2^-$), hydroxyl radical ($\cdot OH$) and hydrogen peroxide (H_2O_2) are generated by normal metabolic processes or from exogenous factors and agents that can easily initiate the peroxidation of membrane lipids, leading to the accumulation of lipid peroxides (Finkel & Holbrook, 2000). ROS can cause damage to a wide range of essential

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biomolecules, such as DNA, and they have been associated with carcinogenesis, coronary heart disease and many other health problems related to advancing age (Zou et al., 2008). Polysaccharides have exhibited strong antioxidant properties and can be explored as novel potential antioxidants (Tseng, Yang, & Mau, 2008).

Potentilla anserina L. is distributed widely in the western areas of China, especially in Qinghai-Tibetan Plateau. For thousands of years, it has been used frequently as a crude substance, taken orally as a functional food and folk medicine. It is documented in the Chinese Tibetan Material Medical that the rhizomes of *P. anserina* contain carbohydrates, protein, fat, tannin, flavonoids, lysine, histidine, etc. (Chen, Zhang, & Huang, 2000). It is reported that triterpenoid saponin extracted from rhizomes of *Potentilla anserina* exhibited antihepatitis B virus (HBV) activities in vivo (Zhao, Cai, Hong, Shan, & Xiao, 2008).

To the best of our knowledge, the investigation of the microwave effects on polysaccharides structure and the corresponding antioxidant activities is rather limited. In the present study, Box–Behnken design (BBD) was employed to optimize the process parameters (ratio of water to raw material, microwave power, extraction temperature and extraction time) of crude polysaccharides from *P. anserina* by MAE (PAP_{MAE}). Furthermore, we attempted to study the solution properties of PAP_{MAE} and polysaccharides from *P. anserina* using HWE (PAP_{HWE}) in an aqueous solution using SEC combined with laser light scattering (SEC-LLS). The antioxidant properties of both PAP_{MAE} and PAP_{HWE} were evaluated and compared for the purpose of assessing the efficacy of traditional Chinese herbs obtained by different extraction methods. Antioxidant properties were assayed in terms of antioxidant activities in vitro, by testing the scavenging abilities on superoxide radicals, hydroxyl radicals, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), reducing power and chelating ability on ferrous ions.

2. Materials and methods

2.1. MAE

MAE was carried out using microwave experiment equipment (NJC 03-2, 2450 MHz, Nanjing, China) with adjustable power settings ranging from 100 to 800 W. It was equipped with one 3000 ml closed polytetrafluoroethylene (PTFE) vessel, a power sensor, a temperature sensor, a temperature controller and cooling system. The rhizomes of *P. anserina* were ground and put into PTFE extraction vessel extracted under different MAE conditions. The ranges of the variables studied are listed in Table 1. After extraction, the vessel was allowed to cool at room temperature, filtered and freeze-dried to obtain crude PAP_{MAE}.

HWE was conducted in a water bath at 90 °C. An amount of 50.0 g rhizomes of *P. anserina* was placed into a 2000 ml glass flask with 1500 ml distilled water and was extracted for 180 min, filtered and freeze-dried to obtain crude PAP_{HWE}.

2.2. Box–Behnken design (BBD)

BBD was employed to statistically optimize the formulation parameters and evaluate main effects, interaction effects and quadratic effects of the formulation ingredients on the yields of polysaccharide. According to the principle of BBD, ratio of water to raw material, microwave power, extraction temperature and extraction time, which were identified to have strong effects on the yields were taken as the variables tested in a 29-run experiment. As shown in Table 1, the four factors chosen for this study were designated as X_1, X_2, X_3, and X_4 and were prescribed into three levels, coded +1, 0, and −1 for high, intermediate and low value, respectively. Test variables were coded according to the following equation:

$$X_i = (X_i - X_0)/\Delta X \quad (1)$$

where x_i is the coded value of an independent variable; X_i is the actual value of an independent variable; X_0 is the actual value of an independent variable at centre point; and ΔX is the step change value of an independent variable. All experiments were performed in triplicate and the averages of polysaccharide yield were taken as response. For predicting the optimal point, a second-order polynomial model was fitted to correlate relationship between independent variables and response (polysaccharide yield). For the three factors, the equation was

$$Y = A_0 + \sum_{i=1}^{4} A_i X_i + \sum_{i=1}^{4} A_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} A_{ij} X_i X_j \quad (2)$$

where Y is the response variables (yields of polysaccharides in real values). The percentage of polysaccharides yield was calculated as the polysaccharides content of extraction divided by dried sample weight. A_0, A_i, A_{ii}, and A_{ij} are the regression coefficients of variables for intercept, linear, quadratic and interaction terms, respectively. X_i and X_j are independent variables ($i \neq j$).

Analysis of the experimental design and data were carried out using SAS software (Version 8.0). ANOVA was performed, and the fitness of the polynomial model equation was expressed by the coefficient of determination R^2. Its statistical significance was checked by F-test at a probability (P) of 0.001, 0.01 or 0.05. The significances of the regression coefficients were also tested by F-test.

Table 1
Levels and code of variable chosen for BBD.

Variables	Symbol		Levels		
	Coded	Uncoded	−1	0	1
Ratio of water to raw material	x_1	X_1	10	15	20
Microwave power (W)	x_2	X_2	200	300	400
Extraction temperature (°C)	x_3	X_3	60	70	80
Extraction time (min)	x_4	X_4	30	60	90

2.3. Purification and components analysis

The protein was removed by the Sevage method, combined with papain, according to the earlier report in our laboratory (Zhang, Huang, Hou, & Wang, 2006). The purified PAP_{MAE} and PAP_{HWE} were obtained by gel permeation chromatography (GPC) on an Ultrahydrogel 500 column (Waters, USA) at a concentration of 1.000 g/l and flow rate of 0.08 ml/min.

Phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) was employed for the measurement of carbohydrate contents of PAP_{MAE} and PAP_{HWE} before and after purified, using glucose as the standard. The protein contents were measured according to Bradford's method, using bovine serum albumin (BSA) as the standard (Chen, Zhang, Qu, & Xie, 2008). Uronic acid contents were determined by measuring the absorbance at 525 nm using the *m*-hydroxybiphenyl colorimetric procedure, with D-glucuronic acid being the standard (Blumenkrantz & Asboe, 1973).

2.4. Molecular weight determination

HPSEC-LLS measurements were carried out on size-exclusion chromatograph combined with multi-angle laser photometer (MALLS, $\lambda = 690$ nm; DAWN EOS, Wyatt Technology Co., USA). Ultrahydrogel™ column (7.8 × 300 mm, Waters, USA) was used as SEC instrument. An optilab refractometer (Dawn, Wyatt Technology Co., USA) was simultaneously connected. The polysaccharides samples were prepared with the desired concentrations, and optical clarification of the samples was achieved by filtrating them into

a scattering cell. The injection volume was 50 μl and the flow rate was 0.5 ml/min. The refractive index increment (dn/dc) value of the sample was determined by using an optilab refractometer at 690 nm (25 °C) to be 0.145 ml/g. The basic light scattering equation was as follows:

$$\frac{K_c}{R_\theta} = \frac{1}{M_w}\left(1 + \frac{16\pi^2\langle S^2\rangle_z}{3\lambda^2}.\sin^2\left(\frac{\theta}{2}\right)\right) + 2A_2C \quad (3)$$

where K is an optical constant equal to $[4\pi^2n^2(dn/dc)^2]/(\lambda^4N_A)$; c, the polysaccharide concentration in mg/ml; R_θ, the Rayleigh ratio; k, the wavelength; n, the refractive index of the solvent; dn/dc, the refractive index increment; N_A, the Avogadro' number; A_2, the second virial coefficient. As the column separated the polymer according to molecular weight, each fraction was led to the light scattering detector for instantaneous measurement of the scattering intensities. The refractive index detector connected in series gave the polymer concentration. In chromatography mode, we had a single and sufficiently low concentration at a particular slice because of the further dilution by the SEC column of the already dilute injected sample.

2.5. Assay for antioxidant activities

2.5.1. Superoxide radical scavenging assay

The superoxide radical scavenging assay was measured according to the method of Qi (Qi et al., 2005). Superoxide radicals were generated in a PMS/NADH system for being assayed in the reduction of NBT. Polysaccharides were dissolved in deionized water at the concentration of 0.02–1 mg/ml. The reaction mixture, containing varying concentrations of samples, Tris–HCl (16 mM, pH 8.0), NADH (338 μm), NBT (72 μm) and PMS (30 μm), was incubated at room temperature for 5 min and the absorbance was read at 560 nm against a blank. The capability to scavenge superoxide radical was calculated using the following equation:

$$\text{Scavenging effect } (\%) = [1 - A/A_0] \times 100\%$$

where A_0 is the absorbance of mixture solution without sample; A is the absorbance of the test sample mixed with reaction solution.

2.5.2. Hydroxyl radical scavenging assay

The hydroxyl radical assay was measured by the method of Ghiselli (Ghiselli, Nardini, Baldi, & Scaccini, 1998) with a minor modification. Polysaccharides were dissolved in deionized water at the concentration of 0.1–10 mg/ml. The sample solution (0.1 ml) was mixed with 0.6 ml of reaction buffer [20 mM phosphate buffer (pH 7.4), 2.67 mM deoxyribose, and 100 μm EDTA], 0.2 ml of 0.4 mM ferrous ammonium sulfate, 0.05 ml of 2.0 mM Vc, and 0.05 ml of 10 mM H_2O_2 was then added to the reaction solution. The reaction solution was incubated for 15 min at 37 °C and then 1 ml of 1% thiobarbituric acid (TBA) and 1 ml of 2% trichloroacetic acid (TCA) were added to terminate the reaction. The mixture was boiled for 15 min and cooled to room temperature. The absorbance of the mixture was measured at 532 nm against blank. The capability to scavenge hydroxyl radical was calculated using the following equation:

$$\text{Scavenging effect } (\%) = [1 - A/A_0] \times 100\%$$

where A_0 is the absorbance of mixture solution without sample; A is the absorbance of the test sample mixed with reaction solution.

2.5.3. Effect of scavenging 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radicals

The free radical-scavenging activity of the polysaccharides was measured by DPPH test according to the method of Shimada with some modifications (Shimada, Fujikawa, Yahara, & Nakamura, 1992). The 0.2 mmol/l solution of DPPH in methanol was prepared daily before UV measurements. One milliliter of the polysaccharides of different addition quantities (0.02–1 mg) in water was thoroughly mixed with 2 ml of freshly prepared DPPH and 2 ml of methanol. The mixture was shaken well, allowed to stand for 30 min in the dark, and the absorbance was then measured at 517 nm against a blank. Lower absorbance of the reaction mixture indicated higher free radical-scavenging activity, which was analyzed from the graph plotted of inhibition percentage against compound concentration. Vc and BHT were used as positive controls. The experiment was carried out in triplicate and averaged. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{Scavenging effect } (\%) = [A_0 - (A - A_b)/A_0] \times 100\%$$

where A_0 is the absorbance of DPPH solution without sample; A is the absorbance of the test sample mixed with DPPH solution and A_b is the absorbance of the sample without DPPH solution.

2.5.4. Reducing power assay

The reducing power was determined according to the method of Qi et al. (2005). Different concentrations of samples (0.1–5 mg/ml, 2.5 ml) were mixed with 2.5 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 ml of potassium ferricyanide (1%). The mixture was incubated for 20 min at 50 °C. The reaction was terminated by TCA solution (10%). Then, the solution was mixed with distilled water and ferric chloride (0.1%), the absorbance was measured at 700 nm against a blank. A higher absorbance indicated a higher reducing power. BHA was used for comparison.

2.5.5. Metal chelating assay

The ferrous ion-chelating ability of polysaccharides extracted by MAE was investigated with slightly modified method of Li (Li, Li, & Zhou, 2007). Samples in different concentrations (0.1–5 mg/ml) were mixed with $FeCl_2$ (0.1 ml, 2 mM) and ferrozine (0.4 ml, 5 mM), shook well, stayed still for 10 min at room temperature. Then the absorbance of the mixture was determined at 562 nm. In the control, the sample was substituted with EDTA. The ferrous ion-chelating activity was given by the following equation:

$$\text{Chelating ability } (\%) = [(A_0 - A)/A] \times 100\%$$

where A_0 is the absorbance of mixture solution without sample; A is the absorbance of the test sample mixed with reaction solution.

2.5.6. Statistical analysis

All the data were shown in means ± SD within significance $P < 0.05$ after subjecting to an analysis of variance (ANOVA) and processed with SPSS 13.0.

3. Results and discussion

3.1. Optimization of extraction conditions by BBD

3.1.1. Extraction model and statistical analysis

Response surface optimization is more advantageous than the traditional single parameter optimization in that it saves time (Yin & Dang, 2008). There were a total of 29 runs for optimizing the four individual parameters in the current BBD which was applied to the production of crude PAP_{MAE}. The values of responses (yield of polysaccharides) at different experimental combination for coded variables are given in Table 2. The percentage yield ranged from 9.39% to 14.68%. By applying multiple regression analysis on the experimental data, the response variable and the test variables were related by the following second-order polynomial equation:

Table 2
BBD and the response values for the yields of crude PAP_{MAE}.

Run	Water/raw material	Microwave power (W)	Temperature (°C)	Time (min)	Yield (%)
1	0	0	0	0	13.68
2	−1	−1	0	0	12.54
3	1	0	0	1	12.18
4	0	−1	1	0	11.72
5	1	0	0	−1	9.85
6	−1	0	1	0	10.76
7	−1	0	−1	0	13.04
8	0	−1	0	1	10.86
9	0	0	−1	1	14.68
10	0	0	0	0	13.76
11	1	1	0	0	12.79
12	−1	0	0	1	11.83
13	0	0	−1	−1	9.91
14	1	−1	0	0	10.24
15	0	1	−1	0	14.28
16	1	0	1	0	10.85
17	0	0	0	0	13.88
18	0	0	1	1	11.31
19	0	0	1	−1	11.57
20	0	0	0	0	14.29
21	1	0	−1	0	12.55
22	0	0	0	0	12.91
23	−1	1	0	0	10.39
24	0	1	0	−1	13.74
25	0	1	0	1	12.73
26	0	1	1	0	9.65
27	0	−1	0	−1	10.63
28	−1	0	0	−1	11.20
29	0	−1	−1	0	9.39

$$Y = 13.7 - 0.1X_1 + 0.69X_2 - 0.67X_3 + 0.56X_4 + 1.15X_1X_2 + 0.14X_1X_3 + 0.42X_1X_4 - 1.74X_2X_3 - 0.31X_2X_4 - 1.26X_3X_4 - 1.19X_1^2 - 1.10X_2^2 - 1.00X_3^2 - 0.90X_4^2 \quad (4)$$

where Y is the polysaccharides yield and X_1, X_2, X_3, and X_4 are the coded values for ratio of water to raw material, microwave power, extraction temperature and extraction time, respectively.

The ANOVA of the quadratic regression model showed that the value of the determination coefficient ($R^2 = 0.9254$). The value of the adjusted determination coefficient (Adj. $R^2 = 0.9037$) was reasonably close to 1, which indicated a high degree of correlation between the observed and predicted values. At the same time, a low value of coefficient of the variation (CV = 6.97%) indicated a high degree of precision and a good deal of reliability of the experimental values.

The P values are used as a tool to check the significance of each coefficient, which in turn may indicate the pattern of the interactions between the variables. The coefficient estimate for the parameter optimization suggested that the independent variables (X_2, X_3, and X_4), quadratic terms (X_1X_1, X_2X_2, X_3X_3, and X_4X_4) and cross product coefficient (X_1X_2, X_2X_3, and X_3X_4) significantly affected the polysaccharides yield ($P < 0.05$). The results of the study showed that the ratio of microwave power was the most significant single parameter which influenced polysaccharides yield followed by extraction temperature and extraction time.

3.1.2. Optimization of the procedure

Eq. (4) allowed the prediction the effects of the four parameters on the polysaccharides yields. Six independent response surface plots and their respective contour plots are shown in Figs. 1 and 2. Two variables within the experimental range were depicted in one 3D surface plots while the two other variables were kept constant at zero level. The shapes of the contour plots, circular or elliptical indicated whether the mutual interactions between the variables were significant or not (Muralidhar, Chirumamila, Marchant, & Nigam, 2001).

In the case of polysaccharides extract, both ratio of water to raw material (X_1) and extraction temperature time (X_3) used had a negative impact on the polysaccharide production. There was a decrease in the yield of polysaccharides with an increase in the ratio of water to raw material and extraction temperature (Figs. 1A and 2A). Meanwhile, increased extraction time (X_4) up to a threshold level led to increased polysaccharides yield as shown

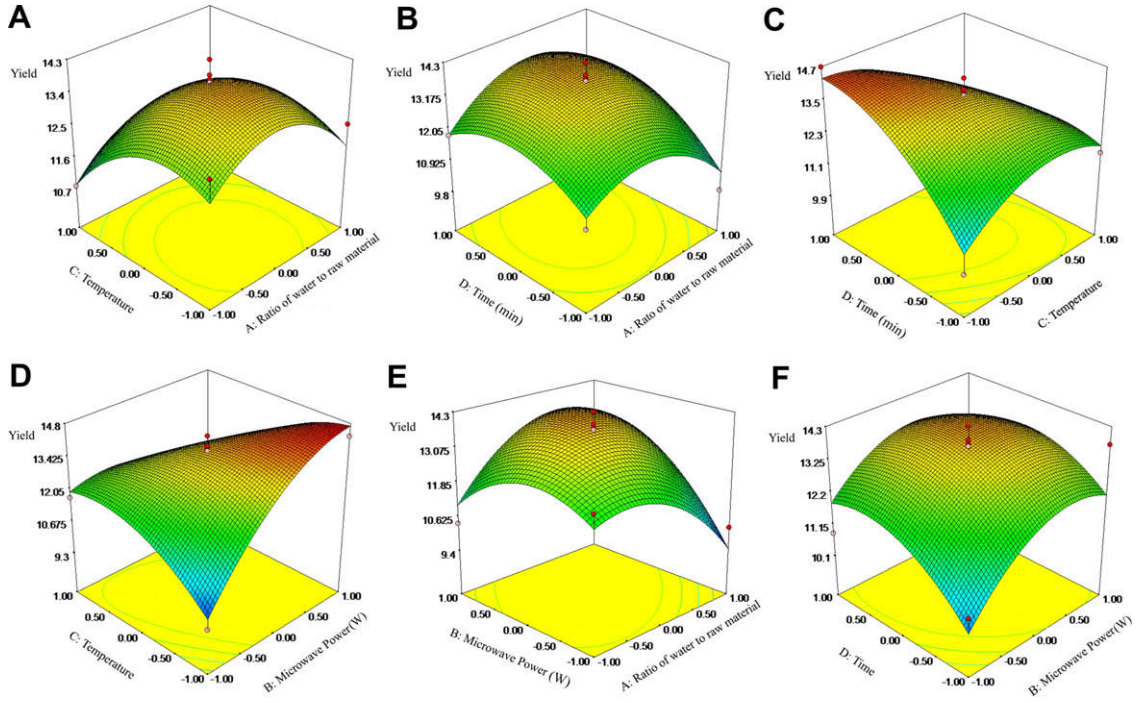

Fig. 1. Response surface plots for the effects of (A) temperature and ratio of water to raw material; (B) time and ratio of water to raw material; (C) time and temperature; (D) microwave power and temperature; (E) microwave power and ratio of water to raw material; and (F) time and microwave power on the yield of polysaccharide.

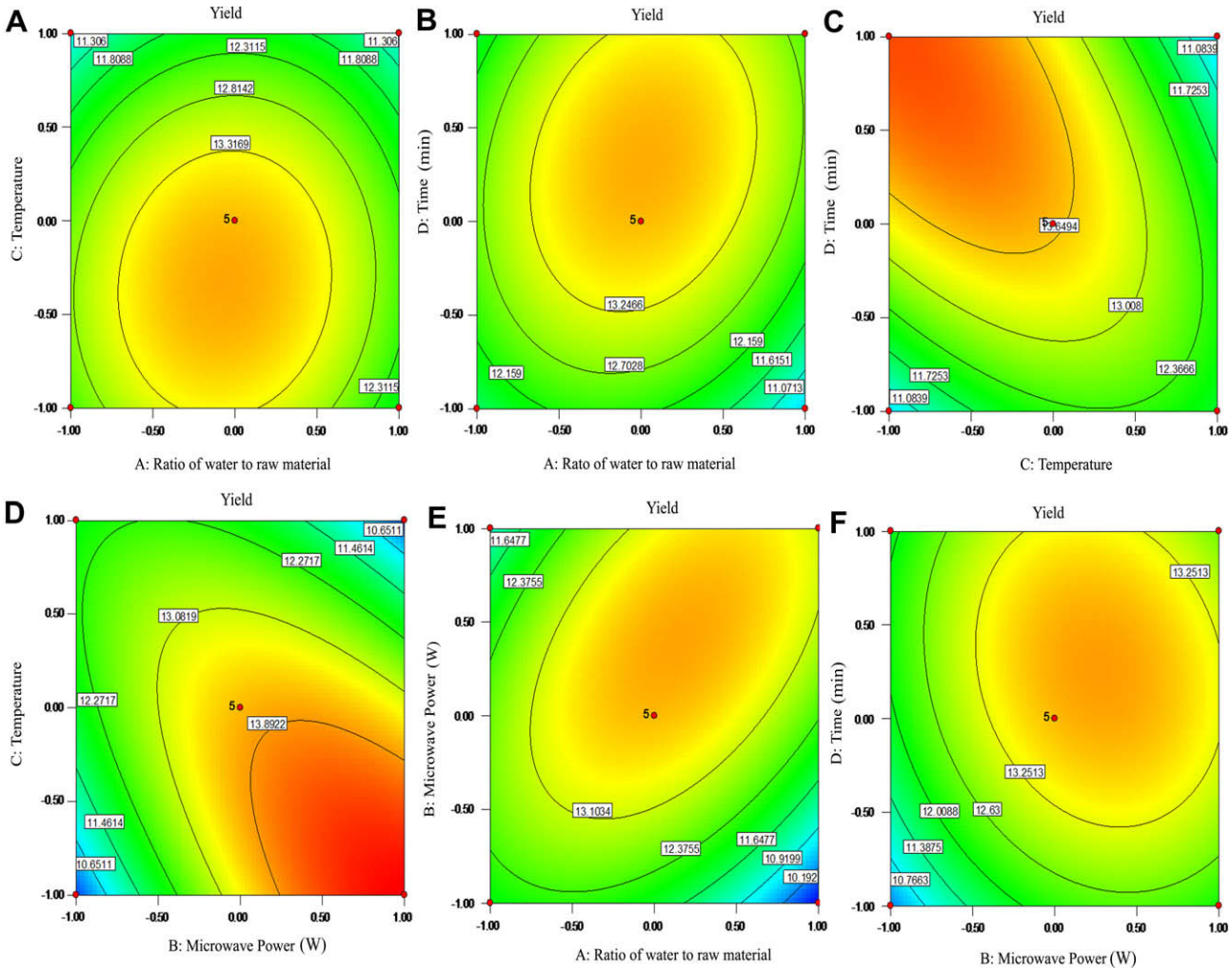

Fig. 2. Contour plots for the effects of (A) temperature and ratio of water to raw material; (B) time and ratio of water to raw material; (C) time and temperature; (D) microwave power and temperature; (E) microwave power and ratio of water to raw material; and (F) time and microwave power on the yield of polysaccharide.

in Figs. 1B and C and 2B and C. Beyond this level, polysaccharides yield slightly decreased.

A great increase in polysaccharides yield resulted when the microwave power (X_2) was increased in the range from 200 to 400 W (Figs. 1D and 2D). At lower temperature (below 70 °C), high yield of polysaccharides was obtained. It indicated that the greater yield could be obtained when the moderate microwave power and a lower level of the extraction temperature were selected. Figs. 1E and F and 2E and F also show that the extraction yield of polysaccharides increased with increase in the extraction time (30–60 min) and microwave power.

It could be concluded that the optimal extraction conditions of PAP_{MAE} were ratio of water to raw material 14.5:1, microwave power 369 W, extraction temperature 63.3 °C and extraction time 76.8 min. Among the four extraction parameters studied, microwave power was the most significant factor to affect yield of polysaccharides, followed by extraction temperature.

3.1.3. Validation of the models

In order to validate the adequacy of the model equations (Eq. (4)), a verification experiment was carried out under the optimal conditions mentioned above. Under the optimal conditions, the model predicted a maximum response of 13.704%. A mean value of 13.332 ± 0.51 (n = 5), obtained from real experiments, demonstrated the validation of the extraction model. The good correlation between these results confirmed that the model was adequate for reflecting the expected optimization.

3.2. Comparison of MAE with classical methods

The efficiency of extraction using MAE was compared with that of classical methods. Clearly, these findings demonstrated that MAE was promising extraction methods that offer improved efficiency by reducing extraction time (Dong, Xie, Wang, Zhan, & Yao, 2009; Hou & Chen, 2008; Li, Ding et al., 2007; Qiao et al., 2009; Yin & Dang, 2008). When considering MAE for 70 min, the results showed that the method was able to give higher yields than HWE at lower temperature. Furthermore, it considerably reduced energy consumption, enhanced the efficiency of the extraction and was environmentally friendly technique. The main mechanism for enhanced yield with MAE was the dipole rotation of the H_2O in the microwave field, which was highly influenced by the solvent dielectric constant and dissipation factor.

3.3. Composition analysis

Protein, carbohydrate and uronic acid contents of PAP_{MAE} and PAP_{HWE} were determined and are given in Table 3. The protein contents decreased after purification and no obvious difference was observed between PAP_{MAE} and PAP_{HWE}. In all the samples, the car-

Table 3
Protein, carbohydrate and uronic acid contents of PAP_{MAE} and PAP_{HWE}.

Samples	Crude		Purified	
	PAP_{MAE}	PAP_{HWE}	PAP_{MAE}	PAP_{HWE}
Protein (%)	10.6	11.4	3.4	4.7
Carbohydrate (%)	76.5	80.8	88.2	93.6
Uronic acid (%)	11.5	10.8	16.3	15.1

Table 4
Molecular characterization of PAP using different extraction methods by HPSEC-LLS.

Sample	Fractions	$M_w \times 10^5$	$M_n \times 10^5$	$M_Z \times 10^5$	M_w/M_n	$\langle S^2\rangle_z^{1/2}$ (nm)
PAP_{HWE}	Peak 1	8.915	3.567	52.37	2.499	32.5
	Peak 1	4.528	1.964	13.43	2.305	25.5
PAP_{MAE}	Peak 2	1.505	1.302	1.739	1.156	52.2
	Peak 3	0.183	1.218	0.448	1.508	54.1

bohydrate contents of PAP_{HWE} were higher than those of PAP_{MAE}. This may be due to the degradation of polysaccharide molecule in MAE.

3.4. Analysis of molecular properties

The characterization of natural polysaccharides having various chemical components, molar mass and chain conformation was important because of their critical effect on end-use structure–property relations (Cui et al., 2008). The molecular weight and chain conformation of the purified PAP_{HWE} and PAP_{MAE} using different extraction methods were determined by SEC-LLS. The weight average molar mass, M_w, polydispersity (PD, M_w/M_n) and z-average radius of gyration ($\langle S^2\rangle_z^{1/2}$) are shown in Table 4 for the two samples. The SEC-LLS chromatograms patterns of PAP_{HWE} and PAP_{MAE} are shown in Fig. 3A and B. The chromatograms of PAP_{HWE} exhibited a single peak indicating that there was no aggregation and the homogeneity of the purified sample. The radius of gyration, M_w, and PD for PAP_{HWE} were measured to be 32.5 nm, 8.915×10^5, and 2.499, respectively.

It was reported that the degradation of polysaccharide occurred during the microwave heating process (Tao & Xu, 2008). This was in accordance with our results that PAP_{MAE} showed three peaks on HPSEC. The M_w for each peak was determined to be 4.528×10^5, 1.505×10^5 and 0.183×10^5, respectively. Compared to PAP_{HWE}, PAP_{MAE} showed a sharp decrease in M_w. The M_w against molar mass differential and integral distribution curves also indicated that PAP_{MAE} contained three components (Fig. 3C and D). According to Galema, microwave heating resulted in polarization of polar bonds (such as the C–O–C glycosidic linkages) and increase in molecules reactivity. Therefore, microwave heating might cause a hydrolytic cleavage of polysaccharide chains as well as breaking intermolecular hydrogen bonds (Tao & Xu, 2008).

Relevant structural information and further insight into the nature of the polysaccharide can be obtained by investigating the fractal dimension (d_f) (Tao, Zhang, & Peter, 2006). The d_f value could be determined from the M_w dependence of $\langle S^2\rangle_z^{1/2}$ and was defined as the inverse of the exponent v:

$$\langle S^2\rangle_z^{1/2} = fM^v \tag{5}$$

$$d_f = 1/v \tag{6}$$

The molecular property of natural polysaccharide was an important parameter influencing bioactivity. On the theory of polymer solutions, the value of d_f was 1 for a rigid rod and linear polymers with Gaussian coil nature had d_f value ranging from 5/3 to 2. A three-dimensional object with a homogeneous density had a mass fractal dimension of 3. The d_f value of monodisperse polymers could be extracted directly from the angular dependence of the scattered light or neutron intensity. This approach had been successfully applied to highly branched polysaccharides with high molecular size such as amylopectin and other synthetic branched polymers (Tao & Xu, 2008).

Fig. 3E shows the plot of M_w versus $\langle S^2\rangle_z^{1/2}$ of PAP_{HWE} and PAP_{MAE}. The resulting relation was expressed as

$$\langle S^2\rangle_z^{1/2} = 0.866M_w^{0.11\pm0.055} \tag{7}$$

$$\langle S^2\rangle_z^{1/2} = -0.087M_w^{0.33\pm0.008} \tag{8}$$

A decrease in the d_f value was observed in MAE. Based on the results, it also showed that degradation occurred in the microwave heating process. The mechanism of degradation was regarded as a hydrolytic cleavage of polysaccharide chains as well as breaking intermolecular hydrogen bonds. In this case, the control of extraction conditions became an essential step in the MAE. This was in accordance with BBD results, longer time and higher microwave power caused a negative effect on yields during MAE.

3.5. Antioxidant activity analysis

3.5.1. Scavenging activity of superoxide radical

The scavenging ability of superoxide anion radicals is extremely important to anti-oxidation work. It is known to indirectly initiate lipid peroxidation as a result of H_2O_2 formation, creating precursors of hydroxyl radicals. In the PMS/NADH-NBT system, superoxide anion derived from dissolved oxygen by the PMS/NADH coupling reaction reduces NBT (Xu et al., 2009).

Fig. 4A shows the inhibitory effect of PAP_{MAE}, PAP_{HWE} and Vc. The results indicated a concentration-dependent radical-scavenging activity at all tested concentrations of the samples. At the amount between 20 and 100 μg/ml, PAP_{MAE} was found to have more scavenging activity than Vc. However, the scavenging effect was 53.92% and 93.47% for PAP_{HWE} and Vc at the concentration of 1 mg/ml, respectively. The scavenging effect was 20.2–40.67% for PAP_{HWE} at the test concentration range. These results indicated that PAP_{MAE} had a stronger superoxide radical-scavenging activity than PAP_{HWE}, which could bear comparison with that of Vc at a relatively lower amount. It was reported that the scavenging activity of polysaccharides from *Aloe barbadensis* against superoxide radicals was less than 40% at the concentration of 1.0 mg/ml (Liu, Wang, Xu, & Wang, 2007). At the amount between 0.25 mg/ml and 2 mg/ml, the effects of *Dioscorea nipponica* polysaccharides on scavenging superoxide radicals were 27.5–35.52% (Luo, 2008). Compared to these results, PAP_{MAE} had a stronger scavenging activity for superoxide radicals. Our data on the activity of scavenging superoxide radicals of PAP_{MAE} suggested that it was likely to contribute towards the observed antioxidant effect.

3.5.2. Scavenging activity of hydroxyl radical

Hydroxyl radicals, generated by the reaction of iron–EDTA complex with H_2O_2 in the presence of Vc, attack deoxyribose to form products that, upon heating with 2-thiobarbituric acid under acid conditions, yield a pink tint. Added hydroxyl radical scavengers compete with deoxyribose for the resulted hydroxyl radicals and diminish tint formation (Qi et al., 2005). The scavenging effect of PAP_{MAE}, PAP_{HWE} and Vc is shown in Fig. 4B. Vc exhibited the strongest scavenging activity against hydroxyl radicals compared with polysaccharide samples. The scavenging effect of PAP_{MAE} and PAP_{HWE} increased with increasing sample concentration. At the concentration of 0.1–10 mg/ml, the scavenging effects were 4.41–50.1% and 5.62–43.7% for PAP_{MAE} and PAP_{HWE}, respectively. Nevertheless, the scavenging effect of both samples was less effective than that of Vc.Previous studies had reported two types of antiox-

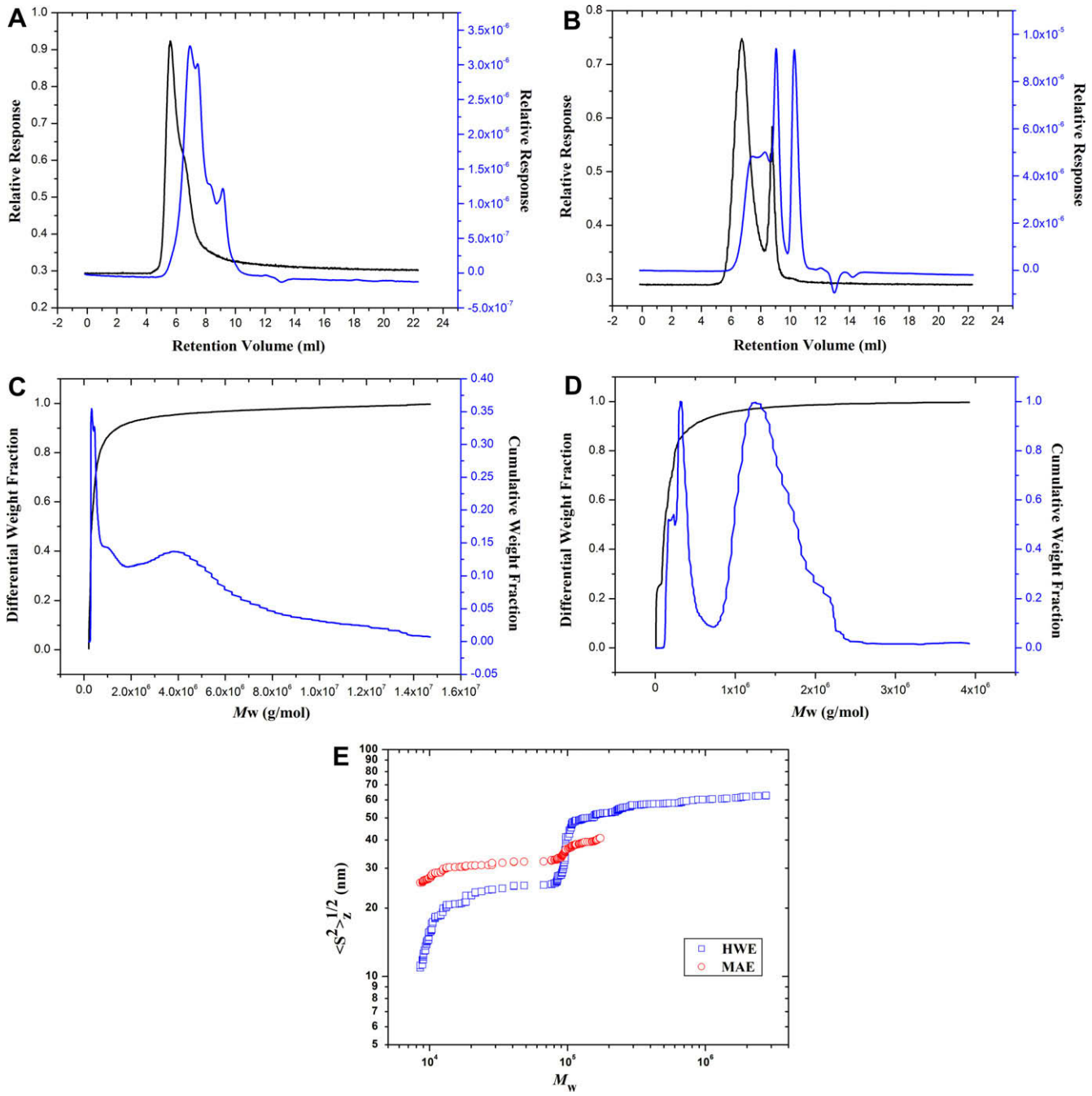

Fig. 3. SEC-LLS chromatograms of the samples. Laser light scattering photometry and RI for PAP_{HWE} (A) and PAP_{MAE} (B) detected at 25 °C. Molar mass distribution analysis of PAP_{HWE} (C) and PAP_{MAE} (D). Plot of log $\langle S^2 \rangle_z^{1/2}$ versus log M_w for PAP_{HWE} and PAP_{MAE} (E).

idant mechanisms: suppression against hydroxyl radical generation and cleaning the hydroxyl radical generated. Earlier researchers suggested both the mechanisms might be responsible for the hydroxyl radical scavenging ability of polysaccharide (Wang, Zhang, Zhang, & Li, 2008). In another assay system in this study, it was demonstrated that the iron chelating ability and the trend of the chelating ability were nearly the same to the order of the scavenging ability to hydroxyl radical. The antioxidant activities of PAP_{MAE} were not a function of a single factor but a combination of several factors.

3.5.3. Scavenging activity of DPPH radicals

The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of natural compounds (Chen, Zhang et al., 2008). DPPH is a stable free radical that shows maximum absorption at 517 nm in methanol. When DPPH encounters a proton-donating substance, for example, an antioxidant, the radical would be scavenged and the absorbance at 517 nm is reduced. Based on this principle, the antioxidant activity of a substance can be expressed as its ability in scavenging the DPPH free radical. In the DPPH test, the antioxidants are able to reduce the stable DPPH radical to the yellow-colored diphenylpicrylhydrazine. The scavenging ability of the samples on hydrogen peroxide is shown in Fig. 4C and compared with Vc and BHA.Fig. 4C shows that the scavenging effects of PAP_{MAE}, PAP_{HWE}, Vc and BHA on the DPPH radical increased dependent on concentration. Both PAP_{MAE} and PAP_{HWE} had a strong antioxidant activity, the scavenging effects of which were 66.01% and 58.23%

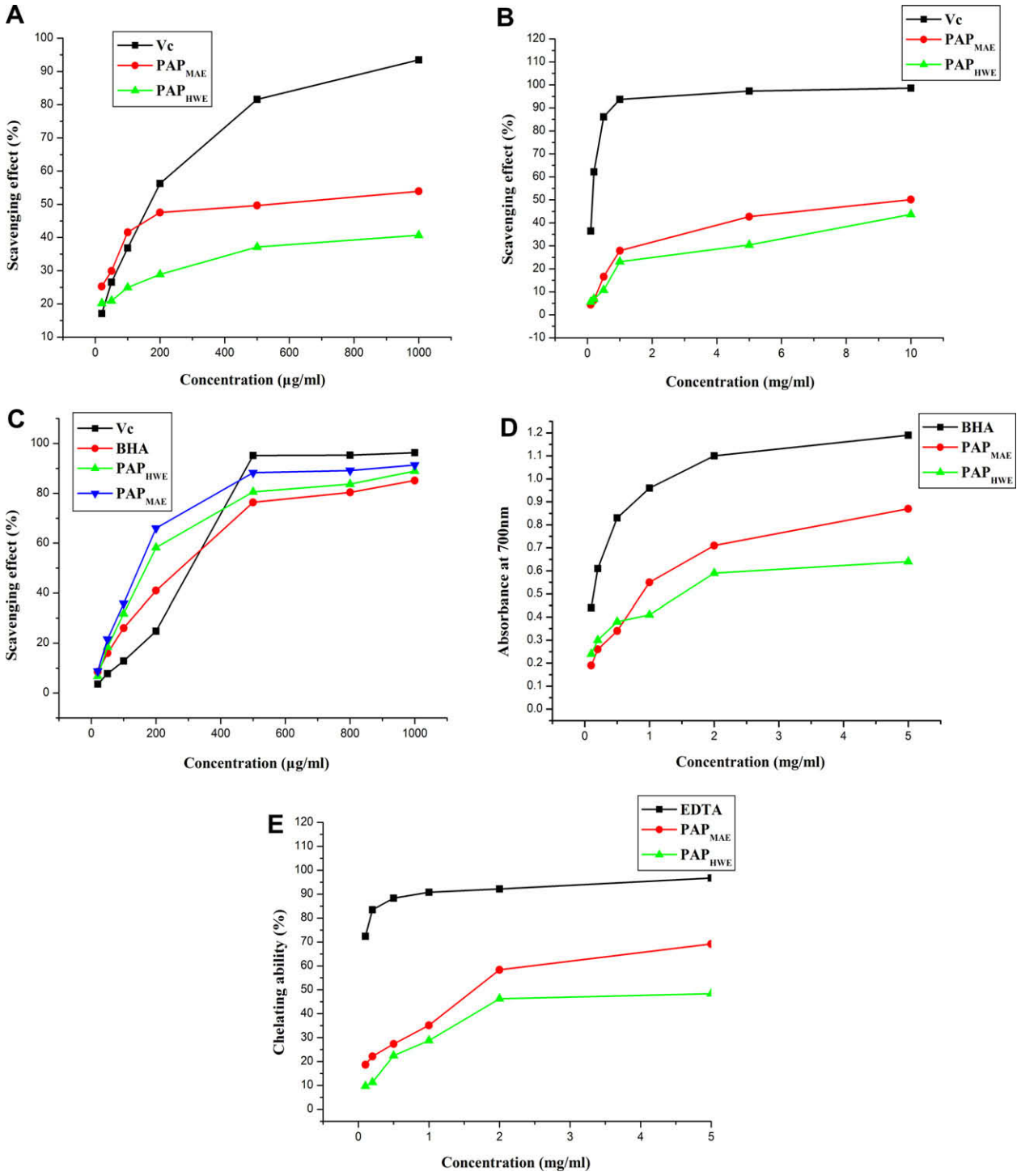

Fig. 4. Antioxidant effect of PAP_{MAE} and PAP_{HWE} with various methods: (A) scavenging activity of superoxide radicals; (B) scavenging activity of hydroxyl radicals; (C) scavenging activity of DPPH radicals; (D) reducing power; and (E) chelating effect on ferrous ions; data are presented as mean values ($n = 3$).

at a dose of 200 μg/ml, higher than those of Vc and BHA. The scavenging effects were 91.33% and 88.94% of PAP_{MAE} and PAP_{HWE} at the concentration of 1 mg/ml, respectively. The radical-scavenging activities of the samples were lower than that of Vc but higher than that of BHA used in this study, 96.27% and 85.13% at the concentration of 1 mg/ml, respectively. Furthermore, PAP_{MAE} exhibited a relatively higher level of radical-scavenging activity than PAP_{HWE}.

3.5.4. Reducing power

The presence of a reluctant such as antioxidant substances in the antioxidant samples causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, Fe^{2+} can be monitored by measuring the formation of Prussian blue at 700 nm (Qi et al., 2006). Fig. 4D depicts the reducing power of PAP_{MAE}, PAP_{HWE} and BHA. The reducing power of the samples correlated well with increasing concentration. In addition, the reducing power of PAP_{MAE} was more pronounced than that of PAP_{HWE}, absorption of 0.87 and 0.64 for PAP_{MAE} and PAP_{HWE} at the concentration of 5 mg/ml, respectively. However, the reducing power was weaker than that of BHA for all the samples. It was reported that polysaccharides from *Ganoderma tsugae* showed reducing power of 0.41 at 5 mg/ml (Tseng et al., 2008). In addition, it was relatively low in

reducing power compared to polysaccharides from *Laminaria japonica*, which showed the absorption of no more than 0.15 at the concentration of 3 mg/ml (Wang et al., 2008).The reducing properties were generally associated with the presence of reductones, which had been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Qi et al., 2005). Reductones were also reported to react with certain precursors of peroxide, thus preventing peroxide formation. Our data on the reducing power of PAP_{MAE} suggested that it was likely to contribute towards the observed antioxidant effect.

3.5.5. Chelating effect on ferrous ions

Ferrum is known as the most important lipid oxidation pro-oxidant due to its high reactivity. The ferrous state of ferrum accelerates lipid oxidation by breaking down hydrogen and lipid peroxidase to reactive free radicals. Fe^{3+} also produces radicals from peroxides, although the rate is tenfold less than that of Fe^{2+}. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of other chelating agents, the complex formation is disrupted with the result that the red color of the complexes decreases. Measurement of color reduction therefore allows estimating the metal chelating activity (Qi et al., 2006). Fig. 4E shows that the ferrous ion-chelating abilities of PAP_{MAE} and PAP_{HWE} were concentration related. The chelating ability of PAP_{HWE} was weak. At a concentration of 0.1–5 mg/ml, the chelating ability ranged from 18.67% to 69.16% for PAP_{MAE}. However, compared with EDTA, the chelating ability of all samples was weaker. Ferrous ion-chelating ability of polysaccharides from *G. tsugae* was similar to that of PAP_{MAE}, it showed about 70% of chelating effect at 5 mg/ml (Tseng et al., 2008). Compared with PAP_{MAE} and PAP_{HWE}, polysaccharides from *L. japonica* exhibited less chelating ability, a maximum chelating effect of 29.48% at a concentration range from 0.5 to 2 mg/ml (Wang et al., 2008).The chelating agent which forms σ bonds with metal was effective as secondary antioxidants for the reduction of redox potential, thereby stabilizing the oxidized form of the metal ion (Liu et al., 2007). Our results showed that reducing power of PAP_{MAE} probably plays a role in the anti-oxidation observed.It was reported that the molecular weight of polysaccharides was an important parameter influencing antioxidant activities. The polysaccharide fraction (TPC-3) isolated from *Camellia sinensis* with the lowest molecular weight (4.2×10^4 Da) showed the highest antioxidant activities (Chen, Xie, Nie, Li, & Wang, 2008). One sulfated lacquer polysaccharide, with moderate M_w (1.27×10^4 Da) showed the best antioxidant capacities. Its reducing capacity was 0.61 at 500 μg/ml, scavenging abilities for superoxide and hydroxyl radical were 56.4% at 500 μg/ml and 55.6% at 1000 μg/ml, respectively (Zou et al., 2008). Although it was not mentioned by the author, sulfated fungus polysaccharide (YSP-S2) with low molecular weight (0.45×10^6 Da) showed the best antioxidant capacities (Yang, Gao, Han, & Tan, 2005). The present study confirmed the results that PAP_{MAE}, which was degraded during microwave heating processing, showed higher antioxidant activities than polysaccharide extracting using the conventional methods.The antioxidant mechanism may be due to the supply of hydrogen by PAP_{MAE}, which combines with radicals and forms a stable radical to terminate the radical chain reaction. The other possibility is that PAP_{MAE} can combine with the radical ions which are necessary for radical chain reaction; then the reaction is terminated. However, the exact explanation of mechanism underlying the free radical-scavenging activity exerted by polysaccharides is still not fully understood (Chen, Xie et al., 2008).

4. Conclusion

An efficient process of MAE had been developed for the extraction of polysaccharides from *P. anserina* with enhanced yield. BBD was used for optimizing extraction parameters in this work. Results showed that a ratio of water to raw material 14.5:1, microwave power 369 W, extraction temperature 63.3 °C and extraction time 76.8 min were the best conditions to produce crude PAP_{MAE}. Under the most suitable conditions, maximum yield of polysaccharides 13.332% can be achieved.Compared with PAP_{HWE}, a sharp decrease in M_w was observed. Three fractions with different molecule weight and polydispersity were detected in SEC-LLS measurement. Furthermore, the changes in d_f values showed a conformation transition in microwave heating process. A d_f value of 3.03 indicated that PAP_{MAE} exhibited a compact hard sphere conformation in an aqueous solution.Compared with PAP_{HWE}, PAP_{MAE} showed better antioxidant activities. On the basis of the results in this study, it was clearly indicated that PAP_{MAE} had antioxidant activity against various oxidative systems in vitro and may be comparable to that of synthetic antioxidants Vc and BHA. The antioxidant mechanisms of PAP_{MAE} may be attributed to strong hydrogen donating ability, a metal chelating ability, and their effectiveness as scavengers of superoxide and free radicals. Overall, the present experiment on MAE and bioactivity of PAP_{MAE} show that it is useful as a functional food as well as a potential therapeutic agent.

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