Optimization of Ultrasonic-assisted Extraction Technology of *Sargassum fusiforme* Polysaccharides and Evaluation of Their Antioxidant Activity

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An ultrasonic-assisted extraction method was employed to get high quality bioactive polysaccharides from *Sargassum fusiforme* (SFP), with the technology optimized in light of an orthogonal test design $L_9(3^3)$. Being the major factors that influence the yield of the extracts, the ultrasonic power, water/solid ratio, and extraction time were selected on the basis of a single-factor test. The extraction yield and the antioxidant activity of SFP were used as dependent variables in the analysis of the orthogonal test results. The antioxidant activity of SFP was evaluated using IC_{50} of scavenging 1,1-diphenyl-2-picrylhydrazyl (DPPH) as the activity monitoring parameter. The optimum extraction conditions were obtained when the ultrasonic power, the extraction time, and the ratio of water to solid were 800 W, 10 min and 20, respectively. Under the optimized conditions, the SFP extracted with the optimized ultrasonic-assisted method exhibited strongest DPPH radical scavenging activity ($P < 0.05$) compared with conventional hot water extracted SFP and other two antioxidants.

Keywords: *Sargassum fusiforme*, polysaccharides, orthogonal experiment, extraction, antioxidant

Introduction

Sargassum fusiforme, the brown alga, is one of the most widely consumed seaweeds in China, Japan and Korea, not only as food, but also as herbal medicine. As an important economic alga, it is now cultured on a vast scale in coastal zone in China's Zhejiang and Fujian provinces (Huang *et al.*, 2006). The polysaccharides extracted from *Sargassum fusiforme* (SFP) in medicine are polymeric carbohydrate structures and have widely been suggested as antioxidants (Siriwardhana *et al.*, 2004), anti-coagulants (Kim *et al.*, 1998), and tumor inhibitor (Han *et al.*, 2009) because of their chemical properties and biological activities (Zhu *et al.*, 2010). However, the utilization of SFP currently is restricted by its polysaccharides extraction technology, which is fundamental for its application or further research and development.

The conventional and most common extraction method for polysaccharides is the hot water extraction (HWE) method and many papers have been 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 (Wang *et al.*, 2009a, 2010). It should be noted that HWE of polysaccharides is always time-consuming, laborious and polysaccharides losing. To solve these problems, the acid extraction method came into being. This method is not without its flaws, however. It may easily alter the structure of the polysaccharide related immunocompetence (Abdel-Fattah *et al.*, 1974). Therefore, alternative extraction techniques like the microwave-assisted extraction (MAE) and ultrasonic assisted extraction (UAE) technologies have attracted considerable interests and have been employed to extract polysaccharides from different plant materials, including *Cyclocarya paliurus* (Xie *et al.*, 2010), jujube (Li *et al.*, 2007a), longan fruit pericarp (Yang *et al.*, 2008), *Poria cocos* (Wang *et al.*, 2009b), Lingzhi (Chen *et al.*, 2010a), and *Inonotus obliquus* (Chen *et al.*, 2010b). More specifically, ultrasound has been reported for the extraction of *Sargassum pallidum* (Ye *et al.*, 2008), which belongs to the same genus as *Sargassum fusiforme*. It has been proved that the ultrasonic treatment has mechanical effects that facilitate the disruption of cell walls, and enhances mass transfer of the cell contents as a result of cavitation bubble collapse (Vinatoru *et al.*, 1997). The problem is that ultrasonic wave has degradation effects on polysaccharides

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(Bao *et al.*, 2008). It has been figured out that the changes in the structure and the degradation of polysaccharides depend on power and operating parameters (Zhou and Ma, 2006), which needs to be optimized.

However, there are few reports on the optimization of extraction technology and the bioactivity of polysaccharides. The objective of this study was to investigate the significant variables (ultrasonic power, extraction time, water/solid ratio), and further to optimize the levels of the extraction variables for SFP production by employing the orthogonal $L_9(3^3)$ test design. It was also attempted to compare the yield and DPPH radical scavenging activity of polysaccharides by the classical and ultrasound-assisted extraction.

Materials and Methods

Materials and equipments Commercially available fresh *Sargassum fusiforme* was purchased from Huiyuan Marine Biotechnology (Dongtong, Zhejiang, China). They were washed with distilled water for several times and then dried at 50–60°C. Afterwards, the dried samples were grounded in a high disintegrator to pass 80 mesh sieve prior to storage in a silica dryer for future use. A JY2D -ultrasonic generator (Xinzhong Bio-technology Institute, Shanghai, China) was used for extraction.

Chemicals 1,1-diphenyl-2-picrylhydrazyl (DPPH), Vitamin E and Vitamin C were purchased from Sigma chemical Company (St. Louis, MO, USA). All other chemicals used were of analytical grade.

Extraction of polysaccharides from *Sargassum fusiforme* Fifty gram of *Sargassum fusiforme* dry powder mixed with distilled water was homogenized in an Erlenmeyer flask using an ultrasonic generator, with a different ultrasonic power, temperature and time. The flask then was treated with water bath at 50°C and 80°C for 1 h respectively, and centrifuged at 4000 rpm for 10 min. The supernatant was collected for further use. The precipitate was added by 2% sodium carbonate solution with a ratio of 1:10 and lixiviated 2 h at room temperature, and centrifuged again to obtain the supernatant which were adjusted to pH 7.0 by using 0.1M hydrochloric acid. All the supernatant from the above procedure were mixed and added with chloroform: n-butylalcohol mixture (5:1) to remove the protein. The total mixture was then centrifuged again. The produced precipitate was removed and the supernatant was added with 60% ethanol to make sedimentation, which was collected by applying the same centrifugation process. The crude polysaccharides were obtained after the procedures of ethanol reflux extraction, acetone wash, and vacuum drying. The percentage polysaccharides yield (%) is calculated as the polysaccharides content of extraction divided by dried pretreated sample weight.

Table 1. Factors and levels for orthogonal test.

Variable	Level		
	1	2	3
A, ultrasonic power (W)	400	800	1200
B, extraction time (min)	4	6	10
C, ratio of water to solid (n)	20	30	40

Optimization of polysaccharides extraction An orthogonal $L_9(3^3)$ test design was used to investigate the optimal extraction condition of polysaccharides from *Sargassum fusiforme*. As seen from Table 1, nine extraction were carried out at sonic power of 400, 800, and 1200 W, ratio of water to solid 20, 30, 40, and extraction time 4, 6, 10 on the basis of the single-factor test, which was a small scale study with only 5 levels concerned. The yield (%) of SFP was the dependent variable. The SFP obtained from the above 9 tests was operated following the method in Section 2.3.

Assay of DPPH radical scavenging effect The free radical scavenging activity of the polysaccharides was measured by IC_{50} of DPPH scavenging test with some modifications (Larrauri *et al.*, 1998; Yokozawa *et al.*, 1998). The absorption peak of mauve in DPPH solution was detected by spectrophotometry. The reduction of OD_{517} resulted in adding antioxidant indicates the capacity to eliminate the organic free radicals. A 1×10^{-4} mol/L ethanolic solution of DPPH was prepared. A 0.001 g/mL polysaccharide solution also was made by using polysaccharide powder. 2 mL DPPH solution was fully mixed with 2 mL polysaccharide solution. After standing in dark for 30 min, the absorption of OD_{517} was detected. The DPPH radical scavenging activities were calculated from the equation: $[1 - (A_i - A_j) / A_0] \times 100$, where A_0 is the absorbance of the solution including 2 mL of DPPH solution and 2 mL of ethanol; A_i is the absorbance of the solution including 2 mL DPPH solution and 2 mL polysaccharide solution; A_j is the absorbance of the solution including 2 mL of polysaccharide solution and 2 mL of ethanol. The 50% inhibiting concentration of DPPH (IC_{50}) by polysaccharides consumption was chosen to be the antioxidation parameter. The IC_{50} value (mg of extract per mL) is the effective concentration at which the DPPH radicals were scavenged by 50% and was obtained by interpolation from linear regression analysis. The lower the IC_{50} the higher the antioxidant activity.

Statistical analysis All statistical analysis was performed by software SPSS 13.0. Differences among means were tested using Duncan's new multiple range test. The orthogonal test statistical analysis was conducted by using DPS software. Significant difference among groups was shown as $P < 0.05$.

Results and Discussion

Effect of ultrasonic power on extraction yield of polysaccharides from *Sargassum fusiforme* The yield (%) of SFP affected by different ultrasonic extraction power (400 – 2000 W) was seen in Fig. 1 (a), where other two factors (extraction time, ratio of water to solid) were fixed at 8 min and 30:1. The yield (%) of SFP increased when the ultrasonic power increased from 400 to 800 W, and reached the high value (7.81) at 800 W and then dropped from 800 to 1600 W. The decreased SFP yield when a high ultrasonic power was used was possibly due to the degradation of polysaccharides by ultrasonic wave. According to the reports of Li *et al.* (2005), as well as Sivakumar and Pandit (2001), application of high ultrasonic power results in degradation effect. However, strangely, then the yield reached the maximum value (7.94)

Fig. 1. Effects of the main three factors on extraction yield of polysaccharides from *Sargassum fusiforme* by ultrasonic-assisted method: (a) ultrasonic power; (b) extraction time; (c) ratio of water to solid.

when ultrasonic power ranged from 1600 to 2000 W.

Effect of extraction time on extraction yield of polysaccharides from *Sargassum fusiforme* The yield (%) of SFP affected by different extraction time (4 – 12 min) was seen in Fig. 1 (b), where other two factors (ultrasonic power, ratio of water to solid) were fixed at 800 W and 30:1. The yield (%) of SFP increased as extraction time increased from 4 to 6 min. And then there is a little drop when the extraction time increased to 8 min. With the time increasing from 8 to 12 min, the yield (%) of SFP continued to increase and reached the peak value (7.87) at 12 min.

Effect of ratio of water to solid on extraction yield of polysaccharides from *Sargassum fusiforme* The yield (%) of SFP affected by different ratio of water to solid (10 – 40) was seen in Fig. 1 (c), where other two factors (ultrasonic power, extraction time) were fixed at 800 W and 8 min. The yield (%) of SFP increased with the increasing ratio of water to solid and reached the maximum value (7.55) at 30 and then dropped in a mild slope from 30 to 40.

Therefore, in this study, we adopted ultrasonic power of 400, 800, 1200, extraction time of 4, 6, 10 and ratio of water to solid of 20, 30, 40, for further study objects in the orthogonal test design experiment.

Optimization of the extraction parameters of polysaccharides from *Sargassum fusiforme* Various parameters affect the optimization of the experimental conditions for the development of ultrasonic extraction method. The ultrasonic power, the ratio of water to solid, and the extraction time are generally considered to be the most important factors that affect the yield (%) of SFP. The investigated levels of each factor were selected depending on the above experimental results of the single-factor and examined using an orthogonal test design $L_9(3^3)$. Independent variables with three variable levels, the sonic power (400, 800, 1200 W), the extraction time (4, 6, 10) and the ratio of water to solid (20, 30, 40) are listed in Table 1. The total evaluation index was used for analysis by statistical method. The orthogonal test results and extreme difference analysis are presented in Table 2 and Table 3, with SFP yield and DPPH scavenging activity (IC_{50} of DPPH) as dependent variables respectively.

As seen from the results of Table 2, the maximum yield of SFP (8.52%) was obtained when ultrasonic power, extraction time and ratio of water to solid were A1B3C3 (400 W, 10 min and 40). However, we cannot directly choose the corresponding extraction conditions as the best technology. Results of Table 3 showed that the minimum of IC_{50} of DPPH (2.26 mg) was achieved when the ultrasonic power, the extraction time and the ratio of water to solid were A3B3C2 (1200 W, 10 min and 30). According to the R values, the influences to the mean extraction yield and IC_{50} of DPPH de-

Table 2. Analysis of $L_9(3^3)$ test results with the yield of SFP as dependent variable.

No.	A, ultrasonic power (W)	B, extraction time (min)	C, ratio of water to solid (n)	Yield of SFP (%)
1	A ₁	B ₁	C ₁	7.11 ± 0.17
2	A ₁	B ₂	C ₂	7.60 ± 0.14
3	A ₁	B ₃	C ₃	8.52 ± 0.047
4	A ₂	B ₁	C ₂	7.66 ± 0.15
5	A ₂	B ₂	C ₃	7.12 ± 0.06
6	A ₂	B ₃	C ₁	8.13 ± 0.081
7	A ₃	B ₁	C ₃	6.86 ± 0.062
8	A ₃	B ₂	C ₁	7.15 ± 0.085
9	A ₃	B ₃	C ₂	7.39 ± 0.12
K_1^a	23.23	21.63	22.39	
K_2	22.91	21.87	22.65	
K_3	21.40	24.04	22.50	
k_1^b	7.74	7.21	7.46	
k_2	7.64	7.29	7.55	
k_3	7.13	8.01	7.50	
R^c	0.61	0.80	0.09	

^a $K_i^A = \Sigma$ extraction yield at A_i .

^b $k_i^A = K_i^A / 3$.

^c $R_i^A = \max\{k_i^A\} - \min\{k_i^A\}$.

Table 3. Analysis of $L_9(3^3)$ test results with IC_{50} of DPPH as dependent variable.

No.	A, ultrasonic power (W)	B, extraction time (min)	C, ratio of water to solid (n)	IC_{50} of DPPH (mg)
1	A ₁	B ₁	C ₁	3.96 ± 0.4
2	A ₁	B ₂	C ₂	3.59 ± 0.11
3	A ₁	B ₃	C ₃	3.29 ± 0.31
4	A ₂	B ₁	C ₂	3.25 ± 0.43
5	A ₂	B ₂	C ₃	2.96 ± 0.14
6	A ₂	B ₃	C ₁	2.67 ± 0.067
7	A ₃	B ₁	C ₃	3.26 ± 0.2
8	A ₃	B ₂	C ₁	2.72 ± 0.39
9	A ₃	B ₃	C ₂	2.26 ± 0.33
K_1^a	10.84	10.47	9.35	
K_2	8.88	9.27	9.10	
K_3	8.24	8.22	9.51	
k_1^b	3.61	3.49	3.12	
k_2	2.96	3.09	3.03	
k_3	2.75	2.74	3.17	
R^c	0.86	0.75	0.14	

^{a, b, c} the same as Table 2.

crease in the order of $B > A > C$ and $A > B > C$, respectively. Besides, results (Table 4) indicate that the yield of SFP was significantly influenced by the ultrasonic power and the extraction time ($P < 0.001$). In view of considerations to both the SFP yield and the DPPH scavenging activity, optimum

conditions of the ultrasonic power and the extraction time were 800 W and 10 min respectively. With regard to the ratio of water to solid, in order to save production cost and operate conveniently for industrialization, the ratio of 20 was selected.

Table 4. Analysis of orthogonal test variance.

	Source	Sum of squares	Degree of freedom	Mean square	F value	Significance
Yield of SFP	A	1.913	2	0.957	77.910	< 0.001
	B	3.548	2	1.774	144.489	< 0.001
	C	0.033	2	0.017	1.354	0.283
	Error	0.221	18	0.012		
IC ₅₀ of DPPH	A	3.672	2	1.836	21.497	< 0.001
	B	2.558	2	1.279	14.974	< 0.001
	C	0.084	2	0.042	0.491	0.620
	Error	1.537	18	0.085		

We finally make the optimum technology as follows: ultrasonic power, 800 W, extraction time, 10 min, and the ratio of water to solid, 20. Under the optimized conditions, an 8.26% yield of SFP was obtained, which was 2.22 and 1.88% higher than that of SFP extracted by HWE and MAE method, respectively. In terms of extraction yield, MAE was less suitable than UAE for the extraction of polysaccharides from *Sargassum fusiforme* because of its lower extraction efficiency. The present result was inconsistent with the work of Xie *et al.* (2010), in which the MAE proved to be the best method for extracting polysaccharides.

Effect of different methods on DPPH radical scavenging activity The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of natural compounds (Chen *et al.*, 2008). This method is based on the reduction of ethanolic DPPH solution in the presence of a hydrogen donating antioxidant, leading to the formation of non-radical form DPPH-H. The polysaccharide extract is able to reduce the stable radical DPPH to yellow-coloured diphenylpicrylhydrazine (Yang *et al.*, 2008). The positive correlation between polysaccharide concentration and its antioxidant activity is well documented (Li *et al.*, 2007b).

In this study, effect of DPPH radical scavenging activities were investigated using three SFP samples obtained by the conventional HWE, MAE and the optimized UAE methods, respectively, and compared with two antioxidants, Vit C and Vit E. The results are shown in Fig. 2. As shown in Fig. 2, the IC₅₀ of scavenging DPPH free radicals of SFP extracted by the UAE method was significantly lower than that of the HWE method ($P < 0.01$), and was significantly lower than Vit C ($P < 0.05$). The results indicated that polysaccharides extracted by the optimized UAE method exhibited strong DPPH scavenging activity. Yang *et al.* (2008). reported similar results for polysaccharides from longan fruit pericarp (PLFP), of which the DPPH radical scavenging activity could be improved by application of various ultrasonic power, time and temperature. According to Chen and Yan (2005),

Fig. 2. Effect of DPPH radical scavenging activities of different *Sargassum fusiforme* polysaccharides extracted. * $P < 0.05$, ** $P < 0.01$. HWE, hot water extraction method, MAE, microwave-assisted extraction, UAE, ultrasonic assisted extraction method.

the antioxidant activity of polysaccharides was related to their degree of polymerization and structure. Under various ultrasonic conditions, the molecular weight and structure of polysaccharides would be modified. In the present study, the improved DPPH radical scavenging activity was possible due to the degradation of SFP to different extent.

Conclusions

Based on the above-mentioned facts, we can draw a conclusion that SFP, obtained by the optimum ultrasonic-assisted extraction technology through orthogonal experiment method, exhibited a strong DPPH scavenging activity. Further studies should be carried out to elucidate bioactivity through animal experiments with the purpose of applying the polysaccharides in food and medicine industry.

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