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Extraction, preliminary characterization, antioxidant and anticancer activities *in vitro* of polysaccharides from *Cyclina sinensis*

Changxing Jiang[a,b], Mingchun Wang[a], Jun Liu[a], Dan Gan[a], Xiaoxiong Zeng[a,*]

[a] College of Food Science and Technology, Nanjing Agricultural University, Nanjing 210095, Jiangsu, China
[b] College of Life Science and Chemical Engineering, Huaiyin Institute of Technology, Huaiyin 223001, Jiangsu, China

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ABSTRACT

In the present study, we investigated the extraction, purification, preliminary characterization, antioxidant and anticancer activities *in vitro* of polysaccharides from *Cyclina sinensis* (CSPS). Firstly, the optimal parameters for extraction of CSPS were obtained by using a central composite design as follows: extraction temperature, 90 °C; extraction time, 250 min; ratio of water to raw material, 29; and extraction times, two times. Then, the crude CSPS was sequentially purified by chromatography of DEAE-52 and Sephadex G-100, resulting in three purified fractions of CSPS-1, CSPS-2 and CSPS-3. We found that the composition and property of CSPS-3, having relatively higher contents of protein, uronic acid, sulfate and more complicated monosaccharide composition, were quite different from those of CSPS-1 and CSPS-2. Furthermore, we demonstrated that CSPS-3 had strong scavenging activities *in vitro* on superoxide radical and hydroxyl radical and strong inhibitory effect *in vitro* on the growth of human gastric cancer BGC-823 cells.

1. Introduction

Cyclina sinensis, a well-known bivalve mollusk in the family of Veneridae, is widely distributed along the coastal waters of China, Korea, and south eastern Asia and found in muddy sand beaches (Zhao, Li, Kong, & Mao, 2009). It is one of the most important bivalve species in Chinese aquaculture (Liu, Ma, Hu, Miao, & Li, 2002; Yu, Gu, & Yang, 2005). It has been reported that *C. sinensis* can be used for the treatment of inflammation, asthma and dental ulcer in traditional Chinese medicine (Wang et al., 2007). In addition, it has been demonstrated that it is rich in protein, amino acid, lipid and polysaccharides that may contribute to the biological functions, such as anti-tumor, anti-inflammation and immune-regulation (Abdulkadir & Tsuchiya, 2008; Gu, Yu, & Cai, 2006; Liu, Zhang, Dou, Lu, & Guo, 1997). However, little attention has been devoted to the extraction and biological activities of polysaccharides from *C. sinensis* (CSPS) compared with those of some other bivalvia species such as *Callista chione*, *Mytilus coruscus*, *Mixylla rosacea* and *Hyriopsis cumingii* (Cimino et al., 2001; Gomes et al., 2010; Luppi, Cesaretti, & Volpi, 2005; Pomin, 2009; Qiao, Hu, et al., 2009; Qiao, Ke, et al., 2009; Saravanan & Shanmugam, 2010; Volpi & Maccari, 2005; Xu et al., 2008). Therefore, we report here the extraction, preliminary characterization, antioxidant and anticancer activities *in vitro* of CSPS.

In the present study, we firstly investigated the effects of extraction temperature, time, ratio of extraction solvent (water) to raw material, and extraction times on the extraction yields of CSPS. Then, we optimized the conditions for CSPS extraction by using central composite design (CCD) (Hsu, 1995), one of response surface methodology (RSM). Since RSM is a powerful technique for testing multiple variables and widely used to optimize the extraction of polysaccharides (Guo, Zou, & Sun, 2010; Qiao, Hu, et al., 2009; Sun, Liu, & Kennedy, 2010). Furthermore, we purified the crude CSPS by chromatography of DEAE-52 and Sephadex G-100 and characterized the purified fractions of crude CSPS. Finally, we investigated the antioxidant and anticancer activities *in vitro* of crude CSPS and its purified fractions by determining their superoxide radical scavenging activities, hydroxyl radical scavenging activities and inhibitory rates against human gastric cancer BGC-823 cells.

2. Materials and methods

2.1. Materials

C. sinensis was purchased from Nanjing Aquatic Product Market (Nanjing, China). Human gastric cancer BGC-823 cells were obtained from the Cell Bank of Shanghai Institute of Cell Biology (Shanghai, China). Arabinose, rhamnose, fucose, xylose, galactose, glucose, mannose, ferrozine, nitroblue tetrazolium (NBT), phenazine methosulfate (PMS), reduced nicotinamide adenine dinucleotide (NADH), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), DEAE-52 cellulose and Sephadex

* Corresponding author. Fax: +86 25 84396791.
E-mail address: zengxx@njau.edu.cn (X. Zeng).

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Table 1
Central composition design with four independent variables for the extraction of polysaccharides from *Cyclina sinensis* (CSPS) and the response values (yield of CSPS).

Block	Run	Extraction temperature (°C)		Extraction time (min)		Water to raw material ratio (ml/g)		Yield (%)
		X_1	Code x_1	X_2	Code x_2	X_3	Code x_3	
2	1	75	−1	175	−1	24	−1	11.2
	2	90	1	250	1	24	−1	14.0
	3	90	1	175	−1	36	1	12.5
	4	75	−1	250	1	36	1	12.8
	5	82.5	0	212.5	0	30	0	15.4
	6	82.5	0	212.5	0	30	0	15.6
3	7	90	1	175	−1	24	−1	12.6
	8	75	−1	250	1	24	−1	12.9
	9	75	−1	175	−1	36	1	13.2
	10	90	1	250	1	36	1	13.8
	11	82.5	0	212.5	0	30	0	15.9
	12	82.5	0	212.5	0	30	0	14.6
4	13	70	−1.68	212.5	0	30	0	13.5
	14	95	1.68	212.5	0	30	0	14.8
	15	82.5	0	150	−1.68	30	0	14.0
	16	82.5	0	275	1.68	30	0	15.5
	17	82.5	0	212.5	0	20	−1.68	11.3
	18	82.5	0	212.5	0	40	1.68	10.8
	19	82.5	0	212.5	0	30	0	15.2
	20	82.5	0	212.5	0	30	0	15.1

G-100 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum and RPMI-1640 media were purchased from Gibco/Invitrogen (Grand Island, NY, USA). All other reagents were all of analytical grade.

2.2. Extraction of polysaccharides

The crude CSPS was prepared according to the reported method with some modifications (Qiao, Hu, et al., 2009). Briefly, fresh *C. sinensis* was collected and washed carefully with cold water. After removing the shells and impurities, the flesh was crushed by a high speed disintegrator and the homogenate was kept in 90% of ethanol (v/v) for 2 weeks. Then, the collected flesh was air-dried at 50 °C. For extraction, the dried flesh was extracted by water in a designed extraction temperature, time and ratio of water to raw material. After treatment, the mixture was centrifuged at 5000 rpm for 20 min, and the insoluble residue was treated again as mentioned above. The supernatants were collected, concentrated to a proper volume by using a vacuum rotary evaporator, deproteinated by the method of Sevag, Lackman, and Smolens (1938) and mixed with three times volume of absolute ethanol. The mixture was stirred vigorously and then kept overnight at 4 °C. The precipitate was collected by centrifugation at 5000 rpm for 20 min and air-drying at 50 °C to a constant weight, affording the crude CSPS. The yield of extraction was calculated according to the following formula:

$$\text{Extraction yield}\,(\%) = \frac{W_1}{W_0} \times 100$$

where W_1 is the weight of crude CSPS and W_0 is the dried weight of flesh.

2.3. CCD design for the extraction of polysaccharides

In the present study, CCD with three independent variables (X_1, extraction temperature; X_2, extraction time; X_3, ratio of water to raw material) at five levels and one block (extraction times) at three levels was applied to determine the best combination of extraction variables for extraction of CSPS. For statistical calculation, the variables were coded according to Eq. (1):

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

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 center point, and ΔX_i is step change value of an independent variable. The range of independent variables and their levels are presented in Table 1. The whole design consisted of 20 experimental points carried out in random order, and the experimental data (Table 1) were fitted to the following second-order polynomial model:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (2)$$

where Y is the predicted response (yield of CSPS), β_0, β_i, β_{ii} and β_{ij} are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively, X_i and X_j are the independent variables ($i \neq j$).

2.4. Purification of crude CSPS

The crude CSPS was sequentially purified by chromatography of DEAE-52 and Sephadex G-100 according to the reported methods with slight modifications (Qiao, Hu, et al., 2009). Briefly, the solution of crude CSPS (3 ml, 15 mg/ml) was applied to a column (2.6 cm × 30 cm) of DEAE-52 cellulose. Then, the column was stepwise eluted with 0, 0.1 and 0.5 M sodium chloride solutions at a flow rate of 60 ml/h. The obtained elute (10 ml/tube) was collected automatically and the carbohydrates were detected by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). As results, three fractions of polysaccharides (Fig. 1) were obtained. Each fraction was collected, concentrated, dialyzed and further purified through a column (2.6 cm × 60 cm) of Sephadex G-100, respectively, resulting in three purified fractions of CSPS-1, CSPS-2 and CSPS-3. Finally, CSPS-1, CSPS-2 and CSPS-3 were lyophilized for further study.

2.5. Characterization of CSPS-1, CSPS-2 and CSPS-3

The carbohydrate content in CSPS-1, CSPS-2 and CSPS-3 was determined by phenol–sulfuric acid method. The protein content was determined by the method described by Bradford (1976), using bovine serum albumin for the standard curve. The content of uronic acid was determined according to the method of Blumenkrantz and Asboe-Hansen (1973) using D-glucuronic acid as the standard. The

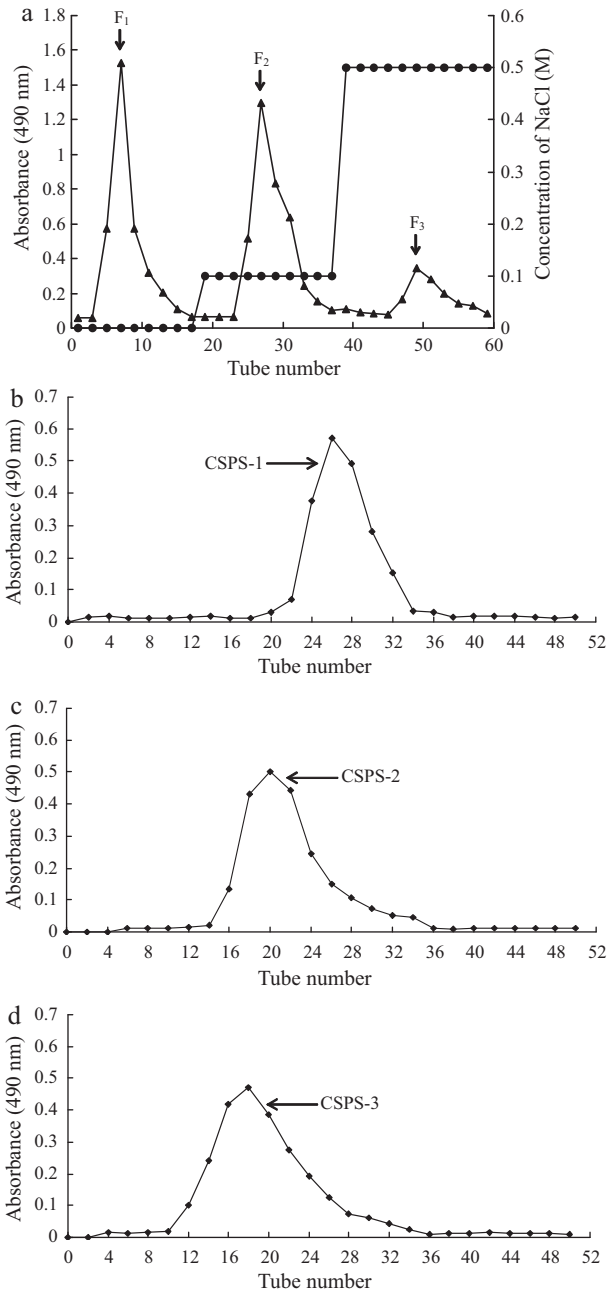

Fig. 1. Stepwise elution curve of crude CSPS on anion-exchange chromatography column of DEAE-52 cellulose (a) and elution curve of polysaccharide fractions (F_1, F_2 and F_3) from DEAE-52 cellulose on size-exclusion chromatography column of Sephadex G-100 (b–d).

content of sulfate radical was determined according to the reported method (Doigson & Price, 1962).

The monosaccharide compositions of CSPS-1, CSPS-2 and CSPS-3 were analyzed by gas chromatography (GC). Each sample (5 mg) was hydrolyzed with 2 ml of 2 M trifluoroacetic acid at 120 °C for 2 h. The hydrolyzate was repeatedly co-distilled with methanol to dryness and conventionally converted into aldonitrile acetates. The aldonitrile acetate derivatives of standard monosaccharides were prepared in the same way. Then, all the derivatives were analyzed by a 6890N GC (Agilent Technologies, Santa Clara, CA, USA) equipped with flame ionization detector and an HP-5 fused silica capillary column (30 m × 0.32 mm × 0.25 mm). The operation conditions of GC were as following: flow rates of N_2, H_2 and air were 25, 30 and 400 ml/min, respectively; the temperatures of oven, detector and inlet were set at 210, 280 and 250 °C, respectively.

2.6. Assay of antioxidant activity in vitro of CSPS

2.6.1. Assay of superoxide radical scavenging activity

The superoxide radical scavenging activity of CSPS was determined according to the reported method (Kanatt, Chander, & Sharma, 2007) with some modifications. Briefly, each 1.0 ml of NBT solution (156 μmol/l of NBT in 0.1 M phosphate buffer, pH 7.4) and NADH solution (468 μmol/l of NADH in 0.1 M phosphate buffer, pH 7.4) were mixed with 1 ml sample solution with different concentrations (0, 0.4, 0.8, 1.6, 3.2 and 4.0 mg/ml). The reaction was started by adding 1.0 ml of PMS solution (60 μmol/l PMS in 0.1 M phosphate buffer, pH 7.4), and then incubated at 25 °C for 5 min. The absorbance at 560 nm was measured against the blank (water and 0.1 M phosphate buffer instead of sample and NBT solution, respectively). The scavenging activity on superoxide radical was calculated according to the following formula:

$$\text{Superoxide radical scavenging activity}\,(\%) = 100 \times \frac{A_0 - A_1 + A_2}{A_0}$$

where A_0 is the absorbance of control without tested samples, A_1 is the absorbance in the presence of tested samples, and A_2 is the absorbance of tested sample without NBT solution.

2.6.2. Assay of hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was determined according to the method of Liu, Wang, Xu, and Wang (2007). Briefly, 1 ml sample solution with different concentrations (0, 0.4, 0.8, 1.6, 3.2 and 4.0 mg/ml) was added to 2 ml of sodium phosphate buffer (150 mM, pH 7.4) containing 10 mM $FeSO_4$, 2 mM sodium salicylate and 6 mM H_2O_2. The mixture was incubated at 37 °C for 30 min and the absorbance of the solution was detected at 510 nm. The hydroxyl radical scavenging activity was calculated according to the following formula:

$$\text{Hydroxyl radical scavenging activity}\,(\%) = 100 \times \frac{A_0 - A_1 + A_2}{A_0}$$

where A_0 is the absorbance of control sample, A_1 is the absorbance in the presence of tested samples, and A_2 is the absorbance of tested sample without sodium salicylate solution. The ascorbic acid was used as positive control.

2.7. Assay of inhibitory activity in vitro of CSPS on BGC-823 cell proliferation

The inhibition rates of crude CSPS and its purified fractions on the growth of human gastric cancer BGC-823 cells were determined by MTT-based colorimetric method (Mosmann, 1983). Briefly, BGC-823 cells were suspended in RPMI-1640 medium supplemented with 10% FBS, 100 units/ml of penicillin and 100 μg/ml of streptomycin at a density of 1×10^5 cells/ml. The cell suspension was pipetted into a 96-well plate (50 μl/well) and inoculated at 37 °C in a humidified 5% CO_2 incubator (Sanyo, Japan) for 24 h. Then, 50 μl of test sample with different concentrations (0, 50, 100, 200 and 400 μg/ml in fresh growth medium) was added into each well separately. After 72 h incubation, MTT reagent (5.0 mg/ml) was added to each well (10 μl/well), and the plate was further incubated for 4 h. Then, 100 μl of 10% SDS in 0.01 M HCl was added into each well and kept overnight for the dissolution of formazan crystals. The absorbance of each well was read at 570 nm using an ELISA plate reader (TECAN Infinite F200, Switzerland). The inhibitory rate was

Table 2
Variance analysis of response surface quadratic model for the extraction of polysaccharides from *Cyclina sinensis*.

Source	Sum of squares	df	Mean square	*F*-Value	*P*-Value
Block	0.209	2	0.104	0.413	0.694 [b]
Model	43.641	9	4.849	20.035	0.0001 [a]
Lack of fit	1.066	5	0.213	0.735	0.645 [b]
Pure error	0.870	3	0.290		
Corrected total	45.786	19			
X_1	1.821	1	1.821	7.523	0.0253 [a]
X_2	3.115	1	3.115	12.872	0.0071 [a]
X_3	0.042	1	0.042	0.174	0.6873 [b]
X_1^2	3.276	1	3.276	13.537	0.0062 [a]
X_2^2	1.010	1	1.010	4.174	0.0753 [b]
X_3^2	35.631	1	35.631	147.222	0.0001 [a]
X_1X_2	0.245	1	0.245	1.012	0.3438 [b]
X_1X_3	0.605	1	0.605	2.5	0.1525 [b]
X_2X_3	0.605	1	0.605	2.5	0.1525 [b]

$R^2 = 0.9575$, adjusted $R^2 = 0.9097$.
[a] 5% significance level.
[b] Not significant relative to the pure error.

calculated according to the following formula:

$$\text{Inhibition rate}\,(\%) = \left(1 - \frac{A_1}{A_0}\right) \times 100$$

where A_1 and A_0 are the absorbance of test sample and control, respectively.

2.8. Statistical analysis

Analysis of the experimental design and data was carried out using Design Expert software of version 7.0 (Stat-Ease Inc., Minneapolis, USA). Analysis of variance (ANOVA) was carried out and the fitness of the polynomial model equation was expressed as the coefficient of determination R^2. The significances of the regression coefficients were tested by *F*-test. One-way ANOVA was performed using the SPSS 13.0 for windows (SPSS, Chicago, IL, USA). Multiple comparisons of means were done by the least significance difference (LSD) test. *P*-Values of less than 0.05 were regarded as significant.

3. Results and discussion

3.1. Model fitting and optimization for extraction of CSPS

3.1.1. Model fitting

By using the software of Design Expert version 7.0, a polynomial model describing the correlation between the extraction yield of CSPS and the three variables was obtained as follows:

$$\begin{aligned} Y = &- 111.4 + 1.5258X_1 + 0.0794X_2 + 3.4566X_3 \\ &+ 0.0006X_1X_2 - 0.0062X_1X_3 - 0.0012X_2X_3 \\ &- 0.0086X_1^2 - 0.0002X_2^2 - 0.0445X_3^2 \end{aligned} \quad (3)$$

where Y represents the yield of crude CSPS, X_1, X_2 and X_3 represent extraction temperature, time and ratio of water to raw material, respectively.

The results of ANOVA, lack-of-fit and the adequacy of the model are summarized in Table 2. The model *F*-value of 20.035 implied that the model was highly significant. There was only a 0.01% chance that a model *F*-value could occur due to noise. The determination coefficient R^2 of the model was 0.9575, indicating that 95.75% of the variability in the response could be explained by the model. In addition, the *P*-value of 0.645 for lack-of-fit implied the lack-of-fit was not significant relative to the pure error, indicating the model equation was adequate to predict the extraction yield of CSPS within the range of experimental variables.

The significance of the regression coefficients was tested by *F*-test, and the corresponding *P*-values for the model terms are also listed in Table 2. The *P*-value is used as a tool to check the significance of each coefficient, which in turn may indicate the pattern of the interaction between the variables. Smaller the *P*-value is, more significant the corresponding coefficient is. Accordingly, X_1, X_2, X_1^2 and X_3^2 were significant ($P < 0.05$), while X_3, X_1X_2, X_1X_3, X_2X_3, X_2^2 were not significant ($P > 0.05$). In addition, the block was not significant ($P > 0.1$), indicating that extraction times within the experimental range had no influence on the extraction yield of CSPS. Therefore, 2 times extraction was better than the others considering the reduction of energy consumption and process waste.

3.1.2. Optimization for extraction of CSPS

The fitted response surface plots and contour plots for the model were generated by the Stat-Ease Design-Expert software in order to better understand the interactions of the variables. The shape of the contour plots indicates whether the mutual interactions between variables are significant or not. A circular contour plot indicates that the interaction between related variables is negligible, while an elliptical contour plot indicates that the interaction between related variables is significant (Muralidhar, Chirumamila, Marchant, & Nigam, 2001). The response surface plots and contour plots as shown in Fig. 2 were generated using Design-Expert, which depicted the interactions between two variables by keeping the other variables at their zero levels for crude CSPS production. It is evident that these three-dimensional plots and their corresponding contour plots provided a visual interpretation of the interaction between two variables and facilitated the location of optimum experimental conditions. By employing the software Design-Expert, the solved optimum values of the tested variables for the extraction of CSPS were extraction temperature 90 °C, extraction time 250 min and ratio of water to raw material 29. Using the optimal conditions, the maximum predicted extraction yield of CSPS was 15.62%, which corresponded fairly well to that of real extraction ($15.52 \pm 1.26\%$). The result suggested that the regression model was accurate and adequate for the prediction of CSPS extraction.

3.2. Purification and characterization of CSPS

By using the optimal extraction conditions, crude CSPS was prepared. Then, the crude CSPS was firstly separated through an anion-exchange column of DEAE-52 cellulose. As a result, three independent elution peaks (F_1, F_2 and F_3) as shown in Fig. 1a were obtained. The three fractions were collected, concentrated, dialyzed and loaded onto a column of Sephadex G-100, respectively. The column was eluted with deioned water, and the resulting elute was collected. As shown in Fig. 1b, each fraction generated one single elution peak, affording CSPS-1, CSPS-2 and CSPS-3, respectively.

Data in Table 3 show the monosaccharide compositions and the contents of carbohydrate, protein, uronic acid and sulfuric radical of CSPS-1, CSPS-2 and CSPS-3. Notably, CSPS-3 was quite different from CSPS-1 and CSPS-2. CSPS-3 contained 6.34% of protein. In addition, it contained relative higher contents of uronic acid and sulfuric radical than CSPS-1 and CSPS-2. Furthermore, the monosaccharide composition of CSPS-3 was more complicated than that of CSPS-1 or CSPS-2. According to GC analysis, CSPS-1 was composed of xylose and glucose in a molar percent of 4.92 and 95.08, respectively, and CSPS-2 was only composed of glucose. However, CSPS-3 was composed of rhamnose, fucose, mannose, glucose and galactose with a molar percent of 11.48, 17.15, 12.44, 21.57 and 37.36, respectively.

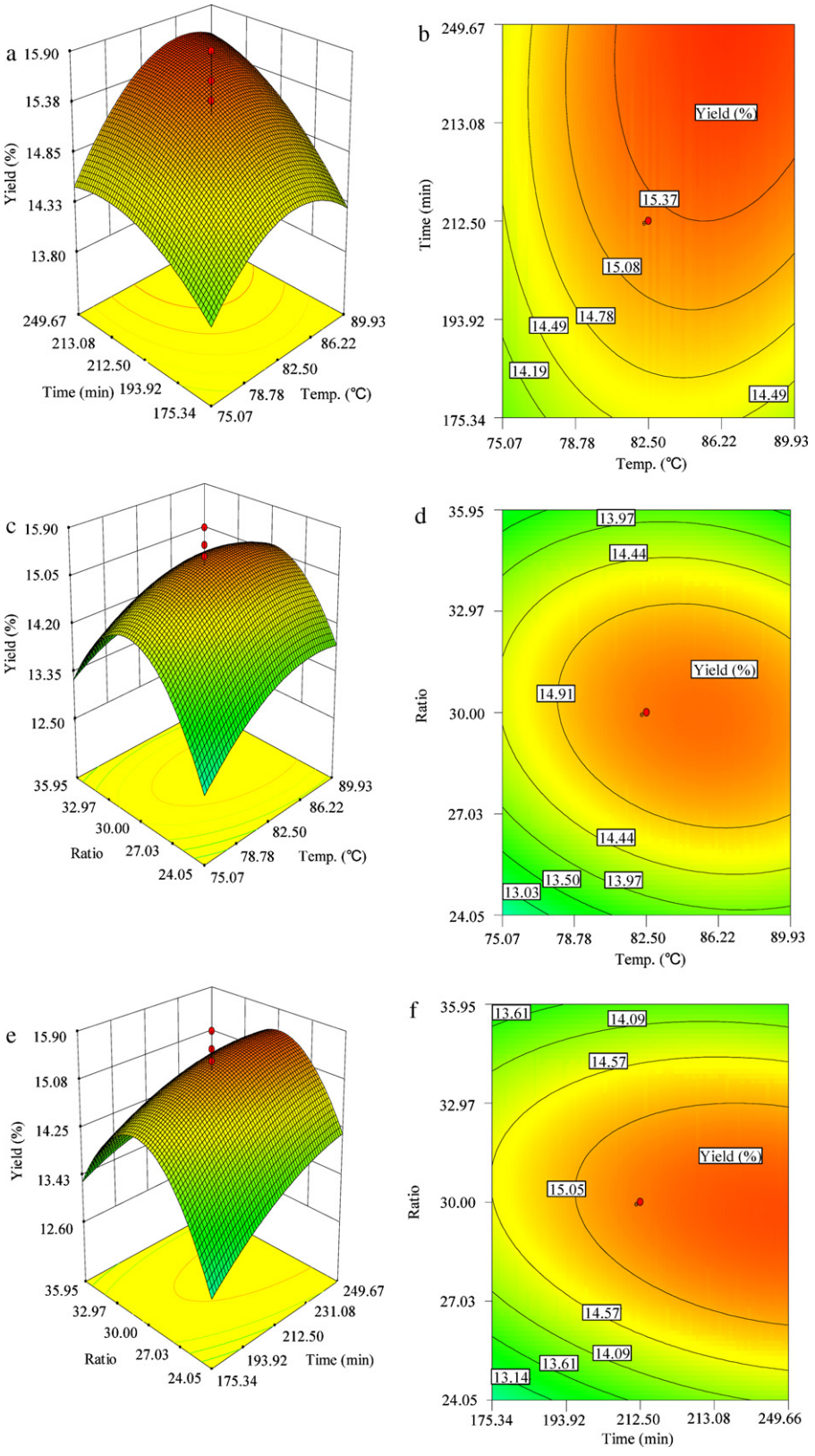

Fig. 2. Response surface plots (a, c and e) and contour plots (b, d and f) showing the effects of extraction temperature, extraction time, ratio of water to raw material and their mutual effects on extraction yield of CSPS.

CSPS-1 was a neutral polysaccharide as it was eluted with water from a DEAE-52 cellulose anion-exchange column, while CSPS-2 and CSPS-3 were acidic polysaccharides since they were eluted out with NaCl solution (Hokputsa et al., 2004; Yang et al., 2008). It could be verified by the fact that CSPS-2 and CSPS-3 had relative higher contents of uronic acid and sulfate (Table 3). In addition, the present results were similar to our previous report on polysaccharides from *H. cumingii* (Qiao, Hu, et al., 2009).

Table 3
The chemical compositions and the contents of carbohydrate, protein, uronic acid and sulfate for CSPS-1, CSPS-2 and CSPS-3.

Item	CSPS-1	CSPS-2	CSPS-3
Carbohydrate (%)	98.75	95.58	84.71
Protein (%)	–[a]	–	6.34
Uronic acid (%)	0.16	0.96	2.13
Sulfuric radical (%)	1.22	2.08	3.58
Sugar components (mol%)			
Rhamnose	–	–	11.48
Fucose	–	–	17.15
Xylose	4.92	–	–
Mannose	–	–	12.44
Glucose	95.08	100	21.57
Galactose	–	–	37.36

[a] Not detectable.

3.3. Antioxidant activity in vitro of CSPS

3.3.1. Superoxide radical scavenging activity of CSPS

Superoxide radical is a reduced form of molecular oxygen created by receiving one electron from mitochondrial electron transport systems. The superoxide radical plays an important role in the formation of hydrogen peroxide, hydroxyl radical, or singlet oxygen, which induces oxidative damage in lipids, proteins and DNA (Lee, Koo, & Min, 2004). Therefore, the scavenging of superoxide radical is very important for protecting bio-macromolecules in living cells from oxidative damage.

Data in Fig. 3a show the scavenging activities of crude CSPS, CSPS-1, CSPS-2 and CSPS-3 on superoxide radical. The scavenging effects of crude CSPS and its purified fractions were evident at all of the tested concentrations. The scavenging effects of crude CSPS, CSPS-1, CSPS-2 and CSPS-3 were correlated well with the increase of concentration up to 4.0 mg/ml. CSPS-3 showed stronger scavenging effect than crude CSPS, but the other two purified fractions (CSPS-1 and CSPS-2) had weaker activity than crude CSPS. At a concentration of 4.0 mg/ml, the scavenging activity of crude CSPS, CSPS-1, CSPS-2 and CSPS-3 was 67.8, 52.5, 39.6 and 75.6%, respectively. The results indicated that CSPS had strong scavenging activity on superoxide radical. The strong scavenging activity of CSPS-3 might be attributed to its high contents of protein, uronic acid and sulfuric radical.

3.3.2. Scavenging activity on hydroxyl radical of CSPS

Hydroxyl radical is one of the most reactive free radicals and can react with all bio-macromolecules in living cells (Huang, Ou, & Prior, 2005; Sun et al., 2009). However, no enzymes found in organisms are known to degrade hydroxyl radical (Akashi, Nishimura, Ishida & Yokota, 2004). Therefore, screening some specific non-enzymatic or dietary antioxidant with hydroxyl radical scavenging effect from natural resources is extremely important for the increased function of the antioxidant defense system in the living cells.

The hydroxyl radical, generated by the Fenton reaction in the system, was scavenged by crude CSPS, CSPS-1, CSPS-2, CSPS-3 and ascorbic acid. The hydroxyl radical scavenging effects of crude CSPS, CSPS-1, CSPS-2, CSPS-3 and ascorbic acid were increased with the increase of concentration up to 4.0 mg/ml (Fig. 3b). The scavenging effect of crude CSPS and CSPS-3 was higher than that of CSPS-1 and CSPS-2. At a concentration of 4.0 mg/ml, the hydroxyl radical scavenging activity was 86.2, 12.4, 19.1 and 74.5% for crude CSPS, CSPS-1, CSPS-2 and CSPS-3, respectively. However, ascorbic acid showed higher hydroxyl radical scavenging activity than crude CSPS. The results demonstrated that crude CSPS and CSPS-3 possessed strong scavenging activities on hydroxyl radical.

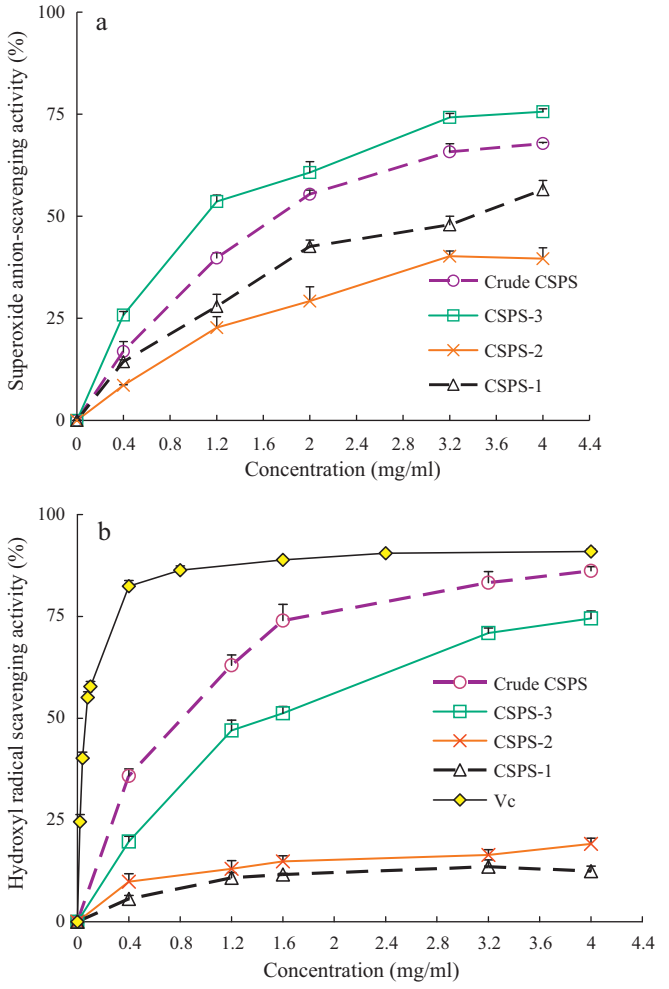

Fig. 3. Scavenging effects on superoxide radical (a) and hydroxyl radical (b) of crude CSPS, CSPS-1, CSPS-2 and CSPS-3.

3.4. Inhibitory effect of CSPS on BGC-823 cell proliferation

Human gastric cancer line BGC-823 is an ideal model for the study of cell proliferation (Chen et al., 2008). In this study, the inhibitory effects of CSPS, CSPS-1, CSPS-2 and CSPS-3 on the growth of BGC-823 cells were investigated by MTT assay. As showed in Fig. 4, all polysaccharides exhibited a dose-dependent activity within the concentration range of 50–400 μg/ml, and the inhibitory effects of CSPS-1, CSPS-2 and CSPS-3 increased significantly ($P < 0.05$) with the increase of sample concentration. At a

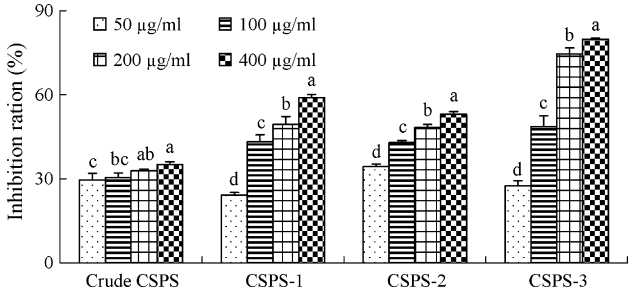

Fig. 4. Inhibitory effects *in vitro* of crude CSPS, CSPS-1, CSPS-2 and CSPS-3 at different concentrations against human gastric cancer BGC-823 cells. Values are presented as means ± standard deviation (SD) of three independent experiments. Different alphabets (a–d) in superscript for each treatment (crude CSPS, CSPS-1, CSPS-2 or CSPS-3) denote significant difference ($P < 0.05$).

concentration of 400 μg/ml, the inhibitory effects of crude CSPS, CSPS-1, CSPS-2 and CSPS-3 were 35.16, 58.98, 42.91 and 79.89%, respectively. Obviously, CSPS-3 showed strong inhibitory effect on the growth of BGC-823 cells. The higher activity of CSPS-3 might be attributed to its high contents of protein, uronic acid and sulfuric radical and relative more complicated monosaccharide composition.

The level of anti-oxidation and reactive oxygen species is correlated well with the generation and malign transformation of cancer cells. If compounds can enhance the level of anti-oxidation and clear the reactive oxygen species in cancer cells, they may inhibit the cells growth (Leng, Liu, & Chen, 2005). Therefore, the higher anticancer activity of CSPS-3 might be attributed to its higher scavenging activity on free radicals, such as superoxide radical and hydroxyl radical.

4. Conclusions

In this study, CCD was employed to optimize the parameters for the extraction of CSPS. As a result, the maximum yield of crude CSPS (15.52 ± 1.26%) was obtained under the following conditions: extraction temperature, 90 °C; extraction time, 250 min; ratio of water to raw material, 29; times of extraction, two times. Then, three purified fractions of CSPS-1, CSPS-2 and CSPS-3 were obtained from the crude CSPS through sequential purification purified by chromatography of DEAE-52 and Sephadex G-100. The chemical and GC analytic results demonstrated that CSPS-3 was quite different from CSPS-1 and CSPS-2. CSPS-3 contained 6.34% of protein, relatively large amounts of uronic acid and sulfate and had more complicated monosaccharide composition than the other two fractions. Furthermore, the antioxidant and anticancer activities *in vitro* crude CSPS and its purified fractions were evaluated. The results demonstrated that crude CSPS and CSPS-3 possessed strong scavenging activities on superoxide radical and on hydroxyl radical. For anticancer activity, all polysaccharides exhibited a dose-dependent activity within the concentration range of 50–400 μg/ml. At a concentration of 400 μg/ml, the inhibitory effects of crude CSPS, CSPS-1, CSPS-2 and CSPS-3 were 35.16, 58.98, 42.91 and 79.89%, respectively. The results indicate that CSPS may be a new source of natural antioxidants with potential value for health food and therapeutics. Further works on the structure and biological activity *in vivo* of CSPS is in progress.

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