Ultrasonically Assisted Extraction and HPLC Determination of Chondroitin Sulfate from Fish Heads

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(Received on 2nd December 2011, accepted in revised form 27th February 2012)

Summary: In the present study, we investigated the ultrasonically assisted extraction(UAE) and high-performance liquid chromatography (HPLC) determination of chondroitin sulfate(CS) from fish heads. Firstly, the optimized parameters for CS extraction were obtained by using Box–Behnken design(BBD) and response surface methodology(RSM) based on the single-factor experiments. As results, the optimum conditions were ultrasonic time 45min, ultrasonic power 280W and solvent/material ratio 6ml/g. Under these conditions, the experimental yield of CS was 4.623%. Secondly, the CS was purified by DEAE-Cellulose 52 chromatography and Sephadex G-100 chromatography to afford purified CS. Finally, we developed a simple method for the determination of glucuronic acid (GlcA) and N-acetylgalactosamine (GalNAc) in purified CS by HPLC with a diode array detector (DAD). The results showed that there were obvious saccharides peaks in CS.

Introduction

In the past decades, it has been found that the polysaccharides in animals were a potential source of bioactive products. Plysaccharides have attracted much attention in recent years, due to their strong biological activities[1,2]. A large amount of cartilage in the fish heads is produced as a by-product from fishery industries. Cartilage matrix is composed of glycosaminoglycans which are mainly chondroitin sulfate(CS), presenting in the form of proteoglycans[3]. CS is a polymeric carbohydrate which comprises a repeating disaccharide motif of glucuronic acid (GlcA) and N-acetylgalactosamine (GalNAc), often modified by sulphate groups replacing one, or more, of the OH groups on C4 and C6 of GalNAc and C2 and C3 of GlcA. Over the last decade, published literatures have indicated that CS possessed a number of biological activities such as anti-inflammatory, antioxidant, immunoregulatory, antibacterial, antiviral and antitumor activities[4]. And CS has attracted much attention by consumers and researchers due to its broad spectra of therapeutic properties and good safety. Therefore, it is necessary to develop some methods to extract and detect CS from fish heads.

However, conventional extraction of polysaccharides from animal materials required long time, high temperature and exhibited low efficiency. Ultrasonically assisted extraction(UAE) has been employed for preparing bioactive compounds from plants and different materials in numerous studies[5], which has been proved to be effective[6]. In this decade, a great amount of research has developed to state the applicability of ultrasound in diverse processes. Nowadays it is accepted that the ultrasound can facilitate diverse processes of extraction. Usually, UAE was used in plants active ingredients extraction[7,8,9], but there was limited number of publications on UAE from animal material. Attempt was made to extract chitin from fresh water prawn shells[10] by using sonication. However, there is little information about the preparation of CS from fish heads by UAE technology. The great extraction efficiency by ultrasonic treatment is mainly due to ultrasonics involves superficial tissue disruption, increasing surface mass transfer intraparticle diffusion and loading of the extraction chamber with substrate[6]. Cartilage is a tissue formed by a matrix of collagen associated with proteoglycans, macromolecules with an axial protein to which the CS is covalently attached. Furthermore, the CS is more easy to dissociate from the polysaccharide-protein complexes by UAE technology[11]. Usually, the carbazole assay[12] and Elson Morgan's assay[13] were used to determine the contents of GlcA and GalNAc in the CS, respectively. However, these methods are less sensitive and likely interference with other saccharides in the polysaccharide samples[14]. Therefore, determination of GlcA and GlcNAc in the CS produced from fish heads by more sensitive method would be important for the elucidation of

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function and utilization of the polymers.

The main objective of the present study was to develop an response surface methodology to optimize the extraction parameters of CS from fish heads through UAE technology, and a sensitive method for the determination of GlcA and GalNAc in the CS by high-performance liquid chromatography (HPLC) with a diode array detector (DAD). It is to help us to take action for future study the polymers.

Results and discussion

Single Factor Experiment for Extraction of CS

Effect of Ultrasonic Time on the Yield of CS

Ultrasonic time was one factor that would influence the extraction efficiency and selectivity of the fluid. It has been reported that a long ultrasonic time favored the production of polysaccharides[8]. However, excessive lengthening of ultrasonic time may induce the change of polysaccharides molecule structure[9]. In the present study, the effect of ultrasonic time on the yield of CS was investigated using different ultrasonic time (15, 30, 45, and 60min), while other extraction variables were set as follows: ultrasonic power 200W, extraction ratio of solvent to material 3 and extracting times 1. The yield sharply increased and varied from 2.709% to 4.549% when the ultrasonic time increased from 15min to 45min (Fig. 1A). The yield decreased slightly when the ultrasonic time over 45min,which may be due to the increase in some protein with increasing ultrasonic time that decreased the yield of total CS. Statistical analysis showed that significant differences were existent among 15, 30, 45, and 60min ($p < 0.05$). Thus, extraction time of 30–60min was favorable for the production of CS. For saving of energy and lowering of cost, 45min was selected as the centre point of ultrasonic time in the RSM experiments.

Effect of Ultrasonic Power on the Yield of CS

To investigate the effect of ultrasonic power on the yield of CS, extraction process was carried out using different ultrasonic power of 120, 200, 280, and 360W, while other extraction parameters were fitted as follows: ultrasonic time 60min, extraction ratio of solvent to material 3 and extracting times 1. Ultrasonic treatment has mechanical effects that facilitate mass transfer between immiscible phases through a super agitation, especially at low frequency[9]. As shown in Fig. 1B, there was an increasing trend in the yield of CS from 120 to 280W. However, application of high ultrasonic power results in degradation effect, which further affects the yield when the ultrasonic power was exceed 280W, such as 360W. Statistical analysis showed that significant differences were existing among 120, 200, 280, and 360W($p < 0.05$). Thus, ultrasonic power of 200–360W was favorable for the production of CS. Therefore, 280W was selected as the centre point of ultrasonic power in the RSM experiments.

Effect of Solvent/ Material Ratio on the Yield of CS

Ratios of solvent to material were set at 1, 3, 6, and 9 in order to investigate the effect of different extracting ratio of solvent to material on the yield of CS. In the range of 1–9 for the ration of water to raw material, significant differences were existent between 1 and 3, 3 and 6, 6 and 9 ($p < 0.05$) as shown in Fig. 1C. The yield of CS significantly increased from 2.753 to 4.631% as the ratio of solvent to material increased from 1 to 6. This may due to the increase of the driving force for the mass transfer of the polysaccharides[15]. Therefore, 6 was selected as the centre point of extracting ratio of solvent to material in the RSM experiments.

Effect of Extraction Times on the Yield of CS

Fig. 1D showed the effect of extraction times on the yield of CS while other extraction parameters were fitted as follows: ultrasonic time 60min, ultrasonic power 200W, and extraction ratio of solvent to material 3. From Fig. 1D, we found that there was an increasing trend in the yield of CS accompanying the increase of extracting times, but there was not significant difference ($p > 0.05$) between 2 times, 3 times and 4 times. Taking the yield and processing cost into consideration, 2 times was sufficient for the extraction of CS. Thus, 2 times was selected as the extracting times in the next experiments.

Model Fitting and Optimization for Extraction of CS

Model Fitting

By using the software of Design Expert version 7.1.3, a polynomial model describing the correlation between the extraction yield of CS and the three variables was obtained as follows:

$$Y=4.58+0.21\ A+0.26\ B+0.22C+0.29AB-0.072\ AC+0.25BC-0.70A^2-0.60B^2-0.41C^2$$

Where Y represents the yield of CS, A, B

and C represent ultrasonic time, ultrasonic power and solvent to material ratio, respectively.

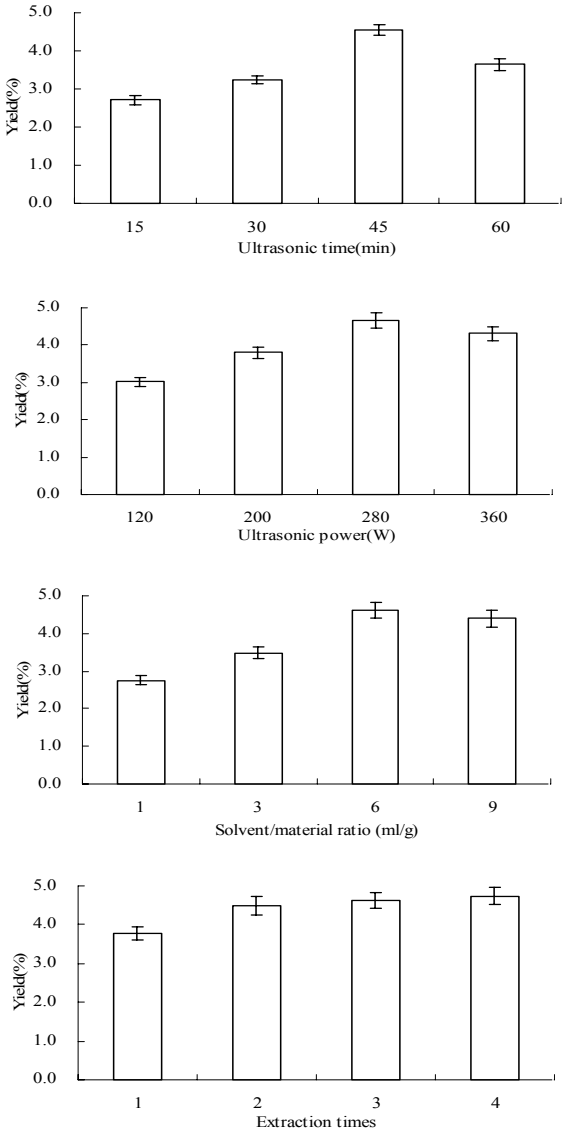

Fig.1.: Effects of ultrasonic time (A), ultrasonic power (B), solvent/material ratio (C) and extraction times (D) on the yield of CS.

The results of ANOVA, lack-of-fit and the adequacy of the model were summarized in Table-1. The model *F*-value of 30.021 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 0.9747, indicating that 97.47% of the variability in the response could be explained by the model. In addition, the p-value of $p = 0.0139$ for lack-of-fit implied the lack-of-fit was significant relative to the pure error, indicating the model equation was adequate to predict the extraction yield of CS within the range of experimental variables.

Table-1: Analysis of variance for the response surface quadratic model for CS yield[a].

Source	Sum of squares	Degrees of freedom	Mean square	F-value	P-value
Model	6.622	9	0.736	30.021	< 0.0001
A	0.360	1	0.360	14.706	0.0064
B	0.539	1	0.539	22.003	0.0022
C	0.391	1	0.391	15.961	0.0052
AB	0.328	1	0.328	13.397	0.0081
AC	0.020	1	0.020	0.834	0.3914
BC	0.244	1	0.244	9.938	0.0161
A^2	2.047	1	2.047	83.520	< 0.0001
B^2	1.496	1	1.496	61.024	0.0001
C^2	0.723	1	0.723	29.515	0.0010
Residual	0.172	7	0.025		
Lack of fit	0.157	3	0.052	13.921	0.0139
Pure error	0.015	4	0.004		
Total	6.793	16			

[a] A, Ultrasonic time; B, Ultrasonic power; C, solvent/material ratio.
$R^2 = 0.9747$, adjusted $R^2 = 0.9423$.

The significance of the regression coefficients was tested by *F*-test, and the corresponding *P*-values for the model terms were also listed in Table-1. 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, A, B, C, AC, BC, A^2, B^2, and C^2 were significant ($p < 0.05$), while AC was not significant ($p > 0.05$).

Optimization for Extraction of CS

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 was negligible, while an elliptical contour plot indicates that the interaction between related variables was significant[16]. 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 CS production. It was 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 CS were ultrasonic time 45min, ultrasonic power 280W and ratio of solvent to material 6mL/g. Using the optimal conditions, the maximum predicted extraction yield of CS was 4.580%, which corresponded fairly well to that of real extraction (4.576 ± 6.12%). The result suggested that the regression model was accurate and adequate for the prediction of CS extraction.

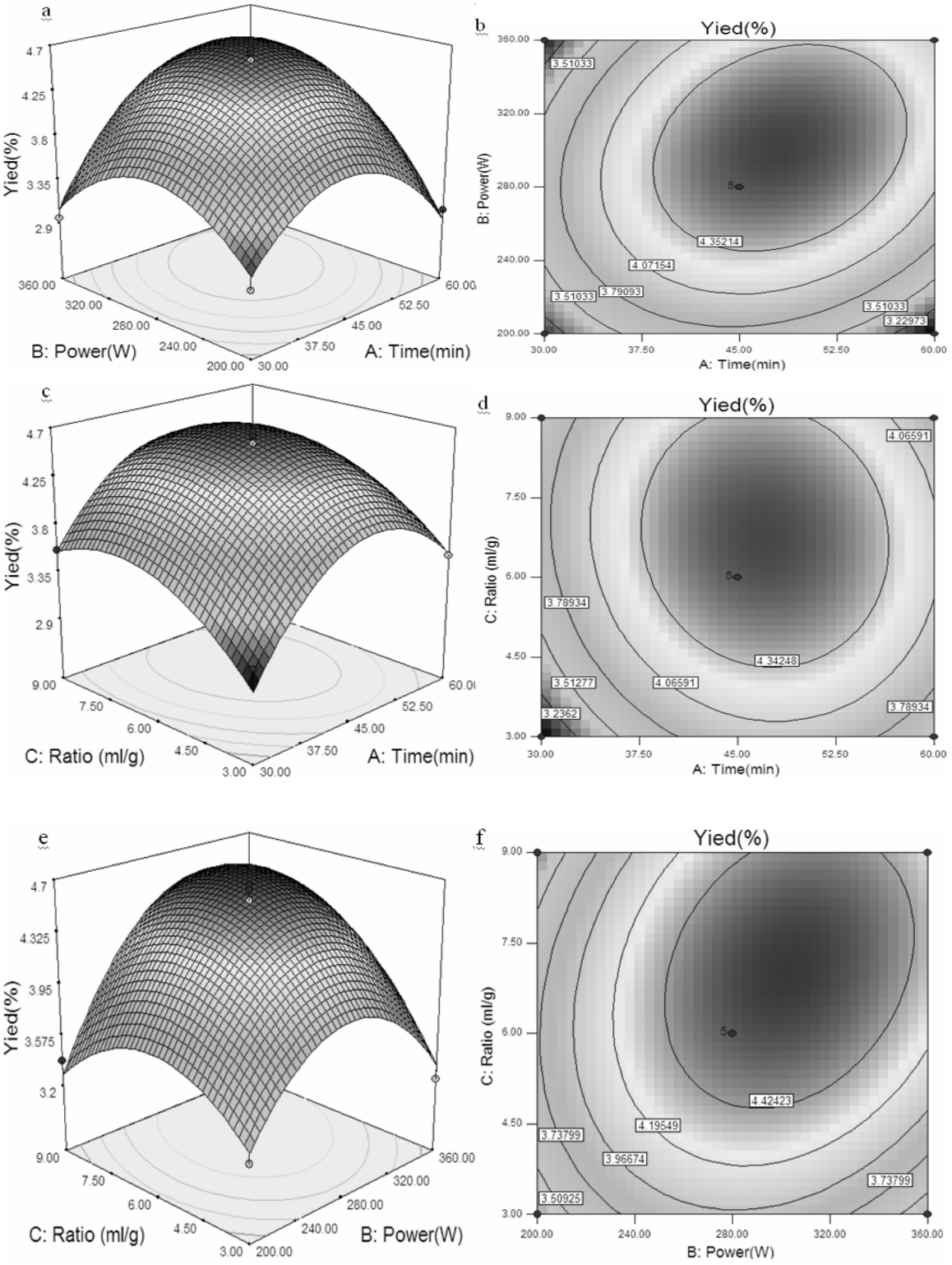

Fig.2: Response surface plots (a, c and e) and contour plots (b, d and f) showing the effects of ultrasonic time, ultrasonic power, solvent/material ratio and their mutual effects on CS.

Determination of Chondroitin Sulfate by HPLC

Determination of glucuronic acid in CS

Methods to quantify glucuronic acid have been reported, anion exchange chrom-
atography was the most common HPLC mode for GlcA analysis[17]. Method for uronic acid microanalysis by normal-phase partition chromatog-raphy (NPPC) with postcolumn fluoresceence deriva-teization has been reported[18]. Heiss has detected the GlcA by extracted ion chromatograms of the methylation analysis with Hakomori permethyl-ation and prereduction[19]. However, these methods would be added to the cost and time consuming. Therefore, we decided to utilize a DAD for the quan-tifycation of glucuronic acid using an Shodex SUGAR KS-801 column. Standard GlcA prepared as the used stock solution, was determined using the suggested HPLC procedures. Results were found to be reproducible, Fig.3A, the chromatograms of standard glucuronic acid and Fig.3B, glucuronic acid obtained from acid hydrolysed chondroitin sulphate came from fish heads.

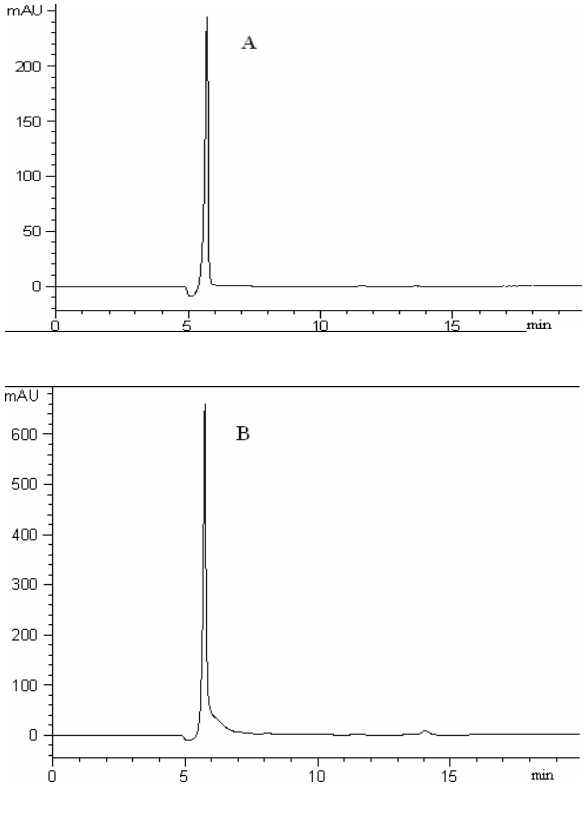

Fig.3: HPLC chromatograms of (A) standard glucuronic acid (1.0 mg/ml), and (B) acid hydrolysed chondroitin sulphate from fish heads (3.0 mg/ml).

Determination of N-acetylglucosamine in CS

Method to quantify glucosamine by HPLC with evaporative light scattering detector (ELSD) has been reported. Recently, glucosamine was analyzed by HPLC with fluorometric or UV detection after derivatization reagents, or high pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) [20]. Furthermore, a quantitative densitometric high-performance thin-layer chromatography (HPTLC) [21] method and a HPLC–ESI-MS/MS method[22] were developed for the determination of glucose-amine, but these methods were expensive and laborious. Thus we used a Sugar-D column with DAD to quantify the content of glucosamine sulphate. The typical chromatograms of glucosamine standards and glucosamine obtained from acid hydrolysed chondroitin sulphate came from fish heads were showed in Fig.4A and Fig.4B, respectively.

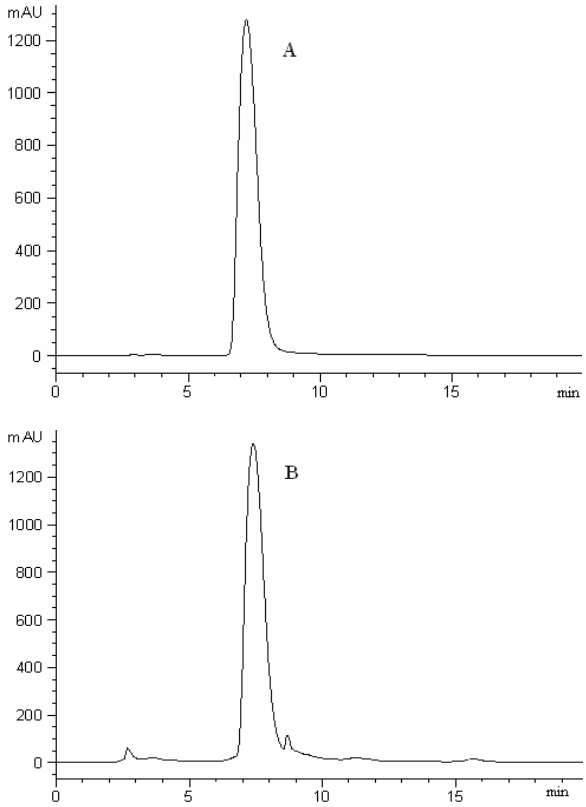

Fig.4: HPLC chromatograms of (A) standard glucosamine (1.0mg/ml), and (B) acid hydrolysed chondroitin sulphate from fish heads (3.0 mg/mL).

Experimental

Materials and Reagents

D-Glucuronic acid (GlcA) sodium salt monohydrate and N-acetylgalactosamine (GalNAc) were purchased from Sigma Chemical Co. (St. Louis, USA). All other chemicals were of analytical grade.

Instrumentation

Agilent 1100 HPLC system equipped with a diode array detector (DAD). Sugar-D column (Nacalai Tesque Inc., Kyoto). Shodex SUGAR KS-801 column (Showa denko KK, Tokyo).

Extraction of CS

Carassius auratus (Linnaeus) were obtained from Longzi Lake, Bengbu, Anhui province of China. Chondroitin sulfate was extracted according to the method reported by Nakano[23-25] with slight modification. Briefly, the fresh fish heads were heated at 80 ℃ water bath for 60 min in order to remove the protein and fat, then the skeletons were washed with pressurized water, dried at 50 ℃ and pulverized into powder, finally extracting with alkaline solution. The extraction process was performed at different ultrasonic time, ultrasonic power and solvent/material ratio. The extract was filtered through a Whatman No. 1 filter paper and the filtrate was then concentrated with a rotary evaporator at 60 ℃ under vacuum. And more anhydrate ethanol was added to the supernatant to a final concentration of 80% (v/v). It was kept overnight at 4℃ and centrifuged to collect precipitate, all the samples were lyophylized.

The CS yield of extraction was calculated according to the formula below:

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

where $W_1$is the weight of CS and W_0 is the dried weight of fish heads.

Box–Behnken Design for the Extraction of CS

On the basis of single-factor experiment for CS production, proper ranges of ultrasonic time, ultrasonic power, extraction times and solvent/material ratio were preliminarily determined. A three-level, three-variable Box–Behnken design (BBD) (software Design-Expert v.7.1.3, Stat-Ease, Inc, Minneapolis, USA) was applied to determine the best combination of extraction variables for the production of CS. Based on the investigations on single-factor experiment, three variables used in this study were ultrasonic time (30–60min, A), ultrasonic power (200–360W, B) and solvent/material ratio (3–9 mL/g, C), with three levels for each variable, while the dependent variable was the yield of CS. The symbols and levels were shown in Table-2. Five replicates at the center of the design were used to allow for estimation of a pure error sum of squares. Experiments were randomized to maximize the effects of unexplained variability in the observed responses due to extraneous factors. A full quadratic equation or the diminished form of this equation, shown as follows, was used for this model.

$$Y = \beta_0 + \sum_{j=1}^{k}\beta_j X_j + \sum_{j=1}^{k}\beta_{jj} X^2_j + \sum\sum_{i<j}\beta_{ij} X_i X_j$$

where Y is the estimated response, β_0, β_j, β_{jj} and β_{ij} are the regression coefficients for intercept, linearity, square and interaction, respectively, while X_i and X_j are the independent variables coded ($i \neq j$).

Table-2: Box–Behnken design matrix and the response values for the yield of CS.

Standard order	Ultrasonic time (min)	Ultrasonic power (W)	Solvent /material Ratio (ml/g)	Polysaccharide yield (%)	
				Experimental	Predicted
1	60(1)	280(0)	9(1)	3.631	3.828
2	30(-1)	280(0)	3(-1)	3.155	2.968
3	30(-1)	360(1)	6(0)	2.955	3.040
4	60(1)	200(-1)	6(0)	3.038	2.940
5	30(-1)	200(-1)	6(0)	2.973	3.100
6	45(0)	280(0)	6(0)	4.657	4.580
7	60(1)	360(1)	6(0)	4.166	4.040
8	60(1)	280(0)	3(-1)	3.509	3.532
9	45(0)	280(0)	6(0)	4.623	4.580
10	45(0)	280(0)	6(0)	4.536	4.580
11	30(-1)	280(0)	9(1)	3.563	3.552
12	45(0)	360(1)	9(1)	4.364	4.300
13	45(0)	360(1)	3(-1)	3.251	3.360
14	45(0)	280(0)	6(0)	4.512	4.580
15	45(0)	280(0)	6(0)	4.553	4.580
16	45(0)	200(-1)	3(-1)	3.261	3.340
17	45(0)	200(-1)	9(1)	3.387	3.280

Purification of CS

The CS (50 mg) dissolved in de-ionized water was applied to a DEAE Cellulose-52 column (2.6 × 20 cm). The column was eluted with 0.5 M sodium chloride solution at a flow rate of 1 mL/min. And fractions (5 ml/tube) were collected automatically and checked by the carbazole method[12].

The fractions containing CS were collected, concentrated, dialyzed, lyophilized and further purified through Sephadex G-100 column (2.6 × 60 cm) to afford purified CS. Finally, purified CS was lyophilized for further study.

Determination of CS come from Fish Heads by HPLC

Analysis of Glucuronic Acid

The purified CS came from fish heads was weighed and finely powdered. A 3-5 mg of CS was suspended in 5 ml of 2M trifluoroacetic acid and refluxed at 120℃ for 2h. The generated monosaccharide co-concentrated with methanol, filtrated and analyzed on Aglient 1100 HPLC System equipped with a DAD and a Shodex SUGAR KS-801 column. The column was eluted with 100% water at a flow rate of 0.8 ml/min, and oven temperature was set at 40 ℃. The total run time was 10 min between each routine injection. And a 1-2mg GlcA standard was performed at the same condition as mentioned above. Then, peaks of samples were identified by comparing retention time with GlcA standard.

Analysis of N-acetylgalactosamine

The purified CS came from fish heads was weighed and finely powdered. A 3-5mg of CS was suspended in 5 ml of 6M hydrochloric acid at a 25ml measuring flask, boiling waterbath for 2h. The generated monosaccharide co-concentrated with methanol, filtrated and analyzed on Aglient 1100 HPLC System equipped with a DAD and a Sugar-D column. The column was eluted with 80:20 of acetonitrile:water (v/v) at a flow rate of 1.0 ml/min, and oven temperature was set at 40℃. The total run time was 18 min between each routine injection. And a 1-2mg GalNAc standard was performed at the same condition as mentioned above. Then, peaks of samples were identified by comparing retention time with GalNAc standard.

Statistical Analysis

Analysis of the experimental design and data was carried out using Design Expert software of version 7.1.3 (Stat-Ease Inc., Minneap-olis, USA). 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 16.0 for windows (SPSS, Chicago, IL, USA). Multiple comparisons of means were done by the least significance difference (LSD) test. Differences were considered to be statistically significant if $p < 0.05$.

Conclusions

In the present study, the CS from fish heads was prepared by UAE technology. Based on the single-factor experiments, BBD from RSM was used for optimizing extraction parameters in this work. The optimal conditions for the production of CS were as the following: ultrasonic time 45min, ultrasonic power 280W and solvent/material ratio 6ml/g. Under these conditions, the experimental yield of CS was 4.623%. Then, the purified CS was obtained through DEAE-Cellulose 52 chromatography and Sephadex G-100 chromatography. Furthermore, a simple method for determination of GlcA and GalNAc in purified CS by high-performance liquid chromatography with a diode array detector was developed. The results showed that there were obvious saccharides peak in CS.

Acknowledgements

This work was partly supported by the Universities Natural Science Research Project of Anhui Province (KJ2012B094) and the Dr. Scientific Research Foundation of Bengbu College.

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