EXTRACTION OF CHONDROITIN SULFATE FROM CHICKEN KEEL CARTILAGE BY COMBINED APPLICATIONOF ULTRASOUND TREATMENT AND ALCALASE HYDROLYSIS

Truong Ngoc Thao*, Vu Nguyen To Nhu, Nguyen Thi Nguyen, Dong Thi Anh Dao*

Dept. Food Technology, Ho Chi Minh city University of Technology, 268 Ly Thuong Kiet, 10 District, Ho Chi Minh City

*Email: *dtanhdao@hcmut.edu.vn; truongngocthao1995@gmail.com*

Received: 13 July 2018; Accepted for publication: 9 October 2018

ABSTRACT

Chondroitin sulfate (CS) is a glycosaminoglycan composed of *N*-acetyl-D-galactosamine and D-glucuronic acid sulfated at positions 4 or 6. Chondroitin sulfate is used in functional food for supporting treatment of arthritis. Chicken keel cartilages, a by-product of the poultry slaughter industry, can be used as a source for CS production. Our study was undertaken to extract CS from chicken keel cartilages by the combined application of ultrasound treatment and Alcalase hydrolysis. The variables of the ultrasound treatment process, such as ratio of material and buffer, pH, temperature, and time were investigated. The optimal conditions were obtained by the Circumscribed Central Composite design method using the Modde 5.0 software. Optimal values for this process were found at pH9, ratio of material to buffer 1:9 ($w_{material}/v_{buffer}$), temperature 47.34 ^{o}C, and time within 9.2 minute. After ultrasound treatment, hydrolysis using the enzyme Alcalase was performed under the following conditions: ratio enzyme to substrate 4 % (v/w), temperature 55 ^{o}C, and time 60 minutes. HPLC analysis showed that the powder product contained 80.45 % CS with an average molecular weight of 159.53 kDa (determined by GPC). The overall yield of CS was 24.29 % of the absolute dry weight, and 61.02 % of the total carbohydrate content of the starting material.

Keywords: glycosaminoglycans, chondroitin sulfate, alcalasehydrolysis, chicken keel cartilage, ultrasound treatment.

1. INTRODUCTION

Glycosaminoglycans (GAGs) are large linear polysaccharides constructed of repeating disaccharide units of an amino sugar (either GlcNAc or GalNAc) and an uronic acid (either glucuronic acid or iduronic acid). GAGs' primary role is to maintain and support collagen, elastin and turgidity in the cellular spaces. Based on core disaccharide structures, GAGs are classified into five groups: chondroitin sulfate (most prevalent GAGs), dermatan sulfate, heparan sulfate, keratan sulfate, andhyaluronic acid. Chondroitin sulfate (CS) is a polymeric carbohydrate which comprises a repeating disaccharide motif of glucuronicacid (GlcA) and *N*-acetyl-galactosamine (GalNAc), often modified by sulfate groups replacing one, or more, of the

OH groups on C4 and C6 of GalNAc6 and C2 and C3 of GlcA [1]. It has been reported to have a wide range of applications in the pharmaceutical, cosmetic, and food industries for its anti-degenerative arthritis, anti-inflammation, antiatherogenic, antitumor and hypolipidemic capacities [2]. Thus, it is necessary to release the CS from chicken keel cartilage.

Although common sources of CS include bovine tracheal cartilage and shark cartilage, it is also useful to explore other sources for the CS production. The chicken keel cartilage appears to be a potential source, because it remains on the frame of the carcass after removal of the chicken breast fillets, it can be easily collected for the CS isolation [3]. In Viet Nam, agricultural statistics showed a rapid growth of the poultry meat industry; chicken keel cartilage was discarded as waste from chicken processing. Therefore, by investigating a combined use of ultrasound treatment and Alcalase hydrolysis, this study aimed to determine the extraction method that yields is the most of CS from the chicken keel cartilages.

2. MATERIALS AND METHODS

2.1. Materials

Chicken keel cartilage was provided from Pham Ton Co. Ltd, Go Vap district, Ho Chi Minh City, stored at -18 ^{o}C in sealed Polypropylene bags. The chemical compositions of chicken keel cartilages were determined and shown in Table 1. The content of the basic components similar to the analysis results of Shin *et al.* [4].

Table 1. Chemical compositions of chicken keel cartilage.

Compositions	Content (%)
Moisture	77.74±1.35
Protein	11.8±0.12
Lipid	0
Ash	1.6 ± 0.27
Total carbohydrate	8.86 ± 0.86

Standard CS from shark cartilage (assay ≥ 90 %, USP standards), 1,9-dimethylmethylen blue (DMMB) (Sigma-Aldrich, USA), Folin-Ciocalteu (Merck), Acid trichloacetic (TCA), $Na_2HPO_4.12H_2O$, KH_2PO_4, $CuSO_4$ (China), Ethanol 96 % (v/v) (Viet Nam).

Enzyme Alcalase 2,4 L (a bacterialendoprotease of *Bacillus licheniformis*) were obtained from Novozymes (USA), stored at 4 ^{o}C. The unit activity of Alcalase is 2.4AU-A/g.

2.2. Methods

2.2.1. Experimental design

2.2.1.1. Pretreatment of chicken keel cartilage by heat treatment

Chicken keel cartilages was pretreated by blanching that affects CS content through two factors of the blanching temperature and time: surveyed temperature range from 60 to 90 ^{o}C with step 5 ^{o}C and duration time from 2 to 10 minutes [5-9]. The function of the heat treatment is to inhibit available enzyme activities in material and to remove easily the surrounding tissues before proceeding to the next stages.

2.2.1.2. Preparation of crude CS from chicken keel cartilage by ultrasound treatment

After removing the surrounding tissues, a sample mass of 50 g chicken keel cartilage was used for each experiment**,** washed in NaCl solution 0.9 %, pretreated by blanching in hot water with survey parameters such as temperature, time and conducted milling to reduce particle size. After adding in buffer of sodium phosphate solution, carrying out the ultrasound treatment by ZZLINKER 5L at ultrasonic frequency 40 kHz with power of 90 W, surveyed variables such as: the ratio of material sample and phosphate buffer from 1:6 to 1:14 (v/v); buffer pH was adjusted in the range 7÷11; ultrasonic temperature from 30 to 70 ^{o}C at the time thresholds as follows: 0, 5, 10, 15 and 20 minutes [5 -12].

2.2.1.3.Extraction of CS from chicken keel cartilage by Alcalase hydrolysis

After ultrasound treatment, cartilage was hydrolyzed by Alcalase, withstirring at the rate 120rpm. The hydrolysis variables were investigated with enzyme concentration from 0 to 6 % (v/w_{pro}), incubation temperature was varied from 40 to 65 ^{o}C for the time of 0 to100 minutes [12]. At the end of hydrolysis, enzyme was inactivated at 80 ^{o}C for 10 minutes, then using trichloacetic acid 4 % w/v (TCA) to precipitate protein; the samples were filtered by vacuum filtration method through Whatman filter paper of 45 µm. Then the filtrate was dialyzed by dialysis tubing cellulose membrane with MWCO14 kDa for 3 hours to remove out impurities like TCA, amino acids, and minerals. Then the filtered solution was precipitated by ethanol 96 % (v/v), centrifuged to obtain solid CS; continually that was dissolved in water to form CS solution 5 % (w/v). The powdered CS was obtained by spray drying by the Lab-Plant SD-06AG laboratory scale spray dryer at 120 ^{o}C and flow rate 285 mL/h. The chemical composition of CS was analyzed by HPLC. The molecular weight of CS was determined by GPC.

2.2.2. Analytical method

2.2.2.1. Determination of the physicochemical and chemical characteristics of chicken keel cartilage

Moisture content, protein, lipid, and ash were determined by the following methods: ISO 7514:1990; AOAC 2001.11; AOAC 2003.06; TCVN 7142:2002.

2.2.2.2.Analysis of CS content by UV-Vis absorption spectrophotometry

The method to determine CS is the use of dimethylmethylene blue developed by Farndal e*et al*. [13].

The powdered CS was dissolved in the de-ionized water and determined according to Farndale et al. method [13]. Using ΔDi4s(ΔUA-[1→3]-GalNAc-4s) as a standard; 1,9-DMMB was used to react with CS. Then using spectrophotometer, immediately measured at wavelength

λ = 525 nm (The test tube should be covered with silver paper to avoid directly exposure to light).

CS content formula:

$$H_{CS}\ \% = \frac{OD - 0{,}3083 \,.k.V}{2{,}7322 \,.m\,.\,100 - h\ .1000} \cdot 100\ \% \quad (1)$$

OD: absorption at wavelength λ = 525 nm; k: dilution factor; V: Volume of liquid sample; m: weight of kneel sample (g); h: humidity of sample; the values of 2.7322 and 0.3083 are coefficients of calibration curve equation: y = 2.7322x + 0.3083 with R^2 = 0.9934.

2.2.2.3. Optimization of ultrasound treatment by Circumscribed Central Composite (CCC) designs and Modde 5.0 software

Response surface methodology (RSM) with star distance of CCC (Circumscribed Central Composite) designs and Modde 5.0 software were used to carry out the experiments to optimize the ultrasonic conditions. The variables were coded according to the following equation:

$$x_i = \frac{Z_i - Z_{0_i}}{\Delta Z_i}, i = 1,2,3,4 \quad (2)$$

where x_i is the dimensionless coded value, Z_i is the actual value of variables, Z_{0i} is the actual value of variables at the center point, and ΔZ_i is the step change value.

2.2.2.4. Analysis of CS content by HPLC (High Performance Liquid Chromatography)

HPLC method was used to seperate, identify and quanlify each component in the mixture, based on the different affinity between substances with two phases that are always exposed but not mixed together.Applied condition of determination of CS content by HPLC according with High Performance Hitachi Liquid Chroma Detector with UV-Vis-5420 detector, with the Column of InertSustain ODS C18 (250 × 4.6 mm, 5 µm); the wavelength detection is 260 nm; and using buffer solution pH 4.0 by salt sodium octane sulfonic acid (0.6g/L) + TEA (triethylamin) (1.5 ppm), it was adjusted to pH 4 with phosphoric acid.

2.2.2.5. Molecular weight analysisof CS by GPC (Gel permeation Chromatography)

GPC method determines the average molecular weight based on the retention time in the chromatogram. In addition, GPC allows the determination of Number - avarage molecular weight (M_n), peak molecular weight (M_p) and Z- average molecular weight (M_z).

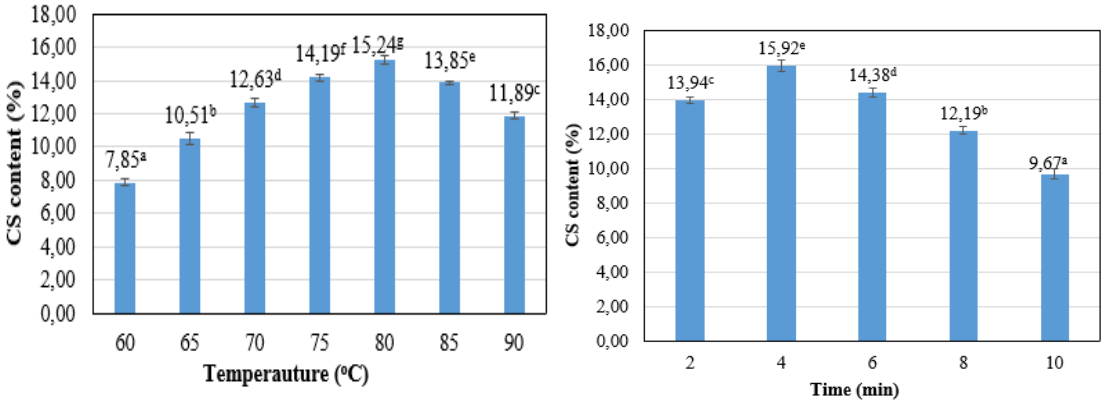
2.2.2.6. Statistical analysis

The experiments were performed in triplicates. The results were examined by analysis of variance (ANOVA) followed by Tukey's test (p-value < 0.05) and were presented as means of two determinations ± SD (standard deviation).

3. RESULTS AND DISCUSSION

3.1. The effects of heat treatment

The results of Figure 1 showed that the heat treatment of material at 80 ^{o}C for 4 minutes obtained the highest CS content, approximately 15.92 %. If the temperature rises, the protein molecules are dilated and denatured, the tertiary and quaternary structure of the protein can be transferred to the primary and secondary structureleading to therelease of CS compound from core protein. On the other hand, heating in the boiling waterforalong time, over 4 minutes leaded the denaturation of protein, released free CS into blanching watercausedCS lost. Therefore, blanching at 80 ^{o}C for 4 minutes was chosen for the next experiments.



a) *b)*

Figure 1. Effects of heat treatment: temperature (a)and time (b)on CS content.

3.2. The effects of ultrasound treatment

The results in Figure 2 showed that the ultrasound treatment affected the yield of CS. This can be significantly improved by the use of ultrasound treatment, as the energy generated from collapsing cavitational bubbles provides greater penetration of the solvent into the cellular material and improves mass transfer to and from interfaces. Therefore, the ultrasound treatment conditions would influence the extracted CS content.

The dynamics of extraction was the difference in protein concentration between solid phase and liquid phase. If a high amount of buffer was added in the sample, it would dilute the concentration of enzymes in followed hydrolysis. Thus, there was an optimum dilution ratio to achieve the maximum result. Besides, the temperature of ultrasound treatment affected the vapor pressure, surface tension, and viscosity of liquid medium. Increased temperature can cause a decrease in viscosity which allows for a more violent collapse and resultin increasing hydrolysis efficiency. The increase in ultrasonic time leads to longer cavitation bubbles, so that the cell structure of chicken keel cartilage was more broken, thus resulting in more extraction of CS.

After treatment with the ultrasonic bath of 40 kHz, there was a significant reduction in the size of particles and molecular weight of protein fraction. Therefore, the dilution ratio of material and buffer 1:10, pH 9 of sodium phosphate buffer, temperature 50 ^{o}C for 10 minutes, were chosen for the next experiments.

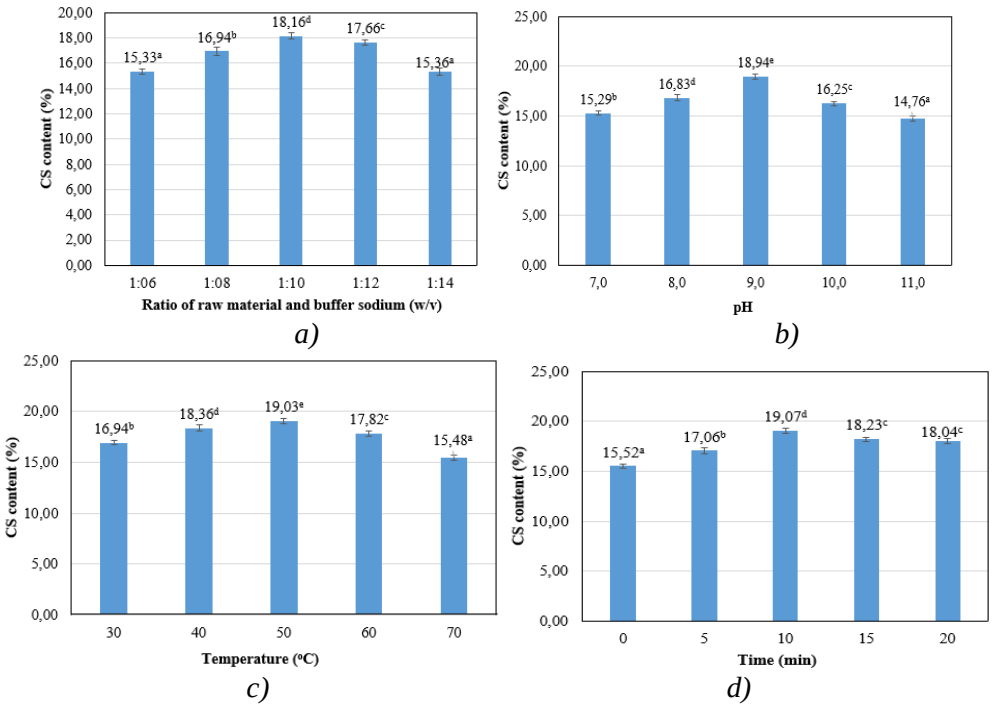


Figure 2. Effects of ultrasound variables: dilution ratio (a), pH (b), temperature (c), and time (d) to CS content.

3.3. Ultrasound treatment optimization usingCircumscribed Central Compositedesign

Experiments were sought to establish the relationship with four independent variables, including dilution ratio of raw material and buffer sodium (Z_1), pH (Z_2), ultrasonic temperature (Z_3) and ultrasonic time (Z_4).The levels of each variable were coded as -α, -1, 0, +1, +α (α = 2) in Table 2.

Table 2. Independent variables and their coded in actual level used in (CCC)design for optimizing.

Factor	Unit	Symbol	Levels				
			-α	**-1**	**0**	**1**	**+α**
Ratio material and buffer	% $w_{material}/v_{buffer}$	Z_1	6	8	10	12	14
pH		Z_2	7.0	8.0	9.0	10	11
ultrasonic temperature	^oC	Z_3	30	40	50	60	70
ultrasonic time	Minute	Z_4	0	5	10	15	20

Basing on the Circumscribed Central Composite design, Ultrasound treatment was carried out with 31 combinations of four independent variables, as per experimental designs were presented in Table 3.

Table 3. Actual levels of independence variables along with the observed value (CS content).

Experiments	Coded Variables				Response	Experiments	Coded Variables				Response
	x_1	x_2	x_3	x_4	Y (%)		x_1	x_2	x_3	x_4	Y (%)
1	-1	-1	-1	-1	15.8	17	- 2	0	0	0	15.39
2	1	-1	-1	-1	7.39	18	+2	0	0	0	9.62
3	-1	1	-1	-1	14.75	19	0	- 2	0	0	8.35
4	1	1	-1	-1	7.84	20	0	+2	0	0	11.83
5	-1	-1	1	-1	9.11	21	0	0	- 2	0	12.76
6	1	-1	1	-1	7.81	22	0	0	+2	0	9.38
7	-1	1	1	-1	12.1	23	0	0	0	- 2	8.8
8	1	1	1	-1	14.27	24	0	0	0	+2	7.81
9	-1	-1	-1	1	12.83	25	0	0	0	0	19.83
10	1	-1	-1	1	8.65	26	0	0	0	0	19.42
11	-1	1	-1	1	9.71	27	0	0	0	0	18.94
12	1	1	-1	1	10.34	28	0	0	0	0	20.06
13	-1	-1	1	1	8.2	29	0	0	0	0	19.42
14	1	-1	1	1	7.64	30	0	0	0	0	18.64
15	-1	1	1	1	9.51	31	0	0	0	0	20.31
16	1	1	1	1	11.28						

Regression model was used to calculate the predicted extracted CS content and compared with experimental result. High value of coefficient of determination (R^2 = 0.975; Q^2 = 0.738) shows the adequacy of the applied model. The ANOVA analysis also revealed that there was a non-significant (probability P = 0.061 > 0.05) lack of fit that further validated the model. The regression equation for the responsible function *Y* was as follow, with x_1, x_2, x_3, x_4 were coded variables:

$$Y(\%) = 19.513 - 1.063x_1 + 0.725x_2 - 0.531x_3 - 0.484x_4 + 0.610x_1x_2 + 1.054x_1x_3 + 0.610x_1x_4 + 0.827x_2x_3 - 0.268x_2x_4 - 0.121x_3x_4 - 1.420x_1^2 - 1.887x_2^2 - 1.697x_3^2 - 2.233x_4^2 \quad (3)$$

Predicted CS content (%) (of absolute dry material)can be calculatedby the following equation:

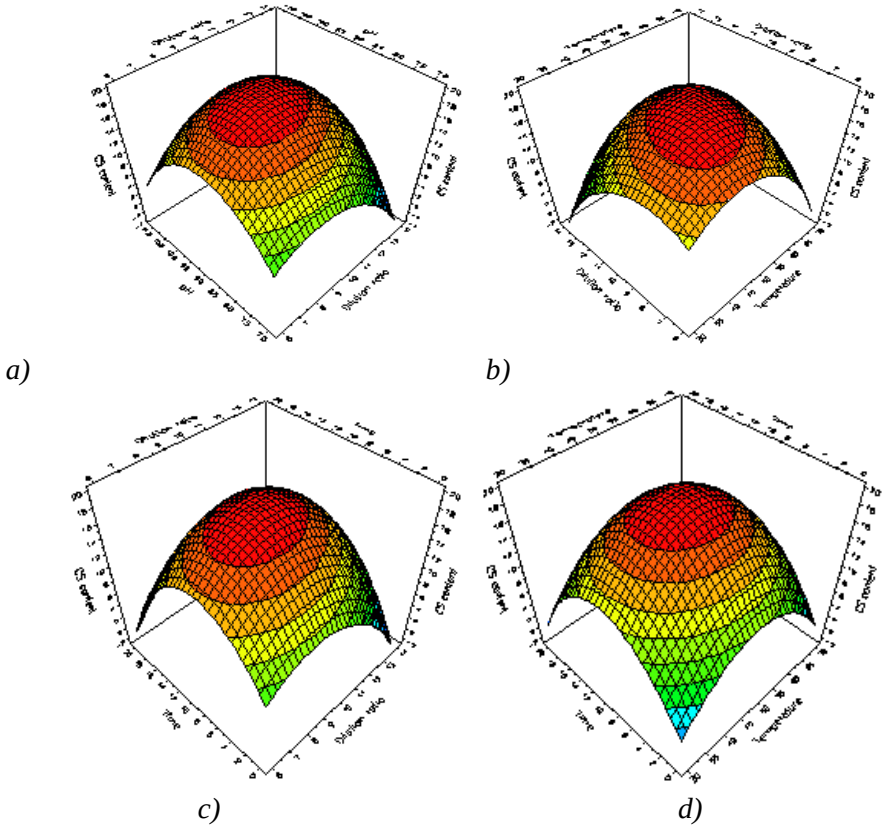
$$CS\ \% = -124.335 + 0.769Z_1 + 27.501Z_2 + 0.401Z_3 + 1.094Z_4 + 0.31Z_1Z_2 + 0.054Z_1Z_3 + 0.06Z_1Z_4 + 0.083Z_2Z_3 - 0.361Z_1^2 - 1.887Z_2^2 - 0.017Z_3^2 - 0.094Z_4^2 \quad (4)$$

where Z_1 = dilution ratio of raw material and buffer, Z_2 = pH, Z_3 = ultrasonic temperature, Z_4 = ultrasonic time.

The response surfaces were illustrated very homogeneous in the experimental domain executed (Figure3).

The optimal condition for the ultrasound treatment from the experiment was determined: the ratio dilution was 1:9, pH 9, the temperature was 47.34 ^oC, and incubation time was 9.2 minutes. For these conditions, the CS content in the prediction model was 19.28 %. This result was of no significant difference in comparison with the experimented value (19.92± 0.1 %, p <

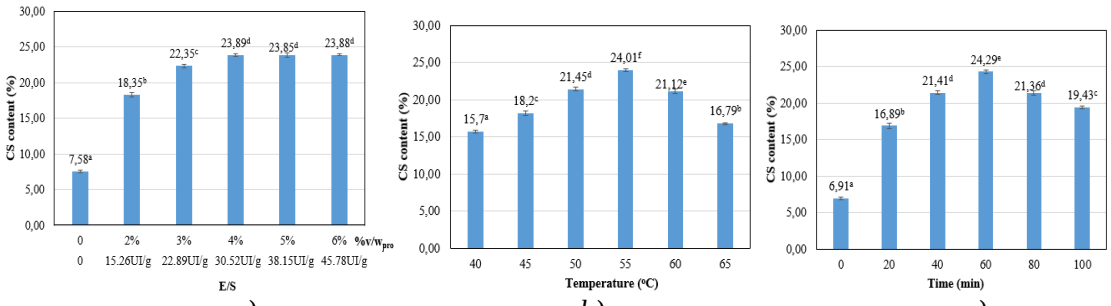
0.05), which showed a close relationship between the experimented values and the predicted values, that indicated the satisfaction of the developed model.



a) *b)* *c)* *d)*

Figure 3. The interactive effect of pH and ratioof material:buffer (a); ratioof material: buffer and ultrasonic temperature (b); ultrasonic time and ratio of material:buffer (c); ultrasonic time and temperature (d) on CS content.

3.4. Effects of hydrolysisconditions



a) *b)* *c)*

Figure 4. Effects of hydrolysis condition: E/S content (a), temperature (b) and time (c) to CS content.

The results revealed that the highest CS content was significantly affected by the E/S ratio (v/w_{pro}) at 4 %.Therefore, the Alcalase content 4 % was used in the next experiments.

If hydrolysis occurs at the high temperature for along time, the enzyme will be denatured or decreased activity. Besides, the amount of substrates will also decrease, and the interaction of enzyme and substrate is limited, leading low CS content. Through the results in Figure 4, the temperature at 55 ^{o}C of hydrolysis for 60 minutes were chosen to obtain the highest CS content 24.29 % (w/w dry material).

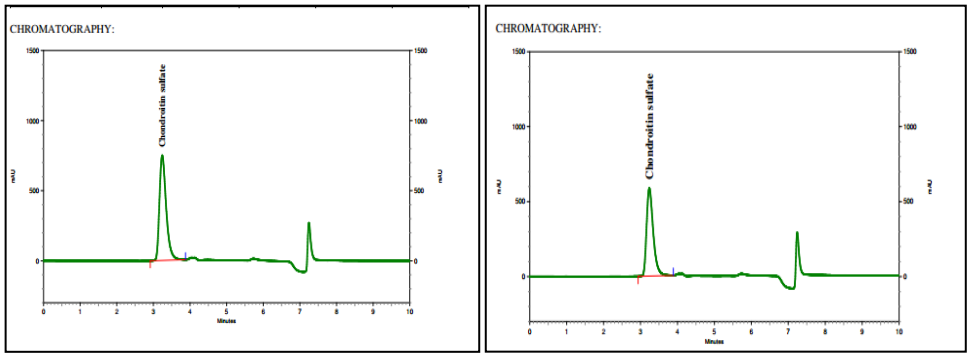
3.5. Physicochemical and chemical characteristics of the obtained CS

The result in Table 4 showed that moisture content 7.4 % is similar to the result of the study of S.C. Shin [4], protein content 6.05 % is lower, the content of carbohydrate is similar.

Table 4. Composition of researched CS.

Analytical criteria	Unit	Content	Method
Protein	% (w/w)	6.05±0.21	AOAC 2001.11
Carbohydrate	% (w/w)	79.11±0.35	AOAC 974.06
Lipid	% (w/w)	0	AOAC 2003.06
Moisture	% (w/w)	7.4±1.42	AOAC 934.01
Ash	% (w/w)	7.3±0.12	ISO 7514:1990

By HPLC method, researched CS obtained the purity of 80.45 % (Figure 5) with the average molecular weight (M_w) of 159.53kDa by GPC (Table 5 and Figure 6). The yield of obtained CS reached 24.29 % of absolute dry material; 61.02 % in comparison with carbohydrate of dry material. This value is higher than the result of Luo *et al.*(16.8 %) [5]; and higher than the result of research by Garnjanagoonchorn *et al.*(14.08 %) [6].

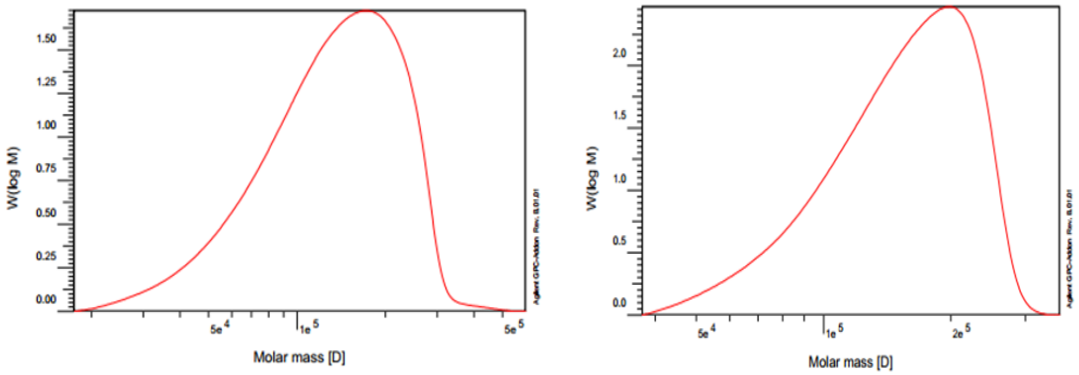


a) *b)*

Figure 5. Chromatogram of CS content consisted in CS sample standard (a) and CS preparation (b).

Table 5. Parameters of GPC analysis.

Sample	Time (minute)	M_w (Da)	M_n(Da)
CS standard	7,457	145120	108460
Researched CS	6,941	159530	136290



a) *b)*

*Figure 6.*Molecular weight of CS standard (a) and researched CS (b).

The result of GPC in Table 5 and Figure 6 showed an average molecular weight of 159.53kDa for the dried CS product, this value is similar the CS standard with M_w value of 145.12kDa.

4. CONCLUSION

By the combined application of ultrasound treatment and enzymatic hydrolysis with Alcalase, chondroitin sulfate (CS) was extracted from chicken keel cartilage with high yields. At the optimal conditions found for the ultrasound treatment by using Circumscribed Central Composite design and Modde 5.0 software, the yield of CS was 24.29 % of the absolute dry weight, and 61.02 % of the total carbohydrate content of the starting material. Thus, the chicken keel cartilage is a potential source for the extraction of chondroitin sulfate with application in dietary supplements.

Acknowledgements. Financial support from Science and Technology Department - Ho Chi Minh City People's Committee and Vietnam National University - Ho Chi Minh City for this research is acknowledged.

REFERENCES

1. Jackson R. L., Busch S. J. and Cardin A. D. - Glycosaminoglycans: molecular properties, protein interactions, and role in physiological processes, Physiological Reviews **71** (1991) 481-539.
2. Robert M. L. - Chondroitin sulfate: A complex molecule with potential impacts on a wide range of biological systems, Complementary Therapies in Medicine **17** (2009) 56-62.
3. Xie J., Ye H. Y., Luo X. F. - An efficient preparation of chondroitin sulfate and collagen peptides from shark cartilage, International Food Research Journal **21** (2014) 1171-1175.
4. Shin S. C., You S. J., An B. K., Kang C. W. - Study on extraction of mucopolysaccharide-protein containing chondroitin sulfate from chicken keel cartilage, Asian-Australasian Journal of Animal Sciences **19** (2006) 601-604.
5. Luo X. M., Fosmire G. J., Leach R. M. - Chicken keel cartilage as a source of chondroitin sulfate, Poultry Science **81** (2002) 1086-1089.

6. Garnjanagoonchorn W., Wongekalak L., Engkagul A.- Determination of chondroitin sulfate from different sources of cartilage, Chemical Engineering and Processing **46** (2007) 465-471.
7. Anchalee Srichamroen, Takuo Nakano, Zeb Pietrasik, Lech Ozimek, Mirko Betti- Chondroitin sulfate extraction from broiler chicken cartilage by tissue autolysis, Food Science and Technology **50** (2013) 607-612.
8. María B., Javier F., Carmen G. S., Ricardo I. P., José A. V. - Production of chondroitin sulphate from head, skeleton and fins of scyliorhinus canicula by-products by combination of enzymatic, Chemical Precipitation and Ultrafiltration Methodologies **13** (2015) 3287-3308.
9. Nakano T., Pietrasik Z., Ozimek L., Betti M. - Extraction, isolation and analysis of chondroitin sulfate from broiler chicken biomass, Process Biochemistry **47** (2012) 1909-1918.
10. Lignot B., Lahogue V., Bourseau P.- Enzymatic extraction of chondroitin sulfate from skate cartilage and concentration-desalting by ultrafiltration, Journal of biotechnology **103** (2003) 281-284.
11. Nakano T. and Ozimek L. - Chondroitin sulphate distribution in broiler chicken carcasses, Bristish Poultry Science **55** (2014) 54-58.
12. Xiong S. L., Wu Z., Li A., Wei M. –Extraction, separation and purification of chondroitin sulfate from chicken keel cartilage, Chin Soc Agric Eng **25** (2009) 271-275.
13. Farndale R.W., Buttle D. J., Barrett A. J. - Improved quantitation and discrimination of sulfated glucosaminoglycan, Biochimica et Biophysica Acta **883** (1986) 173-177.