Study on the determination of chitosan by enzymatic hydrolysis-spectrophotometry

Sihui Wu[1,a], Yonghe Jiang[2,b], Shang Kong[3,c], Haitao Pan[4,d], Danyuan Xu[5,e], and Zhengquan Su[5,f]

[1]Guangdong Food and Drug Vocational Technical School, 510663, Guangzhou, Guangdong, China

[2]Dongsheng Hospital of Guangzhou, 510000, Guangzhou, Guangdong, China

[3]Key Research Center of Liver Regulation for Hyperlipidemia SATCM/Class III Laboratory of Metabolism SATCM, Guangdong TCM Key Laboratory for Metabolic Diseases, Guangdong Pharmaceutical University, 510006, Guangzhou, Guangdong, China

[4]School of Pharmaceutical Sciences，Centre for Cellular & Structural biology, Sun Yat-Sen University, 510000, Guangzhou, Guangdong, China

[5]College of Public Health, Guangdong Pharmaceutical University, 510240, Guangzhou, Guangdong, China

[a]wsh2709@163.com, [b]191342366@qq.com, [c]932326791@qq.com, [d]1484521210@qq.com, [e]494595467@qq.com, [f]suzhq@scnu.edu.cn

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Abstract. Chitosan (CTS) is a natural basic polysaccharide, which have no a very effective way to determine its content now. In this paper, we used cellulase combined with mixed acid under ultrasound assisted (CAU) method to hydrolyze CTS into glucosamine, which can react with diaminobenzene formaldehyde acid alcohol solution to form red compound under alkaline condition acetyl acetone. The red compound has the UV absorption at 525nm measured by spectrophotometry, which can further calculate the content of CTS. The results showed that the optimal conditions were that the sample amount was 3mg, enzyme-substrate ratio was 80mg/g and pH 5.2 as well as water bath at 50 ºC for 30min and then supersonic at 50ºC for 7hr in the enzymolysis step, 0.168mol/L hydrochloric acid was 0.8ml, 75% sulfuric acid was 3.5ml, the boiling water bath for 30min and ultraphonic at 50ºC for 60 min in the acid hydrolysis step. The average recovery was 100.02±5.51%, and the content of CTS was 96.58±1.54%. The results indicated that the mentioned methods are easy and effective for the quantitative analysis of CTS.

Introduction

Chitosan (CTS), the derivative of chitin, is a natural polymer of glucosamine and N-acetylglucosamine derived from the shells of crustaceans such as crab, lobster and shrimp [1]. CTS and its derivatives, such as chitosan oligosaccharides and glucosamine, are non-toxic, harmless, and shares the characteristics of good permeability, moisture retention, degradation, electrolytic, adsorption and hygroscopicity, therefore which have been widely used in agriculture, food industry, water treatment, textiles, chemicals, cosmetics and other fields [2-3]. CTS has biological activity of antibacterial, bactericidal, anti-cancer, immunity improvement, accelerating tissue repair and hemostasis, biodegradability and biocompatibility, so the CTS possesses widely application foreground in the pharmaceutical and biotechnological industries[4-5]. Therefore, the establishment of quantitative analysis of CTS is particularly important.

At present, the main content determine methods for CTS include HPLC, electrochemistry, spectrophotometry, titrimetry and infrared spectroscopy, etc [6-8]. We have published several research papers on the determination for CTS [7-14]. However, due to restrictions of domestic experimental conditions and the lack of pure CTS, the content of CTS has not been determined directly. That is should be to say, CTS hydrolyzed into glucosamine, which is determined by HPLC and Spectrophotometry, should be recognized as a kind of good detected method [6]. The main

method for hydrolysis of CTS into glucosamine is realized by sulfuric acid currently, which is ineffective and disadvantages of determination. In this paper, we used cellulose, mixed acid, and ultrasound assisted (CAU) method to hydrolyze CTS into glucosamine, which can react with diaminobenzene formaldehyde acid alcohol solution to form red compound under alkaline condition acetyl acetone. The red compound has the UV absorption at 525nm measured by spectrophotometry, which can further calculate the content of CTS.

Materials and Reagents

CTS (CTS, Lot, 10321A; viscosity, >200 mP/s; degree of deacetylation, 96.2%) was purchased from Shandong AoKang Biotechnology Co. Ltd. China; Glucosamine hydrochloride (Lot, T0024) was supplied by Zhejiang Kangle pharmaceutical Co. Ltd. China; Cellulase (Lot, 41530) and was obtained from Guangzhou Chemical Reagent Co. Ltd., China; Acetylacetone was obtained from Tianjin Baishi Chemical Co., Ltd., China; Diaminobenzene formaldehyde was supplied by Sinopharm Chemical Reagent Co. Ltd., China; All other reagents and solvents were of analytical grade.

SHA-B-type water bath oscillator, Jiangsu Jintan Honghua Instrument Factory, China; Spectrum Lab 22pc UV visible spectrophotometer, Analytik Jena AG, Germany; SB-120DT-type Ultrasonic cleaner, Ningbo Xinzhi Biological Technology Co., Ltd., China; CP124C electronic scale, OHAUS (Shanghai) Instrument Co., Ltd., China; 101A-1ET electric constant temperature drying oven, Shanghai Experimental Instrument Factor Co., Ltd., China.

Experimental Design and Methods

Hydrolysis of CTS

Firstly, 10ml CTS solution was sucked by 10ml colorimetric tube, and added cellulase in certain concentration, then ultraphonic a certain period under certain temperature. The enzymes were inactivated in boiling water and removed by centrifugation with retaining the supernatant for the next experiment.

Secondly, 1.0ml CTS solution was sucked by 10ml colorimetric tube, and added hydrochloric acid and 75% sulfuric acid in certain concentration and volume, then mixed well, bathed with boiling water in certain period and ultraphonic a certain period in certain temperature. After ultraphonic, the sample was cooled to room temperature and was transfered to 100ml volumetric flask. Regulating the pH of the solution as 7.2-7.5, and lastly diluted with distilled water to 100ml, mixed well for the following step.

Construction of glucosamine hydrochloride calibration curve

Glucosamine hydrochloride standard solution were prepared in distilled water in the quality range from 0 to 0.1mg. The aliquot portions (0, 1.00, 2.00, 3.00, 4.00, 5.00ml) of stock solution prepared at 3.2 were quantitatively transferred into 10ml measuring flasks, completed to the mark of 5ml with water and which was added to acetylacetone solution 1.00ml, shaken, placed in 90°C with water bath for 25min, removed, cooled quickly with running water, added anhydrous ethyl alcohol 3.00ml, placed in 60°C with water bath for 10min, then added diaminobenzene formaldehyde solution 1.00ml, shaked hard and mix well, placed in 60°C with water bath for 35min, removed, immediately cooled to the room temperature with cold water, which was measured in 525nm. The linear regression equation was calculated between the area under the curve and the quality of Glucosamine hydrochloride in mg.

Measurement of CTS

1.00ml CTS solution was transferred into 10ml colorimetric tube, the absorbance of which was determined according to the method in *3.3*. The concentration of glucosamine hydrochloride in the above solution was also determined as described in *3.3*, from the computed regression equation.

The mass fraction of CTS is ω1, according to equations:

$$w_1 = \frac{m_1}{m} \times 0.8309 \times 100\% \quad (1)\ [15]$$

$$w_2 = \frac{m_1}{m} \times 0.7466 \times 100\% \quad (2)$$

Where m_1 is the amount of of glucosamine hydrochloride from the linear regressive equation(mg); m is the quality of CTS of acid hydrolysis(mg); 0.7466 is the coefficient which glucosamine hydrochloride converted to CTS; 0.8309 is the coefficient which glucosamine hydrochloride converted to glucosamine.

We found that the calculated results of euqation(1) results constrast the content of CTS samples to big errors. Consequently this paper revised the equation (1) to the equation(2), calculated the content of CTS by the coefficient which glucosamine hydrochloride converted to CTS, as shown in equation (2).

Results and discussion

Glucosamine hydrochloride calibration curve

The quantitative determination of glucosamine hydrochloride by applying the suggested spectrophotometry procedure is valid in the quality range from 0 to 0.1mg. The linear regression equation was found to be:

$A=6.0171m +0.0081$, $R_2=0.9991$.

Different blind authentic samples, prepared as the used stock solution, were determined using the suggested spectrophotometry procedures.

Optimization of hydrolysis conditions

Water bath time in 50°C

0.5ml cellulase solution (whose mass concentration was 8mg/ml) were added to 10ml CTS (5mg/ml) solutions under pH 5.2 respectively, the mixed solutions were placed in 50°C water bath for 5min, 10min, 20min. 30min, 35min, 50min, and then supersonic at 50 °C for 7hr, water bath at 100°C for 10min, cooled and centrifuged them to take the supernatant. 0.6ml CTS solution processed above were added to 0.168mol/L hydrochloric acid 0.8ml, 75% sulfuric acid 3.5ml, shaken the mixture solution well, placed in 100°C with water bath for 30min, removed, supersonic 60min at 50 °C, cooled to room temperature, neutralized with saturated sodium carbonate solution to pH 7.2-7.5 and completed to the mark with water in 100 ml measuring flask. The solution was taken 1ml to determine the content of CTS.

Fig. 1 showed that, with the extension of water bath time, the CTS gradually hydrolyzed, the quality of CTS reached the highest content when the water bath time was 30min. Because with the extension of time, the CTS glycosidic bond gradually fractured, but in the case of concentrated acid and prolonged high temperature, CTS may be carbonized, resulting in decreased production rate of glucosamine.

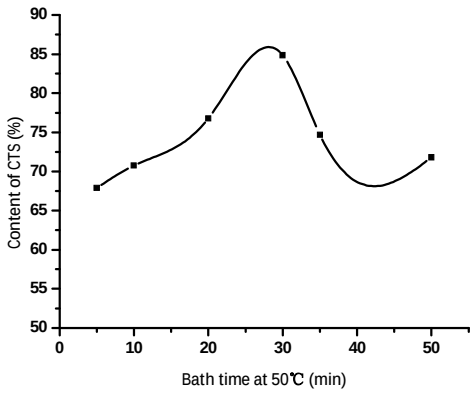

Fig. 1 Influence of water bath time at 50°C

Sampling weight

The steps were determined as described in *4.2.1*, placed the CTS solution processed by cellulase solution 0.4ml, 0.5ml, 0.6ml, 0.7ml, 0.8ml for the acid hydrolysis step, respectively.

Fig. 2 showed that the content of CTS reached the biggest when the sampling mass of CTS was 0.4ml, and the second biggest when 0.6ml, the hydrolysis gradually decreased with the increase of the quality of CTS. However, it's prone to generate interruption on absorbance of CTS when the sample weight is small (0.4ml), therefore we confirm the sampling weight is 0.6ml.

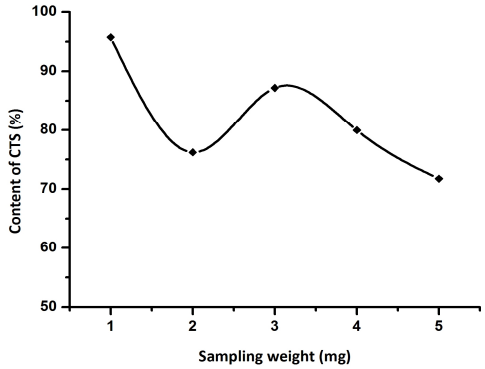

Fig. 2 Influence of sampling dosage (weight)

Dosage of cellulase

The steps were determined as described in *4.2.1*, the concentrations of cellulase solutions taken (0.5ml/sample) were 2mg/ml, 4mg/ml, 6mg/ml, 8mg/ml, 10mg/ml and 12mg/ml, respectively.

Fig. 3 showed that the content of CTS reached the biggest when the dosage of enzyme was 4mg. The CTS content was significantly reduced with the increase of the amount of cellulase.

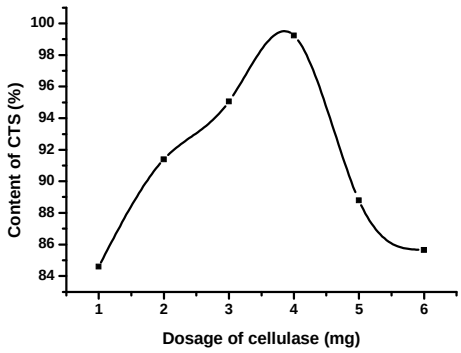

Fig. 3 Influence of dosage of enzyme

Cellulase hydrolysis time

The steps were determined as described *4.2.1*, the supersonic time at 50 ºCwas 4h, 5h, 6h, 7h and 8h, respectively.

Fig. 4 showed that 7hr was the optimizing cellulase hydrolysis time. However, the CTS in smaller degree of polymerization obtained in the longer cellulase hydrolysis time, such as CTS oligosaccharide and monosaccharide, can be destroyed by strong acid and high temperature in the acid hydrolysis step, which make the smaller content of CTS.

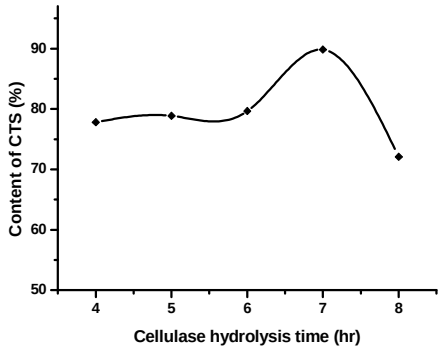

Fig. 4 Influence of enzyme hydrolysis time

pH

The steps were determined as described in *4.2.1*, the cellulase hydrolysis reactions were placed under pH 4.6, 4.8, 5.0, 5.2 and 5.4, respectively.

Because of the wrong solubleness when pH > 6.0, we merely investigated the influence of different pH on enzymatic reaction when pH < 6.0. The influence of pH on enzymatic reaction embodies the dual function between CTS dissolved state and the ionization of enzymatic active group. Therefore, we should confirm a optimum pH. Fig. 5 showed that the quality of CTS reached the highest when pH 5.0-5.2, and the decreased CTS content displayed when pH < 5.0 or pH > 5.2.

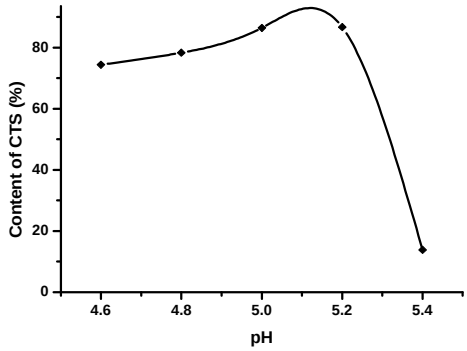

Fig. 5 Influence of pH on determination of CTS

Dosage of H_2O_2

The steps were determined as described in *4.2.1*, in the cellulase hydrolysis step, after supersonic at 50 ^{o}C for 6hr, continue supersonic for 1hr that added to 30% H_2O_2 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6mL, respectively.

Fig. 6 showed that the content of CTS was decrease with the increase of H_2O_2. The reason is that H_2O_2 can oxidate CTS oligosaccharides and react with acid in the acid hydrolysis when it degrade CTS. According to the results, we choose 0.1ml as the optimizing dosage.

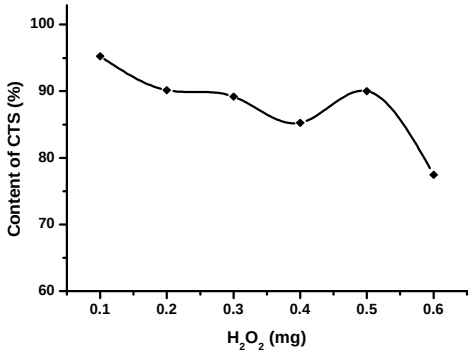

Fig. 6 Influence of H_2O_2 dosage

Orthogonal test

Based on the results and analysis of single factor test in *4.2*, the best condition for preparing glucosamine was studied by the orthogonal test. Three controllable variables, dosage of cellulase (ml), pH, and cellulase hydrolysis time were selected, each at three levels. The results indicated that the dosage of cellulase is the main factor, the optimum reaction condition was obtained as follows: cellulase hydrolysis time: 7hr; dosage of cellulase: 4mg; pH 5.2.

Determination of CTS

According to the results and analysis of single factor experiments and the orthogonal experiments investigated, the optimal CTS experimental conditions for six parallel verification experiment were as follows: the sample amount was 3mg, enzyme-substrate ratio was 80mg/g and pH 5.2 as well as water bath at 50 °C for 30min and then supersonic at 50 °C for 7hr in the enzymolysis step, 0.168mol/L hydrochloric acid was 0.8ml, 75% sulfuric acid was 3.5ml, the boiling water bath for 30min and ultraphonic at 50 °C for 60 min in the acid hydrolysis step. the average content of CTS was 96.58±1.54%. All samples were analyzed six times (n = 6).

The role of cellulase

Nine parallel samples were hydrolyzed by acid, cellulase-acid and cellulase-H_2O_2-acid (three sample per method) for determining the content of CTS. The results of t-test showed that t=23.806>t (0.05, 3) =3.182， P=0.002<0.01. There has been a significant difference between acid and cellulase-acid hydrolysis, which indicated that cellulase can effectively improve the content of CTS by hydrolysis.

Precision

Precision of the assay was determined by analyzing the samples at three different concentrations. For assessment of the variation, samples were analyzed six times (n = 6). The results showed that RSD was 2.06%, 1.04%, and 0.46%, the assay was precise as the RSD was less than 3.00%. These results indicated acid-cellulase hydrolysis assay is a good precision of the analytical method.

Adding standard recovery

Twelve 10ml parallel samples of 5mg/ml CTS solution, thereinto six were added to 5mg/ml glucosamine hydrochloride standard solution 5ml, the other six were control, which added to 8mg/ml cellulase solution 0.5ml, the follow-up procedure were described in *4.2.1*. The yield of glucosamine hydrochloride was calculated by formula (2), The adding standard recovery calculated formula is shown as follows:

$$Y = \frac{m_1 \times M_2 \times 96.2\%}{m_2 \times M_1} \times 100\% \quad (3)$$

Where m_1 is the amount of glucosamine hydrochloride from the linear regressive equation(mg); M_1 is the relative molecular mass of glucosamine hydrochloride; m_2 is the amount of CTS of acid hydrolysis(mg); M_2 is the relative molecular mass of CTS; 96.2% is the degree of deacetylation (DD) of CTS.

The results showed that the mean recovery of CTS was 100.02±5.51%, which meet the requirement.

Conclusion

In this paper, CTS was hydrolyzed to glucosamine by cellulase combined with mixed acids (hydrochloric acid and sulfuric acid) under ultrasound-assisted (CAU), CTS was indirectly determined by glucosamine hydrochloride. By examining the effects on CTS hydrolysis, we tested the following factors: bath time in 50C, sample dosage of CTS, dosage of cellulase, cellulase hydrolysis time, pH and H_2O_2 or not etc. to determine the appropriate hydrolysis conditions (Single factor test), and the method precision and recovery were researched. The results showed that the optimal conditions were that the sample amount was 3mg, enzyme-substrate ratio was 80mg/g and pH 5.2 as well as water bath at 50 °C for 30min and then supersonic at 50 °C for 7hr in the enzymolysis step,

0.168mol/L hydrochloric acid was 0.8ml, 75% sulfuric acid was 3.5ml, the boiling water bath for 30min and ultraphonic at 50 °C for 60 min in the acid hydrolysis step. The average recovery was 100.02±5.51%, and the content of CTS was 96.58±1.54%. The results of CAU hydrolysis of CTS was significantly higher than that of the only mixed acid hydrolysis, which is simple, high accuracy and precision, and has a significant role in increasing the content of CTS. Therefore, CAU hydrolysis is applicable for the determination of CTS.

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