Ciência Rural, Santa Maria, v.52:1, e20200723, 2022
http://doi.org/10.1590/0103-8478cr20200723
ISSNe 1678-4596
FOOD TECHNOLOGY

The effect of deproteinization methods on the properties of glucosamine hydrochloride from shells of white leg shrimp (*Litopenaeus vannamei*) and black tiger shrimp (*Penaeus monodon*)

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ABSTRACT: *The effect of methods to remove protein content on the properties of glucosamine hydrochloride from the shells of white leg shrimp (Litopenaeus vannamei) and black tiger shrimp (Penaeus monodon) was investigated. Chitin from shrimp shells was obtained by demineralization in 6% HCl for 12h, deproteinization by two different methods (first group soaked in 8% NaOH for 36h and second group treated in Alcalase enzyme at the concentration of 0.2% for 36h). Two group samples were converted to glucosamine hydrochloride by soaking in 36.76% HCl solution for 5h at 85 °C. The results of fourier transform infrared spectroscopy (FTIR), solubility and recovery yield analysis showed that deproteinization methods did not significantly affect the properties of glucosamine hydrochloride. However, glucosamine hydrochloride from white leg shrimp shells contained higher recovery yield and solubility than black tiger shrimp shells.*
Key words: *glucosamine hydrochloride, shrimp shells, deproteinization method, recovery yield, solubility.*

Efeito de métodos de desproteinização nas propriedades do cloridrato de glucosamina de cascas de camarão de pernas brancas (*Litopenaeus vannamei*) e cascas de camarão de tigre preto (*Penaeus monodon)*

RESUMO: *Investigou-se o efeito de métodos para remover o conteúdo de proteínas nas propriedades do cloridrato de glucosamina das conchas de camarão de pernas brancas (Litopenaeus vannamei) e camarão de tigre preto (Penaeus monodon). A quitina das cascas de camarão foi obtida por desmineralização em HCl a 6% por 12 h, desproteinização por dois métodos diferentes (primeiro grupo embebido em NaOH a 8% por 36 h e segundo grupo tratado na enzima Alcalase na concentração de 0,2% por 36 h). Duas amostras de grupo foram convertidas em cloridrato de glucosamina por imersão em solução de 12M HCl por 5 h a 85 °C. Os resultados das análises de FTIR, solubilidade e rendimento de recuperação mostraram que os métodos de desproteinização não afetaram significativamente as propriedades do cloridrato de glucosamina. No entanto, o cloridrato de glucosamina de cascas de camarão de pernas brancas continha maior rendimento e solubilidade de recuperação do que as cascas de camarão tigre preto.*
Palavras-chave: *cloridrato de glucosamina, cascas de camarão de pernas brancas, cascas de camarão tigre preto, FTIR.*

INTRODUCTION

White leg shrimp and black tiger shrimp have been the main products in exportation of Vietnam in recent years. According to Vietnam Association of Seafood Exporters and Producers (VASEP), the production of exported shrimp in the first six months in 2018 reached US $1.6 billion, up 7.6% over the same period in 2017. However, during shrimp processing, a large amount of by-product, such as heads, legs, shells, and tails, accounts for 40-50% of the total weight (XU et al., 2008). Utilization of shimp waste, including shrimp shells, is necessary from the viewpoints of both environmental conservation and the development of new valuable products such as chitin, chitosan and glucosamine hydrochloride.

Glucosamine is naturally present in the shells of shellfish, animal bones, bone marrow, and fungi. Glucosamine can be produced commercially in many different ways by the hydrolysis of crustacean exoskeletons or, less commonly, by fermentation of grain such as corn or wheat (SHAHIDI et al., 1999). Glucosamine in the form of glucosamine sulphate, glucosamine hydrochloride, or N-acetyl-glucosamine is extensively used as a dietary supplement in the treatment for osteoarthritis, knee pain, and back pain (HOUPT et al., 1999. LUO et al., 2005), and a critical evaluation indicated that glucosamine is safe under

Received 08.04.20 Approved 04.15.21 Returned by the author 06.05.21
CR-2020-0723.R1
Editors: Rudi Weiblen Levy Carvalho Gomes

current conditions of use and does not affect glucose metabolism (ANDERSON et al., 2005).

Glucosamine hydrochloride (G-HCl) production from commercial chitin and chitosan (LEITE et al., 2002; LI et al., 2007), crustacean shells (BENAVENTE et al., 2015), crab exoskeletons (JORGE et al., 2019) has been characterized. However, little information is available regarding the effect of deproteinization methods on the properties of glucosamine hydrochloride from shrimp shells. Therefore, the purpose of this study was to produce G-HCl by investigating the changes of G-HCl properties from shrimp shells by different deproteinization methods, i.e., alkaline method and enzyme method.

MATERIALS AND METHODS

Preparation of shrimp shells

White leg shrimp shells and black tiger shrimp shells were collected from a frozen seafood company in Can Tho city, Vietnam. The samples were transported to our laboratory under iced condition, washed with chilled water before being cut into small pieces by scissors, placed in polyethylene bags and then stored at –20 °C until use.

Chitin extraction

Chitin was extracted from shrimp shells by demineralization in 6% HCl (analytical grade, 36 - 38%, Xilong Scientific Co., Ltd., Guangdong, China) for 12h with gentle stirring at a sample/HCl solution ratio of 1:10 (w/v) and deproteinization by two different methods (alkaline method by soaking in 8% NaOH (analytical grade, 96.0%, Guangdong Guanghua Sci-Tech Co., Ltd., Guangdong, China) for 36h with continuous stirring at a sample/NaOH solution ratio of 1:10 (w/v) and enzyme method by treating in 0.2% Alcalase enzyme 2.4 T (Novozymes, Denmark) for 36h at 40 °C. Two groups of chitin obtained were washed several times with distilled water until pH neutral and dried at 50 °C for 10h. Finally, chitin was milled and screened to select the fraction of particles with a size lower than 0.22 mm.

Glucosamine hydrochloride production

Glucosamine hydrochloride production was performed following the method described by BENAVENTE et al. (2015) with minor modifications by reducing the dried time at 50 °C from 12h to 10h. Chitin collected from two different methods of removing protein content was dissolved in 36.76% HCl at 85 °C for 5h at a sample/HCl solution ratio of 1:20 (w/v). After dissolving, the coarse solids were removed by filtration, and the collected solution was left to crystallize by adding ethyl alcohol (15mL, w = 95%), and kept at 4 °C for 15 days. The mixture was once more filtered, and the solid crystals were washed with ethyl alcohol and dried at 50 °C for 10h.

Determination of moisture, protein and ash content

Moisture, ash and protein content in the shrimp shells after demineralization and deproteinization were analyzed according to the Association of Official Analytical Chemists methods (AOAC 2000). The moisture content was determined by drying the samples in an air oven at 105 °C for 24 h until constant weights were obtained, cooled in a desiccator and re-weighed. The difference between fresh and dry weights was taken as the amount of water present and was converted to percentage. The crude protein was analyzed by the Kjeldahl method to determine nitrogen content, using 6.25 as the conversion factor to get crude protein from total nitrogen (WANG et al., 2008). Ash content was determined by using samples (pre-dried) from the analysis of moisture content analysis was heated in a furnace at 650 °C for 4 h. The final weight was subtracted from the initial weight and converted to percentage to give an estimate of the ash content.

Fourier transform infrared spectroscopy (FTIR) of glucosamine hydrochloride

FTIR spectra of glucosamine hydrochloride from two chitin groups was analyzed using Omnic 6.0 sofware (Thermo-Nicolet, Madison, Wisconsin). The spectra was recorded using Bruker Optics ALPHA FT-IR spectrometer with spectra wavenumber from 4000 to 500 cm^{-1}.

Yield of extracted glucosamine hydrochloride (G-HCl)

The yield of G-HCl was determined using the following equation:

$$\text{Yield (\%)} = \frac{\text{The weight of G-HCl collected after extracting (g)} \times 100\%}{\text{The weight of chitin before converting to G-HCl (g)}}$$

Solubility of G-HCl

For solubility checking, 1g G-HCl was dissolved in 100 ml of distilled water at ambient temperature for 2h with stirring. The solution was filtered through a pre-weighed Whatman No. 1 filter paper. After removing of all the solvent, the filter paper was dried at ambient temperature and re-weighed. The percent solubility was calculated from the ratio of weight gain of filter paper x100.

Statistical analysis

All experiments were repeated triplicate. All data were shown as standard deviation of the mean (S.D.M). All data were analyzed by using SPSS software (SPSS 16.0 for Windows) and using Duncan's multiple range tests for evaluating differences between variables.

RESULTS AND DISCUSSION

Proximate composition of white leg shrimp shells and chitin

The proximate composition of white leg shrimp shells and black tiger shrimp shells are shown in table 1. The percentage of protein in white leg shrimp shells and black tiger shrimp shells was 49.43±0.45 and 49.51±0.54, respectively while the ash content in these shells was 25.32±0.24 and 28.37±0.35, respectively. The high amount of protein found in white leg shrimp shells and black tiger shrimp shells, was similar to that in shrimp waste (SHAHIDI, 1994).

Similar to common method, for the production of crude chitin, shrimp shells were soaked in acidic solution and continuously in alkaline solution to remove ash and protein content, respectively. Shrimp shells after treating with HCl 6% for 12h effectively removed the ash content (from 25.32 to 0.87% in white leg shrimp shells and from 28.37 to 0.89% in black tiger shrimp shells). All shrimp shells showed the ash content less than 1% after demineralization in HCl, it is the important condition to produce high quality chitin (NO & MAYER, 1995).

For removing protein content, samples after demineralization were separated into 2 groups; first group soaked in alkaline solution (NaOH 8% for 36h) and second group treated in enzyme solution (enzyme Alcalase 0.2% for 36h). The effectiveness of removing protein in first group by using traditional method (alkaline method) could be observed in table 2 (the protein in white leg shrimp shells reduced from 49.43 to 3.26% and decreased from 49.57 to 3.43% in black tiger shrimp shells). With purpose of reducing the chemical waste in environment, second group replaced for NaOH 8% by enzyme Alcalase 0.2% was also shown the ability of reducing protein in shrimp shells (from 49.43 to 4.12% in white white leg shrimp shells and from 49.57 to 4.29% in black tiger shrimp shells).

Extraction yield of G-HCl from chitin

Extraction yields of G-HCl from two types of shrimp shells chitin, which were removed protein by alkaline method and enzyme method, are presented in table 3. The G-HCl yield from white leg shrimp shells chitin using alkaline solution to remove protein (60.31±2.06%) was slightly higher than that from black tiger shrimp shells chitin (57.32±1.79%). The similar tendency could be observed in chitin treated in enzyme Alcalase, which had higher yield in white leg shrimp shells chitin (58.64±1.82%) when compared to black tiger shrimp shells chitin (57.07±2.02%). The yield of G-HCl depends on extracted temperature and the ratio of chitin/HCl solution (w/v). The increase of extracted temperature leads to faster dissolvability of chitin and this can contribute to a more effective hydrolysis (BENAVENTE et al., 2015). The extraction yield of G-HCl from shrimp shells (57.07 – 60.31%) was similar to yield of extracted G-HCl from crustacean shells (58%) (BENAVENTE et al., 2015) but lower than yield of G-HCl from tiger prawn waste (65.33%) and Persian Gulf shrimp (87.3%) (EKO et al., 2014; MOJARRAD et al., 2007). In present study, there was no significant difference between the extraction yields of G-HCl from two types of shrimp shells chitin in the similar conditions of temperature and ratio of chitin/HCl solution.

Solubility of G-HCl

The solubility of the glucosamine produced from two types of chitin in water at room temperature can be seen in table 4. The tests of glucosamine hydrochloride solubility were carried out using water at room temperature. The solubility

Table 1 - The chemical compositions of shrimp shells.

Sample	----------Composition (%) on dry weight basis----------		
	Moisture content	Ash content	Protein content
White leg shrimp shells	6.21±0.22	25.32±0.24	49.43±0.45
Black tiger shrimp shells	5.25±0.17	28.37±0.35	49.51±0.54

Data are expressed as mean ± standard deviation (n=3).

Table 2 - The proximate compositions of crude chitin from shrimp shells.

Deproteinization method	-------Chitin from white leg shrimp shells--------		-----Chitin from black tiger shrimp shells----	
	Ash content (%)	Protein content (%)	Ash content (%)	Protein content (%)
Deproteinization by alkaline method	0.87±0.03	3.26±0.04	0.89±0.08	3.43±0.06
Deproteinzation by enzyme method	0.87±0.03	4.12±0.05	0.89±0.08	4.29±0.09

Data are expressed as mean ± standard deviation (n=3).

of G-HCl produce removed protein content by alkaline method and enzyme method was 87.71 and 84.07% in white leg shrimp shells chitin and 84.67 and 82.59% in black tiger shrimp shells chitin, respectively. It can be explained that this process not only removes the acetyl group but also splits up the chitosan polymer into shorter units. As a result, the chloride ions (Cl-) from the hydrochloric acid HCl can easily to bind with the chitosan amine groups in the formation of NH_3Cl. The hydroxyl bond between O-H and NH_3Cl makes glucosamine hydrochloride readily soluble in water (EKO et al., 2014). Furthermore, KRALOVEC & BARROW (2008) reported that solubility increases or decreases with the temperature of the solvent and that glucosamine can easily be dissolved in water at a temperature of 20 ºC. If a substance can be readily dissolved at low temperatures, this indicates that it is highly soluble. Overall, the deproteinization method was not affected the solubility of G-HCl from shrimp shells.

Fourier transform infrared spectroscopy (FTIR) of G-HCl

Figure 1 shows the FTIR spectra of glucosamine hydrochloride extracted from two types of chitin removed protein by alkaline method and by enzyme method. The FTIR spectra of G-HCl from both types of chitin indicated an extremely high degree of similarity with G-HCl commercial reference, which are essentially identical with regard to the band-positions of G-HCl main groups. The FTIR spectrum of G-HCl produced from white leg shrimp shells (deproteinization by NaOH and enzyme Alcalase) appeared at 3290.4 and 3289.67 cm^{-1} in association with the O–H and N–H stretching (BRUGNEROTTO et al., 2001), and from a NH_2 scissoring band at 1616.39 and 1615.98 cm^{-1} and at 1096.56 and 1094.37 cm^{-1} due to secondary alcohol –OH. The data of FTIR analysis in G-HCl from black tiger shrimp shells showed the similar wavelength number at 3293.37 and 3292.08 cm-1, at 1618.93 and 1618.41 cm^{-1} and at 1095.06 and 1094.31 cm^{-1}, respectively for two group samples removed protein by two different methods. In agreement with this result, the study of BENAVENTE et al. (2015) and EKO et al. (2014), reported that the FTIR spectra of G-HCl made from crustacean shells and Persian Gulf shrimp was similar to standard glucosamine with wavelength number of 3370-3300, 1615 and 1094 cm^{-1}, respectively. The similar in wavelength number between G-HCl from shell of white leg shrimp, black tiger shrimp and crustacean shells, Persian Gulf shrimp in research of BENAVENTE et al. (2015) and EKO et al. (2014) are considered normal and acceptable as long as the values are still within the wavelength ranges for each functional group.

CONCLUSION

Glucosamine hydrochloride could be extracted from the white leg shrimp shells and black tiger

Table 3 - The extraction yields of G-HCl from two types of chitin.

Sample	----------Extraction yield (%)----------	
	Deproteinization by using alkaline	Deproteinization by using enzyme Alcalase
Chitin from white leg shrimp shells	60.31 ± 2.06[a]	58.64 ± 1.82[a]
Chitin from black tiger shrimp shells	57.32 ± 1.79[a]	57.07 ± 2.02[a]

Data are expressed as mean ± standard deviation (n=3).

Different superscripts in the same column indicate statistical differences (P <0.05).

Table 4 - The solubility of G-HCl from two types of chitin.

Sample	----------Solubility (%)----------	
	Deproteinization by using alkaline	Deproteinization by using enzyme Alcalase
Chitin from white leg shrimp shells	87.71±1.69[a]	84.07±2.16[a]
Chitin from black tiger shrimp shells	84.67±2.23[a]	82.59±1.77[a]

Data are expressed as mean ± standard deviation (n=3).
Different superscripts in the same column indicate statistical differences (P <0.05).

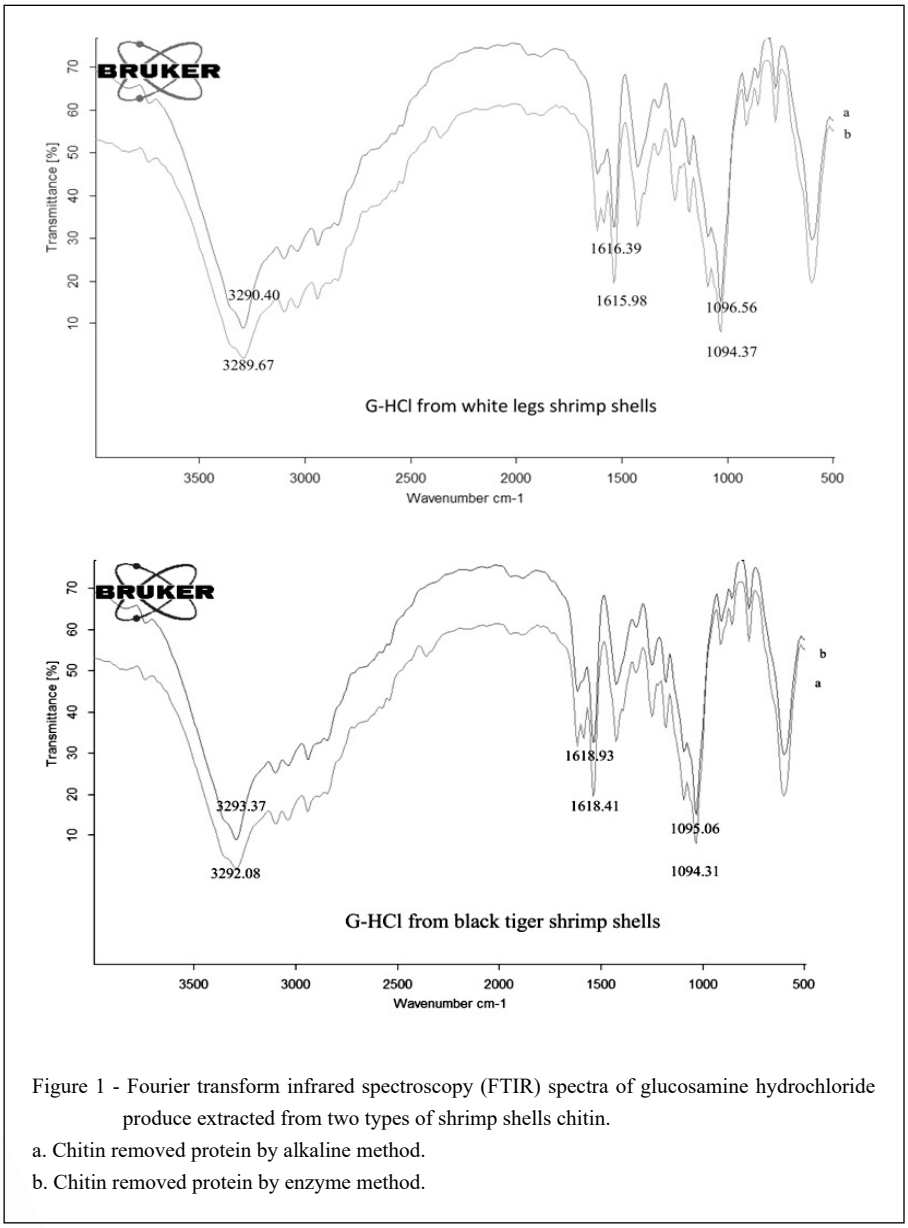

Figure 1 - Fourier transform infrared spectroscopy (FTIR) spectra of glucosamine hydrochloride produce extracted from two types of shrimp shells chitin.
a. Chitin removed protein by alkaline method.
b. Chitin removed protein by enzyme method.

shrimp shells by different methods to remove protein content. The results of extraction yield, the solubility and FTIR spectrum showed no significantly difference between glucosamine hydrochloride produced from chitin removed protein by alkaline and enzyme method. These results suggest that glucosamine hydrochloride could be effectively obtained from the processing by-products of some commercial shrimp species from Vietnam, and using enzyme method of removing protein to have potential as a realistic alternative to alkaline method.

ACKNOWLEDGEMENTS

This study is funded in part by the Can Tho University Improvement Project VN14-P6, supported by a Japanese ODA loan.

DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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