SYNTHESIS AND THERMAL STABILITY INVESTIGATION OF GLUCOSAMINE HYDROCHLORIDE FROM SHRIMP SHELLS

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TÓM TẮT

TỔNG HỢP VÀ NGHIÊN CỨU ĐỘ BỀN NHIỆT CỦA GLUCOSAMINE HYDROCHLORIDE TỪ VỎ TÔM

Glucosamine, một amino monosaccharide thiết yếu có liên quan đến khả năng phục hồi sụn và bôi trơn khớp, có thể được chiết xuất từ chitin có trong bộ xương ngoài của loài giáp xác và chân đốt. Nghiên cứu này tập trung vào việc sử dụng vỏ tôm sú Penaeus Việt Nam để tổng hợp glucosamine hydrochloride (GlcN-Cl) thông qua xử lí hydrochloric acid đậm đặc ở tỉ lệ rắn-lỏng 1:9 (w/v) trong 3,0 giờ ở 85 °C. Phân tích cho thấy sự biến mất của các tín hiệu đặc trưng tương ứng với các nhóm amide I, amide II và amide III trong chitin, được thay thế bằng các đỉnh mới biểu thị các dải uốn và cắt kéo NH_2 trong cấu trúc GlcN-Cl thu được. Sự thay đổi cấu trúc của chitin là rõ ràng, chuyển từ cấu trúc lớp có tổ chức đồng nhất với bề mặt rắn, không có kênh mao quản sang dạng hình trụ được đặc trưng bởi kích thước hạt lớn hơn, không đồng đều và bề mặt nhẵn, tròn, cung cấp bằng chứng thuyết phục về sự chuyển hoá thành công từ chitin thành GlcN-Cl. Hàm lượng khoáng chất, protein và chitin được xác định lần lượt là 47,91±0,28%, 23,13±0,17% và 27,98±0,32%. Tỷ lệ thu hồi GlcN-Cl là 75,67±1,72%, độ tinh khiết là 99,03±0,55%. Mặt khác, ngoài các yếu tố ảnh hưởng đến quá trình thủy phân chitin, nghiên cứu này còn khảo sát độ bền của GlcN-Cl trong môi trường acid do quá trình thủy phân chitin trong dung dịch HCl đặc. Phát hiện này tăng tiềm năng tái sử dụng dư lượng P. monodon với khả năng chuyển hoá của chúng thành GlcN-Cl có giá trị, hứa hẹn cho các ứng dụng thực phẩm và y tế.

Từ khoá: *Chitin, glucosamine hydrochloride, thuỷ phân chitin, Penaeus monodon.*

1. INTRODUCTION

Chitin, the second most abundant biopolymer in nature after cellulose, is a polysaccharide. Its annual growth reaches an astonishing 100 billion tons [1]. Chitin is found in both animals and fungi like *Aspergillus niger* and *Mucor rouxii* [2]. In animals, chitin forms the primary structural component of arthropod shells in shrimps, crabs, and other related species. The chitin content in these

organisms ranges from 7% to 36% by weight [3]. Vertebrates also contain chitin in the form of fish cuticles [2,4].

Structurally, chitin closely resembles cellulose, and contains a chain of *N*-acetylglucosamine (NAG) units. The *N*-acetyl group is linked to the glucosamine group through a covalent β-(1→4) bond, resulting in a molecule known as 2-acetoamido-2-deoxy-β-D- glucose [5]. Due to the presence of the *N*-acetyl group binding to glucosamine, chitin exhibits very low solubility and is challenging to process chemically, thereby limiting its potential applications. To overcome this limitation, the *N*-acetyl group is chemically reduced, resulting in the formation of chitosan. When the degree of deacetylation of chitin reaches about 50%, it becomes soluble in weak aqueous acidic solutions [2]. The first method to obtain chitosan from chitin was developed by *C. Rouget* and involved the addition of chitin to a concentrated sodium hydroxide solution [6]. Chitosan demonstrates solubility in certain dilute organic acids like formic acid or acetic acid, which enhances its ability to undergo hydrolysis as compared to the insoluble chitin polymer.

Glucosamine (2-amino-2-deoxy-D-glucose, GlcN) is an amino monosaccharide that serves as a precursor for the disaccharide unit of glycosaminoglycan, which is the essential component of proteoglycans found in articular cartilage [7]. The human body synthesizes GlcN by combining glucose with the amino acid glutamine [8]. GlcN products, commonly used as joint cartilage supplements for arthritis patients, are often available as GlcN-Cl or GlcN sulfate salts [9]. Numerous studies have been conducted to optimize GlcN production due to its wide range of applications. Besides the traditional HCl hydrolysis method, researchers have employed enzymes, deep eutectic solvents, or molten salt hydrate solvents [10-12] to convert chitin into *N*-acetyl glucosamine (NAG) before producing GlcN. Compared to NAG, enzymatic production of GlcN is less explored due to the scarcity of enzymes catalyzing the hydrolysis of chitin to GlcN. Therefore, research on enzymatic GlcN production primarily focuses on using chitosan as a starting material [13]. While each method has its own advantages and disadvantages, on an industrial scale, hydrolyzing chitin with concentrated HCl solution remains the most popular method [14-16]. A gap identified is that the hydrolysis process and crystallization of the product occur in a strong acid solution, after phase separation at low temperature, GlcN-Cl crystals can dissolve in excess acid, reducing the efficiency of the GlcN-Cl production process.

In this study, the mineral, protein, and chitin content of shrimp shell are investigated. The main focus is on developing a process for preparing GlcN-Cl directly from chitin, without involving chitosan as an intermediate product. The stability of GlcN-Cl crystals after crystallization was examined in acid HCl solution. Solutions were found to minimize GlcN-Cl loss and increase process efficiency.

2. MATERIALS AND METHODS

2.1. Materials

Shrimp shells are collected from the Investment and Trade Fisheries Joint Stock Company, located in Vinh Loc Industrial Park, Ho Chi Minh City. In the initial processing phase, shrimp shells are separated from the product, gathered, and carefully processed by removing the meat from the raw materials, followed by

washing, drying, and mincing, and stored in a dry place.

Hydrochloric acid (HCl), sodium hydroxide (NaOH), and hydrogen peroxide (H_2O_2) are purchased from Xilong, China; ethanol (EtOH), activated carbon, and litmus paper are bought from Ho Chi Minh City chemical and Sci-Tech materials Co. Ltd., Viet Nam.

2.2. Characterization

The morphology of samples is studied by Scanning electron microscope (S-4800, Hitachi, Japan). Before measurement, samples are coated with a thin Pt layer. Chemical structure of chitin and glucosamine is analyzed by FTIR spectroscopy (Bruker, Alpha II) in the range of 4000-500 cm^{-1}. The GlcN-Cl content is determined using an HPLC-Shimadzu with a C18 column (0.15 m × 4.60 mm × 5.00 µm). A sample volume of 20 µL is injected into the instrument. The HPLC column lysing solvent is a mixture of acetate buffer (pH = 5.9) and MeOH (v/v = 7/3). The flow rate is 1.0 mL/min, and the temperature is stabilized at room temperature. The UV detection wavelength is set at 195 nm.

2.3. Extraction of chitin

Chitin is extracted from shrimp shells by demineralization and deproteinization [17]. The process undergoes adjustments concerning the solid/liquid ratio, processing time, and concentration of substances. The shrimp shells are soaked in 10% HCl solution (1:20 w/v) at room temperature for 8 h and are washed several times with water until neutral pH. The shells are then treated with 5% NaOH solution (1:10 w/v) at 90 °C for 4 h for deproteinization. The shells are afterward separated from the solution and washed with water several times to obtain a light pink chitin product. To enhance the purity, chitin is immersed in a mixture of 1.0% H_2O_2 and 0.2% NaOH catalyst (1:1 w/w) at room temperature for 4 h. Subsequently, the solid is filtered and washed till neutral pH. After each step, the sample is dried at 105 °C to determine volume and recovery rate (H_i) by Eq. 1. The mineral and protein components represent the values loss during the processing steps and the remaining component is chitin.

$$H_i\% = \frac{m_i}{m_o} \times 100\% \quad (1)$$

Where,

m_i = weight of recovered solid after step

m_o = weight of raw material

The treatment of shrimp shells by diluted HCl and NaOH solutions creates soluble ionic salts that do not pose any environmental harm. In this study, the chitin bleaching phase avoids the use of NaClO and chlorine due to their potential influence on the environment.

2.4. Preparation of GlcN-Cl

Chitin is treated with a 36% HCl solution in a round-bottom flask quipped with a condenser. As the chitin is heated, it dissolves in the concentrated HCl, resulting in a yellow solution that gradually turns black. A solid-to-liquid ratio (SLR), hydrolysis time, and temperature are investigated according to **Table 1**.

Table 1. Experimental design

SLR (w/v)	Time (h)	Temperature (°C)
1:4 - 1:12	2.5	85
1:9	1.5 - 4.0	85
1:9	3.0	70 - 95

The solution is decolorized by adding activated carbon (chitin-to-carbon ratio = 1:4 w/w). Subsequently, the hot solution is filtered, and the filtrate is allowed to crystallize at 0 - 4 °C for 12 h. The obtained crystals are filtered, washed with EtOH and dried at 105 °C, followed by cooling in a desiccator, and weighed to

determine the final weight of the solid. The recovery rate is calculated by Eq. (2):

$$H\% = \frac{\text{weight of solid product}}{\text{weight of chitin}} \times 100\% \quad (2)$$

The yield of product is calculated by Eq. (3):

$$H\% = \frac{\text{weight of solid product} \times \text{purity}}{\text{weight of GlcN - Cl}} \times 100\% \quad (3)$$

Where, purity of product is determined by HPLC method. The equation used for calculating weight of GlcN-Cl:

$$m_{\text{GlcN-Cl}} = \frac{\text{weight of chitin}}{\text{mol. weight of chitin}} \times \text{mol. weight of GlcN - Cl}$$

After crystallization, GlcN-Cl crystals are filtered and heated at 105 °C to investigate their thermal stability in an acidic environment. Every 10 minutes, the solution is decanted from the solid, and the mass loss is determined.

3. RESULTS AND DISCUSSION

3.1. Components of shrimp shell

The composition of minerals, protein, and chitin in shrimp shells is determined to be 47.91±0.28%, 23.13±0.17%, and 27.98±0.32%, respectively. The collected chitin is white or light pink (Figure 1a). According to the previous work, the chitin recovery rate ranges from 7% to 36% [3].

3.2. GlcN-Cl recovery rate

The experiments were conducted under the same conditions, which involved using 36% HCl solution at 85 °C for 2.5 h. The resulting mixture is decolorized using activated carbon (Figure 1b).

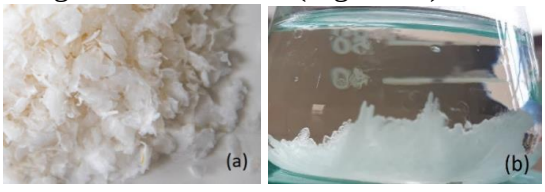


Figure 1. Chitin (a) and GlcN-Cl crystal (b)

As a result, the SLR at 1:9 achieves the highest solid recovery rate after crystallization (Figure 2a). In Figure 2b of the graph, the recovery rate reaches its highest value at the 3-hour mark. The hydrolysis of chitin is conducted under the same conditions regarding HCl concentration, hydrolysis time at 3.0 h, and the SLR at 1:9 (w/v). However, different temperatures are used for the hydrolysis, specifically 70 °C, 85 °C, and 95 °C. The experimental results indicate that the highest product recovery rate is achieved at 85 °C (Figure 2c). At elevated temperatures, GlcN-Cl crystals exhibit reduced stability in acidic media (Figure 2d). The standard curve showcases a linear correlation between the quantity of mass loss and time. This correlation is expressed through the regression line equation: $y = -0.0141x + 2.5516$, accompanied by a correlation coefficient of $R^2 = 0.9702$. The results demonstrate that factors such as the SLR of chitin/HCl solution, reaction temperature, and reaction time significantly influence the efficiency of the GlcN-Cl synthesis process. A high SLR can lead to incomplete hydrolysis, which is also observed at 70 °C and reaction times less than 3 hours. If the SLR is lower than 1:9, excess HCl can dilute the GlcN-Cl solution, hindering crystal formation or reducing chitin conversion to GlcN-Cl. Conversely, temperatures exceeding 85 °C or reaction times longer than 3 hours can significantly decrease reaction efficiency. This may be attributed to the instability of GlcN-Cl at elevated temperatures or prolonged reaction times. This finding is consistent with the results of our investigation into the stability of GlcN-Cl crystals in acid at high temperatures. Therefore, after the crystallization process, it is essential to separate the crystals from the mixture and then concentrate the solution for recrystallization. GlcN-Cl should be washed with ethanol before drying and storage.

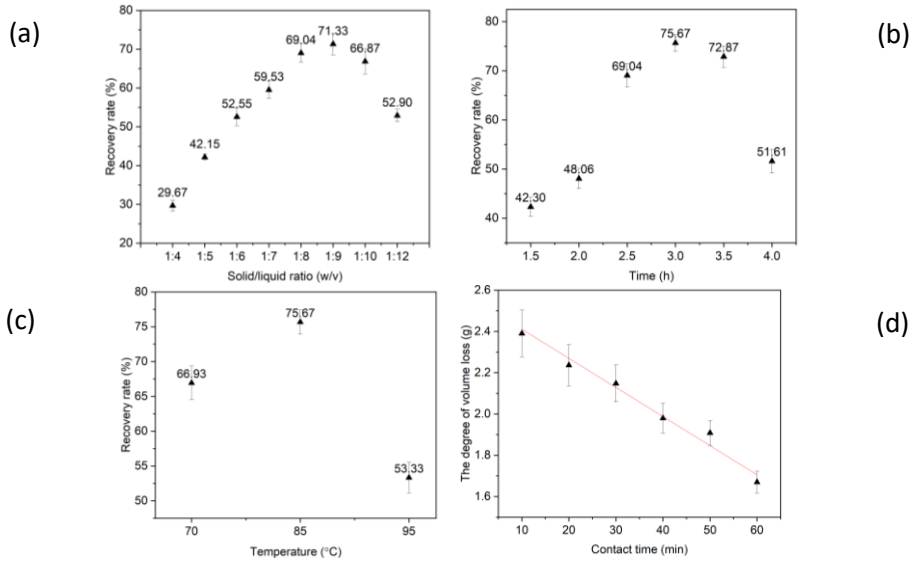
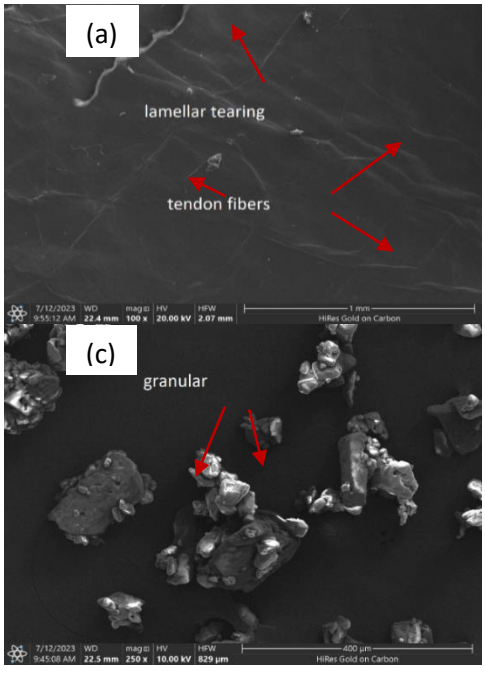
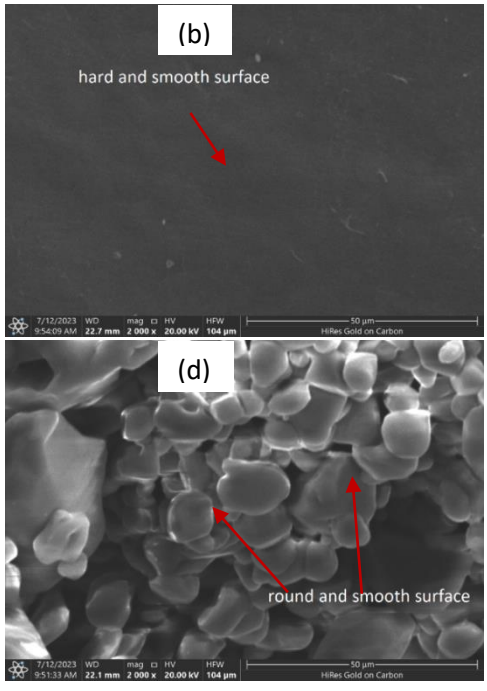


Figure 2. The recovery rate at different solid/liquid ratios (a); contact time (b); temperatures (c) and the extent of volume loss varies over time (d)

3.3. Morphology (SEM)

The surface morphology of chitin changes according to the species used as the chitin source, and it plays a role in determining the usage area of chitin. According to the previous chitin isolation studies, chitin exhibited three surface morphologies: a hard and rough surface without pores or nanofibers, a surface solely composed of nanofibers, and a surface with both pores and nanofibers [18]. Figure 3a and b show that the surface of chitin from the shrimp shell exhibits a uniform structure with a lamellar organization and many tendon fibers, presenting a hard and smooth surface without pores. In addition, the morphology of GlcN-Cl is cylindrical in shape, featuring large irregular particle sizes and a round, smooth surface (Figure 3c and d).





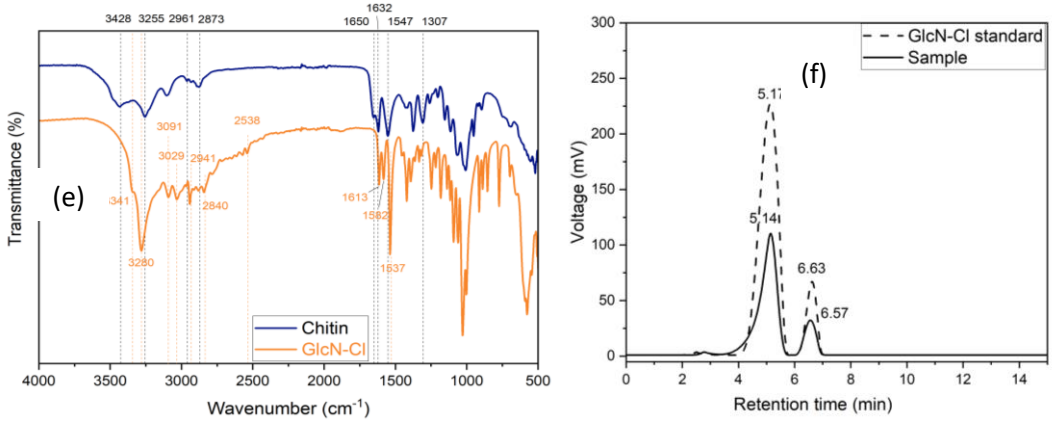


Figure 3. SEM images of chitin at (a) x100 and (b) x2000 magnification, GlcN-Cl at (c) x250, (d) x2000 magnification, FTIR spetra of chitin and GlcN-Cl (e) and chromatogram of GlcN-Cl standard and sample (f)

3.4. FTIR spectral analysis

Spectra of chitin and GlcN-Cl are shown in Figure 3e. Chitin and GlcN-Cl presents some characteristic peaks. The peaks at 3428 cm^{-1} and 3255 cm^{-1} (chitin), 3341 cm^{-1} and 3280 cm^{-1} (GlcN-Cl) are corresponded to N-H and O-H stretching vibrations and intermolecular hydrogen bonding. Absorption bands at a range of wavenumbers 2961-2873 cm^{-1} (chitin) and 2941-2840 cm^{-1} (GlcN-Cl) indicating the presence of vibration span C-H on -CH_2- on the aliphatic group.

In chitin spectra, because chitin has three crystalline forms, either alpha, beta or gamma [18], the FTIR spectra peak between 1650 and 1632 cm^{-1}, known as the amide I band, showing that chitin from shrimp shell is in the alpha crystalline form, due to the two peaks observed at 1650 and 1547 cm^{-1}. These two peaks represent alpha chitin's intramolecular and intermolecular hydrogen bonds forming its antiparallel chain arrangement [19,20].

The hydrogen bonds between amide I (-C=O) and amide II (-NH-) are responsible for the peak at 1650 cm^{-1}, while the second peak occurring at 1632 cm^{-1} is due to hydrogen bonding between the –CH_2O side chain and -C=O [21]. The absorption bands at 1547 and 1307 cm^{-1} correspond to amide II (N-H bending) and amide III (C-N stretching) respectively [22].

In GlcN-Cl spectra, the primary amino group usually give two peaks, from symmetric and antisymmetric stretching, the characteristic absorptions at 3091 and 3031 cm^{-1}. After hydrolysis of chitin, the peaks at 1650, 1632, 1547 and 1307 cm^{-1} disappear along with the appearance of new strong peaks at 1613, 1528 and 1537 cm^{-1} (NH_2 bending and scissoring band) in spectra of GlcN-Cl [23]. The absorption bands at 1613-1537 cm^{-1} are due to the NH_3^+ in GlcN-Cl for asymmetrical, symmetrical NH_3^+ bending and scissoring in the region 3341-2538 cm^{-1}, which proves the formation of NH_3^+ in GlcN-Cl [24,25]. The FTIR spectrum confirmed the successful transformation of chitin into GlcN-Cl.

3.5. Determination of the GlcN-Cl content

Figure 3f represents the HPLC chromatograms of GlcN-Cl standard and sample. The chromatograms of sample are detected distinct peaks, with respective retention times of 5.14 and 6.57 min, close to that found in GlcN-Cl standard, at the retention time of 5.17 and 6.63 min. The two peaks in the HPLC spectrum of

GlcN-Cl are labeled as 5.14 and 6.57. These peaks likely correspond to different forms or isomers of GlcN-Cl that are separated by the chromatography column, α-GlcN-Cl và β-GlcN-Cl, respectively [26]. The retention time of each peak can be used to identify and quantify the specific compounds present in the sample. The amount of GlcN-Cl content in the prepared glucosamine from shrimp shell is estimated to be 990.33±5.51 mg/g. Fawwaz *et al.* [16] reported that the amount of GlcN-Cl isolated from the initial chitin produced from *P. monodon* was 698.41 mg/g. The purity of GlcN-Cl in this study is comparatively greater than that of exoskeletons in the previous study. Therefore, the yield of GlcN-Cl obtained is about 70.59±1.61%, higher than the previous values of 51.01, 63.50, and 66.64% [15,27,28].

Many previous studies suggested that the recovery rate after hydrolysis was also indicative of the yield of the GlcN-Cl preparation process, or using the equation (3) without considering the purity value of the product, is incorrect. In this study, measures are taken to prevent such issues from occurring.

4. CONCLUSIONS

This study represents a significant step forward in the realm of green chemistry, demonstrating a sustainable approach to the synthesis of GlcN-Cl from biomass residues. By minimizing the use of chemicals, bypassing the requirement for chitosan intermediate products, and averting the generation of harmful waste, our methodology aligns closely with the principles of environmentally conscious research and development. Analysis of *P. monodon* shrimp shells revealed substantial mineral, protein, and chitin content, quantified at 47.91±0.28%, 23.13±0.17%, and 27.98±0.32%, respectively. Optimal conditions for GlcN-Cl production from chitin were determined as a SLR of 1:9 (w/v), treatment duration of 3.0 h, and a temperature of 85°C, yielding a purity of 99.03±0.55% and efficiency of 70.59±1.61%. Importantly, this process capitalizes on abundant waste resources from seafood processing, transforming them into high-value functional foods, thereby contributing significantly to sustainable development objectives. Through our innovative approach, we not only enhance resource efficiency but also pave the way for the creation of valuable products with promising applications in food and medicine, thus reinforcing the importance of sustainability in scientific endeavors.

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