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Physicochemical Characteristics of Glucosamine from Blue Swimming Crab (*Portunus pelagicus*) Shell Prepared by Acid Hydrolysis

Jittrakan KRAISANGSRI[1], Sitthipong NALINANON[1,*], Siriporn RIEBROY[2], Suthasinee YARNPAKDEE[3] and Palanivel GANESAN[4]

[1]*Faculty of Agro-Industry, King Mongkut's Institute of Technology Ladkrabang, Ladkrabang, Bangkok 10520, Thailand*
[2]*Food and Nutrition Program, Faculty of Agriculture, Kasetsart University, Bangkok 10900, Thailand*
[3]*Division of Marine Product Technology, School of Agro-Industry, Faculty of Agro-Industry, Chiang Mai University, Chiang Mai 50100, Thailand*
[4]*Nanotechnology Research Center, Department of Biotechnology and Applied Life Science, College of Bio-medical and Health Science, Konkuk University Glocal campus, Chungju 380701, Republic of Korea*

(*Corresponding author's e-mail: sitthipong.na@kmitl.ac.th)

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Abstract

The aim of this research was to characterize glucosamine hydrochloride (GluHCl) from the shell of blue swimming crab (*Portunus pelagicus*). The crab shell was finely milled and processed to chitin prior to HCl hydrolysis using 30 % HCl for 30 min at 100 ºC for glucosamine production. The resultant glucosamine was recovered by crystallization using 95 % ethanol and was dried in a hot air oven. The color of the glucosamine crystals, expressed as L*, a*, and b*, was 83.01, 5.03, and -3.38, respectively. Crab shell glucosamine had high purity, which could be strongly stained by ninhydrin and presented at the same R_f of standard D-glucosamine using thin layer chromatography. Furthermore, prepared glucosamine exhibited similar Fourier transform infrared (FTIR) spectrum as standard D-glucosamine. Glucosamine from blue swimming crab shell had high purity as determined by HPLC and contained 808.15 mg D-glucosamine/g sample. The maximal transition temperature (T_{max}) and the total enthalpy (ΔH) of prepared glucosamine were 194 ºC and 754.42 J/g, respectively. As a consequence, with the presented method, the resultant glucosamine was characterized to be D-glucosamine. Therefore, blue swimming crab shell, a byproduct from crab meat processing, has high potential as a raw material to produce glucosamine for food and nutraceutical applications.

Keywords: Blue swimming crab, characterization, crab shell, FTIR, glucosamine, HPLC

Introduction

Glucosamine is an aminomonosaccharide, which is the principal component of the *O*-linked and *N*-linked glycosaminoglycans which form the matrix of all connective tissues and cartilages [1], and has recently received much attention due to its therapeutic activity against osteoarthritis [2]. The preparation of glucosamine hydrochloride (GluHCl) from chitin is a simple hydrolysis reaction [3]. During this reaction, chitin is deacetylated and depolymerized to glucosamine hydrochloride in the presence of hydrochloric acid [4]. Chitin is a natural polymer, which is found in crustaceans including lobsters, shrimps, and crabs. Glucosamine hydrochloride is an important derivative of chitin. About 40 - 50 % of the total weight of crustaceans is removed as waste or byproduct during processing [5,6], particularly in shrimp and crab shells. The production of glucosamine from processed crustacean byproducts has been

studied previously. Wanichpongpan and Attasat [6] prepared GluHCl through acid hydrolysis of chitosan from shrimp-shell with 14.4 % HCl (w/v) at 80 °C for 6 h prior to crystallization with ethanol at a ratio of 1:1, which was then dried in a hot air oven. To produce glucosamine sulfate ($GluSO_4$), the obtained GluHCl was then reacted with sodium sulfate at 40 °C for 30 min [6]. Mojarrad [7] reported that the optimum conditions for GluHCl preparation from Persian Gulf shrimp (*Metapenaeus monoceros*) were 30 and 37 % HCl at a ratio of 1:9 (w/v) at 100 °C for 4 h. Blue swimming crab (*Portunus pelagicus*), a commercially important species, are distributed throughout the coastal waters of the tropical regions of the Western Indian Ocean and the Eastern Pacific [8]. In Thailand, *P. pelagicus*, for direct consumption and for use as a raw material in the processing industry, is generally caught in the Andaman Sea and the Gulf of Thailand [9]. Crab shell, the byproduct from crab processing, is generated in large quantities annually [9]. Sibi *et al.* [5] prepared glucosamine hydrochloride (GluHCl) from the crustacean shells of *Penaeus monodon* (Indian shrimp), *Portunus pelagicus* (blue swimming crab), and *Portunus sanguinolentus* (3 spot crab) by acid hydrolysis. They reported that, with HPLC analysis, crab shell was a better source of GluHCl than shrimp shell. In addition, production of GluHCl from crustacean shells minimized environmental pollution from their processing plants [10]. Therefore, the aim of this study was to isolate and characterize glucosamine from blue swimming crab shell prepared by hydrochloric acid hydrolysis.

Materials and methods

Chemicals

Ethanol, sodium hypochlorite, and hydrochloric acid were obtained from Merck (Darmstadt, Germany). Ninhydrin, activated charcoal, D-glucosamine, and N-acetyl-D-glucosamine were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sodium hydroxide was obtained from Ajax (NSW, Australia). All chemicals used were of analytical grade.

Preparation of blue swimming crab shell

Blue swimming crab shell from fresh crab, with an average size of 6 - 7 crabs/kg, was obtained from Viriya Crab Product Co., LTD, Surat Thani, Thailand. The crab shell was washed thoroughly with tap water and dried in a hot air oven at 100 ºC for 4 h. Dried crab shells were finely milled using a hammer mill prior to sifting through a 0.75 mm sieve. The milled crab shell was packed in polyethylene bags and kept at 4 ºC until use.

Isolation of glucosamine

Glucosamine from blue swimming crab shell was isolated according to the method of Sibi *et al*. [5] with some modifications. The milled crab shell was deproteinized with 3.5 M NaOH at a ratio of 1:9 (w/v) at 65 ºC and stirred using an overhead stirrer for 2 h. The suspension was then cooled to room temperature prior to filtration with Whatman No. 1 filter paper. The residue was washed 3 times with distilled water at a ratio of 1:50 (w/v) prior to neutralization using 0.1 M HCl and then washed with distilled water. After filtration, deproteinized crab shell was demineralized by soaking in 1 M HCl at a ratio of 1:15 (w/v) and stirred at room temperature for 18 h. The demineralized sample was washed with distilled water, neutralized and soaked with 0.35 % sodium hypochlorite, washed with distilled water, dried in a hot air oven at 60 ºC for 4 h, and referred to as 'chitin'. The chitin was hydrolyzed with 30 % (w/v) HCl at a ratio of 1:19 (w/v) at 100 ºC for 30 min, followed by decolorization with activated charcoal and filtration. The filtrate was concentrated by evaporation at 45 °C prior to crystallization with 95 % ethanol at a ratio of 1:9 (w/v). After centrifugation at 5,000×g for 15 min, the glucosamine crystals were washed with 99 % ethanol, dried in a hot air oven, and referred to as 'glucosamine'.

Yield

The yield (% dry weight basis) of glucosamine was calculated by the following equation;

$$\text{Yield (\%)} = [\text{weight of dried glucosamine (g)}/\text{weight of milled crab shell (g)}] \times 100 \quad (1)$$

Chemical composition of blue swimming crab shell

The chemical composition, including moisture, fat, ash, and protein contents of blue swimming crab shell was determined according to the AOAC [11] methods.

Color and whiteness

The color of glucosamine from blue swimming crab shell was determined by measuring the L*, a*, and b*. Three replicates were carried out for each sample. Whiteness was then calculated using the following equation [12];

$$\text{Whiteness} = 100 - \left[(100 - L^*)^2 + a^{*2} + b^{*2}\right]^{1/2} \quad (2)$$

Thin layer chromatography (TLC)

The purity of glucosamine was determined by TLC according to the method of Esters *et al.* [13]. The analysis was performed on 20×10 cm^2 TLC plate. The plate was developed 6 cm beyond the origin with 1-butanol-acetic acid-deionized water (3:1:1) as the mobile phase and silica gel as the stationary phase. The plate was strained with ninhydrin reagent. The chromatogram of glucosamine prepared from crab shell was visualized and compared with standard N-acetyl-D-glucosamine and D-glucosamine.

Fourier transform infrared spectroscopy (FTIR)

FTIR analysis was performed according to the method of Ahmad *et al.* [14]. The glucosamine samples (0.1 g) were placed onto the crystal cell, and the cell was clamped into the mount of the FTIR spectrometer (Model Equinox 55, Bruker, Germany). Spectra in the range of 400 - 4000 cm^{-1} with automatic signal gain were collected in 32 scans at a resolution of 4 cm^{-1} and ratioed against a background spectrum recorded from the clean, empty cell at 25 ºC. Analysis of spectral data was carried out using the OPUS 3.0 data collection software program (OPUS 3.0, Bruker, Germany).

High performance liquid chromatography (HPLC)

HPLC analysis was performed according to the method of Sibi *et al.* [5] using the 1260 infinity series LC system installed with a G1311C pump, a G1329B autosampler, a G13166 column compartment, and a G4212B DAD detector. Chromatographic separation was carried out on a Zorbax Eclipse XDB-C8 analytical column (4.6×250 mm^2, 5 μm; Agilent Technologies). The mobile phase used was orthophosphoric acid (pH 2.5): acetonitrile (70:30) with a flow rate of 1.8 ml/min in a column at ambient temperature with infusion volume of 20 μl in each experiment. Injection volume was 20 μl and detection was by UV absorbance at 195 nm. The retention times of N-acetyl-D-glucosamine, D-glucosamine, and glucosamine from blue swimming crab shell were determined by the absorbance of the refractive index (RI) signal. This RI signal was expressed as a dimensionless number in nano-Refractive Index Units (nRIU), and indicated the differences between the RI of the sample in the sample cell and the mobile phase in the reference cell.

Differential Scanning Calorimetry (DSC)

DSC was performed, using Model DSC 7 (Norwalk, USA). Temperature calibration was done using an indium thermogram. Glucosamine samples (3 - 6 mg) were accurately weighed into aluminum pans and sealed. The samples were scanned at 5 ºC/min over a range of 50 - 300 ºC under nitrogen gas. An empty pan was used as the reference. Total denaturation enthalpy (ΔH) was estimated by measuring the area in the DSC thermogram. The maximum transition temperature (T_{max}) was estimated from the thermogram.

Results and discussion

Chemical composition of blue swimming crab shell and yield of glucosamine

The chemical composition of blue swimming crab shell, after being dried and milled, is shown in **Table 1**. The results showed that blue swimming crab shell contained 43.15 ± 12.53 % carbohydrate, 29.78 ± 0.43 % ash, 18.14 ± 0.79 % protein, 8.68 ± 0.42 % moisture, and 0.25 ± 0.08 % fat on a dry weight basis. Carbohydrate was the major component found in the crab shell, indicating chitin as a major component. Chitin is the most abundant polysaccharide, containing a derivative of glucose as a backbone, and has nitrogen and acetamido groups in its molecule [5]. Generally, the chemical composition of crab shells varies with species, seasons, and many other factors [15]. Jung *et al.* [16] reported that littoral crab shells (*Carcinus mediterraneus*) contained 12.1 ± 0.5 % protein, 59.8 ± 0.2 % ash, and 0.7 ± 0.2 % fat based on dry weight, which was slightly different from that of the protein and ash contents of blue swimming crab shell (**Table 1**). Overall, the percentages of proteins and ash in crab shell were shown to vary within a broad range of 13 - 15 % protein and 18 - 23 % ash [17]. After HCl hydrolysis, chitin in crab shell was mostly converted to glucosamine. The yield of resultant glucosamine was calculated to be 36.47 % based on the milled crab shell. Benavente *et al.* [18] found that the best condition for the highest yield (58%) to produce GluHCl from shrimp shell by acid hydrolysis was obtained at solid/liquid ratio of 1:20, temperature of 85 °C, and with agitation. Nurjannah *et al.* [19] reported that the yield of GluHCl production from crab shell hydrolyzed with 27 % HCl at 90 °C for 4 h was found to be 18.39 %. The variable factors that can influence the yield of crustacean shell hydrolysis are acid concentration, acid solution to solid ratio (v/w), and hydrolysis time [7]**.** Therefore, blue swimming crab shell could be a good source for glucosamine production.

Table 1 Chemical composition of blue swimming crab shell after being dried and milled.

Compositions	Content (% db)*
Protein	18.14 ± 0.79
Fat	0.25 ± 0.08
Ash	29.78 ± 0.43
Moisture	8.68 ± 0.42
Carbohydrate	43.15 ± 2.53

*Values are given as mean ± SD (n = 6).

Color and whiteness of glucosamine from blue swimming crab shell

The color of glucosamine from blue swimming crab shell, measured in the CIE system, is shown in **Table 2**. Glucosamine crystal showed high L^* values (L* = 83.01 ± 0.35), and that a* > 0 and b* < 0. L*, a* and b* values correspond to black/colorless component (L*; L* = 0 represents black, and L* = 100 represents colorless), the red/green color component (a*; a* > 0 is associated with red, and a* < 0 is associated with green) and the yellow/blue color component (b*; b* > 0 is associated with yellow, and b* < 0 is associated with blue) [20]. Glucosamine crystals were visualized as light gray in color, even though the whiteness was estimated to be 81.96 %. The result was in accordance with Rosmiati *et al*. [21] who reported that the whiteness of GluHCl from chitosan of silkworm pupae shell (*Bombyx mori* L.) was estimated to be 83.56 ± 0.03 %. Remarkably, the use of ethanol to recover glucosamine led to a change in glucosamine crystal color from colorless to light grey. Benavente *et al.* [18] found that the thinner, clearer, and brighter glucosamine crystal was obtained by cooling the glucosamine mixture at 5 °C and adding 95 % ethanol for crystallization.

Table 2 Color and whiteness of glucosamine crystal measured in the CIE system.

Color	Glucosamine crystal [†]
L*	83.01 ± 0.35
a*	5.03 ± 0.01
b*	-3.38 ± 0.08
Whiteness	81.96 ± 0.34

[†] Values are given as mean ± SD (n = 6).

Purity of glucosamine from blue swimming crab shell

TLC

The purity of glucosamine was assessed by using TLC. TLC chromatograms of different glucosamines are shown in **Figure 1**. The result revealed that standard N-acetyl-D-glucosamine, a typical monomer of chitin, had a very low intensity band after ninhydrin staining. This was possibly due to there being no free -NH_2 group in its structure [22]. In contrast, glucosamine from blue swimming crab shell could be stained with ninhydrin, showing high intensity at the same R_f of standard D-glucosamine. Since D-glucosamine is the major component in the chitosan structure, the results suggested that chitin might be converted, to some extent, to chitosan during glucosamine isolation. The conversion of chitin to chitosan was previously shown to depend on the reaction temperature, time, and the concentration of the alkaline solution [23].

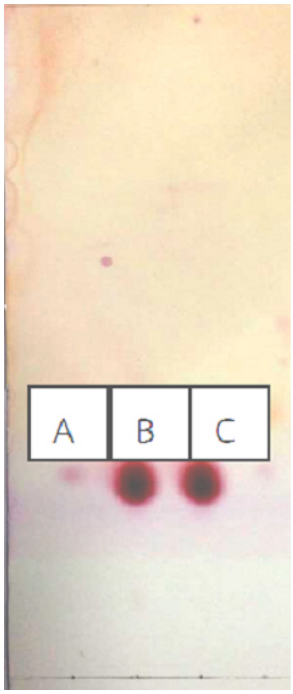

Figure 1 TLC chromatograms of N-acetyl-D-glucosamine (A), glucosamine prepared from crab shells (B), and D-glucosamine (C).

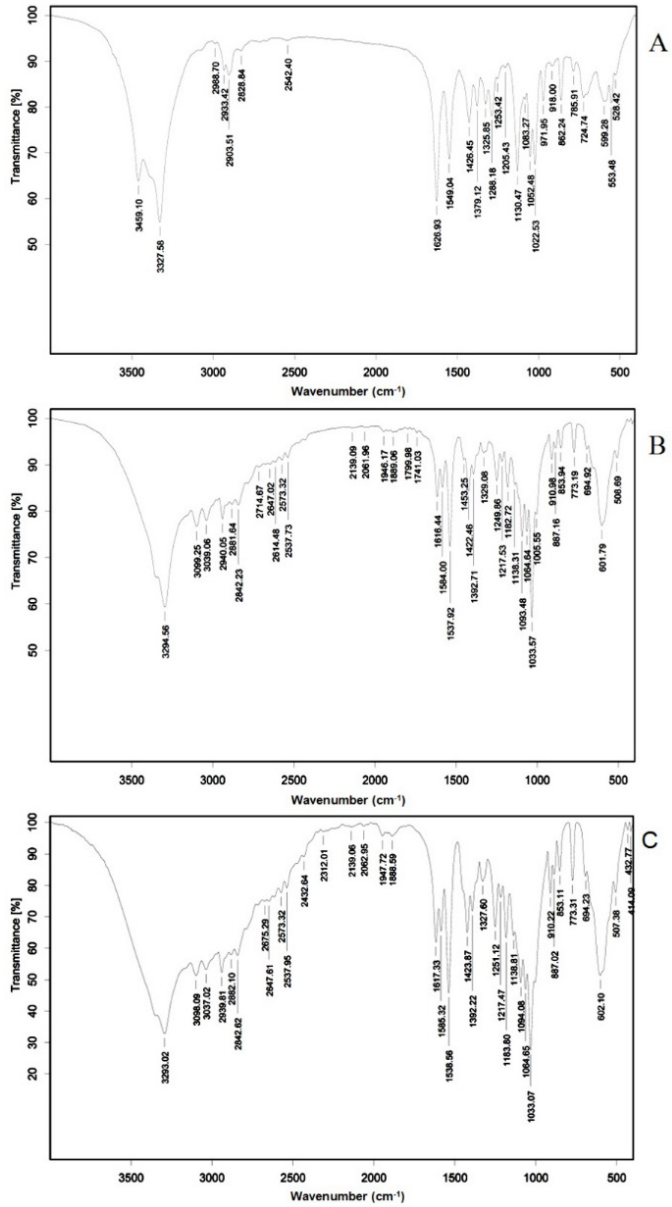

Figure 2 FTIR spectra of N-acetyl-D-glucosamine (A), D-glucosamine (B), and glucosamine prepared from blue swimming crab shell (C).

FTIR

FTIR spectra of N-acetyl-D-glucosamine, D-glucosamine, and glucosamine prepared from blue swimming crab shell are depicted in **Figure 2**. The FTIR spectra of N-acetyl-D-glucosamine, D-glucosamine, and glucosamine prepared from blue swimming crab shell were displayed in the amide region. Generally, the FTIR spectra of D-glucosamine and glucosamine from crab shell were similar. N-acetyl-D-glucosamine exhibited different spectra from other glucosamines. At the amide A region, N-acetyl-D-glucosamine had 2 absorption peaks at wavenumbers of 3327.58 and 3459.10 cm^{-1}, whereas D-glucosamine and glucosamine from blue swimming crab shell showed a distinct absorption peak at

wavenumbers of 3294.56 and 3293.02 cm^{-1}, respectively. Benavente *et al*. [18] reported that glucosamine from crustacean shells exhibited an intense band at 3,370 - 3,300 cm^{-1}, associated with O-H and N-H stretching, and an NH_2 scissoring band at 1,615 and at 1,094 cm^{-1} due to secondary alcohol -OH. Furthermore, The FTIR spectrum of glucosamine from crab shell showed a deacetylation due to the wavenumber at about 1726 cm^{-1} for C=O stretching absorption, which exists in chitin, having disappeared [24].

HPLC

Figure 3 represents the HPLC chromatograms of different glucosamines. The results showed that the chromatograms of standard N-acetyl-D-glucosamine and D-glucosamine detected distinct peaks, with retention times (RT) of 4.731 and 6.776 min, respectively. Glucosamine from blue swimming crab shell exhibited only one peak, at the retention time of 6.770 min, close to that found in standard D-glucosamine. The amount of D-glucosamine content in the prepared glucosamine from blue swimming crab shell was estimated to be 808.15 ± 4.25 mg/g. Therefore, glucosamine from blue swimming crab shell had high purity and contained D-glucosamine as the major constituent. Sibi *et al.* [5] reported that, based on HPLC analysis, the amount of GluHCl isolated from *Penaeus monodon* (Giant tiger prawn), *P*. *pelagicus* (blue swimming crab), and *P*. *sanguinolentus* (3 spot crab) was 3.32, 21.83, and 21.64 mg/g, respectively. The purity of glucosamine from *P*. *pelagicus* in this study was comparatively greater than that of crustacean shell in the previous study.

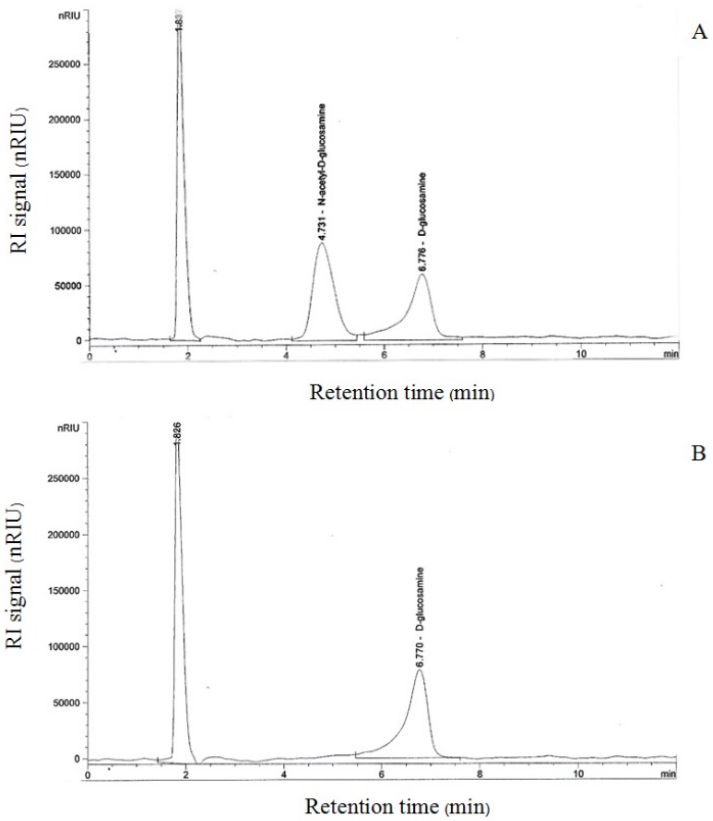

Figure 3 HPLC chromatograms of N-acetyl-D-glucosamine and D-glucosamine (A) and glucosamine from blue swimming crab shell (B).

DSC

DSC thermogram of the glucosamine from blue swimming crab shell is depicted in **Figure 4**. Maximal transition temperatures (T_{max}) and the total enthalpy (ΔH) of glucosamine were 194 ºC and 754.422 J/g, respectively. T_{max} of glucosamine from blue swimming crab shell was comparable to that found in standard D-glucosamine (190 - 194 ºC), but lower than that found in N-acetyl-D-glucosamine (211 ºC) (Sigma, St. Louis, MO, USA). The results were in accordance with Islam *et al.* [10], who reported that GluHCl from chitin extracted from indigenous shrimp shells exhibited a melting point of 192 - 194 ºC. Similar results were reported by Rosmiati *et al.* [21], who reported the melting point of D-glucosamine from silkworm pupae shell (*Bombyx mori* L.) (189 - 193 ºC). However, the melting point of glucosamine from crab shell was lower than that of N-acetyl-D-glucosamine (221 ºC) [25]. Therefore, it was confirmed that the major component in glucosamine from blue swimming crab shell was D-glucosamine.

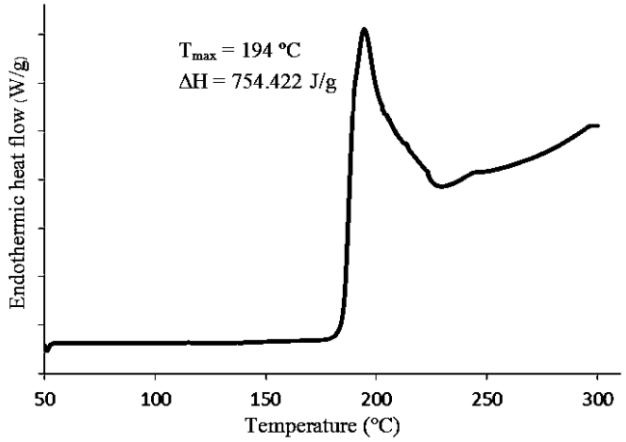

Figure 4 DSC thermogram of glucosamine prepared from blue swimming crab shell.

Conclusions

Glucosamine from blue swimming crab shell could be effectively prepared by acid hydrolysis. Based on the results from TLC, FTIR, HPLC, and DSC analysis the isolated glucosamine contained D-glucosamine as the major constituent, with high purity. The byproduct from crab processing, such as crab shell, could be a future alternative source for glucosamine production, which could be used in food and pharmaceutical applications with high therapeutic value.

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