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ISOLATION OF GLUCOSAMINE HCL FROM *SCYLLA PARAMAMOSAIN* AND DETERMINATION BY HPLC

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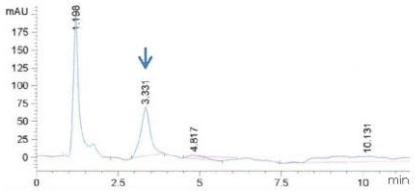
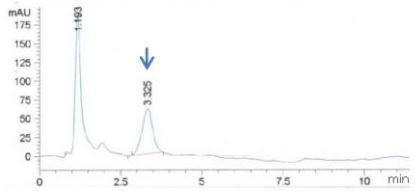
Graphical abstract



Chromatogram of Glu-HCl standard from *Sigma-Aldrich*



Chromatogram of Glu-HCl sample from Experiment 1

Abstract

Scylla paramamosain is one of crustaceans that has economic value in Indonesia. About 40-50% of the total weight of crustaceans goes as waste after being processed to be human food. The Crustacean waste needs to be reduced in order to minimize the negative impact on the environment. Whereas products derived from crustacean shell also has medical value (e.g. Chitosan, Glucosamine HCl). Glucosamine is an amino monosaccharide acting as a substrate for the production of aggrecan and proteoglycans and thus have therapeutic activity in osteoarthritis. The present study has been aimed to prepare glucosamine hydrochloride (Glu-HCl) from *S. paramamosain* waste by acid hydrolysis in four treatment groups and their quantitation by high-performance liquid chromatography (HPLC). The best condition from this experiment is shell deproteination with 3% NaOH solution (w/v 1:6), at 85ºC, for 30 minutes, demineralisation with 1N HCl (w/v 1:10), at 75ºC, for 1.5 hours, depigmentation with NaClO 0.38%, at room temperature, for 1 hour, and hydrolysis with conc. HCl (w/v 1:20), at 85ºC, for 18 minutes, resulted in the yield of 6.15 ± 0.62% and quantitation by HPLC analysis of obtained glucosamine hydrochloride was 98.48 ± 0.74%. Further, this study describes the recycling of *S. paramamosain* waste to a value-added product which is having potential applications in the field of food and medicine.

Keywords: *Scylla paramamosain*, glucosamine hydrochloride, crustaceans, chitin, chitosan

1.0 INTRODUCTION

D-glucosamine ($C_6H_{13}NO_5$) is an amino sugar (hexosamine) which naturally presents in human body and crustacean shells. It is a precursor of biochemical synthesis of the GAGs (glycosaminoglycans) found in cartilage. Premature loss of cartilage is part of the clinical syndrome recognized as OA (osteoarthritis) [1]. Thus, glucosamine in the form of glucosamine sulfate, glucosamine hydrochloride (Glu-HCl), or N-acetyl-glucosamine is extensively used as a dietary supplement in the treatment for osteoarthritis, knee pain, and back pain [2]. Glucosamine can be prepared by acid hydrolysis of chitin or chitosan from crustacean using strong mineral acids [3, 4, 9], and can be prepared by hydrolizing chitosan using crude enzyme (i.e chitosanase and β-d-glucosaminidase obtained from the fermentation broth of Microbacterium sp. OU01.) [26].

Active Pharmaceutical Ingredients (API) Industry has produced Glu-HCl through hydrolysis of chitin of shrimp shells [5, 6]. *Scylla paramamosain* known as Mud Crab (Indonesian - *kepiting super*) is one of the crustaceans that is widely cultivated and consumed in Indonesia. However, about 40-50% of the weight of crustaceans goes as waste after being processed into human food [7]. Every year Indonesia produces around 56,200 tons of waste containing chitin [8]. Crustaceans waste in the environment is slowly

degraded and can cause pollution problems [4], therefore it needs attention to overcome this. Based on the dispute, it is expected that *S. paramamosain* shells can be processed into glucosamine which has a higher economic value.

In a previous study, Sibi *et al.* [4] conducted a study of producing Glu-HCl from various types of crustaceans (*Penaeus monodon, Portunus pelagicus,* and *Portunus sanguinolentus*) by hydrolyzing their chitosan. Benavente *et al.* [3] and Pesek *et al.* [9] conducted a study of producing Glu-HCl from shrimp shells by hydrolyzing their chitin. The present study aims to get the optimum condition of preparing Glu-HCl from *S. paramamosain* shells waste by dividing the study into four treatment groups as the table is included in the methodology. However, this study is the first report to produce Glu-HCl from *S. paramamosain* shell waste and to evaluate this new source of Glu-HCl, which is important for the preservation of the ecosystem from crustaceans waste pollution.

2.0 METHODOLOGY

2.1 Materials

S. paramamosain was determined by Dr. Bambang Irawan from Faculty of Science and Technology of Universitas Airlangga.

Chitin standard was obtained from BPPT (Agency of Assessment and Application of Technology, Indonesia); Chitosan standard was obtained from *Sigma-Aldrich*.

The D-Glucosamine HCl standard was obtained from Sigma-Aldrich with a ≥99% of purity and a molecular weight of 215.63 g/mol.

2.2 Methods

2.2.1 Step 1: Pre-wash with Water

S. paramamosain shells were initially hand washed with cold tap water by removing dirt and loose tissues. Finally, they were washed with hot distilled water and then dried under the sun for 24 hours [4]. Washed and dried shells were powdered and passed through 100 µm mesh sieves. The powdered shells were weighed approximately 50 grams for each experiment group (Table 1). All experiments were carried out in triplicate.

2.2.2 Step 2: Deproteinization

The *S. paramamosain* shells powder were treated with 3% sodium hydroxide (NaOH) solution (w/v 1:6) at 85ºC for 30 minutes to remove the remaining proteins and other organic materials [23]. After the reaction, the solution was colored and frothy, therefore, the sample was washed repeatedly with water until most of the color and frothing disappeared and the resulting solution was near neutral [22]. The sample was finally washed with distilled water and then dried in an oven at 50ºC to obtain constant weight.

2.2.3 Step 3: Demineralization

The products from step 2 were treated with a 1N hydrochloric acid (HCl) solution (w/v 1:10) at 75ºC for 1 hour to dissolve inorganic minerals, mainly calcium carbonate ($CaCO_3$). Then the filtrated solution was removed and replaced with 130 mL of 1N HCl solution (w/v 1:10) at 75ºC for 30 minutes, resulted in chitins. The chitins were then washed several times with water to remove $CaCl_2$ and other water-soluble impurities [22]. The chitins obtained were filtered and dried in an oven at 50ºC until constant weight.

2.2.4 Step 4: Decolouration

The chitins were treated with 0.38 wt% NaClO solution at room temperature for 1 hour to remove pigments [3]. The washed chitins were filtered and dried in an oven at 50ºC until obtaining constant weight. The resultant chitins were obtained in the form of a white powder. Furthermore, the chitin was identified by FT-IR.

2.2.5 Step 5: Extraction of Glu-HCl from Chitin or Chitosan

In step 5, chitins resulted from the first to fourth steps have become the intermediate material and been converted to Glu-HCl can be carried out in different four groups of treatment. For experiment 1 and experiment 2, chitins were hydrolyzed directly into Glu-HCl using HCl conc. at 85ºC and 90ºC, respectively. For experiment 3 and experiment 4, chitins were processed into chitosans first, then they were hydrolyzed to Glu-HCL using HCl conc. at 85ºC and 90ºC, respectively.

2.2.5.1 Step 5.1: Conversion of Chitin to Glu-HCl

Chemical hydrolisis was achieved by the treatment of extracted chitins from step 4 with HCl conc. (w/v 1:20) into a 250 mL of reflux vessel [3] at elevated temperature. After reached 85ºC or 90ºC, respectively. The mixture was kept at the given temperatures for 18 minutes (until the solid was completely dissolved). The process of purification was carried out further on step 6.

2.2.5.2 Step 5.2: Conversion of chitins – chitosans to Glu-HCl

Deacetylation of chitins to chitosans was achieved by the treatment of chitin from step 4 using 45% NaOH (w/v 1:20) at 110ºC for 2 hours with constant agitation. The products were neutralized until reached pH 6-7 by being washed several times using distilled water and dried at 50ºC for 24 hours. The chitosans obtained were identified by FT-IR. The resulted chitosans then were hydrolyzed into Glu-HCl.

Chemical hydrolysis was achieved by the treatment of chitosan obtained with HCl conc. (w/v 1:20) into a 250 mL of reflux vessel [3]. After reached 85ºC or 90ºC respectively, the mixture was kept at the given temperatures for 18 minutes (until the solid

was completely dissolved). The process of purification was carried out further in step 6.

2.2.6 Step 6: Purification of Glu-HCl Crystals

Each of the resultant brownish black materials from step 5 was decolorized with activated charcoal (1:0.1 w/w) in the hot condition [4] and stirred until the temperature dropped to 40°C.

The filtrate was filtered by gravity through a Whatman™ 1091-935 Grade 91 filter paper to remove the solid particles presented in the solution, and it was left to crystallized at 40 ± 5°C. In order to increase the crystallization rate, ethyl alcohol (15 mL, w = 95%) was added to the filtrate. The solid crystals obtained then were washed with ethyl alcohol 95% at 5ºC [3] and dried at 40 °C in an oven for 2 days. The Glu-HCl was recrystallized by diluting it in aqua demineralised (1:10 w/v), then decolorized again with activated charcoal (1:0.1 w/w), stirred, filtered twice trough Whatman™ 1091-935 Grade 91 and then used Whatman filter 0,2 µm. The Glu-HCl product was dried in an oven at 50°C until obtaining constant weight. The Glu-HCl obtained was identified by FT-IR.

Table 1 Experimental procedure for the extraction of Glu-HCl from *S. paramamosain* shells

Treatment groups	Experimental details
Experiment 1	Chitin*) was hydrolyzed with HCl conc. (w/v 1:20) at 85°C, Glu-HCl then was purified as described in step 6.
Experiment 2	Chitin*) was hydrolyzed with HCl conc. (w/v 1:20) at 90°C, Glu-HCl then was purified as described in step 6.
Experiment 3	Chitin*) was deacetylated with 45% NaOH (w/v 1:20) at 110°C for 2 hours, chitosan obtained was identified by FT-IR, hydrolyzed with HCl conc. (w/v 1:20) at 85°C, Glu-HCl then was purified as described in step 6.
Experiment 4	Chitin*) was deacetylated with 45% NaOH (w/v 1:20) at 110°C for 2 hours, chitosan obtained was identified by FT-IR, hydrolyzed with HCl conc. (w/v 1:20) at 90°C, Glu-HCl then was purified as described in step 6.

*Each chitin was obtained by processing *S. paramamosain* through step 1 until step 4

Effects of various parameters such as production pathway (chitin hydrolysis and chitosan hydrolysis) and temperature (85°C and 90°C) on the extraction of Glu-HCl were investigated.

2.3 FT-IR Spectrum

The FT-IR spectrum of chitin, chitosan, and Glu-HCl was recorded on a Perkin Elmer Spectrum One FT-IR spectrometer in the form of Kbr discs.

2.4 HPLC Method

HPLC analysis was performed using Agilent: 1100 Series LC system installed with a pump, an autosampler, a column compartment, and DAD Detector. Analysis method preferred to USP 41 [10] with solvent composition, temperature, and injection volume adjustment by carrying out chromatographic separation on a Zorbax *Carbohydrate Analysis* Analytical column (4.6 x 150 mm, 5 µm; Agilent Technologies). The mobile phase used was dibasic potassium phosphate (pH 7.5): acetonitrile (32.5:67.5) with the flow rate of 1.5 ml min in a column at 39ºC. Injection volume was 8 µl and detection was by UV absorbance at 195 nm.
Stock standard solutions were prepared by accurately adding 380 mg of Glu-HCl standard to 20 ml of diluent (water: acetonitrile - 50:50) in a 25 ml of volumetric flask, sonicated and made up to final volume. Working standards were prepared in the mobile phase, sonicated, and filtered through a 0.22 µm filter.

2.5 Loss of Drying and Residue on the Ignition of Glu-HCl from *S. Paramamosain*

Method of Loss on Drying and Residue on the Ignition of Glu-HCl was performed by preferring to USP 41 [10].

2.6 Arsenic Impurities

Method for the determination of arsenic impurities was performed preferred to USP 41. The requirement for arsenic impurities was not more than 3 ppm [10].

2.7 Optical Rotation

Method of the spesific rotation of Glu-HCl was performed preferred to USP 41. The spesific rotation of Glu-HCl was +70.0° to +73.0° [10].

3.0 RESULTS AND DISCUSSION

3.1 Production of Chitin, Chitosan, and Glu-HCl from *S. paramamosain*

Glucosamine is part of the structure of chitosan, and chitin which composes the exoskeletons of crustaceans, arthropods, and fungi [4]. The composition of the shells of selected crustaceans was reported to consist of 30-40% protein, 30-50% calcium carbonate & calcium phosphate, and 20-30% chitin [11].

Chitin is the second most ubiquitous natural biopolymer after cellulose on earth; it is a linear amino-polysaccharide consisting mainly of β-(1 → 4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose units and partially of β-(1 → 4)-linked 2-amino-2-deoxy-β-D-glucopyranose. The most important derivative of chitin is chitosan obtained by partial deacetylation of chitin under alkaline conditions, and thus composed of N-acetyl-D-glucosamine and D-glucosamine units. The degree of N-acetylation is basically employed to differentiate chitin from chitosan, and the presence of amino groups, in the chitosan structure, gives this polymer many peculiar properties [24]. The process of

deacetylation of chitin into chitosan and hydrolysis of chitin into glucosamine is described on Figure 1. The normal procedure for preparation of chitin from crustacean shells includes the use of sodium or potassium hydroxide for deproteination, hydrochloric acid for demineralization and agents to remove the remaining proteins, calcium, and color, respectively [25]. Deacetylation is a step to convert chitin to chitosan by the removal of acetyl group. The preparation of chitosan is generally achieved by treatment with concentrate of sodium or potassium hydroxide solution at elevated temperature [24]. Furthermore, acid hydrolysis is the primary mechanism involved in thermal depolymerization of chitosan chloride in the solid state [25].

Sibi *et al.* [4] and Sun *et al.* [26] prepared D-glucosamine by hydrolysing the chitosan. Benavente *et al.* [3] prepared D-glucosamine by hydrolysing the chitin.

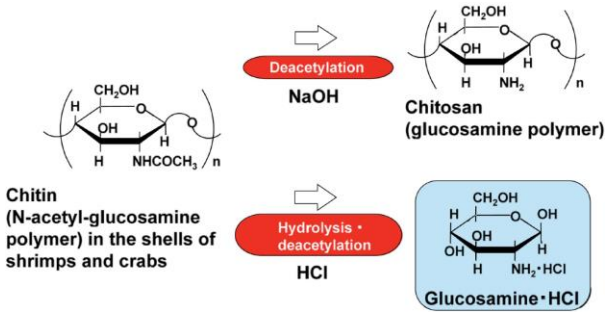


Figure 1 Manufacturing processes of chitosan (a glucosamine polymer) and glucosamine (a chitosan monomer) from chitin (an N-acetyl-glucosamine polymer) [30]

In the present study, crustacean wastes were used to produce Glucosamine HCl by acid hydrolysis of chitin and compared it via chitosan. The yield of chitin after demineralization was 24.94 ± 1.85% for experiment 1 and 24.64 ± 1.79% for experiment 2. In the same treatment condition, the yield of chitin from experiment 3 was 25.11 ± 2.13% and experiment 4 was 26.19 ± 1.42%. The yield of chitosan after deacetylation of chitin with 45% NaOH solution was 18.42 ± 1.25% for experiment 3 and 18.68 ± 2.29% for experiment 4. The yield of Glu-HCl after hydrolysis of chitin with HCl conc. was 6.15 ± 0.62% for experiment 1 and 6.38 ± 0.77% for experiment 2, whilst hydrolysis of chitosan with HCl conc. resulted in 3.02 ± 0.46% for experiment 3 and 2.92 ± 0.44% for experiment 4 (Table 2).

Table 2 The yield of chitin, chitosan, and Glu-HCl from *S. paramamosain* waste

Experiment Group Name	Yield of Chitin (%)	Yield of Chitosan (%)	Yield of Glu-HCl (%)
Experiment 1	24.94 ± 1.85	-	6.15 ± 0.62
Experiment 2	24.64 ± 1.79	-	6.38 ± 0.77
Experiment 3	25.11 ± 2.13	18.42 ± 1.25	3.02 ± 0.46
Experiment 4	26.19 ± 1.42	18.68 ± 2.29	2.92 ± 0.44

Varun *et al.* [12] described demineralization of crab shells powder using 2N hydrochloric acid (w/v 1:15) at room temperature for 12 hours produced 12.2% yield of chitin. The optimized condition in this study was the demineralization of *S. paramamosain* shells powder using IN HCl treatment at 75ºC for 1.5 hours that produced 24.64% until 26.19% yield of chitin. It was reported that the use of high temperature accelerated the demineralization reaction by promoting the penetration of the solvent into the chitin matrix. Thus, some demineralization reactions were carried out at higher temperature [13]. Furthermore, it was reported that the penetration of solvent into chitin strongly depended on the particle sizes. The decisive factor in the demineralization was related to the contact area between the chitin matrix and the solvent [14]. From the literature above, we can conclude that the yield of chitin can be affected by the species and the condition of demineralization.

3.2 Characterization of Chitin and Chitosan from *S. paramamosain*

Figure 2 showed the FT-IR spectrum of the functional group of the molecular structure of chitin produced from BPPT and from *S. paramamosain* shells can be identified referred to Pavia *et al.* [15]. The fundamental vibrations were due to O–H and N–H stretching band (in the range 3,650 - 3,000 cm^{-1}), C–H stretching band (in 3,000 -2,840 cm^{-1}), carbonyl group (in 1,680 -1,630 cm^{-1}), and C–O–C band (around 1120 cm^{-1}).

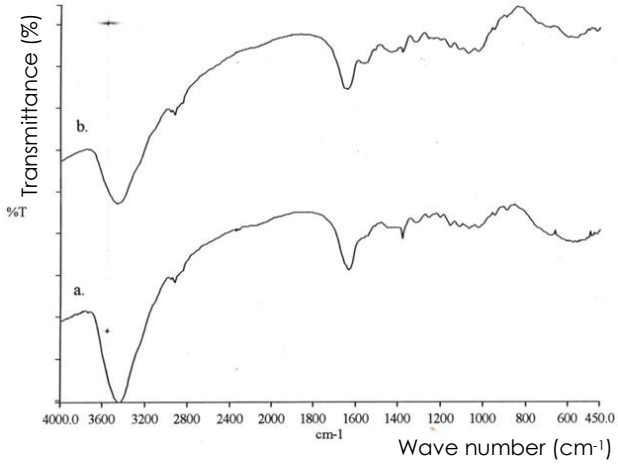


Figure 2 (a) FT-IR spectra of chitin standard from BPPT, Indonesia (b) FT-IR spectra of chitin produced from *S. paramamosain* shells

Figure 3 showed the FT-IR spectrum of the functional group of the molecular structure of chitosan produced from *Sigma-Aldrich* and from *S. paramamosain* shells could be identified referred to Pavia *et al.* [15]. The fundamental vibrations were due to O–H and N–H stretching band (in the range 3,650 - 3,000 cm^{-1}), C–H stretching band (in 3,000 - 2,840 cm^{-1}), residue of carbonyl group (in 1,680 - 1,630 cm^{-1}), and C–O–C band (around 1120 cm^{-1}).

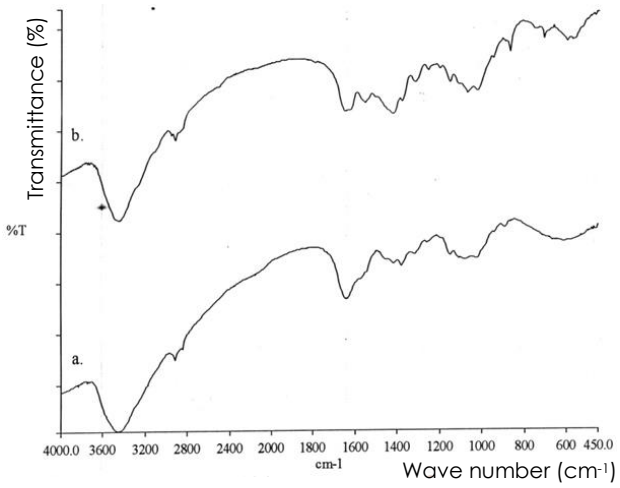


Figure 3 (a) FT-IR spectra of chitosan standard from *Sigma-Aldrich* (b) FT-IR spectra of chitosan produced from *S. paramamosain* shells

3.3 Characterization and Purity Determination of Glu-HCl from *S. paramamosain*

The influence of the intermediate material and temperature on acid hydrolysis was studied. It should be considered that HCl was previously heated to 60°C before the addition of chitin or chitosan and constant agitation must be carried out during the hydrolysis process [3]. After the chitin or chitosan was completely dissolved in a period of 18 minutes [3], the solution darkened and became a brown color immediately. The dissolution of chitin or chitosan was influenced by temperature and agitation. The change to brown color in solutions could be caused by the Maillard reaction which involveed amino groups reacting with an aldehyde and could lead to color change associated with hydrolysis of chitin [16].

The hydrolysis process involved two acid-catalyzed hydrolysis reactions: the glycosidic linkage (depolymerization) and the *N*-acetyl linkage (deacetylation) [3]. Hackman [17] observed that most of the degradation of the chitin chain occurred during the first few minutes and that the products formed were oligosaccharides.

3.3.1 FT-IR Characterization

The FT-IR spectra were used to identify the product and determine the degree of similarity. The results of the comparison between the spectra of Glu-HCl from *S. paramamosain* and the reference standard from *Sigma-Aldrich* revealed that a coincidence between 96.50 and 99.35% was obtained. The highest correlation corresponded to the hydrolysis product obtained from chitin experiment group at a solid/liquid ratio of 1:20 with agitation (experiment 1 and experiment 2). Lower values were obtained from chitosan experiment groups (experiment 3 and experiment 4). These results showed that in the range examined, Glu-HCl with better quality was produced from chitin hydrolysis.

Figure 4 showed the FT-IR spectrum of the functional group of the molecular structure of Glu-HCl produced from *Sigma-Aldrich* and from *S. paramamosain* shells could be identified referred to Pavia *et al.* [15]. The fundamental vibrations were due to O–H and N–H stretching band (in the range 3,650 - 3,000 cm^{-1}), C–H stretching band (in 3,000 - 2,840 cm^{-1}), $–NH_2$ scissoring band (in 1,640 -1,560 cm^{-1}), C–O–H band (in the range 1440-1220 cm^{-1}), C–O–C band (around 1120 cm^{-1}).

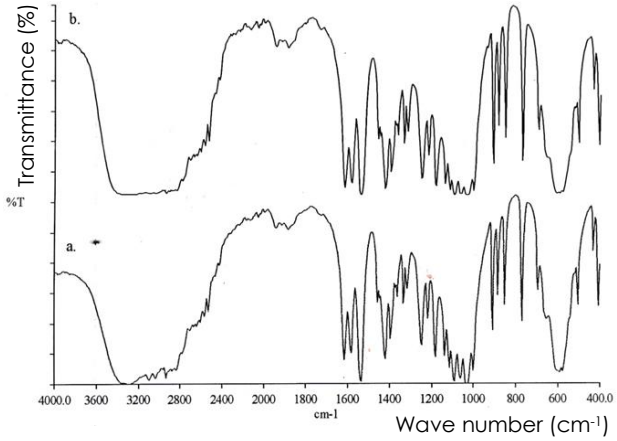


Figure 4 (a) FTIR spectra of Glu-HCl standard from *Sigma-Aldrich* (b) FTIR spectra of Glu-HCl produced from *S. paramamosain* shells

3.3.2 HPLC Analysis

In previous study, Pesek [9] isolated glucosamine by hydrolyzing shrimp shell chitin, treating the glucosamine obtained by solid phase extraction (SPE), and creating an HPLC protocol to analyze the glucosamine extracted by using HPLC with mass spectrometry (MS) detection. In present study, the method of glucosamine extraction and determination was cheaper because it did not involve SPE, used isocratic eluation, and was detected by (Diode-Array-Detector) DAD.

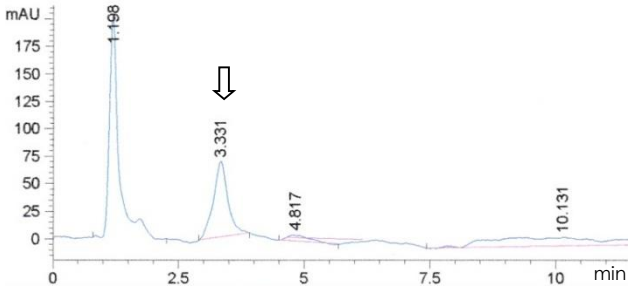
HPLC method was verified according to USP 41 [10], using Glu-HCl from *Sigma-Aldrich* as reference standard. The linearity of Glu-HCl was achieved in the range 2.1743 to 10.0452 mg/mL, r = 0.9998, Vx0 = 1.74%; accuracy 98.98 – 100.10%, n = 12, precision as RDS = 0.23 – 0.9%, n = 12. This result meet the requirement [19].

Peak identification of Glu-HCl was done by using spiking method and comparing with the retention time of pure standard. Figure 5 represented the chromatograms Glu-HCl prepared from crustacean shells. Purity and match factor of the peaks were confirmed with the characteristic of spectra obtained from the detector (Table 3). The Match Factor Value meet the requirement referred to Maio [18] whilst Peak Purity Value meet the requirement referred to Yuwono and Indrayanto [19]. From the result of FT-IR spectrum analysis and peak identification from HPLC, the compound could be confirmed as Glu-HCl.

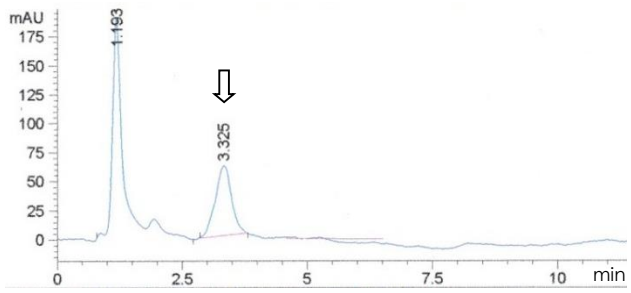
Table 3 The retention time of chromatogram peak and match factor spectrum between Glu-HCl from *Sigma-Aldrich* and Glu-HCl from *S. paramamosain* shells and their Peak Purity

Experiment Group Name	Rt	Match Factor	Peak Purity
Glucosamine Standard	3.331	999.8172	999.923
Experiment 1	3.325	998.7249	999.988
Experiment 2	3.195	996.5859	999.912
Experiment 3	3.311	999.625	996.950
Experiment 4	3.388	999.5131	999.792

There was one peak with retention time 3,1 – 3,3 minutes which were related to adsorption of Glu-HCl in the standard chromatogram (Figure 5). From the HPLC analysis, the purity of Glu-HCl prepared from *S. paramamosain* shells was 98.48 ± 0.74% for Experiment 1 and 79.45 ± 0.72% for Experiment 2, whilst the purity of Glu-HCl from Experiment 3 was 77.41 ± 0.90%, and from Experiment 4 was 74.28 ± 1.03% (Table 4).



(a) Chromatogram of Glu-HCl standard from *Sigma-Aldrich*



(b) Chromatogram of Glu-HCl sample from Experiment 1

Figure 5 Comparison between chromatogram of Glu-HCl standard from *Sigma-Aldrich* with chromatogram of Glu-HCl sample from Experiment 1

3.3.3 Loss on Drying and Residue on Ignition Test

The result of *Loss on Drying* test of Glu-HCl was 0.96 ± 0.05% for Experiment 1 and 0.93 ± 0.06% for Experiment 2. In the same test condition, the loss percentages of Glu-HCl were 0.86 ± 0.05% for Experiment 3 and 0.91 ± 0.05% for Experiment 4 (Table 4). The *Loss on Drying* test results met the requirement referred to USP 41 [10].

The result of *Residue on Ignition/ Sulfated Ash* test of Glu-HCl was 1,26 ± 0.05% for Experiment 1 and 2.30 ± 0.07% for Experiment 2. In the same test condition, the residue percentages of Glu-HCl were 3.49 ± 0.05% for Experiment 3 and 4.56 ± 0.07% for Experiment 4 (Table 4). The *Residue on Ignition* test results exceeded the requirement of USP 41 [10].

Based on XRF analysis of the *S. paramamosain* shells in two replications, it was found that calcium in the form of CaO present in 93,08 ± 0,10% and the others were P_2O_5, SO_3, MnO, Fe_2O_3, CuO, Br, SrO, MoO_3. Probably, the high percentage of the residue was caused by the high content of calcium presented in the *S. paramamosain* shells. Therefore, it is necessary to optimize the demineralization stage, related to the amount of $CaCO_3$ contained in the shell.

Table 4 FT-IR spectrum correlation (%) Data, Purity Data, Loss on Drying Data and Residue on Ignition Data of Glu-HCl from *S. paramamosain* shells

Experiment Group Name	Glu-HCl FTIR Spectrum correlation* (%)	Purity of Glu-HCl (%)	Loss on Drying of Glu-HCl (%)	Residue on Ignition of Glu-HCl (%)
Exp. 1	99.10	98.48 ± 0.74	0.96 ± 0.05	1.26 ± 0.05
Exp. 2	99.35	79.45 ± 0.72	0.93 ± 0.06	2.30 ± 0.07
Exp. 3	97.23	77.41 ± 0.90	0.86 ± 0.05	3.49 ± 0.05
Exp. 4	96.50	74.28 ± 1.03	0.91 ± 0.05	4.56 ± 0.07

*With regard to Glu-Hcl from *Sigma-Aldrich*.

Previous studies have revealed that acid hydrolysis is a more preferred method to release glucosamine from chitin material [20]. Several factors such as hydrolysis time, temperature, pH, and acid concentration are attributed to the release of glucosamine from chitin or chitosan. Acid hydrolysis of chitin with concentrated HCl for longer time leads to the breakdown of glucosamine and decreases recovery [21]. Low concentrations of hydrochloric acid slows down the chitin hydrolysis.

Holme *et al.* [27] reported that chitosan is thermally degraded at 60, 80, 105, and 120°C, the degradation rate of chitosan increases with the increasing temperature and degree of acetylation during acid hydrolysis. Novikov [28] reported that glycoside bond in acetylated monomeric chains is more readily hydrolyzed than the bond in deacetylated chains. Therefore, chitin is more easily hydrolyzed into Glu-HCl than chitosan. The glycoside bond is hydrolyzed by the S_N1 mechanism in which the rate-determining step is the proton attack to form carbocation.

Furthermore, the deacetylation rate in acid solution is almost independent of the initial degree of deacetylation (DD). This is also the case for the deacetylation of chitin and chitosan in alkaline solution. The deacetylation course depends only on the experimental hydrolysis conditions and is independent of DD of chitin and chitosan. Hence, the steps of deproteinization and demineralization of chitin production should be performed with care

to avoid uncontrolled deacetylation affecting the glucosamine yield [28].

Chitin and chitosan also contain the glucose groups. The decomposition of sugar leads to the formation of volatile (caramel aroma) and brown-coloured compounds (caramel colours). The reaction can be affected by heat and is catalysed by acids and bases. The colours and aromas depend on the sugar used (i.e. whether mono-, oligo- or polysaccharide) [29]. Chitosan was treated with more heat and base than chitin, therefore it is possible that the glucose group in chitosan is more degraded than the glucose group in chitin which causes low glucosamine purity. Also, Glu-HCl derived from chitosan was treated through more steps than Glu-HCl derived from chitin so that there must be a loss of product in the isolation and purifying processes. Active ingredient products probably dissolved in the washing or netralizing processes.

This study aims to get the optimum condition for extraction of Glu-HCl from *S. paramamosain* shells. The level of Glu-HCl in this study ranges from 74.28% to 98.48%, in which this result is higher than Sibi's [4] study.

Glu-HCl produced from Experiment 1 has approached the USP 41 requirements. As additional data, arsenic impurity and optical rotation of Glu-HCl from experiment 1 (optimal method) were determined. The results of the Glu-HCl produced from experiment 1 group were arsenic impurities not detected (Limit of detection As was 5 ppb) in Glu-HCl and the optical rotation of the Glu-HCl extract was 75.33 ± 1.53 while the standard Glu-HCl (Sigma Aldrich) was 73.67 ± 3.06, as a comparison. To the best of our knowledge, *S. paramamosain* shells have never been reported before as a source of Glu-HCl production.

4.0 CONCLUSION

In conclusion, these results showed the optimized condition for producing Glu-HCl from *S. paramamosain* is deproteination shell with 3% NaOH solution (w/v 1:6), at 85ºC, for 30 minutes, demineralisation with 1N HCl (w/v 1:10), 75ºC, 1.5 hours, depigmentation with 0.38% NaClO, at room temperature, for 1 hour, and hydrolysis with HCl conc. (w/v 1:20), at 85ºC, for 18 minutes with agitation (experiment 1) resulted in the yield 6.15 ± 0.62% and HPLC analysis of obtained Glu-HCl level is 98.48 ± 0.74%. Glu-HCl produced from Experiment 1 has approached the USP 41 requirements, except optical rotation and ash level. Although it is possible to convert *S. paramamosain* shells waste into valuable products such as Glu-HCl, more experimental works should be carried out to optimize the process of extraction Glu-HCl from *S. paramamosain*. Further, the HPLC method allows the detection and quantitation of glucosamine prepared by acid hydrolysis of chitin and chitosan from the exoskeleton of *S. paramamosain* and can be used as a routine method for the analysis of Glu-HCl in raw materials.

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