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Physicochemical Properties of Silkworm Pupae Shell (*Bombyx mori* L.) Glucosamine Hydrochloride

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Abstract

Glucosamine has been widely used to treat osteoarthritis (OA). In industrial scale, glucosamine commonly provides from chitin or chitosan of shrimps and crustaceans, however, the silkworm (*Bombyx mori* L.) pupae shell is predicted as an alternative source of glucosamine. This study aimed to analyze the optimal concentration of HCl in the production of the silkworm pupa shell glucosamine based on its characteristics. Glucosamine were prepared using the pressurized hydrolysis method by HCl at 0%, 2%, 4%, 5%, 6% and 8% (v/v) for an hour in a vacuum pressure (±0.8-1 atm). The result of study showed that the optimal HCl concentration was 4% with characterized by yield of product 89.25±11.86%, loss on drying 0.44±0.26%, degree of whiteness 83.56±0.03%, solubility 93.89±2.23%, a pH value 5.12±0.01, loss on ignition 0.04±0.01%, melting point 191.00±1.41^{0}C, degree of deacetylation 84.34±7.07% .

Keywords: glucosamine; *Bombyx mori*; silkworm; pupae shell.

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1. Introduction

Glucosamine (*2-amino-2-deoxy-d-glucose*) with the molecular formula $C_6H_{13}NO_5$ is a natural amine sugar and an essential component of glycoproteins and proteoglycans [1]. Amine sugar has the different farmasetikal and biological effect [2] such as the application of osteoarthritis and inflammatory bowel disease treatment [3], accelerating wound healing [4], a beneficial effect on disturbances spatial learning and disorder memory induced by scopolamine [5], as anti inflammatory on joints [6] as well as maintaining strength, flexibility and elasticity of cartilage tissue and joints [7] and increasing the production of hyaluronic acid [8]. In addition, glucosamine acts as a stimulant of immunity, antitumor, nerve and heart protective substances [9]. Several studies have also shown that glucosamine has an ability as antioxidants to inhibit radical superoxide/radical hydroxyl and stress carbonyl, the chelating effect firmly on iron ions and protecting macro molecules such as proteins, fats and the oxidative damage of deoxyribose and protecting erythrocytes from free radicals [10,11,12,13,14]. Studies of in vitro and in vivo tests carried out by the authors in [15] showed that glucosamine could be absorbed by intestine.

So far the raw material of chitosan and glucosamine are shrimp shells [16], but the study results of the authors in [17] showed that silkworm pupae shell (*Bombyx mori* L.) could be used as chitosan. Chitosan that produced on a laboratory scale results are quite good with the degree of deacetylation (DD) of 84%. Previously, the authors in [18] have conducted a similar study about utilization of silkworm pupae shell becomes chitosan with DD value of 83%. Furthermore, the chitosan products could be developed to be glucosamine [19].

Wajo is one of regencies in South Sulawesi that famous as a potential region of Bugis silk fabrics in Indonesia. In Wajo, there are 4982 crafters with a total production of 99640 gloves and silk fabrics of 1,589,000 meters per year with cocoon production average of 37 tons per year. A total of 5,000 cocoons produced 5 kilograms of silk thread, and a kilogram of silk yarn could produce three pieces of cloth gloves [20]. The spinning process from cocoon to be silk yarn produces silkworm pupae shell as by-products. The pupae is usually directly discharged and considered as waste that is not useful. Meanwhile pupae can be processed into flour and used as a Pury powder [21], nuggets, flakes and some traditional snacks [22]. The process of making pury powder leaves pupae shell which can be utilized as chitosan [17, 18].

The manufacture of glucosamine from silkworm pupae shell has not been studied before. Therefore, the test to optimize the manufacture of glucosamine from silkworm pupae shell is required. This study will develop a glucosamine hydrochloride from silkworm pupae shell as a raw material of supplement food. The objective of this study was to to analyze the optimal concentration of HCl in the production of the silkworm pupa shell glucosamine hydrochloride based on its characteristics.

2. Materials and Methods

2.1. Materials

The materials used are silkworm pupae shell, 12 N HCl (technical), NaOH (technical), Isoprophyl Alcohol / IPA (technical), distilled water, concentrated H_2SO_4, H_3BO_3, methyl red indicator, selenium mix, 0.1 N HCl pa, 40%

NaOH, HNO_3, $HClO_4$.

2.2. Manufacturing glucosamine hydrochloride process

Pupae shell as a raw material for making the chitosan was washed and dried. Proximate analysis is then performed to measure moisture content, ash content, protein content (total nitrogen), fat content measured using AOAC methods [23]. The manufacturing process include the demineralization using HCl 1 N, the deproteinization process using 3N NaOH and deasetilisation process using NaOH 50% [24]. Yield of chitosan then calculated and moisture analyzed, as well as ash content, protein content (total nitrogen) and fat content using AOAC method [23] and the degree of deacetylation [25].

Manufacture of glucosamine hydrochloride from chitosan was done by pressurized hydrolysis method. The method used refers to the authors in [26, 27] using acid concentration treatment. Glucosamine hydrochloride manufacturing process started from soaking 5 grams of chitosan in HCl solution (sample: HCl = 1: 9) with HCl concentration of 0%, 2%, 4%, 5%, 6% and 8% (v / v). The determination of HCl concentration based on the result of selected glucosamine hydrochloride by the authors in [26] of 5% and the authors in [27] of 8% with 2% range of the lower interval. The heating time is 1 hour at vacuum pressure ± 0.8 - 1 atm.

The samples were hydrolyzed in pressurized heater washed with IPA until the pH reached 3-5. Samples then put into oven at 40 ° C until dry. Furthermore, the calculation of the yield [23], degree of whiteness [28], loss on drying [29] the pH value [29], the level of solubility [30], loss on ignition [29], melting point [23], degree of deacetylation [25] and analysis of in vitro antioxidant activity using DPPH method refers to the method of the authors in [31] with several modifications.

2.3. Degree of whiteness

Color analysis performed using an Chromameter Minolta CR-200. Color system was done by placed the samples on provided space, then the start button is pressed and values of L, a, and b of samples will be obtained. The measurement results then converted into the Hunter system whereas L stated parameters of brightness from black (0) to white (100). Notation a stated chromatic colors red-green mix with a value of + a (positive) from 0 to +100 for red color and value - a (negative) from 0 to -80 for the green color. Notation b states chromatic color mixture of blue-yellow whereas a value + (positive) from 0 to +70 for theyellow color and -b value (negative) from 0 to -80 for the blue color. Furthermore, by using the data L, a and b the whiteness level (degree of whiteness) could be calculated using the following equation:

$$\text{Degree of whiteness} = 100 - [(100-L)^2 + a^2 + b^2]^{1/2}$$

2.4. Loss on drying analysis

Loss on drying (LoD) test conducted by placing one gram sample on oven at temperature of 105^0C for 2 hours. LoD percentage is calculated by the formula as follows:

$$LoD\ (\%) = \frac{\text{initial weight of sample} - \text{weight of samples after oven)}}{initial\ weight\ of\ sample}\ x\ 100\%$$

2.5. pH value analysis

A total of 400 mg of sample was dissolved in 20 ml of distilled water, stirred with a magnetic stirrer until homogeneous, then the pH measured using a pH meter.

2.6. Test of solubility

500 grams of powder dissolved in 100 ml of water, then filtered by Whatman 42 paper using vacuum filtration. Before the filter paper is dried at 105^0C for 30 minutes (on the oven) and weighed. After filtration on vacuum, filter paper and precipitate dried at 105^0C for 3 hours and cooled in a desiccator for 15 minutes, then measured.

$$\%\ \text{Solubility} = 100 - \frac{weight\ of\ precipitate\ sample - weight\ of\ filter\ paper}{\frac{(100 - \%water\ content)}{100}\ x\ weight\ of\ sample}\ x\ 100\%$$

2.7. Loss on ignition analysis

Ignition residue measurement performed by heating the porcelain cup in an electric furnace temperature at 600^0C for 30 minutes then measured. A gram of sample was weighed in a porcelain cup and added with 1 mL of H_2SO_4 then heated on a Bunsen with low heat level until overall samplecharred. 1 mL of H_2SO_4 was added to the sample and reheated until emitting white smoke. Samples were put in an electric furnace at a temperature of 600^0C for 2 hours then LoI calculated using the formula:

$$LoI\ (\%) = \frac{Weight\ after\ heating - weight\ of\ empty\ cup}{weight\ of\ sample}\ x\ 100\%$$

2.8. Test of FTIR absorption to determine the degree of deacetylation of chitosan and glucosamine hydrochloride

A gram of sample and each standard mixed with KBr with a ratio of 1: 200 and then crushed using a mortar. The mixture is placed in a pressing tool and pressed with pressure load of 800 kg for 5 minutes. The absorbance of pieces pressed results measured using FTIR. Scanning range used is between 450 cm-1 to 4000 cm-1. Measurements were made by observing the graph or spectrum formed.

2.9. Melting point test

Melting point test conducted by MelTemp. Glucosamine hydrochloride powder inserted into the capillary tube through the open end of tube. Basic pipe then tapped on the bottom or dropped through a long narrow tube. It is intended to make glucosamine become solid so that the melting process takes place evenly. It processes were done repeatedly to get a solid sample in a tube of 1.5-3 mm. Capillary tube then inserted into an electric heater with a 400^0C thermometer for melting point determination. The machine wass on while the temperature was

raised slowly until it reachs the melting point.

3. Results and Discussion

3.1. Characteristics of silkworm pupae shell chitosan

Raw materials used of this study is waste of silkworm pupae shell (*Bombyx mori* L.) that obtained from the process of making silk thread in Wajo, South Kalimantan and Pati, Central Java. Pupae shell contains several components such as pigments, minerals, protein, fat and chitin [32]. The analysis of dry pupae shell obtained moisture content of 5:24 ± 4.68%, ash content of 5:06 ± 4:35%, protein of 73.77 ± 1.97%, fat of 4:43 ± 0:04% and carbohydrate of 2:30 ± 11:50 %.

3.1.1. The yield of chitosan

Efficient and effective extraction process of raw materials in making chitosan could be seen by the resulted yield. The greater yield indicated the treatment applied is more efficient. The study results of chitosan yield was 14%. The same result was shown by study conducted by the authors in [26] that made from shrimp shells, however the bigger results shown by the study results of the authors in [33] that ranged from 15.21 to 18%; 15.14% [34]; 17% [35]. The yield result might be caused by the differences of temperature and time. Yield and the molecular weight of chitosan is influenced by the temperature and time used [36, 37].

3.1.2. Characteristics of chitosan

Characterization of chitosan categorized into color, odor, shape, moisture content, ash content, fat content, nitrogen total and degree of deacetylation. The results of chitosan characteristics analysis made from silkworm pupae shell can be seen in the Table 1.

Table 1: Characteristics of silkworm pupae shell chitosan

Spesification	The results od analysis (mean±SD)	EFSA 2010 (db)	GRAS 2012 (db)
Color	Off white	-	White to off white powder
Odor	Netral	-	Netral
Shape	Flakes	-	18-120 mesh
Moisture content (%)	6.58±0.37	≤10	≤10
Ash content (%)	0.09±0.04	≤3	≤0.5
Fat content (%)	1.01±0.05	≤1	-
Total Nitrogen (%)	6.02±0.02	≤6	0.02 g/100 g
Degree of deacetylation (%)	79.88±0.62	≥90	75-95

Chitosan obtained from this study showed the color of brownish white, odorless and flake-shaped. The resulted

color might be caused by the rest of organic matter during the demineralization and deproteinization. The results are consistent with the previous study of the authors in [24] that produced yellowish-white chitosan and flake-shaped or fine powder. In addition, chitosan results of this study were able to compensate the quality of chitosan based on the author in [38].

Moisture, ash, fat and nitrogen total are the quality parameters of chitosan. Based on the data in the Table 1, it conluded that the chitosan results of this study meet the chitosan quality in the market [38, 39]. except for fat and total nitrogen that slightly higher. It might be influenced by the demineralization and deproteinization process that not perfect as well as the high protein and fat content of raw materials. The authors in [40] stated that high protein levels of chitosan can be associated with immersion time, and the methods used during the chitosan manufacturing process. The study results of the authors in [41] showed that fat content of chitosan was 0.70-4.01%, while the total protein content of chitosan on the previous study was 2,20% [26] and $\leq$ 1% [27].

Degree of deacetylation is a parameter of the release of acetyl and chitin groups that affecting the change of chitosan properties such as solubility, chemical reactivity and biodegradability. It determined by calculations based on the amide bond and the amine group of FTIR spectrum . The degree of deacetylation of chitosan in this study was 79.88 ± 0.62 % that concluded meet the chitosan standards quality based on the author in [38] of 75-95 % . Based on the previous study results, the degree of deacetylation of chitosan ranged from 80-90 % [24]; 98.65 % [26]; 74.82 % [35]; 82.3 ± 05 % [42]; 81.24 % [34]; and 75-85 % [43].

3.2. Characteristics of silkworm pupae shell glucosamine hydrochloride

The analyzed parameters of glucosamine hydrochloride characteristic are presented in the Table 2 .

Table 2: Characteristics of silkworm pupae shell glucosamine hydrochloride

Characteristics	The HCl addition treatment (mean±SD)					p-value
	2%	4%	5%	6%	8%	
The yield (%)	95.28±4.43^{a}	89.25±11.86^{a}	87.10±15.04^{a}	88.44±14.04^{a}	89.58±13.77^{a}	0.966
Loss on drying (%)	1.24±0.19^{c}	0.44±0.26^{a}	0.87±0.01^{bc}	0.80±0.08^{ab}	0.73±0.06^{ab}	0.026
Degree of whiteness (%)	87.37±0.01^{d}	83.56±0.03^{c}	83.56±0.03^{c}	78.48±0.04^{a}	80.46±0.50^{b}	0.000
Solubility (%)	95.00±2.18^{a}	93.89±2.23^{a}	94.77±1.92^{a}	95.40±3.36^{a}	92.67±6.46^{a}	0.940
The pH value	5.05±0.01^{bc}	5.12±0.01^{c}	5.04±0.05^{bc}	4.76±0.28^{ab}	4.60±0.06^{a}	0.036
Loss on Ignition (%)	0.12±0.01^{b}	0.04±0.01^{a}	0.08±0.01^{ab}	0.12±0.03^{b}	0.11±0.01^{b}	0.020
Melting point (^{0}C)	189.00±1.41^{a}	191.00±1.41^{a}	190.50±0.70^{a}	193.50±0.00^{a}	190.80±1.87^{a}	0.121
Degree of deacetylation (%)	80.73±2.74^{a}	84.34±7.07^{a}	81.63±9.06^{a}	81.86±1.74^{a}	86.59±11.10^{a}	0.918

3.2.1. The yield of glucosamine hydrochloride

The yield is the percentage of a certain part of the integral parts of the material . The glucosamine yield of this

study can be seen in the Table 2, which showed that no significant difference between the concentration acid treatment ($p > 0.05$). The highest glucosamine yield of concentration treatment is HCl 2 % while that the lowest is 5 % HCl concentration treatments . Overall study showed that yield of this study was higher than the results of the previous study were 65.33% [26] and 69.80 % [27].

3.2.2. Loss on drying

Test of loss on drying (LoD) was done to measure the amount of water and volatile materials contained in the sample by drying under certain conditions or temperature . LoD value of glucosamine by HCl concentration treatment range from 0:44 to 1:24 ± 0:26 % ± 12:19 %. Analysis of variance showed that the pressurized hydrolysis method with different HCl concentration treatments gave a significant effect to LoD ($p<0.05$). Based on the Duncan test, the best LoD value of glucosamine using HCl concentration treatment was 4 %HCl concentration. LoD value of this study met the standards required by the author in [39, 44] which is 1%.

3.2.3. Degree of whiteness

Whiteness is a major quality factor of starchy or powder . Whiteness of material is ability to reflect the light of the material to the light of surface. The whitenessdegree of powder products in general is one of the quality parameters that usually expected to have a high whiteness level [44]. Whiteness level of glucosamine in this study ranged from 78.48 % to 87.37 ± 12:04 ± 12:01 %. Analysis of variance showed that the distinct concentration of HCl effected the significant differences on the value of whiteness level ($p< 0.05$). The optimal treatment of this parameter was glucosamine with HCl addition of 2% .

3.2.4. The level of solubility

Solubility is the maximum quantity of a chemical substance dissolved (the solute) to be dissolved in certain solvents to form a homogeneous solution . Broadly solubility of a substance in a particular solvent is a measurement of the saturation concentration by adding little by little of solute (substance) in a solvent until the solute become precipitates (insoluble). Solubility level of glucosamine using HCl concentration treatment showed no significantly different ($p>0.05$). The similiar results have been demonstrated by the authors in [26] which resulted the solubility level of glucosamine ranged from 91.87 to 96.33%. The results indicated that glucosamine that produced by the pressurized hydrolysis method is accordance with standard of the author in [39, 44] that is 90.00 %.

3.2.5. The pH value

Values of pH or acidity used to express the acidity or alkaline degree of a substance , solution or objects . The pH value of glucosamine in this study ranged from 4.60±0.06 to 5.12±0.01. Analysis results of of variance showed a significant difference between the pH values ($p<0.05$). Based on the Duncan test, the different concentrations of HCl showed significant influence to the level of pH values. The optimal glucosamine pH value of HCl concentration treatment is 4%. A decrease of pH value was in line with the increase of acid concentration in the extraction process of glucosamine. The pH value of glucosamine produced is still tend to

acid but it categories to be consumption product by the author in [29, 39] that ranged between 3-5 . Previously the authors in [26] using the same method to produce glucosamine hydrochloride with pH values ranged from 5.60 to 5.66.

3.2.6. Loss on ignition

Loss on Ignition (LoI) or the residue of ignition is an inorganic residue from the combustion or oxidation of organic components of food. LoI is part of the proximate analysis that aimed to evaluate the nutritional value of a product or foodstuffs, especially the total of mineral . Loss on ignition total of glucosamine using HCl concentration treatment ranged from 0.04±0.01% to 0.12±0.03%.. Analysis of variance showed that the concentration of HCl significantly effectted the LoI value of glucosamine ($P < 0.05$). Based on Duncan test, the optimal LoI value of glucosamine was the treatment using HCl concentration of 4% and it met the standard required by the author in [29, 39] that is <0.1%. The LoI value of glucosamine in this study was lower than the study results of the authors in [26] which ranged from 0.23 to 0.75%.

3.2.7. Melting point

The melting point illustrates the number of impurities or foreign substances contained in the ingredients [45]. The analysis results of of glucosamine melting point in this study ranged from 189.00±1.41^{0}C to 193.50±0.00^{0}C. The Analysis of variance showed that the concentration of HCl did not give a significant impact on the melting point of glucosamine ($p > 0.05$). The results are consistent with the study result of the authors in [27] which showed the glucosamine melting point ranged from 190-193^{0}C at HCl concentration of 8% and the authors in [46] of 190-192^{0}C in acid concentration of 32%.

3.2.8. Degree of deacetylation

The degree of deacetylation is the parameter that indicates the molar percentage of monomeric units that have amino groups and vary from 0 (chitin) to 100 (fully deacetylated chitin) [47]. It is determined by calculating the amide bond and the amine group of the FTIR spectra. The degree of deacetylation of glucosamine manufacture varies depending on the amount of acid used, reaction time, and reaction temperature. High quality of the glucosamine products have a high purity level that expressed by the degree of deacetylation value [47, 48].

The degree of deacetylation of glucosamine in this study ranged from 80.73±2.74 - 86.59±11.10%. Analysis of variance showed that the concentration of HCl has no significant effect on the degree of deacetylation of glucosamine ($p>0.05$). The result was lower than the study results of the authors in [26] in the amount of 99.44% that using the same method, but it was accordance with quality standard of commercial glucosamine and predefined [29] which ranged from 75-95%.

3. Conclusion

Based on the characterization parameters of glucosamine, the optinal HCL concentrations treatment is 4% that had a yield of 89.25 ± 11.86%, LOD of 0:44 ± 0:26%, whiteness of 83.56 ± 0.03%, solubility level of 93.89 ±

2:23%, pH value of 5:12 ± 0:01, LoI of 0:04 ± 0:01 %, the melting point of 191.00 ± 1.410C and degree of deacetylation of 84.34% ± 7:07. This product still has to go through a series of food safety analysis to be consumed widely by public.

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