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Characteristics of Chitosan Nanoparticles extracted from Sea Cucumber (*Holothuria scabra*) as Source Materials for Glucosamine

Sumarto*, Bustari Hasan, Rahman Karnila and Mery Sukmiwati

Department of Fishery Products Processing, Faculty of Fisheries and Marine Science, Riau University, Pekanbaru, Riau, 28293, Indonesia

ABSTRACT

Sea cucumber has a thick layer of skin consisting of lime components. In fact, the components contain chitin and chitosan, which have been recognized as potential sources materials for dietary supplement. This study aimed at evaluating the physical and chemical characteristics of chitosan nanoparticles extracted from sea cucumber *Holothuria scabra* when used as source materials for glucosamine. Chitin were extracted from dried samples, chitosan from chitin, while chitosan nanoparticles were obtained from chitosan with different concentrations (C_1 = 0.1%; C_2 = 0.2%; C_3 = 0.3%) of added sodium tripolyphosphate (NaTPP). Production process in this study resulted in 59.82% of chitosan extracted from chitin. Besides, the amount of chitosan nanoparticles obtained at 0.1%, 0.2%, and 0.3% additions of NaTPP were 90.6%, 92.8%, and 96.4%, respectively. These results were characterized in terms of whiteness degree (85.82%, 87.29%, 88.34%, respectively), deacetylation degree (90.6%, 95.8%, 96.2%), moisture (5.73%, 5.26%, 4.82%), and ash (1.29%, 1.07%, 0.98%). Looking at SEM and PSA tests, chitosan was morphologically found to be heterogeneously distributed with averaged 177-micron particle sizes. They also had larger particle chunks and solid as well as intact forms. Meanwhile, chitosan nanoparticles had smaller and smoother chunks, while they were produced in solid and intact forms. Besides, they were homogeneously distributed with sizes ranging between 134 – 206 nm (C_1), 114-128 nm (C_2), and 97-108 nm (C_3). Then, increments in NaTPP concentrations were discovered to contribute to the reduction of *H. scabra*-sourced chitosan nanoparticles size.

Keywords: Chitosan, *Holothuria scabra,* nanoparticles, sodium tripolyphosphate

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E-mail addresses:
sumarto1976@yahoo.co.id (Sumarto)
bustarih@yahoo.com (Bustari Hasan)
kumans_69@yahoo.co.id (Rahman Karnila)
merysarmin@yahoo.com (Mery Sukmiwati)
* Corresponding author

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INTRODUCTION

Sea cucumber is a marine animal originated from Indonesia. It has been recognized to offer various advantages in different aspects of human life, which may be either related or unrelated to dietary. In most situations, they are utilized as a functional food in health and biopharmaceutical fields, or as an ingredient in other chemical-related industries.

In particular, the commercial values of sea cucumber *H. scabra* J have been acknowledged due to its high nutrient contents, which in the form of flour offering a protein content of 60-70% with high essential amino acid components, containing complete fatty acids, carbohydrates, and minerals for human health. In the food industry, its uses begin with handling and weeding processes, in which parts of sea cucumber, particularly its layers that contain a lot of lime, are separated.

Technically, the anatomy of sea cucumber consists of 44.2% body mass (except viscera and gonads), 40% skins, 10.3% stomach contents (viscera and gonads), and 5.5% others including impurities. Despite having a similar proportion to general body parts, the skin has not been fully utilized yet, hence adding wasted parts of sea cucumber remains (Amri et al., 2018). Therefore, there is an opportunity to make it commercially available by increasing its economic values. Among others, the skin of sea cucumber may potentially be processed to produce chitin and chitosan, which is conducted by extracting the skin of sea cucumber to produce these two commercial products.

In general, the process of extracting chitosan was conducted by first soaking chitin shells/skins with NaOH 50% solution using a magnetic stirrer at 100 °C for 90, 120 and 150 minutes. The mix was then filtered and washed using distilled water to produce neutral pH. Then, chitosan being produced was dried in an oven at 45-47 °C for 6 hours. In the literature, extraction time that resulted in the most desired chitosan characteristics was 120 minutes, which produced chitosan in the form of a white fine powder with 37.59% yield, 6.48% water content, 8.19% ash content, and 69.29% deacetylation degree (Amri et al., 2018).

Practically, chitosan is applicable in various modern industries, including pharmacy, biochemistry, cosmetics, food and textile (Berger et al., 2004). The vast potentials of chitosan have been encouraging researches to expand the utilizations of chitosan products by modifying their chemical and physical properties. One of the physical modifications is applied by changing the size of its particles to smaller ones for wider utilizations, leading to the formation of nanoparticles. A smaller physical state is more advantageous compared to similar materials in a larger size due to a greater comparative value in terms of surface area and volume, making it more reactive. In fact, it fits with an established finding that refers to material reactivity as being determined by atoms on the surface, which come in a direct contact with other materials (Suwarda & Maarif, 2012).

In recent advances, chitosan nanoparticles have continued to be under investigation in terms of both determining their composition and finding an appropriate production method. In general, the production of high-quality chitosan nanoparticles requires a suitable, effective and simple method for obtaining a uniform size and desired stability. As a matter of facts, ionic gelation method has become a preferred method to obtain nanoparticles due to its simple process.

During an ionic gelation process, the formation of chitosan nanoparticles is conducted by reacting chitosan with sodium tripolyphosphate, which is known as a multivalent anion, to form cross-linked bonds with cationic chitosan. A method as such produces an interaction between the positive charge at chitosan's amino groups and the negative charge at tripolyphosphate, resulting in smaller particles (Lin et al., 2008). In fact, the production technology of chitosan nanoparticles offers critical advantage in producing good quality drugs by delivering desired characteristics and pellet size. Besides, chitosan is an important ingredient for producing glucosamine, therefore, producing the nanoparticles is expected to improve the quality of these products.

Looking at current literature, the production of chitosan nanoparticles by extracting chitosan shells from tiger shrimp through ionic gelation processes had been conducted by Nadia et al. (2014). In their study, the nanoparticle making process began with the mix of 0.1% Tripolyphosphate (TPP) solution with chitosan solution for then being homogenized by using a magnetic stirrer at 25°C temperature for 1 hour. The production of chitosan nanoparticles from tiger shrimp shells resulted in 80.67% yield rate, 98.65% deacetylation degree, 228.74 nm average particle size. In fact, the particle size is quite uniform, with a ball-like shape, and relatively stable.

In the literature, researches on chitosan nanoparticles from sea cucumber have not been conducted yet to utilize it as the primary ingredient for health-supplementing glucosamine production. Technically, glucosamine is a monomer of chitin and chitosan, which is often found in the shells/skins of various marine biota. Besides, glucosamine has been taken as food supplements to prevent and cure osteoarthritis. In human body, it is a precursor for the biosynthesis of glycosylate proteins and lipid to produce synovial fluid, which is used as a lubricant in the cartilage (Husskison, 2008).

Therefore, it is necessary to conduct a research on the production and quality characterization of chitosan nanoparticles by ionic gelation method using different concentrations of emulsifying solutions. This work hence aimed at determining the physical and chemical characteristics of chitosan nanoparticles extracted from the skins of *H. scabra* sea cucumber using different concentrations of sodium tripolyphosphate (NaTPP).

MATERIALS AND METHODS

Materials

As the focus of this research, several sea cucumbers (*H. scabra*) measuring 525±83.6 grams were obtained from the waters surrounding Terung Island, Batam, Indonesia. Besides, chemicals such as NaOH (Merck, German), acetic acid (Merck, German), distilled water (Bratachem, Indonesia), HCl (Merck, German), Sodium Tripolyphosphate (NaTPP; Merck, German), Tween 80 as emulsifier (Indonesia) and other ingredients were taken for proximate analyses. Equipment used for the preparation and handling of samples, the production of chitin and chitosan, and the manufacturing of chitosan nanoparticles included a magnetic stirrer (BIG LAB 79-1), oven, Particle Size Analyzer (PSA) (Beckman Coulter), Viscometer (Brookfield LV), Fourier Transform Infrared Spectrophotometer (FTIR) (MBQ00 Bruker Tensor Type), and Scanning Electron Microscopy (SEM) (JSM-35C).

Preparation and Flour-making Process

The dissection of sea cucumber being observed was done by using knife to split its abdomen, while fillets were used to separate gonad innards, other body mass and skin. The gonads, skin, other body mass and offal were dried separately in an oven at 40-45°C temperature for 48 hours. They were then grinded in a mixing machine (laboratory blender, model 32BL79, USA), which was set to produce 80 flour mesh size. Then, it was tapped repeatedly to ensure evenly grinded materials.

Chitin Extraction (No et al., 1989)

The sea cucumber flour was placed in a container. NaOH 3.4% was added to the container with 1:10 b/v ratio of ingredients and solutions. After that, the mixture was heated at 65°C for 2 hours while being continuously stirred. Next, the heated mixture was left to cool, then filtered and washed by using distilled water to reach a neutral pH. After the results were weighed, 1 N HCl was added into the tube with 1:10 b/v ratio of materials and solutions. The tube was re-heated at 65°C for 2 hours while being continuously stirred. Then, the precipitate was filtered and washed with distilled water to achieve a neutral pH, and dried at 60°C. The resulting product was chitin with determined chemical and physical characteristics.

Chitosan Extraction (Suptijah, 2004)

Furthermore, chitosan was obtained by making a highly concentrated solution of chitin deacetylation with 50% NaOH. In practice, chitin flour was weighed and 50% NaOH was added at 1:10 (b/v) ratio between chitin and solvent. The mixture was continuously stirred while being heated at 100°C for 120 minutes. Results of the deacetylation process were

then deposited in a centrifuge for 15 minutes to separate solids from liquids. Obtained solids were repeatedly washed by using distilled water to achieve a neutral pH, and dried in an oven at 60°C for 6 hours.

Formation of Chitosan Nanoparticles (Iswandana et al., 2013)

Chitosan solution was produced by dissolving 200 mg of previously extracted chitosan in 100 ml of acetic acid 1% using a magnetic stirrer. The acetic acid 1% was obtained by mixing 10 ml of glacial acetate in 1000 ml of distilled. Next, a 0.1% concentration of sodium tripolyphosphate (NaTPP) solution was produced by dissolving 400 mg of NaTPP in 40ml of distilled and demineralized water by using a magnetic stirrer. Besides, a 0.2% concentration was made by dissolving 800 mg of NaTTP in 40 ml of distilled water, while a 0.3% concentration was produced by dissolving 1200 mg of NaTTP in 40 ml of distilled and demineralized water with a magnetic stirrer at 3000 rpm for 30 minutes to form a nanoparticle suspension. Next, the prepared chitosan solution was poured into a glass beaker while being stirred by using a magnetic stirrer. Then, separated NaTPP solutions (one for each concentration) was added slowly to the chitosan solution to form a nanoparticle suspension. The stirring was continued for 60 minutes to ensure a completed cross-linking process. In general, the whole process for making chitosan nanoparticles from sea cucumber took around 90 – 120 minutes.

Statistical Analysis

In this study, a statistical analysis on the results of experiments was conducted by applying a Completely Randomized Design (CRD). In practice, the data were analyzed by applying a one-way analysis of variance (ANOVA) in SPSS software version 22.0. Treatments being analyzed included NaTTP additions at different concentrations (C1 = 0.1 %; C2 = 0.2%; C3 = 0.3%) and a control chitosan (without any added NaTPP). Besides, test parameters covered moisture, ash, fat, protein, carbohydrate content (Association of Official Analytical Chemist – AOAC, 2005), yield, appearance, color, whiteness degree (Ernawati, 2012), the degree of acidity (Indonesian National Standards, 2004), the degree of deacetylation (Swann and Patwardhan, 2011), Scanning Electron Microscopy (SEM; Masooti et al., 2007) and Fourier Transform InfraRed (PSA; Yang et al., 2014).

Test Parameters

Moisture Content. This parameter was determined by applying the gravimetric method with an oven. The method involved weighing the moist sample after being dried in oven at 105 °C for 24 h. The water mass being produced was determined by comparing the weights of samples before and their constant weights after drying. Moisture content was calculated by using the following equation:

$$\% \text{ Moisture content} = \frac{\text{Wet weight (g)} - \text{Dry weight (g)}}{\text{Wet weight (g)}} \times 100$$

Ash Content. First, a tared crucible was dried and cooled. To discover the amount of ash in the prepared chitosan, 2 g of chitosan was placed into the tared crucible. Samples were heated in a muffle furnace at 600°C for 6 hours. The crucible was left in the furnace to naturally cool until reaching <200°C temperature, and then placed into a desicator for 30 minutes. Then, the mass of crucible and ash content was weighted.

Fat Content. Crude fat was determined by weighing 5 g of each sample to be wrapped in a filter paper by a Soxhlet apparatus using petroleum ether. It was conducted for 4 hours each. Next, extracted materials were left to evaporate all solvent content. After ensuring all solvent had evaporated, the extracted materials were weighed, and its fat content was calculated.

Crude Protein Content and Carbohydrate. Crude protein was analyzed by applying the Kjeldahl method (AOAC, 2005). Observed samples went through three essential steps, i.e. digestion, distillation, and titration, with 6.25 conversion factor to convert total nitrogen to crude protein. Thus, protein percentage in the samples could be calculated. Subtracting 100% by the sum of fat content, protein content, ash content, and moisture would then result in the total carbohydrate content (Onyeike et al., 2000).

Whiteness Degree (Ernawati, 2012). Determining the white degree of chitosan was conducted by using KETT Digital Whiteness Meter for Powder model C-100-3 (KETT Electric Laboratory, 1981). Samples were alternately put in measurement dishes until they were full and solid. Value indicated by the monitor referred to the white degree of observed sample (A), by which it was compared to a standardized whiteness value (110.8) according to the following equation:

$$\% \text{ WD} = \frac{\text{A}}{\text{Standard Value } BaSO_4 \text{ (110.8)}} \times 100$$

Degree of Deacetylation of Chitosan. The FTIR spectra of observed chitosan samples (in the forms of KBr disk and film) were obtained by using an I.R Instrument (MBQ00 Bruker Tensor Type) with *e* frequency range of 400-4,000 cm^{-1}. Deacetylation degree (DD) of the chitosan samples was calculated by following Khan et al. (2002) equation:

$$DD\% = 100 - \left[\left(\frac{A_{1655}}{A_{3450}}\right) \chi \left(\frac{100}{1{,}33}\right)\right]$$

Fourier Transform Infrared Spectroscopy (FTIR). Furthermore, the observed samples were characterized in an infrared spectroscopy by using KBr pellets with 400-4,000cm^{-1} scanning range (FTIR MBQ00 Bruker Tensor Type). KBr pellets were prepared (1 mg chitosan with 100 mg of KBr) and stabilized under a relative humidity before acquiring the spectrum (Brugnerotto et al., 2001). Next, transmittance or absorbance percentage was conducted by using an infrared spectrophotometry. Meanwhile, DD calculations of infrared spectrum in chitin and chitosan were conducted by comparing the absorbance of waves for NH-amide groups (1650-1500) cm-1 (A 1655) with that of primary amine group (3500-3200) cm-1 (A 3450) and the absorbance value of 1.33 for a perfect deacetylation process (Bastaman, 1989).

Scanning Electron Microscopy (SEM). Chitin skins, chitosan and its nanoparticles of *H. scabra* were examined by utilizing Scanning Electron Microscopy (SEM; Hitachi Flexsen 1000), which was equipped by EDS (Energy Dispersive X-Ray Spectroscopy) with two different magnification ranges (5000x for chitosan; 15000x for nanoparticles) and an accelerating voltage at 20kV (JSM-35C). It had a considerably large sample chamber and could accommodate samples as large as 300 mm in diameter and 110 mm high.

RESULTS AND DISCUSSION

Characteristics of Raw Materials

Characterization of raw sea cucumber was conducted to understand the proportion of each parts. Looking at the results of weeding on sea cucumber fillets, the discovered proportion included 40.10% skin, 43.53% other body mass, 10.97% stomach contents (viscera and gonads) and 5.40% impurities (Table 1, Figure 1). All proportions referred to their absolute comparison to total body weight.

Table 1
The average proportion of body parts for fresh sea cucumber (H. scabra J*)*

Parts of Raw Materials	Weight of Sea Cucumber* (g)	Fresh Proportion (%)	Weight of flour (g)	Yield of flour (%)
Skin	109.07	40.10	34.75	31.86
Other body mass	118.40	43.53	12.27	10.36
Stomach contents (Viscera and Gonad)	29.84	10.97	2.75	9.23
Impurities/leftovers	14.69	5.40	-	-
	272	100		

*frozen raw material

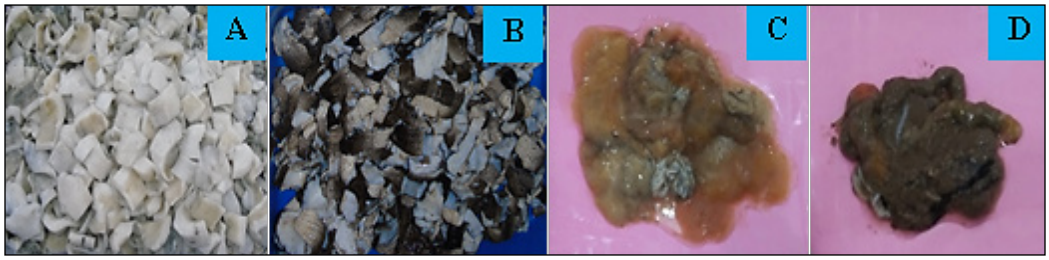

Figure 1. Body parts of sea cucumbers (A. body mass, B. skin, C. viscera and gonads, D. impurities/leftovers)

In fact, there were variations in the values of these characteristics before and after drying. For example, observations on the flour form discovered proportion of the skin at 31.86%, body mass at 10.36%, and stomach contents (viscera and gonads) at 9.23%. Significant differences were observed in the proportion of body mass and stomach contents (viscera and gonads) after drying and shaking processes due to their high level of moisture. Proximate analyses on the chemical composition of sea cucumber *H. scabra* revealed changes in composition between its fresh condition and after being transformed into flour form (Table 2).

Table 2
The chemical (proximate) content of the sea cucumber in fresh and flour raw materials

Chemical compositions	Composition of fresh sea cucumber and flour (%wb)					
	Skin		Other Body Mass		Viscera and Gonad	
	Fresh	Flour	Fresh	Flour	Fresh	Flour
Moisture	23.74 ± 0.52^{a}	8.25 ± 0.13^{a}	77.07 ± 0.51^{b}	9.12 ± 0.25^{b}	82.71 ± 0.22^{c}	16.12 ± 0.74^{c}
Protein	8.75 ± 0.34^{a}	12.12 ± 0.76^{a}	18.08 ± 0.48^{c}	72.25 ± 0.59^{c}	10.08 ± 0.19^{b}	43.47 ± 0.63^{b}
Fat	0.92 ± 0.05^{a}	0.64 ± 0.10^{a}	1.14 ± 0.11^{b}	1.95 ± 0.11^{b}	4.12 ± 0.26^{c}	16.85 ± 0.42^{c}
Ash	46.86 ± 0.27^{c}	55.03 ± 0.24^{c}	2.21 ± 0.14^{a}	5.76 ± 0.37^{a}	1.75 ± 0.19^{b}	11.98 ± 0.76^{b}
Carbohydrate	19.73 ± 0.51^{c}	23.95 ± 1.06^{c}	1.49 ± 0.34^{b}	10.62 ± 0.72^{a}	1.34 ± 0.40^{a}	11.58 ± 1.14^{b}

Numbers followed by same letters mean are not different really ($p<0.05$), data point are mean ± standard deviation ($n=3$)

In fresh condition, the moisture content was discovered to reach 23.74% (skin), 77.07% (body mass) and 82.71% (viscera and gonads). On the other hand, protein content was tested by following AOAC (2005) guidelines, resulting in 8.75% value (skin), 18.08% (other body mass), and 10.08% (viscera and gonads). Meanwhile, fat content was discovered according to the same guidelines, revealing 0.92% value (skin), 1.14% (other body mass), and 4.12% (viscera and gonads). Next, ash content was tested by applying the same guidelines, discovering 46.86% value (skin), 2.21% (other body mass), and 1.75% (viscera and gonads), while carbohydrate content (by-difference) amounted up to 19.73% (skin), 1.49% (other body mass), and 1.34% (viscera and gonads).

This study took the skin parts as raw materials for chitin and chitosan extraction processes. The results of proximate tests for the skin (Table 2), however, revealed variations in terms of its chemical composition between fresh condition and after being transformed into flour form. Technically, a change in form as such was aimed at facilitating the extraction process of chitin and chitosan. Changes occurring in the proportion of chemical composition due to the drying process (wet base) appeared to cause changes in the levels of water, protein, fat, ash and carbohydrates.

Characteristics of Chitin

Table 3 provides a comparison of the characteristics of chitin extracted from the flour form of sea cucumber to other sources. Looking at the results, a chitin yield of 39.08% was obtained from the skin of sea cucumber *H. scabra*. It was in fact relatively close to the result of prior work conducted by Amri et al. (2018) with 40.4% yield, while also higher than 27% yield obtained from crab shells (Nurjannah et al., 2016) and 33.24% obtained from snail shells (Dewi et al., 2016). However, yield discovered in this study was slightly lower to 45.08% yield from the extraction of *Portunus pelagicus* blue crab shell (Syukron et al., 2016). Furthermore, the characteristics of chitin produced by this study from the skin of sea cucumber *H. scabra* were found to mostly meet international standards (Table 3).

Table 3
Characteristics of chitin

Parameters	Chitin of sea cucumber	Chitin of shrimp[1]	Chitin of crab shells[2]	Chitin of blue crab shell[3]	Quality standard[4]
Yield (%)	39.08 ± 1.32	17.36	33.24	45.08	-
Moisture (%)	7.23 ± 0.71	8.50	-	5.72	≤ 10 %
Ash (%)	4.62 ± 0.33	4.25	-	4.84	≤ 2 %
Deacetylation degree (%) (FTIR)	38.84 ± 1.13	-	-	40.47	15-70 %

[1]Hossain and Iqbal (2014), [2]Dewi et al. (2016), [3]Syukron et al. (2016), [4]Bastaman (1989), Bastaman et al. (1990)

Furthermore, the purity of obtained chitin was observed by its low moisture content (7.23%) and DD (38.84%). In general, it was important to state DD as a parameter indicating the percentage of removable acetyl group from the deamination and deacetylation process. A high DD value indicated low acetyl group in the chitin. Technically, a reduction of the acetyl group would result in a stronger interaction between ions and hydrogen bonds (Winarti, 2008). On the other hand, ash content was found to be high (4.62%), exceeding the specified standard (≤ 2%). Then, the mineral content of sea cucumber skin was suspected to also be high, which was considerably not suitable for a demineralization process.

Characteristics of Chitosan and its Nanoparticles

Table 4 provides data on the characterization of chitosan and its nanoparticles. It showed the increasing concentrations of NaTPP from 0.1% to 0.3% to result in increased yield, color, whiteness degree, viscosity, and DD of chitosan nanoparticles. In fact, it was important to note the yield as being calculated from raw chitosan material, by which it was found to increase from 90.6% to 96.4% with respect to the increment of NaTTP concentrations. It appeared to be higher than 81.50% obtained from tiger shrimp shells (Nadia et al., 2014), 76.04% from green mussel shells (Suptijah et al., 2011), and 81.30% obtained from *Vannamei* shrimp shells (Arsyi et al., 2018). Furthermore, a longer stirring time was discovered to deliver a wider time frame for reducing particle sizes. In practices, the homogenization process between chitosan solution and ionic gelation material (NaTPP) could be controlled evenly at a high-speed set for a certain period of time, resulting in smaller chitosan particle sizes and relatively homogeneous particles.

Table 4
Characteristics of chitosan and its nanoparticles

Parameters	Chitosan (control)	C_1 (NaTPP 0.1%)	C_2 (NaTPP 0.2%)	C_3 (NaTPP 0.3%)	Quality standard
Yield (%)	56.84*	90.6**	95.8**	96.4**	-
Color	whiter	whiter	whiter	whiter	whiter
Whiteness degree (%)	80.27[a]	85.82[b]	87.29[b]	88.34[b]	-
Solution color (1.5%) (b/v)	clear	clear	clear	clear	clear
Moisture content (%db)	6.41[c]	5.73[b]	5.26[a]	4.82[a]	≤ 10 %
Ash content (%db)	1.41[b]	1.29[b]	1.07[a]	0.98[a]	≤ 2 %
Viscosity (cP) (1%)	426[c]	198[b]	176[a]	162[a]	Medium (200-799) Low (<200)
Deacetylation degree (%) (FTIR)	77.32[a]	90.6[b]	95.8[c]	96.2[c]	≤ 70 %

* yield of chitin material, ** yield of chitosan material

Furthermore, chitosan produced in this study was found to have characteristics that mostly met international standards (Table 4). Its purity could be observed from moisture and low ash contents despite a relatively near standard DD (≤ 70%). According to Suptijah (2006), a higher DD would result in more amine groups (NH_2) in chitosan molecule chains, making it increasingly reactive. Chitosan obtained in this work was in the form of granules, solid colloid, smooth, and whole round. According to Suptijah et al. (1992), on the other hand, their particle sizes were strongly influenced by raw materials being used. Chitosan derived in the current study from sea cucumber skin had a finer form, making it easy to

get mashed up during chitosan production process (Table 5). Particle sizes as such also affected solubility, by which smaller particle sizes would make chitosan particles easier to dissolve in solvent.

Next, chitosan products in this study were found to be visually white in their powder form with slightly varied degrees of whiteness (80.27%-88.34%). The lowest whiteness degree was found for control chitosan (80.27%), while chitosan nanoparticles had white degrees up to 88.34% (Table 5). It hence emphasized the use of NaTPP concentrations to increase the whiteness degree of a nanoparticle product. Meanwhile, chitosan and chitosan nanoparticle products observed through color tests revealed a clear solution color in comparison to value standard. Prior work by Lisa et al. (2015) suggested color as one of critical parameters to determine the quality of flour products being produced. In general, consumers would prefer flour products with high white degrees. The whiteness degree for a flour product with a vastly brighter white color would reach as much as 100%.

After the solids of chitosan and chitosan nanoparticles were separately dried with 2-3mm thickness in an oven at 45 °C for 6 hours, the values of their water content were found to range from 4.82% to 6.41% (db). These values were in fact lower than the value standard for commercial chitosan products (≤10%). Technically, these values were affected by parameters applied in the drying process, including drying time, amount of dried chitosan, drying area, and drying techniques (Saleh et al., 1994).

The drying process reduced moisture in a material being dried through the evaporation of water during the heating process. Besides, changes occurred in terms of nutrient composition. For example, changes in the amount of ash could increase, while changes in product color might also occurr. In terms of product color, temperature being set should not be too high. The use of an excessively high heating temperature (> 60^{o}C) would damage the color of chitosan being produced, making it yellowish. Furthermore, ash content was a parameter used for determining minerals contained in chitosan, which might affect its solubility, viscosity, and characteristics of final product (No & Meyers, 1995). In this study, the levels of ash content obtained for chitosan and its nanoparticles being produced were in the range of 0.98%-1.41%, which appeared to fulfill specified quality requirements. In fact, it was important to note a low ash content as an indicator of low mineral content. Factors influencing the value might include the demineralization process and the washing technique, which used distilled water for pH neutralization (Angka and Suhartono, 2000). Theoretically, a good washing process would affect both ash and mineral levels released by a material being washed (Benjakula and Sophanodora, 1993).

Moreover, chitosan and its nanoparticles were found to have varying viscosities, *i.e*. 426 cP (control chitosan), 198 cP (0.1% NaTPP), 176 cP (0.2% NaTPP), and 162 cP (0.3% NaTPP). These values in fact met quality standards (200-700 cP in the medium category for raw/control chitosan; <200 cP in the low category for nanoparticles) suggested by Suptijah

et al. (1992). The varied viscosities were considerably influenced by the deacetylation stage during production processes, in which the length of deacetylation process and high concentrations of NaOH would produce reduced molecular weight and viscosity. As the results, chitosan had a shorter chain compared to chitin, which was due to decreased molecular weight caused by the breakdown of polymeric bonds (depolymerization) of its molecular chains (Kolodziejska et al., 2000).

Then, DD value determined the amount of acetyl group lost during a deacetylation process. High DD values ranging from 77.32% to 96.2% indicated the purity of chitosan and nanoparticles being produced. Values as such, especially for chitosan nanoparticles, were found to meet quality standards (≥70%) suggested by Suptijah et al. (1992). These results slightly differed from 98.65% value obtained from tiger shrimp (Nadia et al., 2014). In their work, Muzarelli and Peter (1997) had stated a greater DD would result in a more active chitosan product, which was influenced by a large number of more reactive amine groups containing lone pairs of nitrogen atoms replacing acetyl groups in the structure.

Table 5
Morphological characteristics and the sizes of nanoparticles

Characteristics	Chitosan (raw, not nanoparticles)	C_1 (NaTPP 0.1%)	C_2 (NaTPP 0.2%)	C_3 (NaTPP 0.3%)	Quality standard
Particle shapes (SEM)	granules, solid colloid, smooth, and whole round	granules, solid colloid, smooth, and whole round	granules, solid colloid, smooth, and whole round	granules, solid colloid, smooth, and whole round	granules, solid colloid, and whole round
Particle sizes (PSA)	177^{c} micron	134-206^{b} nm	114-128^{a} nm	97-108^{a} nm	10-1000 nm*
Yield (from raw chitosan,%)	-	90.6	92.8	96.4	≥80% **

*Mohanraj, (2006), **Suptijah et al. (2011)

In terms of morphology, Table 5 provides morphological characteristics of chitosan nanoparticles being produced. These nanoparticles could be visually distinguished by using SEM, which worked according to the nature of electron waves by applying diffractions at very small angles (Masooti et al., 2007).

Figure 2 exhibits the SEM testing of observed samples at different concentrations with magnifications up to 15,000x, while the testing on control chitosan applied up to 5,000x magnifications. Looking at the results, the shape of nanoparticles being observed was in the form of spheres resembling granules, solid colloid, smooth and whole round, and showed relatively homogeneous particle sizes.

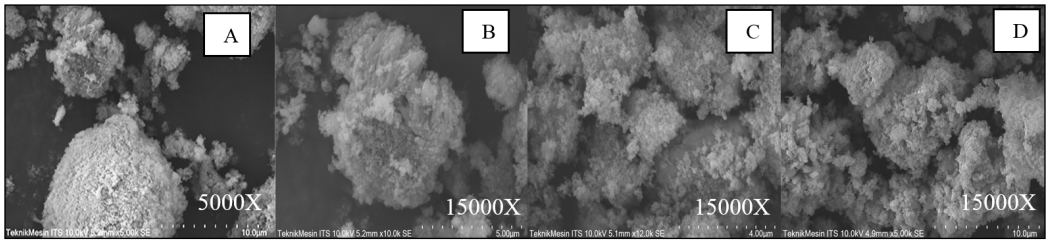

Figure 2. Morphology of chitosan (5,000x) and chitosan nanoparticles (15,000x) (A) Control of chitosan, (B) Nano chitosan 0.1% NaTPP, (C) Nano chitosan 0.2% NaTPP, (D) Nano chitosan 0.3% NaTPP.

In this study particle calculations were conducted by applying an image analysis. Chitosan nanoparticles being produced were tested by using a Particle Size Analyzer (PSA). The results showed average ranges of 134-206 nm (for 0.1% concentration), 114-128 nm (0.2%), and 97-108 nm (0.3%). In an agreement with these results, nanoparticles had been suggested to have solid-shaped particles with sizes ranging between 10-1,000 nm (Mohanraj, 2006). Technically, the method of preparing nanoparticles influenced their sizes. For example, the use of a magnetic stirrer would produce more stable particles with more even sizes under 1,000 nm (Al-Remawi, 2012). Besides, reducing particle sizes by utilizing a magnetic stirrer at a high-speed setting could spread energy received by all parts of a solution, making particle sizes increasingly homogeneous (Nesalin et al., 2009). Moreover, an appropriate NaTTP addition would produce reduced sizes of chitosan nanoparticles and an increased strength of chitosan matrix, making them stronger and harder to split (Du et al., 2009).

Then, obtained yields of chitosan nanoparticles were found to range between 90.6% to 96.4%, meeting the standard threshold (>80%). In fact, these values were greater than those of previous studies. According to Irianto and Muljanah (2011), magnetic stirrers offered advantages during the homogenization process between chitosan solution and NaTTP. Magnetic stirrers could be controlled evenly at high speeds to produce more homogeneous and stable particles with less to no agglomeration(s) for forming nanoparticles in the drying process. It was important to state the less-to-no agglomerated result as being applicable to stable particles only.

CONCLUSIONS

Chitosan produced from the skin of sea cucumber *Holothuria scabra* J offered relatively similar economic values compared to chitosan produced from other sources of raw materials (*e.g.*, shrimp shells and various crab types). This study revealed the use of different NaTTP concentrations to deliver various effects on the characteristics of chitosan nanoparticles obtained from the skin of *H. scabra* sea cucumber. It particularly affected the yield, whiteness degree, moisture content, ash content, viscosity, DD, and particle sizes

of the nanoparticles. Product appearance and solution, on the other hand, were relatively similar, while their shapes were morphologically the same. Looking at the results of this study, the additions of NaTTP concentrations at 0.2% and 0.3% were discovered to deliver insignificant effects, resulting in relatively similar values of parameters being tested. Based on efficiency considerations, the 0.2% concentration of NaTPP was preferable. It was discovered to deliver optimal physical characteristics, resulting in 92.8% yield rate, a whiter color with 77.29% whiteness degree, clear solution color, desired particle shapes (granular, solid colloid, smooth and whole round), and smaller particle sizes ranging from 114-128 nm. The chemical characteristics included 5.26% moisture content (DW), 1.71% ash content (DW), and a low viscosity category (176 cP).

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