Nano collagen of the grouper swim bladder in compliance with quality standard of cosmetics materials

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Abstract. Research of swim bladders on many species revealed that this organ has a high protein content that very potential as a source of collagen. This research aimed to isolate collagen from swim bladders of grouper, convert it into nano collagen, and determine the conformity of collagen with the quality standard of cosmetic materials. Collagen isolation was initiated by the process of elimination all non-collagen protein and other impurities (pre-extraction) with alkaline (NaOH) 0.05 M for 10 hours, followed by extraction with acetic acid 0.5 M (CH₃COOH) for 48 hours with a sample and solvent volume ratio of 1:20 w/v at 4°C. The yield of the extracted collagen was 18.96±1.53%, solubility 81.10±0.88%, and whiteness 90.58±0.13%. Analysis of functional groups showed the presence of amide A, amide B, amide I, amide II, and amide III which indicated as a type 1 collagen. Collagen extract showed positive result for the coloring of Casson’s trichrome. Ultrasonicated nano collagen for 150 minutes had a particle size of 404.1 nm and a polydispersity index of 0.446. The chemical compositions of collagen overall have met the quality requirements of collagen standards as a cosmetic material based on SNI 8076-2014.

Keywords: acid, collagen, grouper, swim bladder, ultrasonic

1. Introduction

Grouper is a reef fish classified as Indonesia’s main export commodity traded in both life and fresh forms (whole, frozen, and fillet). The volume of grouper export in the period of 2012-2016 increased about 30.75% per year with the value in 2016 reached US $ 32.18 million (CBS 2017). The grouper production volume in 2017 raised 404%, from 11,504 tons (2016) to 46,504 tons (MMFA 2018). The high demand of grouper fillets produces a fairly high processing industry by-product (bones, scales, skins, and innards) that reach 20-60% with the proportion of swim bladder approximately 2% (Kartika et al 2016, Gadi et al 2017).

The protein content of fish swim bladders are reported to be quite high in catfish (Pangasius hypophthalmus) 76.75% bk (Trilaksani et al 2006), yellowfin tuna (Tunus albacares) 72.4% bk (Kaewdang et al 2014), and cunang (Muarenesox tabalon) 96.16% bk (Djailani et al 2016). Swim
bladders as collagen sources have several advantages compared to other by-product materials because of its abundant availability as by-product of the processing industry has a protein content dominated by collagen protein and has small impurities. Collagen protein in commercial fish swim bladders can reach 83% (Hickman et al 2000), while in skins, bones, fins, and scales generally only between 30-51% (Nagai and Suzuki 2000).

Collagen has long been utilized in the cosmetics industry, however collagen-based beauty products on the market are still dominated by terrestrial animals-based product, such as bovine, cattle, and pig that still give consumer insecurities about safety and halal assurance due to the possibility of biological diseases transmission such as Bovine Spongiform Encephalopathy (BSE), hand-foot and mouth disease, tapeworm infection, and avian influenza. Commercial collagen in the market has also not been generally accepted by certain religions, such as pig for Moslem and bovine for Hindus (Liu et al 2015). The application of collagen in cosmetic formulas serves to increase skin moisture, maintain skin elasticity, and prevent skin aging (Draelos and Thaman 2006). Smaller particles in the cosmetics are reported have higher penetration potency so that the ability to repair damaged skin is more effective and efficient (Singh and Lillard 2009). The method of reducing size to nanoparticles can be conducted by ultrasonication with several advantages, such as reduce processing time, high accuracy, and low energy consumption (Tiwari et al 2009).

The ultrasonication method in making nanoparticles has been applied to the manufacture of nano chitosan. Sidqi (2011) reported that nano chitosan made by ultrasonication method for 60 minutes with 5 seconds of live pulse and 1-second die pulse resulting particle sizes ranging 470–3000 nm. Li et al (2011) reported an ultrasonication duration of 5 minutes to 30 minutes with a 750 Watt operation and a frequency of 20 kHz resulting smaller size of nano chitosan 1560 nm to 586 nm with a polydispersity index of 0.7 to 0.4. Information related to the manufacture of nano collagen by ultrasonication method has not been widely reported, so information on the use of ultrasound technology in the manufacture of nano collagen from grouper swim bladders is very necessary. This research aimed to determine the collagen extraction by the acid method, nano collagen ultrasonication process, and determine the conformity of collagen with the quality standards of cosmetic materials.

2. Materials and methods

This research was conducted in December 2018-May 2019 at the Laboratory of Biochemistry Department of Aquatic Products Technology IPB University, Laboratory of PT Saraswanti Indo Genetech Indonesia, Laboratory of Histology IPB University, Laboratory of Education and Service Faculty of Fisheries and Marine Science IPB University, Laboratory of Post Harvest Bogor Indonesia, Laboratory of Indonesian Biotechnology and Bioindustry Research Indonesia, Laboratory of Integrated Chemistry IPB, University, Laboratory of Pharmaceutics Faculty of Pharmacy University of Indonesia.

2.1. Materials

The main material in this study is a frozen grouper swim bladders (Epinephelus fuscoguttatus) from fillet processing by-products PT Mahkota Samudera Jaya, North Jakarta which was caught in Fisheries Management Areas (WPP-RI 572) includes the waters of the west Indian Ocean of Sumatra and the Sunda Strait. The chemical materials used, such as acetic acid (CH₃COOH) pro analyst (Fulltime®, China), sodium hydroxide (NaOH) pro analyst (Merck®, USA), sodium chloride (NaCl) pro analyst (HIMEDIA®, India ), 96% ethanol pro analyst (Fulltime®, China), Bovine Serum Albumin (BSA), coomasive brilliant blue G-250, 85% phosphoric acid, Tris-HCl, and casson's trichrome dye.

The tools used, such as ultrasonic cleaner 20 kHz; 200W (Jeken, China), dialysis membrane (Sigma Aldrich, Germany), centrifugation (Hitachi Kiko CR 21G, Japan), freeze dryer (Christ alpha 1-2 ld plus, Australia), High Performance Liquid Chromatography (HPLC) (Shimadzu®-Prominance),
Fourier Transform Infrared (FT-IR) Spectrophotometry (Bruker Tensor 37, Germany), UV-Vis spectrophotometer (2500 Shimadzu®), Olympus CX-31 microscope (Olympus Lifescience, USA), Dino-Eye®, Particle Size Analyzer (PSA) (Malvern, USA), oven (Yamato DV 41), pH meter (HI 2210, UK), and color analyzer RGB-1002 (Lutron, Taiwan).

2.2. Methods

2.2.1. Preparation and characterization of grouper swim bladders (Modification of Sinthusamran et al 2013). Swim bladders were obtained from PT Mahkota Samudera Jaya, North Jakarta on 29 August 2018. Swim bladders during the transportation process were maintained under frozen condition up to the laboratory of aquatic product technology, Dramaga Campus of IPB University Indonesia. Swim bladders were stored in a freezer with a temperature of -20°C and covered with clear plastic. Swim bladders were washed with clean water to remove the impurities. Swim bladders were then cut into 0.5±0.5 cm² and washed thoroughly. Prepared Swim bladders were then characterized includes visual appearance, physical characteristics, proportion, chemical composition, and amino acids profile.

2.2.2. Collagen pre extraction (Modification of Sinthusamran et al. 2013). Pre extraction of collagen was carried out by alkaline solution (NaOH) with concentrations of 0.05 M, 0.10 M, and 0.15 M (M1, M2, and M3) for 12 hours at 4°C with a ratio of 1:10 (w/v). NaOH solution is changed every 2 hours. NaOH solution then measured the dissolved protein by method of Bradford (1976) until the smallest protein content was obtained. The samples were then washed with distilled water until neutral.

2.2.3. Collagen extraction (Modification of Sinthusamran et al. 2013). Extraction of collagen was carried out by acetic acid (CH₃COOH) 0.5 M with solvent volumes ratio of 1:10 (w/v), 1:20 (w/v), and 1:30 (w/v) (V1, V2, and V3) for 48 hours. The samples were then filtered with thin cotton material and precipitated with NaCl 2.6 M against 0.05 M tris-HCl buffer pH 7.5. The precipitation results were then centrifuged at a speed of 10,000 x g for 30 minutes. Centrifuged pellets were then dissolved with CH₃COOH 0.5 M ratio of 1:1 (v/v) and then dialyzed with 14 kDa dialysis membrane against CH₃COOH 0.1 M with the ratio of 1:10 (v/v) for 24 hours. The pellets are then dialyzed again to distilled water ratio 1:10 (v/v) and dialysis water are replaced every 3 hours for 24 hours until pH ≥5. The dialysate was then lyophilized to get a dry collagen extract. All stages were carried out at 4°C. The collagen extract was then characterized includes yield, visual appearance, amino acids profile, functional groups, and staining of collagen tissue.

2.2.4. Nano collagen ultrasonication (Modification of Prince and Smith 1992). Nano collagen ultrasonication was carried out by ultrasonicator with 200 watts, 20 kHz; the temperature of 15°C. Samples were divided into 5 treatments for the time of ultrasonication (0 minutes (control), 60 minutes, 90 minutes, 120 minutes, and 150 minutes) (T0, T1, T2, T3, and T4). The sample was then stirred with a magnetic stirrer while dripping with 96% ethanol 1:1 (v/v) 1,500 rpm for 30 minutes at a temperature of 15°C. Nano collagen was then characterized includes particle size and polydispersity index.

2.2.5. Characterization of collagen conformity with cosmetics material quality. Characterization of compatibility was carried out on collagen extract. Characterization was done by comparing descriptively the chemical characteristics of collagen extract with collagen standards based on SNI 8076-2014 regarding of crude collagen from fish scales. Chemical characteristics of collagen extract compared include water content, protein content, fat content, ash content, and pH.

2.2.6. Yield Analysis. Collagen yield obtained from the ratio between the weight of dry collagen and the weight of grouper swim bladders before pre-extraction (equation 1).

$$\text{Collagen Yield } (\%) = \frac{\text{Collagen in dry weight}}{\text{Raw material in wet weight}} \times 100\% \quad (1)$$
2.2.7. Chemical compositions and amino acids profile analysis. Analysis of chemical compositions and amino acids profile was carried out on swim bladders raw material and collagen extract. Analysis of chemical composition includes protein content, fat content, and ash content according to SNI 01-2891-1992. Amino acids profile was determined by the method of (AOAC 1995) with High-Performance Liquid Chromatography.

2.2.8. Collagen physical characteristics analysis. The physical characteristics of collagen extract analyzed include yield, solubility (Modification of Kittiphattanabawon et al 2005), color and white degree.

2.2.9. Collagen functional group analysis. Functional groups analysis was carried out by Fourier Transform Infrared (FT-IR) Spectrophotometry. 1 mg of dried collagen was mixed with 100 mg KBr and formed into a thin plate. Analysis was carried out at the wavelength of 4,000 to 500 cm⁻¹.

2.2.10. Collagen tissue staining (Kiernan 1990). Collagen tissue staining was done using Casson's Trichrome dye. The dried collagen sample was previously dissolved in an acetic acid (CH₃COOH) 0.1 M. A sample of 0.1 µL was placed in an object-glass and fixed in the air. The collagen is then immersed in Casson's Trichrome dye for 5 minutes, washed with running water for 3-5 seconds and dried by absorbed with water filter paper. Fast dehydration was done in 100% alcohol 3 times. The collagen was then clarified in xylol and attached with a cover glass.

2.2.11. Particle size measurement. Measurement of particles and the distribution of nano collagen was carried out by Particle Size Analyzer (PSA). A sample of 1 mL in kufet was inserted into the PSA. The sample was then fired at the nano wave laser and produces information of particle size and polydispersity index.

2.2.12. Experimental design. The experimental design of the pre-extraction stage was a factorial completely randomized design with two factors (the concentration variation factor and the immersion time variation factor). The experimental design of the extraction stage was a completely randomized design of one factor (the variation in the solvent volume ratio). Data analysis was carried out by software Statistical Analysis System (SAS) version 9.0 to determine variance (ANOVA) and Duncan's Multiple Range Test (DMRT) with a confidence level of 95% if there were significant differences.

3. Results and discussion

3.1. Characteristics of grouper swim bladder (Epinephelus furcoguttatus)
The swim bladder is part of the internal organ in fish. Swim bladders are often found as a by-product along with fish innards. The visual appearance of the grouper and swim bladder of grouper is shown in figure 1.

![Figure 1](image-url)  
Figure 1. Visual appearance (A) grouper fish, (B) grouper swim bladder.
A by-product of grouper fillets processing in PT Mahkota Samudera Jaya, North Jakarta includes heads, bones, skins, scales, innards and swim bladders. The body parts of grouper with the highest percentage is found in the meat, which is 43.29% and the lowest is in the swim bladder 1.67%. Kartika et al (2016) reported that cunang swim bladder (Muaranesox tabalon) had the smallest proportion as part of the fisheries by-products (1.30-1.50%). Effendi (1997) states that the difference in fish body proportion can be influenced by the type of fish, gender, age of fish, fishing ground, season, and type of feed. The proportion of the body parts of grouper is shown in figure 2.

![Figure 2. The proportion of the body parts of grouper.](image)

Grouper swim bladders of this study have the largest chemical composition of water content 71.31±0.84% bb and protein content 25.88±0.00% bb. This chemical composition is similar to all types of fish swim bladders where the highest chemical composition is dominated by water content and protein content. The protein content of grouper swim bladders is higher than swim bladders of yellowfin tuna (Tunnus albacares) and catfish (Pangasius sp.) but lower than swim bladders of cunang (Muaranesox tabalon). Ockerman and Hansen (2000) state that differences in chemical composition of raw material can be influenced by the different species, habitat, age, type of feed, and raw material preparation techniques. Leach (1966) reported that the collagen content of fish swim bladders on a dry basis can reach 98%. High protein content indicates that grouper swim bladder has a potential to be converted into collagen raw materials. The chemical compositions of grouper swim bladder and other swim bladders are shown in table 1.

The results of amino acids profile analysis showed that grouper swim bladder had amino acids profile which was dominated by glycine type 47.19 mg/g, proline 23.71 mg/g, and alanine 22.02 mg/g. Gadi et al (2017) reported that cunang swim bladder (Muaranesox tabalon) have dominant amino acids of glycine, proline, and alanine. Ogawa et al (2004) stated that collagen protein is dominated by glycine, proline, alanine, glutamic acid, and hydroxyproline. The amino acid profile is shown in table 2.

### Table 1. Chemical composition of the grouper swim bladder and several other swim bladders (% bb).

| Chemical compositions | Grouper (Epinephelus fuscoguttatus) | Cunang (Muaranesox tabalon) | Yellowfin Tuna (Tunnus albacares) | Catfish (Pangasius sp.) |
|-----------------------|-------------------------------------|-----------------------------|-----------------------------------|------------------------|
| Moisture              | 71.31±0.84                          | 65.00±0.09                  | 83.33                             | 78.34                  |
| **Protein**           | **25.88±0.00**                       | **33.67±0.71**              | **12.09**                         | **14.73**              |
| Fat                   | 1.29±0.00                           | 0.31±0.11                   | 01.44                             | 00.03                  |
| Ash                   | 0.15±0.01                           | 0.17±0.04                   | 00.29                             | 00.05                  |
| Carbohydrate*         | 1.38±0.84                           | 0.85±0.05                   | 02.85                             | 04.22                  |

*Gadi (2017), Kaewdang (2015), Riyanto (2006), *calculated by the difference
3.2. Pre extraction, collagen extraction, and nano collagen ultrasonication

3.2.1. Pre-extraction. Pre extraction stage aimed at removing non-collagen proteins, fats, minerals, pigments, and other impurities that can affect the extraction result. Zhou and Regenstein (2005) reported that the use of alkaline solvents in the pre-extraction process was better than acid solvents because it was more effective in removing non-collagen proteins, causing low levels of collagen protein loss, and producing insignificant levels of hydroxyproline amino acid loss. The process of removing non-collagen protein when immersed in the pre-extraction stage begins with matrix swelling because most areas of collagen telopeptide have been broken down by alkaline solutions. Swelling in the matrix allows the entry of water molecules so that non-collagen proteins that trapped in the matrix become more easily released (Jaswir et al 2011). Dissolved protein result from the combination of concentration and immersion time in the pre-extraction stage is shown in figure 3.

| Type       | Amino acids | Grouper (Epinephelus fuscoguttatus) | Cunang (Muaranesox tabalon) |
|------------|-------------|-------------------------------------|----------------------------|
| Non-Essential | Gly         | 47.19                               | 95.98                      |
|            | Pro         | 23.71                               | 40.87                      |
|            | Ala         | 22.02                               | 42.99                      |
|            | Glu         | 20.53                               | 40.34                      |
|            | Asp         | 11.61                               | 23.33                      |
|            | Ser         | 07.89                               | 12.19                      |
|            | Tyr         | 01.88                               | 02.37                      |
| Essential  | Phe         | 04.97                               | 11.27                      |
|            | Val         | 04.94                               | 11.10                      |
|            | Arg         | 18.54                               | 38.97                      |
|            | Lys         | 06.93                               | 20.17                      |
|            | Leu         | 06.27                               | 10.87                      |
|            | Ile         | 02.82                               | 05.23                      |
|            | Thr         | 06.89                               | 14.33                      |
|            | His         | 01.55                               | 04.45                      |

Gadi (2017)

The results of variance analysis in the pre-extraction stage showed that differences in concentration, immersion time and interaction between concentration and immersion time had a significant effect of dissolved protein values obtained (mg/mL). Duncan’s further test showed that the combination of 0.05 M NaOH concentration with a time immersion of 10 hours (K1J10) was significantly different (p>0.05) and obtained the lowest concentration of dissolved protein which showed that non-collagen protein was quite effectively dissolved. Liu et al (2015) reported that an alkaline concentration of 0.05-0.15 M was effective in the process of removing non-collagen protein in acid-soluble collagen extracted from grass carp swim bladders (Ctenopharyngodon idella).

The selection of a 0.05 M concentration combined with an immersion time of 10 hours (K1J10) in addition to obtaining the lowest dissolved protein value was also due to an increase of dissolved protein value after 10 hours which indicated that collagen protein started to get dissolved and continue to fluctuate in the following hours. This is due to the excess OH⁻ concentration which caused in a partial breakdown of covalent bonds in the collagen structure. Yoshimura et al (2000) reported that alkaline solvent attacks mainly the telopeptide region of the collagen structure during the pre-extraction process so that it can cause collagen to get dissolve.
3.2.2 Collagen extraction. Extraction was carried out with acetic acid. Analysis of variance on extraction results showed that the different sample and solvent volume ratio showed a significant effect (p>0.05) on the yield of collagen extracted. Duncan's further test showed that there were significant differences in the percentage of yield extracted. The yield of collagen extract of grouper swim bladders with different solvent volumes ratio is shown in figure 4.

Collagen with the highest yield obtained from the solvent volume ratio 1:20 w/v (V2). The high yield produced in collagen V2 caused by the high volume of organic acid used in the extraction process. The use of acids in the extraction process helps in increasing H⁺ ions which causes water molecules to enter the collagen fibers more easily. Water molecules can enter due to the formation of hydrogen bonds between nonpolar groups on collagen fibers with H⁺ ions from acid solvents, thus supporting the destruction of non-covalent bonds in collagen structures which ultimately facilitates the extraction and solubility of collagen in acid solutions (Jaswir et al 2011). The increase in collagen yield from extraction is related to an increase in the breakdown of more hydrogen bonds in collagen molecules due to the large reserves of H⁺ ions in the acid solvent used (Ward and Courts 1977).

3.2.3 Nano collagen ultrasonication. The results of the descriptive analysis based on Particle Size Analyzer (PSA) resulting in an ultrasonication time of 150 minutes (T4) that successfully made the smallest particle size 404.1 nanometer and polydispersity index 0.446. These results have met the standards of collagen nanoparticles. Mohanraj and Chen (2006) stated that nano collagen is collagen with particle size on a scale of 10-1000 nanometers (nm). Nakahira et al (2007) states that the sonication process causes cavitation (bubble formation) which causes the particle size to decrease with increasing
length of sonication. The selection of nano collagen T4 is due to the smallest particle size and polydispersity index value. The polydispersity index shows the spread of particle size distribution. The smaller the polydispersity index value (closer to zero) indicates the more homogeneous level of particle distribution (Yuan 2008).

Table 3. Result of ultrasonication of grouper nano collagen.

| Ultrasonication time (minutes) | Z-average (nm) | PDI* |
|-------------------------------|---------------|------|
| 0                             | 1398          | 0.745|
| 60                            | 1080          | 0.704|
| 90                            | 699.2         | 0.564|
| 120                           | 907.3         | 0.739|
| 150                           | 404.1         | 0.446|

*PDI (Poly Dispersity Index)

The results showed that the longer the ultrasonication time resulted in smaller particle size and the smaller polydispersity index value except at the time of 120 minutes sonication which resulted in particle size and the polydispersity index value returned to enlarge. This is thought to be caused by the amount of energy entering the system so that it can increase the formation of agglomerated particles (Konwarh et al 2011). The difference in particle size and polydispersity index in the process of reducing size to nanoparticles can be influenced by matrix composition, an organic solvent evaporation process, and mixing process (Singh and Lillard 2009). The result of ultrasonication of grouper nano collagen is shown in table 3.

3.3. Characteristics of collagen extract

3.3.1. Yield, visual appearance and physical characteristics of collagen extract. Extraction is an important aspect of the efficiency of the production of a material. The yield of collagen extract produced 18.96±1.53%, higher than collagen extract from cunang swim bladder 14.51±0.43% (Gadi et al 2017) and collagen from yellowfin tuna swim bladder (Tunnus albacares) 1.07% (Kaewdang 2015). The difference yield of collagen extract can be caused by a different type of raw materials, extraction methods, concentration of solvent used, temperature, extraction time, pH, and amount of extract dissolved during the pre-extraction and washing process (Potaros et al 2009, Ratnasari et al 2013).

Wet collagen extract has a transparent white appearance, has a liquid texture at room temperature, and forms a gel at chilling temperature. Wet collagen extract being lyophilized to produce dry collagen extract. Dry collagen has a white appearance with a white degree value of 90.58±0.13% and has a texture like cotton fiber. The lyophilization process is the best drying technique for non-heat-resistant protein materials that can eliminate water content reaching 65-90% (Wang 2000, Reyes et al 2015). The appearance of wet collagen extract, nano collagen, and dry collagen extract are shown in figure 5.

Figure 5. Visual appearance (A) wet collagen extract, (B) nano collagen, and (C) dry collagen.

Solubility value of collagen extract 81±0.88%. The white degree of dried collagen extract is 90.58±0.13%, higher than the collagen extracted from cunang (Muaranesox tabalon) 63.38% (Gadi...
2017) but lower than the skin collagen of yellowfin tuna (*Tunnus albacares* 96.69±0.35% (Hizbullah 2018). The difference in the value of color and a white degree in collagen is influenced by the effectiveness of the alkaline (NaOH) pre-extraction process which has a function to remove impurities, non-collagen protein content, and soluble pigments (Liu *et al* 2015). The physical characteristics of collagen extract from grouper swim bladder are shown in table 4.

**Table 4. Physical characteristics of collagen extract from grouper swim bladder and other swim bladders.**

| Parameter | Collagen from grouper (Epinephelus fuscoguttatus) | Collagen from cunang (Muranesox tabalon)² |
|-----------|--------------------------------------------------|------------------------------------------|
| Solubility (%) | 81.10±0.88                                        | -                                         |
| Whiteness (%) | 90.58±0.13                                        | 63.38                                    |
| Color: L (lightness) | 97.98±0.34                                        | 62.91                                    |
|          | a (green-redness) | -0.97±0.46                               | -1.89                                    |
|          | b (blue-yellowness) | 9.14±0.13                               | 7.44                                     |

Average ± SD from 3 replications in the same sample; ¹Gadi (2017)

3.3.2. Amino acids profile of collagen extract. The amino acid analysis aimed to analyze the specificity of the components of collagen extracted. Collagen has a unique sequence and structure of amino acid molecules that are characteristic of the type of collagen itself. The amino acids profile of collagen from grouper swim bladders and the other swim bladders are shown in table 5.

The results of amino acids profile analysis showed that the collagen extracts from grouper swim bladders were predominantly dominated by glycine 102.04 mg/g, proline 48.20 mg/g, alanine 41.11 mg/g, glutamic acid 35.68 mg/g, and arginine 40.75 mg/g. These results are in accordance with the results of research on collagen swim bladders of cunang fish and swim bladders of white seabass which have dominant amino acids such as glycine, proline, alanine, glutamic acid, and arginine (Gadi *et al* 2017, Sintushamran *et al* 2013). Nalinanon *et al* (2011) stated that the high amino acids glycine, proline, and alanine, as well as the low amino acid tyrosine and histidine indicated that collagen contained in the material was collagen type I

3.3.3. Function groups of collagen extract. Collagen contained functional groups of Amida A, Amida B, Amida I, Amida II, and Amida III which are the constituent structure of collagen protein (table 7). The presence of Amide A in collagen from grouper swim bladder was detected at absorption peak 3440 cm⁻¹. Amida A shows vibrations *stretching* NH detected in the range of absorption areas from 3400 to 3440 cm⁻¹ (Sai and Babu 2001). The presence of Amida B in the collagen from grouper swimbladder was detected at absorption peaks of 2923 cm⁻¹. Amida B shows asymmetrical *stretching* of CH₂ detected in the range of absorption areas of 2935-2915 cm⁻¹ (Coates 2006). The presence of Amida I, Amida II, and Amida III in the collagen from grouper swim bladder was detected at absorption peak 1689 cm⁻¹, 1542 cm⁻¹, and 1249 cm⁻¹, respectively. The FTIR absorption spectrum of collagen from grouper swim bladder is shown in figure 6.

Kong and Yu (2007) stated that Amida I showed a vibration of *stretching* C = O detected in the range of absorption areas 1600-1690 cm⁻¹, Amida II showed CN *stretching* and NH *bending* detected in the range of absorption area of 1480-1575 cm⁻¹, and Amida III shows CN vibrations *stretching* and NH *bending* detected in the range of absorption areas 1229-1301 cm⁻¹. The standard of collagen FTIR spectral absorption area and FTIR spectrum absorption region of collagen from grouper swim bladder results of the study is shown in table 6.
Table 5. Amino acids profile of collagen from grouper swim bladder and another swim bladder (mg/g).

| Type          | Amino acid | Grouper (Epinephelus fuscoguttatus) | Cunang (Muaranesox tabalon) | Seabass (Lutjanus calcafer) |
|---------------|------------|-------------------------------------|-----------------------------|-----------------------------|
| Essential     | Gly        | 102.04                              | 241.06                      | 326                         |
|               | Pro        | 48.20                                | 88.73                       | 111                         |
|               | Ala        | 041.11                               | 86.98                       | 134                         |
|               | Glu        | 035.68                               | 67.26                       | 071                         |
|               | Asp        | 019.98                               | 35.89                       | 046                         |
|               | Ser        | 018.02                               | 25.04                       | 027                         |
|               | Tyr        | 003.48                               | **06.12**                   | **005**                     |
| Non essential | Phe        | 012.09                               | 027.60                      | 013                         |
|               | Val        | 008.18                               | 023.39                      | 022                         |
|               | Arg        | 040.75                               | 094.41                      | 053                         |
|               | Lys        | 011.11                               | 028.89                      | 025                         |
|               | Leu        | 010.31                               | 021.14                      | 023                         |
|               | Ile        | 004.46                               | 010.00                      | 009                         |
|               | Thr        | 0014.8                               | 029.75                      | 024                         |
|               | His        | **003.56**                            | **012.56**                  | **005**                     |

1Gadi (2017); 2Sintushamran et al (2013)

Figure 6. The spectrum of collagen functional groups from grouper swim bladders.

3.3.4. Tissue staining of collagen extract
Detection of collagen tissue was carried out on dried collagen extract. Detection of collagen tissue aims to detect the presence of collagen in the sample using special collagen dye Casson's trichrome (Kiernan 1990). Winarsih et al (2012) stated that trichrome staining technique can not only coloring collagen in histological tissue but also can coloring collagen that has been extracted. The result of collagen tissue staining from grouper swim bladder is shown in figure 7.
Table 6. Standard collagen FTIR absorption area and FTIR spectrum absorption area of collagen from grouper swim bladders.

| Amide  | Absorption area (cm⁻¹) | Absorption peak (cm⁻¹) | Information                          | Reference        |
|-------|------------------------|------------------------|--------------------------------------|------------------|
| Amide A | 3400-3440             | 3440                   | vibrasi stretching NH                 | Sai & Babu (2001) |
| Amide B | 2935-2915             | 2923                   | asymmetrical stretching CH₂           | Coates (2006)    |
| Amide I | 1600-1690             | 1689                   | Vibration stretching C=O              | Kong dan Yu (2007) |
| Amide II | 1480-1575             | 1542                   | CN stretching NH bending              | Kong dan Yu (2007) |
| Amide III | 1229-1301             | 1249                   | CN stretching NH bending              | Kong dan Yu (2007) |

The staining result showed that the sample positive containing collagen. This is indicated by the dominance of blue color in the sample tissue. The trichrome staining method is a staining technique that aims to identify collagen by involving two or more anionic dyes associated with phosphoric acid. This acid can be mixed with dye or solutions of the reagents used. The blue color that shows the presence of collagen tissue in the sample comes from aniline blue dye, while the orange G dye coloring the cytoplasm and cell nucleus is shown in orange or brown (Kiernan 1990).

Figure 7. Results of collagen tissue staining from grouper swim bladders (scale bar of 100 µm).

3.4. Compliance of collagen with quality standard of cosmetics materials
Conformity characterization was done by comparing descriptively the chemical characteristics of collagen extract with collagen standard based on SNI 8076-2014. Chemical characteristics of collagen extract compared include water content, protein content, fat content, ash content, and pH. The chemical characteristic of collagen from grouper swim bladder is shown in table 7.

Table 7. Chemical characteristics of collagen from the grouper swim bladder.

| Parameter   | Collagen from grouper swim bladder (Epinephelus fuscoguttatus) | SNI 8076-2014 |
|-------------|---------------------------------------------------------------|---------------|
| Moisture    | 10.31±0.33                                                   | Maximum 12 %  |
| Protein     | 75.20±0.00                                                   | Minimal 75 %  |
| Fat         | 10.99±0.00                                                   | Maximum 1 %   |
| Ash         | 10.10±0.00                                                   | Maximum 1 %   |
| pH          | 6.62±0.38                                                    | 6.5-8         |

SNI (Indonesian National Standard) 8076-2014 regarding to crude collagen from fish scales.
Chemical composition is one of the determinants of the quality of a material. Water content as a determinant of collagen resistance and consumer acceptance and ash content as a determinant of mineral contained in collagen extract. The results of the analysis of chemical composition (moisture content, protein content, fat content, and ash content) and pH of collagen extracted from grouper swim bladders of the study have met the quality requirements of collagen standards as a cosmetic material based on SNI 8076-2014. The protein content is the highest component of collagen extract among other chemical compositions. Low fat and ash levels from collagen extract showed the success of alkaline solvents in the pre-extraction process in partially destroying covalent, peptide and hydrogen bonds in the complex structure of collagen so that the non-collagen and other impurities components were successfully removed (Ward and Courts 1977).

PH values determine the level of safety and solubility of collagen in their application to various products (Shahiri et al. 2012). The pH value of collagen extract from grouper swim bladder approached a neutral pH of 6.62±0.38. Approximately neutral pH values in collagen extracts are caused by the combination of acids and bases during the extraction process and dialysis process with periodic stirring so as to accelerate the rate of osmosis transfer of materials through the membrane (Zhou and Regenstein 2005, Dennison 2002).

4. Conclusion

Collagen pre-extraction was carried out by alkaline (NaOH) 0.05 M for 10 hours, followed by extraction with acetic acid (CH₃COOH) 0.5 M for 48 hours with a sample and solvent volume ratio of 1:20 w/v at 4°C yielding of 18.96±1.53% collagen extract. Ultrasonicated nano collagen for 150 minutes had a particle size of 404.1 nm and a polydispersity index of 0.446. The chemical compositions of collagen overall have met the quality requirements of collagen standards as a cosmetic material based on SNI 8076-2014. It is necessary to extract nano collagen using ultrasonication method which includes modification of frequency and time of sizing.

References

AOAC 1995 Official Method of Analysis of the Association of Official Analytical of Chemist (Arlington: The Association of Official Analytical Chemist Incorporation)

Bradford M M 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding Anal. Biochem. 72 248-254

CBS 2017 National Production Volume of Grouper 2012-2016 (Jakarta: Central Bureau of Statistics)

Coates J 2006 Interpretation of Infrared Spectra, A Practical Approach (Newtown: Encyclopedia of Analytical Chemistry)

Dennison C 2002 A Guide to Protein Isolation (New York: Kluwer Academic Publishers)

Djailani F, Trilaksani W and Nurhayati T 2016 Optimasi ekstraksi dan karakterisasi kolagen dari gelembung renang ikan cunang dengan metode asam-hidro-ekstraksi JPHPI 19 156-167

Draelos Z D and Lauren A T 2006 Cosmetic Formulation of Skin Care Product (New York: Taylor and Francis Group)

Effendi M I 1997 Biologi Perikanan (Jakarta: Yayasan Pustaka Nusantara)

Gadi D S, Trilaksani W and Nurhayati T 2017 Histologi, ekstraksi dan karakterisasi kolagen gelembung renang ikan cunangMuarenesox talabon JITKT 9 665-683

Gadi DS 2017 Kolagen Larut Asam dari Gelembung Renang Ikan Cunang (Muarenesox Talabon) sebagai Sediaan Krim Pelembab Wajah [Thesis] (Bogor: IPB University)

Hickman D, Sims T J, Miles C A, Bailey AJ, M de Mari and Koopmans M 2000 Isinglass/collagen: denaturation and functionality J. Biotechnol. 79 245-257

Jaswir I, Monsur H A and Salleh H M 2011 Nano-structural analysis of fish collagen extracts for new process development Afr. J. Biotechnol. 10 18847-18854

Kaewdang O, Benjakul S, Kaewmanee T and Kishimura H 2014 Characteristics of collagens from the
swim bladders of yellowfin tuna (*Thunnus albacares*) *Food Chem.* 155 264-270

Kaeuwong O 2015 *Value-Added Products from Yellow Fin Tuna Swim Bladder: Collagen dan Gelatin [Thesis]* (Songkla: Prince of Songkla University)

Kartika I W D, Trilaksani W and Adnyane I K M 2016 Karakterisasi kolagen dari limbah gelembung renang ikan cunang hasil ekstraksi asam dan hidrotermal *JPHIJ* 19 222-232

Kiernan J A 1990 *Histological dan Histochemical Methods: Theory dan Practice* (Oxford: Pergamon Press)

Kittiphitthabawon P, Benjakul S, Visessanguan W, Nagai T and Tanaka M 2005 Characterization of acid-soluble collagen from skin and bone of big eye snapper (*Priacanthus tayenus*) *Food Chem.* 89 363-372

Kong J and Yu S 2007 Fourier transform infrared spectroscopi analysis of protein secondary structures *Acta Biochim. Biophys. Sin.* 39 549-559

Konwarh R, Karak N, Sawian E M, Baruah S and Mandal M 2011 Effect of sonication and aging on the templating attribute of starch for green silver nanoparticles and their interactions at bio-interface *Carbohydr. Polym.* 83 1245-125

Leach A A 1966 Collagen chemistry in relation to isinglass and isinglass finings a review *JIB* 73 8-16

Li J, Kong M, Cheng X J, Dang Q F, Zhou X, Wei Y N and Chen S G 2011. Preparation of biocompatible chitosan grafted poly (lactic acid) nanoparticles *Int. J. Biol. Macromol.* 51 221-227

Liu D, Zhang X, Li T, Yang H, Zhang H, Regenstein M J and Zhou P 2015 Extraction dan characterization of acid dan pepsin soluble collagens from the scales, skins dan swim bladders of grass carp (*Ctenopharyngodon idella*) *Food Biosci.* 9 68-74

Mohanraj V J, Chen Y 2006 Nanoparticle-A Review *TJPR* 5 561-573

MMAF 2018 *Grouper Production of 2017* (Jakarta: Ministry Of Marine Affairs and Fisheries Republic of Indonesia)

Nagai T and Suzuki N 2000 Isolation of collagen from fish waste material-skin, bone and fins *Food Chem.* 68 277-281

Nakahira A, Nakamura S and Horimoto M 2007 Synthesis of modified hydroxyapatite (HAP) substituted with fe ion for DDS application *IEEE T. Magn.* 43 2465-2467

Nalinanon S, Benjakul W, Visessanguan and Kishimura 2007 Use of pepsin for collagen extraction from the skin of bigeye snapper (*Priacanthus tayenus*) *Food Chem.* 104 593-601

Ockerman H W and Hansen C L 2000 *Animal By-product Processing and Utilization* (Lancaster: Technomic Publishing)

Ogawa M, Portier R J, Moody M W, Bell J, Schexnayder M A and Losso J N 2004 Biochemical properties of bone and scale collagens isolated from the subtropical fish black drum (*Archosargus probatacephalus*) *Food Chem.* 88 495-501

Potaros T, Raksakhultai N, Runglerdkreangkrai J and Worawattananaatekul W 2009 Characteristic of collagen from nile tilapia (*Oreochromis niloticus*) skin isolated by two different method *Nat Sci* 43 548-593

Prince GJ and Smith P F 1992 Ultrasonic degradation of polymer solutions-iii The effect of changing solvent and solution concentration *Eur. Polym. J.* 29 419-424

Ratnasari I, Yuwono S S, Nusyam H and Widjanarko S B 2013 Extraction and characterization of gelatin from different fresh water fishes as alternative sources of gelatin *IFRJ* 20 3085-3091

Reyes A, Mahn A, Herrera C and Vasquez J 2015 Freeze-drying of soymilk *IFBE* 1 1-6

Riyanto B 2006 *Pengembangan Lapisan Edible dari I singlass dan Aplikasinya untuk Mempertahankan Mutu Udang Masak* [Thesis] (Bogor: IPB University)

Sai K P and Babu M 2001 Studies on Rana tigerina skin collagen *Biochem. Mol. Biol.* 126 81-90

Shahiri H I, Maghsoudlou Y, Motamedzadeghan A, Sadeghi M A R and Rostamzad H 2012 Study on some properties of acid-soluble collagens isolated from fish skin and bones rainbow trout (*Onchorhynchus mykiss*) *IFRJ* 19 251-257

Sidqi T 2011 *Pembuatan dan Karakterisasi Nanopartikel Ekstrak Temulawak dengan Metode Ultrasonikasi* [Undergraduate Thesis] (Bogor: IPB University)
Singh R and Lillard J J W 2009 Nanoparticle-based targeted drug delivery Exp. Mol. Pathol. (86) 215-223
Sinthusamran S, Benjakul S and Kishimura H 2013 Comparative study on molecular characteristics of acid soluble collagens from skin dan swim bladder of seabass (Lates calcarifer) Food Chem. 138 2435-2441
SNI 1992 SNI 01-2891-1992 Food and Beverage Test Methods (Jakarta: Badan Standardisasi Nasional)
SNI 2014 SNI 8076:2014 Crude Collagen from Fish Scales (Jakarta: Badan Standardisasi Nasional)
Tiwari B K, Donnell O P C and Cullen J P 2009 Effect of sonication on retention of anthocyanins in blackberry juice J. Food Eng. 166-171
Trilaksani W, Nurjanah and Utama H W 2006 Pemanfaatan gelembung renang ikan patin (Pangasius hypophthalmus) sebagai bahan baku isinglass JPHPI 9 13-25
Wang W 2000 Lyophilization and development of solid protein pharmaceuticals Int. J. Pharm. 203 1-60
Ward A G and Courts A 1977 The Science and Technology of Gelatin (New York: Academic Pr)
Winarsih W, Wientarsih I and Sutardi L N 2012 Aktivitas salep ekstrak rimpang kunyit dalam proses persembuhan luka pada mentic yang diinduksi diabetes J. Vet. 13 242-250
Yoshimura K, Terashima M, Hozan D and Shirai K 2000 Preparation and dynamic viscoelasticity characterization of alkali-solubilized collagen from shark skin J. Agric. Food. Chem. 48 685-690
Yuan Y, Gao Y, Zhao J and Mao L 2008 Characterization and stability evaluation of β-carotene nanoemulsions prepared by high pressure homogenization under various emulsifying conditions Food Res Int 41 61-68
Zhou P and Regenstein J M 2005 Effects of alkaline and acid pretreatments on alaska pollock skin gelatin extraction J. Food Sci. 70 392-396