The influence of extraction period on the characteristics of acid soluble collagen from sea catfish (Arius thalassinus) swim bladder

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Abstract. The sea catfish in Indonesia is commonly processed into smoked fish and salted fish. The processing of these two products leaves a by-product, namely a swim bladder of 2%. The study investigated the influence of extraction period on the characteristics of collagen from sea catfish (Arius thalassinus) swim bladder. Collagen was extracted from the swim bladder using 0.5 M citric acid with different extraction periods (8, 12, and 16 h). The extraction period of 12 h produced the highest yield of collagen, namely 40.33%. The results showed that the longer extraction, the more amino acids could be extracted from the swim bladder. Glycine was an amino acid that dominates collagen in the amount of 138544.9 to 175420.0 mg/kg. The electrophoresis pattern of protein fraction indicated that the collagen were of type I because it consists of α1 and α2 chains with a molecular weight of approximately 100 to 150 kDa and β chain of 250 kDa. Fourier Transform Infrared (FTIR) spectra of collagen showed the regions of amide A, B, I, II, and III. However, based on the results of the Differential Scanning Colorimetry (DSC), collagen extracted for 16 h had lower thermal stability than the extraction period of 8 and 12 h. Based on these data, sea catfish swim bladder can be used as an alternative raw material for collagen production because it has a higher thermal stability than mammalian collagen, also can be used in the food, pharmaceutical, and nutraceutical industries.

1. Introduction

Sea catfish (Arius thalassinus) is included in the top ten of marine fish commodities in Indonesia with total production reaching 114,108.70 tons in 2018 [1]. Sea catfish in Indonesia is processed into smoked fish and salted fish. The production of these products produced the by-products of processing, namely head, gut, liver, roes, and swim bladder that have not been optimally utilized. Research related to the use of sea catfish viscera into fish sauce has been conducted [2]. However, research related to the use of sea catfish swim bladder to become collagen has never been carried out.

Swim bladder contains high molecular weight collagen, which is 300 kDa [3]. Currently, alternative sources of collagen from the by-products of the fisheries industry have been developed to replace collagen from mammals and poultries. It is worried that collagen extracted from mammals can transmit mad cow (bovine spongiform encephalopathy) and foot and mouth disease [4], while collagen from poultries can transmit avian influenza [5]. Meanwhile, collagen sourced from fish has advantages over other animals, namely it has a shorter protein fiber [6] and a simpler molecular structure so that it...
is easily absorbed by the human skin and body [7]. In addition, some religions limit the use of products from cows, while Islam prohibits Muslims to consume porcine products. Therefore, study on the use of swim bladders to extract collagen is very important.

Collagen is the main structural component of white connective tissue which can be extracted by several methods, one of which is the acid extraction method. Extraction of collagen with acid method is a widely used because easy to apply than the enzyme method and has a higher collagen purity than the salt method [5]. Collagen has been widely used in food, cosmetics, medicine, therapeutic delivery, diagnostic imaging, and film industries [8]. The types of acids commonly used to extraction of collagen are organic acids, such as acetic acid, citric acid, and lactic acid [9].

Collagen was extracted from scales, skin, and swim bladder of grass carp (Ctenopharyngodon idella) using acid and enzyme method [5] and swim bladder of Rohu fish (Labeo rohita) using pepsin [10]. The effect of acetic acid concentration on the characteristics of collagen from Hybrid catfish (Clarias sp.) skin was also studied [11]. Previous studies also compared collagen from catfish (Ictalurus punctatus) skin which is extracted using acetic acid, citric acid, lactic acid, hydrochloric acid, and pepsin [12]. Meanwhile, the effect of collagen extraction time from sole fish skin using acetic acid was also evaluated [13]. However, no one has reported the collagen characteristics from sea catfish swim bladder which is extracted using citric acid. Thus, the study investigated the influence of extraction period on the characteristics of collagen from sea catfish (Arius thalassinus) swim bladder.

2. Methods

2.1. Proportion of raw material

The sea catfish (Arius thalassinus) was prepared until the parts of fish were obtained, including meat, skin, and bone (for material production of salted sea catfish), head, gut, liver, roes, and swim bladder. The proportion of each piece was expressed as a percentage of the weight total. Material preparation was carried out at a salted sea catfish industry in Lamongan, East Java Province, Indonesia.

2.2. Extraction of collagen

The raw material used was the dried sea catfish swim bladder. Collagen from swim bladder was extracted using citric acid [14]. Swim bladder was cut into 1 cm size and soaked in 0.1 M NaOH with a ratio of 1:8 (w/v) for 6 h at 4 °C. Furthermore, the swim bladder was washed until pH of 7. The swim bladder was soaked in 0.5 M citric acid with a ratio of 1:5 (w/v) at 4 °C. The treatment in this study was the difference of extraction period, namely 8, 12, and 16 h. The sample was squeezed using a nylon cloth and precipitated with NaCl until a final concentration of 2.6 M. The precipitate was obtained by centrifuge (Universal 320 R type 1406 Hettich, Tuttingen, Germany) at 5,000 g for 15 min at 4 °C. Furthermore, the pellets were dissolved in 0.5 M citric acid with a ratio of 1:1 (w/v) and dialyzed with distilled water for 24 h at 10 °C. Collagen was freeze dried (Heto Power Dry LL 1500, Tokyo, Japan) then milled to powder.

2.3. Yield of collagen

The yield of collagen was determined based on the percentage by weight of collagen powder to the weight of dried swim bladder [14].

2.4. Profile of amino acids

The profile of amino acids were observed using Eurospher 100-5 C18 HPLC (Shimadzu LC-6A, Japan). Collagen powder (0.1 g) was hydrolyzed at 100 °C with 5 ml of 6 M HCl for 22 h until homogeneous. An aliquot of 5 µL was injected into amino acid analyzer with AccQ Tag as Eluent A (concentrate) and 60 % acetonitrile as Eluent B. The profiles of amino acids were determined at 36 °C by reverse phase HPLC AccQ Tag column (3.9 x 150 mm) [14].
2.5. Electrophoretic patterns
SDS-PAGE was used to observe electrophoretic patterns based on protein molecular weight [15]. SDS-PAGE was operated using the discontinuous Tris-HCl buffer system, with 4% stacking gel and 7.5% resolving gel. Before incubation for 1 h at 85 °C, collagen powder was suspended in 5% SDS. Furthermore, the mixture was centrifuged at 5,000 g for 10 min. A sample solution of 20 µL was added with the mixture of 60 mM Tris-HCl, 2% SDS, 0.1% bromophenol blue, and 25% glycerol as much as 4:1 (v/v) in the presence of β-ME then put in a well and electrophoresed using electrophoresis (AE-6200 ATTO, Japan) at 100 V for 4 h. Furthermore, the gel was stained with 0.1% Coomassie blue R-250 in 45% methanol and 10% acetic acid.

2.6. Functional group of collagen (FTIR Spectroscopy)
The functional group of collagen was evaluated by FTIR spectrometer (Shimadzu FTIR 8400, Japan). Collagen powder was carried out in the IR spectra with the range 4000 to 400 cm⁻¹.

2.7. Differential scanning calorimetry (DSC)
The stability thermal of collagen was observed using DSC (Shimadzu DSC-60 plus, Japan). Collagen powder were shielded in aluminium containers and heated from 30 to 300 °C with temperature rate of 10 °C min⁻¹.

2.8. Statistical analysis
The data of collagen yield was evaluated in triplicate and analyzed with the analysis of variance (ANOVA) then tested with Duncan’s multiple range tests. The analysis of data in this study used SPSS version 20.0 for windows (SPSS Inc., USA).

3. Results and discussions
3.1. Proportion of raw material
Table 1 and Figure 1 show the proportion by-products from processing salted sea catfish. Swim bladder and liver had the least proportion, namely 2% each part of the average weight sea catfish of 3.13 kg, which is smaller than the swim bladder of tuna, namely 3.5 to 5.5% [16]. The composition of fish greatly varies between species and the chemical composition of each species was influenced by sex, age, environment and season [17].

| Weight of Fish (kg) | Meat, Bone, Skin (kg) | Head (kg) | Gut (kg) | Liver (kg) | Roe (kg) | Swim Bladder (kg) |
|--------------------|-----------------------|-----------|----------|------------|---------|------------------|
| 3.02               | 1.75                  | 0.68      | 0.07     | 0.04       | 0.42    | 0.06             |
| 3.26               | 2.03                  | 0.84      | 0.17     | 0.06       | 0.09    | 0.07             |
| 3.12               | 1.96                  | 0.71      | 0.08     | 0.06       | 0.26    | 0.05             |
| 4.05               | 2.61                  | 1.1       | 0.16     | 0.04       | 0       | 0.13             |
| 3.17               | 2.06                  | 0.83      | 0.14     | 0.07       | 0       | 0.07             |
3.2. Yield of collagen

The results showed that extraction period of collagen using citric acid gave significantly difference (p<0.05) (Fig. 2). The extraction of collagen for 12 h was able to produce higher yield than extraction period of 8 and 16 h. The similar results were shown that collagen extracted from a mixture of threadfin bream scales and fins using 0.5 M citric acid for 24 h produced in a lower yield than extracted for 12 h, namely 8.3% (12 h) and 6.9% (24 h) [14].

3.3. Profile of amino acids

Glycine is the most abundant amino acid in the sea catfish swim bladder (Table 2), namely 22% of the total amino acids. High content of glycine indicated that a material contains collagen. This is due to
tropocollagen molecule, glycine plays an important role in every basic sequence Gly-X-Y- in the formation of triple alpha helical chain in the formation of alpha triple heliks chain, where X is proline and Y is hydroxyproline [20]. The results of amino acids profile analysis of the swim bladder sea catfish has the potential to be a renewable alternative source of by-products from the fisheries industry for the production of collagen powder.

Likewise in the collagen powder extracted from sea catfish swim bladder in this research (Table 2), namely glycine composes 25 to 26% of the total amino acids [21]. The type of amino acids that is also large in number below the amount of glycine is glutamic acid. Aspartic acid and glutamic acid play a role in providing umami taste [22]. In addition to glycine and glutamic acid, collagen powder from sea catfish swim bladder also contained large amounts of proline and alanine, namely 10 to 11% of the total amino acids of each sample. Meanwhile, the type of amino acid that had the lowest content in this study was isoleucine. The three main amino acids composed collagen are glycine, proline, and alanine [23]. Collagen from fish was not contain cysteine and tryptophan and contained small amounts of tyrosine and histidine.

**Table 2. Amino acids profile of sea catfish swim bladder and collagen with different extraction period**

| Amino Acids     | Dried Swim Bladder (mg/kg) | Collagen Powder with Different Extraction Period |
|-----------------|----------------------------|-----------------------------------------------|
|                 | 8 h (mg/kg) | 12 h (mg/kg) | 16 h (mg/kg) |
| Glycine         | 194696.30  | 138544.90    | 161725.16    | 175420.51    |
| Proline         | 84602.00   | 59716.18     | 66268.14     | 77021.01     |
| Alanine         | 81768.17   | 59527.06     | 63841.27     | 77190.41     |
| Glutamic acid   | 85148.89   | 60537.47     | 62243.59     | 77608.05     |
| Arginine        | 69593.91   | 46946.64     | 56340.74     | 57555.07     |
| Aspartic acid   | 45797.86   | 29697.26     | 29248.75     | 37677.63     |
| Threonine       | 41184.07   | 22464.92     | 26437.91     | 28255.78     |
| Phenylalanine   | 39123.31   | 18729.72     | 23042.35     | 21142.94     |
| Serine          | 38832.46   | 23743.09     | 27382.38     | 29636.39     |
| Leucine         | 38558.39   | 18519.40     | 20201.67     | 23005.85     |
| Valine          | 33812.06   | 16430.69     | 17619.42     | 20478.77     |
| Lysine          | 31891.89   | 20595.39     | 22821.44     | 29259.14     |
| Tyrosine        | 30000.21   | 11653.27     | 14550.46     | 12771.43     |
| Histidine       | 22573.81   | 13152.78     | 15770.21     | 15381.88     |
| Isoleucine      | 19253.63   | 10009.96     | 10898.63     | 12424.06     |
| Total Amino Acids| 856836.90  | 550268.70    | 617852.15    | 694827.95    |

Based on Table 2, it can also be seen that increasing the extraction period of collagen using citric acid can increased the total of amino acids that can be extracted from the swim bladder of sea catfish. Extraction for 12 h can produced higher content of each amino acid than extraction for 8 h. However, with a longer extraction period (16 h), it actually produced collagen with lower phenylalanine, tyrosin, and histidine content than extracted 12 h. Although the content of other amino acids increases. Excessive extraction period can cause peptide degradation, namely the acid solution used for collagen extraction can trigger the decomposition of amino acids and other compounds [24].

### 3.4. Electrophoretic patterns

Electrophoretic patterns are used to determine the type and composition of collagen based on the molecular weight of the subunit composition. Collagen produced in this study has a molecular weight of 15 to 250 kDa (Fig. 3) which is higher than collagen from threadfin bream (*Nemipterus japonicas*) scale and fin using calamansi juice, namely 10 to 220 kDa, while collagen extracted with citric acid
only has 2 bands (10 dan 220 kDa) [14]. The high molecular weight of collagen components is closely related to cross-linked molecules [25].

Based on the results of electrophoretic pattern analysis, it can be seen that the collagen from the swim bladder of sea catfish belongs to type I, namely by the presence of a band at a molecular weight of 100 to 150 kDa which indicated \( \alpha_1 \) and \( \alpha_2 \) chains and a molecular weight of 250 kDa indicated the structure of \( \beta_1 \) chain (Fig. 3). The type I collagen consist of \( \alpha_1 \) and \( \alpha_2 \) chains at 100 to 120 kDa [26]. Meanwhile, type I collagen consists of \( \alpha_1 \) dan \( \alpha_2 \) chains at 120 to 150 kDa and \( \beta_1 \) structure at 200 to 250 kDa [27]. Band \( \alpha_1 \) dan \( \alpha_2 \) seen in SDS-PAGE show secondary protein structures (polypeptides) that are two types of \( \alpha \)-helix chains [28]. The results of this study are similar to those of acid soluble collagen (ASC) from yellowfin tuna swim bladder [16]; swim bladder of bighead carp (Hypophthalmichthys nobilis) [6], and swim bladder of seabass (Lates calcarifer) [29].

**Figure 3.** Electrophoretic patterns of collagen with different extraction period. (1) Protein marker; (2) Collagen with extraction period 8 h; (3) Collagen with extraction period 12 h; (4) Collagen with extraction period 16 h

Fig. 3 shows that collagen extracted for 8 h had a 15 kDa band that was more intense than collagen with an extraction period of 12 and 16 h. Collagen with extraction period of 16 h had a more intense 15 kDa than collagen with extraction period of 12 dan 16 h. Meanwhile, collagen with extraction period of 16 h had a band of 100 to 150 kDa and 250 kDa which is more intense. This indicated that the increasing of extraction period results in the degradation of the collagen component which has a molecular weight of 15 kDa. Acidic environmental conditions can cause collagen to be degraded through the breakdown of the salt and Schiff bonds (\(-C=N-\)) [30].

3.5. **Collagen function group (FTIR Spectroscopy)**

The results of FTIR analysis on the three collagen samples showed the presence of amide A, B, I, II, dan III functional groups. Amide A was found at 3444.0, 3448.57, and 3444.35 cm\(^{-1}\) respectively (Fig. 4). Amide A shows the N-H stretching band and the presence of hydrogen bonds, which is in the range of 3400 to 3440 cm\(^{-1}\) for free N-H stretching and about 3300 cm\(^{-1}\) for the N-H group of the peptide involved in hydrogen bonding [30]. Amide B is shown at wavenumber 2933.78, 2934.97, and 2939.73 cm\(^{-1}\) (Fig. 4). Asymmetrical stretch of CH\(_2\) is shown at wavenumber 2928 and 2924 cm\(^{-1}\), representing an amida B on collagen [25].
The wavenumber in the range of 1600 to 1700 cm\(^{-1}\) is owned by amide I with C=O stretching vibration (Fig. 4). This functional group can be used as a marker for the presence of peptide secondary structure [31]. The wavenumber range also shows the presence of hydrogen bonds between C-O (Gly) and N-H stretch which indicates a triple helix structure [25].

Amide II read at wavenumber 1543.02, 1545.32, and 1545.74 cm\(^{-1}\) which indicated the presence of N-H band coupled with C-N stretch (Fig. 4). The lower wavenumber indicates the presence of stronger hydrogen bonds [24, 25]. Meanwhile, amide III read at wavenumber 1231.03, 1231.95, and 1238.11 cm\(^{-1}\) (Fig. 4). This wavenumber explains the existence of molecular interactions in collagen, which consist of C-H and N-H stretching in amide bonds, as well as absorption of CH\(_2\) groups originating from the glycine backbone and proline side chains. This events indicates the presence of hydrogen bonds in collagen [25].

Figure 4. FTIR spectra of collagen with different extraction period. (a) Collagen with extraction period 8 h; (b) Collagen with extraction period 12 h; (c) Collagen with extraction period 16 h

3.6. Differential scanning calorimetry (DSC)

DSC analysis was used to understand the thermodynamic properties of a material. The results of DSC analysis showed that collagen powder with extraction periods of 8, 12, and 16 h had glass transition temperatures of 179.31 °C, 183.60 °C, and 171.74 °C (Fig. 5). The results of this study were lower than the collagen extracted from the swim bladder of Rohu fish (Labeo rohita) using pepsin, which was 197 °C [32]. Collagen-chitosan from swim bladder catfish extracted with pepsin had a
denaturation temperature of 209 °C [33]. The higher of the maximum glass transition temperature, the higher of the denaturation temperature [14].

Collagen extracted from swim bladder and bone has higher thermal stability than collagen extracted from fin, skin, and scale [6]. Meanwhile, collagen from mammals has a denaturation temperature ranging from 39 to 40 °C [34]. The thermal stability of collagen is influenced by species, environment, and imino acid composition [35].
Figure 5. DSC thermograms of collagen with different extraction period. (a) Collagen with extraction period 8 h; (b) Collagen with extraction period 12 h; (c) Collagen with extraction period 16 h

Figure 5 shows that collagen with an extraction period of 16 h had the lowest glass transition temperature, indicated that excessive extraction can reduce the thermal stability of the collagen produced. Meanwhile, collagen extracted for 12 h had a higher glass transition temperature than 8 and 16 h. Amino acid content in collagen affect thermal stability and high amino acid content cause collagen to have high temperature stability [36]. The amino acids proline and hydroxyproline in collagen play a role in maintaining the integrity of collagen thereby increasing thermal stability by means of the pyrrolidin ring helping to strengthen the triple helix structure. Thus, lower content of proline and hydroxyproline lead to lower melting point and thermal stability [25], [37]. However, the case in this study was that collagen with an extraction period of 16 h had the highest content of glycine and proline, but actually had the lowest glass transition temperature. This is thought to be caused by the proportion of glycine to the total amino acids in collagen with an extraction period of 16 h, which is 25.25%, lower than the proportion of glycine to the total amino acids in collagen with an extraction period of 12 h, which is 26.17%. Glycine can affect hydrogen bonding in collagen. This hydrogen bonding occurs when the amino group (NH) of glycine forms a peptide bond with a carboxyl group in an adjacent polypeptide. Thus, glycine is able to hold the triple helix chain together [38].

4. Conclusion
In this study, collagen was extracted from dry sea catfish swim bladder using citric acid with different extraction periods. Extraction period 12 h resulted in the highest yield, which was 40.33 %. Nonetheless, the results in the present study showed that the longer extraction, the more amino acids could be extracted from the swim bladder. Glycine is an amino acid that dominates collagen in the amount of 138544.9 to 175420.0 mg/kg. The electrophoresis pattern of protein fraction and Fourier Transform Infrared (FTIR) spectra indicated that the collagen were of type I because it consists of α1 and α2 chains with a molecular weight of approximately 100 to 150 kDa and β chain of 250 kDa serta FTIR spectra showed the regions of amide A, B, I, II, and III. However, based on the results of the Differential Scanning Colorimetry (DSC), collagen extracted for 16 h has lower thermal stability than the extraction period of 8 and 12 h. Based on these data, sea catfish swim bladder can be used as an alternative raw material for collagen production because it has a higher thermal stability than mammalian collagen, also can be used in the food, pharmaceutical, and nutraceutical industries. Utilization of sea catfish swim bladder can also reduce environmental pollution and increase its economic value.

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