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# Collagen isolation with acid soluble method from the skin of Red Snapper (*lutjanus* sp.)

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**Abstract.** Collagen is an extracellular matrix connective tissue that account for about 30% of the total protein. Red snapper skin has the potential as an alternative source of collagen that can increase the added value of food and fishery industrial waste. This research aimed to isolate collagen by acid-soluble method and collagen characterization. Isolation of red snapper skin collagen consists of two stages, which are pretreatment with 0.1 M NaOH over 12 hours and hydrolysis with 0.5 M acetic acid over 48 hours. The pretreatment process resulted a non-collagen protein content at 0.2576% mg/mL. Hydrolisis with acetic acid yielded 9.71% (wet) and 41.19% (dry). Chemical analysis by amino acid analysis showed the dominant amino acid glycine, proline, arginine and alanine. Physical characterization of collagen was done by FTIR analysis and showed the presence of amide group A, B, I, II and III which belong to typical group of collagen.

## 1. Introduction

Collagen is a main structural white tissue that covers almost 30% of total body protein. It largely used as a substance of cosmetic production and other industries, such as biomedic, pharmacy, and so on. In cosmetic production, collagen functions to reduce wrinkle on face. In biological function, collagen roles on tissue and organ formation, and involved on fission, defend, and cell diferentation [35]. Commercial collagen commonly comes from bovine and porcine, which is banned by certain religion in Indonesia. In addition, collagen derived from bovine is feared to be contaminated by *Bovine Spongiform Encephalopathy* (BSE) also called as mad cow disease, foot and mouth disease, or swine flu if it derived from porcine [8]. Therefore, it need to find other alternative sources of collagen.

Fish are potential as an alternative collagen source which is safe and easy to find. Collagen from fish is derived from its skin, bone, offal, or scales. Fish skin, which is a side-product from fillet industri or factory, is a waste. Fish skin are contained collagen with various rendemen (10-63%), and depends on fish type, extracting material, or extraction techniques [27]. Utilization of fish skin as collagen source can reduce the amount of waste or increase its added value. One of fish skin that can be used as collagen source is red snapper. Red snapper production at 2010 reaches 123.827 tons from fisheries sector and 2300 tons from aquaculture sectore [3]. Increasing of collagen used in comestic indutry makes its demand becomes higher. Therefore, the production of collagen usage from red snapper fish are potential to be developed.

Collagen production from fish skin using isolation process are done on several studies. Ali *et al.* [7] had isolated collagen from red snapper's scale. Peranginangin *et al.* [30] also had isolated collagen from



grouper's skin. Red snapper's skin is used as collagen source through acid-soluble method in this study. The usage of acid soluble method has some advantages, such as only need a few tools and time, produce high rendement with a few waste, and low production cost. The study aims to isolate collagen from red snapper's skin using acid soluble method and to characterize the isolated collagen.

## 2. Material and Method

This research was conducted in three process, that is preparation and sample characterization of red snapper skin, collagen isolation and collagen characterization including yield value, amino acids, and functional groups with *Fourier Transform Infra Red* (FTIR).

### 2.1. Sample Preparation and Chemical Characterization

Red snapper's skin are obtained from Pasar Anyar, Bogor, West Java. The skin of red snapper is cleaned and separated from the meat that is still left on the skin. Red snapper skin samples were cut  $\pm 2 \times 2$  cm and stored in the freezer for the next process. The red snapper to be used is an analysis of chemical composition including determination of water content [2] and determination of protein concentration [1].

### 2.2. Pretreatment (Widowati [39])

The red snapper fish skin was treated with NaOH solution at  $4^{\circ}$  C. Fish skin soaked in 0.1 M NaOH solution with a ratio of 1:10 (b / v) for 12 hours. NaOH solution was changed for every 2 hours. then analysis the protein content quantitatively with Bradford and Bovine Serum Albumin (BSA) tests as standard as well as to determine the concentration of non-collagen protein and the best immersion time [10]. After 12 hours treatment the skin is washed with distilled water to neutralize the pH.

### 2.3. Collagen extraction (Erizal et al. [13])

The fish skin after pretreatment and neutral was then treated with 0.5 M acetic acid by comparing the ratio of skin with acetic acid 1:10 (b / v) for 48 hours at  $4^{\circ}$  C. Next, filtered using cheesecloth. The filtrate obtained was immersed with NaCl until the concentration was 1 M and stored for 12 hours. Then centrifuged 6000 rpm for 1 hour. The resulting pellets were dissolved with 0.5 M acetic acid, then put into a dialysis bag and dialyzed with distilled water for 12 hours. Aquades are replaced every 4 hours. After 12 hours collagen in the dialysis bag was centrifuged at 6000 rpm for 30 minutes. The results of the pellet are then freeze-dried until solid collagen is obtained and the yield is calculated.

### 2.4. Amino Acid Analysis (Nollet dan Leo [26])

Amino acid analysis was carried out using the *Ultra Performance Liquid Chromatography* (UPLC). This method begins with the hydrolysis of sample and 5 mL of 6 N HCl is added and then vortexed. The sample was then hydrolyzed by heating using an oven at  $110^{\circ}$ C for 22 hours. The sample was cooled in a 50 mL volumetric flask then tasted with distilled water. Filter the sample using milipore filter paper 0.45  $\mu$ m. The filtrate was taken and added AABA (alpha amino acid butyric acid) and aquabides. Pipette solution, add Acc flour borate and reagent flour A. Then the mixture is vortexed and left for one minute. The sample was then incubated for 10 minutes at  $55^{\circ}$ C, then injected into the UPLC.

### 2.5. Analisis Gugus Fungsi dengan FTIR (Yan et al. [41])

KBr is ground until smooth, then put into a pellet container to then be printed to form a thin layer to be used as a blank. KBr and mg with ration 1/10 (b/b) of the test sample are mixed, then crushed until smooth and evenly mixed in the agate mortar. Measurement of blanks and test samples is carried out at wave numbers  $4000-400\text{ cm}^{-1}$ .

## 3. Result and Discussion

### 3.1. Sample Preparation and Chemical Characterization

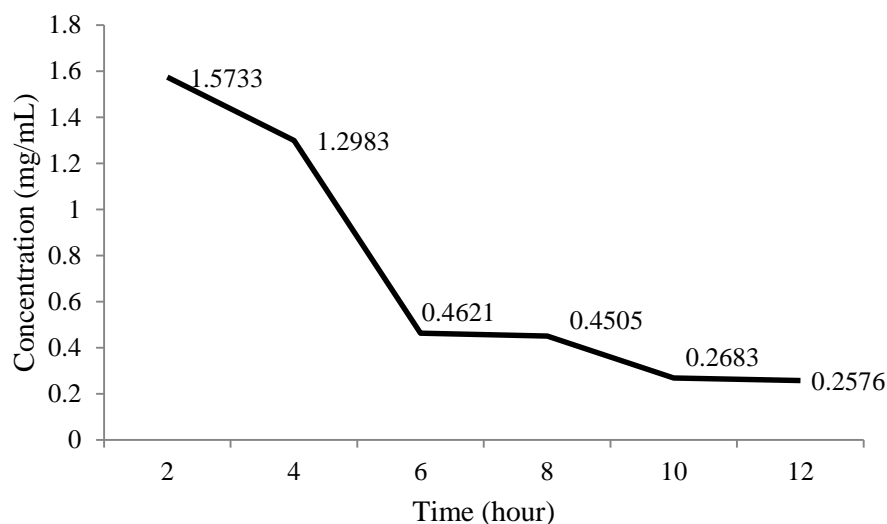
Red snapper's skin are obtained from Pasar Anyar, Bogor, West Java. Sample are characterized using chemical composition analysis. The analysis aims to determine the eligibility of red snapper's skin as

collagen source. Proximate calculation aims to determine pre-treatment process in collagen producing. Fish are known as high protein source which largely used in food industries. Collagen is a protein derivative that usually contains in skin, boned, and scale's skin. The result shows protein content in red snapper's skin has 16.34% (wet) and 68.56% (dry). Nurjanah and Abdullah [29] stated that the protein content of fish ranges from 15-25%. Protein content both in meat and fish skin are relatively same [17]. The protein content of red snapper's skin is lower than white snapper's skin [16], which is  $28.05 \pm 0.41\%$  and higher than patin's skin, which is 4.69% [32]. The results are suspected because species and habitat of the fish are different. The crude protein content of fish skin describes the possible maximum collagen component that can be extracted. High protein levels in skin fish causes the skin to be easily damaged acid or alkaline condition, and microbial activity. So that the skin will easily rot [31].

Water content level in food can lead to ingredient damage, such as microbiological, chemical, and enzymatic processes. The results of the study shows that the water content contained in the red snapper's skin was 76.42%. This water content is higher when compared to bigeye snapper's skin, which is  $68.08 \pm 0.05\%$  and lower than the water content of catfish's skin, which is 79.95% (Kittiphattanabawon *et al.* [21]; Rusli [32]). Water content in skin fish are lower than in its meat. The difference of water content are caused by several factors: habitat differences, environmental condition, and fish species. In a material, water content consists of free water and chemically bound water. The measured water content is the free water in the raw material. The water content also determines the freshness and the durability of a material. Holma *et al.* [18] stated that fish generally consisted of 70-84% water, 15-24% protein, 0.1-22% fat and 1-2% minerals.

#### 4. Pretreatment with NaOH Solution

This pre-treatment process aims to remove contaminants such as fat, minerals, and non-collagen protein on skin fish. The process is done at  $4^\circ\text{C}$  in order to keep the collagen protein of skin fish does not damaged or denatured by hot temperature. Zhou and Regenstein [43] state that there are two alkaline solutions that can be used to remove non-collagen protein, which is NaOH and  $\text{Ca}(\text{OH})_2$ . The usage of alkaline solutions in the pre-treatment process is more effective to remove the non-collagen proteins and has lower level of protein loss than using acid solution [37].



**Figure 1.** Concentration of non-collagen protein in the NaOH solution.

The chemical material changes occur as a result of reaction between fish skin with NaOH in the pre-treatment process. The remaining NaOH solution from soaking process will appear turbid, because process of deproteinase are happened on skin fish. Deproteinase or non collagen protein removal process is caused by NaOH solution which could break the telopeptide of collagen molecule, so that the skin tissue of the fish expands.

Liu *et al.* [24] stated that the usage of 0.1 M NaOH can dissolve non-collagen protein without causing collagen loss in the skin, while the concentration above 0.1 M significantly causes collagen loss in the pre-treatment process. According to the study of Gadi *et al.* [14], the concentration of 0.1 M NaOH solution with 12 hours of soaking is quite stable and effective to dissolve non-collagen protein. High NaOH concentration with long soaking time will cause the amount of the dissolved protein to increase. Therefore it is suspected that not only non-collagen proteins are dissolved, but also collagen proteins.

#### 4.1. Collagen Yield

The collagen yield which produced from red snapper's skin contains 9.71% wet base and 41.19% dry base. The greater the yield produced, the more efficient the treatment is applied [40]. The obtained yield value is greater than in Wibawa *et al.* [38], with the similar usage of raw material which is 5.71%. Moreover, the value is also more smaller than the collagen yield of white snapper's skin without dialysis method in [16], which is 13.87%.

The difference of the yield can be caused by the difference on extraction methods, solution concentration, material type, also the difference both on temperature and extraction times. The reduction of dry yield was accompanied by with increased NaCl concentration. It shows that the higher NaCl concentration, the greater water content and salt that bound together with acetic acid and extracted protein [33].

#### 4.2. Amino acid of Collagen

Amino acid contributes to the stability of the helix collagen structure. Amino acids glycine, proline, and hydroxyproline are the main amino acid that forms collagen, and rarely found in other proteins except collagen and elastin [14]. Aberoumand [4] stated that the content of amino acids in collagen is glycine, with low tyrosine and histidine level and does not contain tryptophan and cysteine. Amino acid composition of collagen are shown in Table 1.

Collagen is formed from three polypeptide chains that are mutually twisted to form triple helix structure with a unique amino acid arrangement, which is Gly-X-Y. X is proline and Y is hydroxyproline. Kittiphaattanabawon *et al.* [22] stated that glycine is an amino acid that forms collagen, and represents about one third of collagen total amino acid. The statement accords to this study result, where glycine has dominant value in red snapper skin collagen, which is equal to 28.28%. The value is greater than both the glycine content in red snapper skin collagen (25.2%) by Jongjareonrak *et al.* [20] and skin collagen of white snapper (21.93%) by [16]. Glycine plays a role in reducing steric resistance and triggering hydrogen bonds in the helix chain [6]. Besides, glycine also functions to form three alpha helix chains into super helical structures.

In the other hand, proline that contained in collagen also plays a role in maintaining the integrity of the collagen structure. Red snapper skin collagen has 12.96% proline, which is relatively large. That amount is lower than the proline content in white snapper's skin, which is 11.93% (Hardiyanti [16]) and smaller than the red snapper skin collagen by Jongjareonrak *et al.* [20], which is 13.1%. High proline content in collagen protein will increase thermal stability. Proline is a unique amino acid in collagen because it plays a role to maintain structural integrity of collagen. Huang *et al.* [19], stated that both the pyrrolidine ring of proline and hydroxyproline limit the conformation of the polypeptide chain and help strengthen the triple helix thermal stability.

Red snapper collagen contains amino acid alanine at 10.42%. This amino acid content of alanine is lower than collagen alanine content in white snapper's skin, which is 11.25% [16]. The ability of collagen to expand is a result of the amount of polar amino acid compositions in collagen, such as arginine, lysine, serine, and threonine, so that it forms a bond with water (Nurhayati *et al.* [28]). Differences in collagen's natural amino acid composition depend on the environment, especially temperature, and habitat of the species (Zhou and Regenstein [43]). Moreover, extraction methods, extracting material concentration, and different amino acid analysis methods will also influence. Hydrophobic amino acid in this analysis result was 62.99% and hydrophilic amino acids was 37%.

**Table 1.** Amino acid composition of collagen from the skin of red snapper.

No	Amino acid	Result (%)
1	Histidine	1.29
2	Serine	4.02
3	Arginine	11.87
4	Glycine	28.28
5	Aspartate	5.00
6	Glutamate	7.17
7	Threonine	3.49
8	Alanine	10.42
9	Proline	12.96
10	Lysine	4.18
11	Tyrosine	1.06
12	Valine	2.49
13	Isoleucine	1.13
14	Leucine	3.10
15	Phenylalanine	3.58

The high concentration of acetic acid that used during the extraction can affect the proportion of amino acids. The research that conducted by Nurhayati *et al.* [28] showed that the amino acids in collagen with acid treatment 1.5 M had a lower proportion than 0.5 M. It happens because the usage of acids with higher concentrations could lead to substitution of negative ions in salts with positive ions in acids faster, so that it can break the structure of the protein.

#### 4.3. Functional Group of Collagen

The collagen infrared spectrum results are shown in Figure 2, while the characteristics of the collagen functional group from FTIR analysis are shown in Table 2. Based on the collagen FTIR spectrum of red snapper's skin shows absorption peaks in the area of amide A, amide B, amide I, amide II, and amide III- which is a typical absorption of collagen. Amide absorption peak of amide A in red snapper's collagen was detected at wavenumber around  $3417.86\text{ cm}^{-1}$ . Amide A shows the stretching vibration of NH which occurs in the range  $3440\text{-}3400\text{ cm}^{-1}$ , when NH groups of peptides involved in hydrogen bonds, the position will shift to a lower frequency of around  $3300\text{ cm}^{-1}$  [36].

Collagen FTIR spectrum showed the presence of an amide B absorption peak detected at wavenumber  $2924.09\text{ cm}^{-1}$ . Amide B group is in the absorption area at wavenumber  $2935\text{-}2915\text{ cm}^{-1}$  [11]. Amide B wavenumber is formed from asymmetrical stretching of CH<sub>2</sub> groups. Higher wavenumbers can be associated with the presence of the free NH<sub>3</sub> from lysine or N-terminus group. Therefore, the smaller wavenumbers in collagen show that the collagen has a lower amount of lysine [9].

The detected wavenumber of amide I in red snapper collagen is  $1647.21\text{ cm}^{-1}$ . Amide I is a typical functional group that composes collagen in range  $1700\text{-}1600\text{ cm}^{-1}$  wavenumber [23]. It was a stretching of C = O groups from peptide bonds and related to the secondary structure of protein. Muyonga *et al.* [25] stated that amide I consists of four components of secondary structure protein, which is  $\alpha$ -helix,  $\beta$ -sheet,  $\beta$ -turn, and random coil that overlap to each others. The peak absorption area of the  $\alpha$ -helix

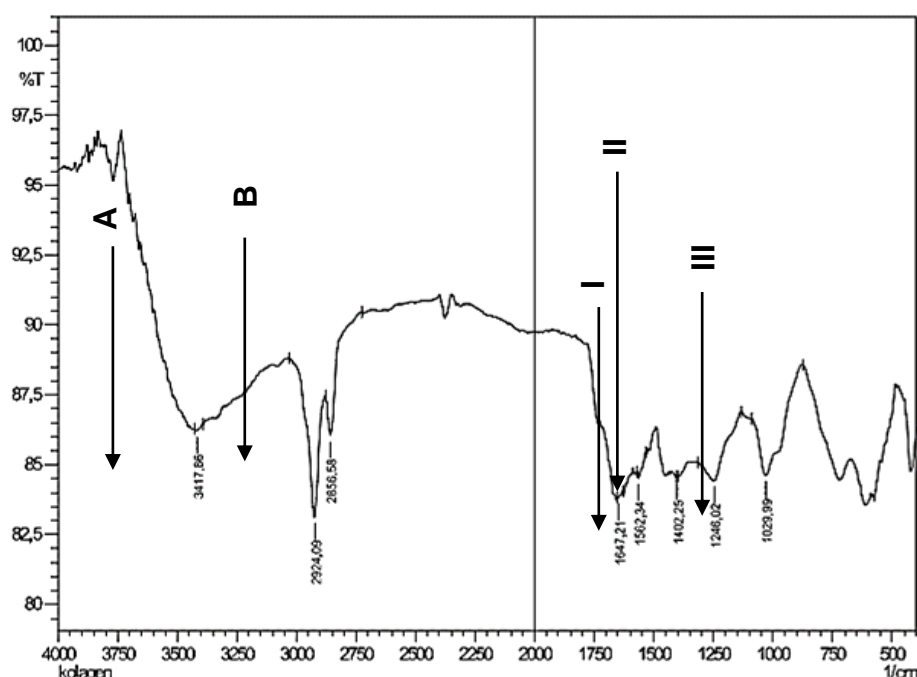
component (1654  $\text{cm}^{-1}$  and 1658  $\text{cm}^{-1}$ ),  $\beta$ -sheets (1624  $\text{cm}^{-1}$  and 1642  $\text{cm}^{-1}$ ),  $\beta$ -turn (1666  $\text{cm}^{-1}$ , 1672  $\text{cm}^{-1}$ , 1680  $\text{cm}^{-1}$ , 1688  $\text{cm}^{-1}$ ), and random coil (1640-1648  $\text{cm}^{-1}$ ).

Amide II, which is a typical functional group of collagen, is detected around 1562.34  $\text{cm}^{-1}$ . Amide II shows the presence both CN stretching and NH bending in the absorption area of 1575-1480  $\text{cm}^{-1}$  (Kong and Yu 2007). The lower absorption indicates that the NH group is involved in bonding with the  $\alpha$  chain [5], a complete triple helix from collagen (Zhang *et al.* [42]) and hydrogen bonds in collagen [12].

**Table 2.** Characteristics of the collagen functional group from FTIR analysis.

Amide	Adsorption of are standard ( $\text{cm}^{-1}$ )	Absorption obtained ( $\text{cm}^{-1}$ )	Characteristic of amide
Amide A	3400-3440 <sup>1</sup>	3417.86	N-H <i>stretching</i>
Amide B	2935-2915 <sup>2</sup>	2924.09	<i>Asimetrikal stretching</i> CH <sub>2</sub>
Amide I	1600-1700 <sup>3</sup>	1647.21	C-O <i>stretching</i>
AmideII	1480-1575 <sup>4</sup>	1562.34	C-N <i>stretching</i> dan N-H <i>bending</i>
Amide III	1200-1400 <sup>4</sup>	1246.02	N-H <i>bending</i> dan C-H <i>stretching</i>

Source: <sup>1</sup>Singh *et al.* [36]; <sup>2</sup>Coates [11]; <sup>3</sup>Kong dan Yu [23]; <sup>4</sup>Kittiphattanabawon *et al.* [22]



**Figure 2.** Spectrum infrared of collagen.

Amide III peak absorption was at 1400-1200  $\text{cm}^{-1}$ , which showed intermolecular interactions of CH stretching and NH bending in collagen [22]. Amide III absorption peak in collagen of red snapper's skin was detected at wavenumber 1246.02  $\text{cm}^{-1}$ . Amide III intensity is related to the existence of triple helix structure which is known from the ratio of amide absorption III with absorption peak at 1450  $\text{cm}^{-1}$  approach to 1. The result showed that the ratio was 0.85, whereas collagen will denaturate by ratio intensity in 0.59. That condition indicates a loss of triple helix structure or known as gelatin. Gomez-Guillen *et al.* [15] stated that the collagen denaturation due to the heating process can cause its triple helix chain to transform completely into a single chain  $\alpha$ -helix (gelatin) with a wavenumber around 1235  $\text{cm}^{-1}$ . It occurs due to the thermal depolymerization process, which is the process of breaking the organized structure of triple helix collagen due to the temperature increased of extraction process [34].

## 5. Discussions

Collagen in red snapper's skin was successfully isolated by acid soluble method with collagen yield 9.71% at wet base and 41.19% of dry base. The characterization shows that it has common collagen functional groups (amide A, amide B, amide I, amide II, and amide III) and the amino acid level are dominated by 28% glycine, 13% proline, 12% arginine and 10% alanine.

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