Characteristics of papain soluble collagen from redbelly yellowtail fusilier (*Caesio cuning*)

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Abstract. Redbelly yellowtail fusilier skin is one of the side products that can be used as raw material for collagen production. Collagen can be extracted by several methods, one of which is through a combination of chemical and enzymatic processes. Useable enzymes was such as papain enzymes. Collagen extracted with this enzyme is known as papain soluble enzyme (PaSC). This study aims to determine the concentration and optimum time of extraction and character of soluble collagen papain (PaSC) from the skin of redbelly yellowtail fusilier. The extraction method of yellow tail skin collagen was divided into three stages: raw material preparation, deproteination using NaOH, and extraction of acid mixture with papain enzyme (PaSC). The results showed that the use of NaOH with a concentration of 0.05 M with an immersion time of 8 hours was able to dissolve the non-collagen protein in an optimal amount. The combination of acetic acid treatment with a concentration of 0.3 M for 3 days and the use of papain enzyme with a concentration of 5,000 U / mg / g of skin was able to produce the highest collagen solubility. The yield of PaSC collagen was 33.28 ± 2.74% (db). The dominant amino acid composition of PaSC collagen was glycine (26.17 ± 0.029%), alanine (13.56 ± 0.025%), and proline (12.34 ± 0.048%). Glycine, proline, and alanine have been the three major amino acids.

Keywords: amino acids, collagen, PaSc, papain, redbelly yellowtail fusilier

1. Introduction

Collagen is one of many proteins found in the skin, bones, and teeth of living things. Collagen is the major structural protein of connective tissue in vertebrate animal bodies with a content of up to 30% of total body protein [1]. Collagen types identified in fish are only type I and V. Type I collagen is found in the skin, bones, and scales of fish [2], while type V collagen is present in connective tissue in the skin, tendons and muscles of fish that also contain collagen type I [3].

Collagen can be produced from various parts of the body of aquatic biota e.g collagen yellow pike conger swimbladder [4], sea cucumber [5], corkskin [6], and redbelly yellowtail fusilier [7]. Fish is one of the biota that can be utilized as raw material of collagen producer. Collagen that comes from the skin and fish bones has a smaller molecular structure compared to collagen made from cattle or pigs so it is easier to be absorbed [8]. Skin and fish bone waste accounts for 30% of the waste with high collagen content [9].

Wang *et al.* [10] stated that fish waste in the form of skin have higher collagen content than other waste that is bone and scales. Fish skin contains collagen with the value of rendemen between 5–30%. This percentage depends on the type of fish, extractor, and collagen extraction technique [1,11]. Each
fish contains collagen and different physicochemical properties depending on the raw material and the way of extraction. Collagen that is enzymatically extracted has an advantage that the yield is greater than the extraction using acid. Jamilah et al. [12] reported that the use of papain enzymes can extract collagen from the skin of snapper fish with a yield of 44% (dry basis or db) while using pepsin enzyme yields a yield of 43.6% (db). This study aims to determine the concentration and optimum time of extraction and character of soluble collagen papain (PaSC) from yellow tail fish skin.

2. Methodology

2.1. Redbelly yellowtail fusilier skin deproteination [7]
Deproteinasi process aims to eliminate non-collagen protein. This process uses NaOH. Yellow-tailed fish skin was immersed in 0.05 NaOH solution with a ratio of 1:10 (w/v) with immersion time up to 8 hours. The alkaline solution was changed every 2 hours at 10°C.

2.2. Soluble Collagen Extraction (PaSC)
The redbelly yellowtail fusilier skin which deproteination then was extracted using 0.3 M acetic acid (1:30 w/v) treatment for 3 days [7]. The samples then were added papain enzyme with concentration 0; 5,000; 10,000; 15,000; 20,000; and 25,000 U / mg / g of skin, then was continued with addition of papain enzyme with concentration of 0; 3,000; 5,000; 7,000; 9,000 U / mg / g skin [12]. The supernatant was then precipitated using NaCl 2.6 M. The result of precipitation with NaCl 2.6 M was separated by 20,000 g of speed centrifugation for 1 hour. The pellets were dissolved in 0.3 M 1:2 (w/v) acetic acid, then dialysis using a 12 KDa dialysis pouch on the aquadest. The pellet was then dried with a freeze dryer to obtain collagen in powder form and calculated yield [13].

2.3. Characterization of Soluble Papain Collagen (PaSC)
Characterization of PaSC includes yield, amino acid composition, and molecular weight estimation.

2.4. Analysis

2.4.1. Determination of collagen yield [7]. The yield of collagen from the skin of the redbelly yellowtail fusilier was calculated on the basis of dryness by comparing the weight of collagen after freeze dry with the dry base of the fish skin initial weight before being processed multiplied by 100%.

2.4.2. Amino acid composition [14]. The amino acid composition was determined by High Performance Liquid Chromatography (HPLC). Analysis of amino acids using HPLC consists of 4 stages, namely making of protein hydrolyzate; drying; derivatisation; and injection and analysis of amino acids. (a). Stage of making protein hydrolyzate. The sample weighed as much as 0.2 g was destroyed. The sample solution plus HCl 6 N is 5–10 mL, heated in an oven at 100°C for 24 hours. The heating process was carried out to remove the gas or air present in the sample so as not to disrupt the resulting chromatogram and to speed up the hydrolysis reaction. (b). Drying stage. The protein hydrolysate was supplemented with a 30 μL drying solution. The drying solution was made from a mixture of methanol, sodium acetate, and triethylamine in a ratio of 2: 2: 1. The drying process was assisted using nitrogen gas to accelerate drying and prevent oxidation. (c). Derivatization step A total of 30 μL derivatization solutions were added to the drying product. The derivatization solution was made from a mixture of methanol, picothiocyanate, and triethylamine solution in a ratio of 3: 3: 4. The derivatization process was performed so that the detector was easy to detect the compound present in the sample, the derivate was diluted by adding 10 mL of acetonitrile 60% or phosphate buffer 0.1 M and then left for 20 minutes. Dilution results were filtered back using milipor measuring 0.45 microns. (d). Injection to HPLC The resultant filter was taken as much as 20 μL to be injected into HPLC. Calculation of amino acid concentration was done by comparing sample chromatogram with standard. Preparation of standard chromatogram was done using amino acids that have the same treatment with the sample.
2.4.3. *Molecular weight estimation* [11]. Dry samples of 2 mg were dissolved in 1 ml of 5% sodium dodecyl sulfate (SDS) and the mixture was incubated at 85°C for 1 hour in a temperature controlled bath. The mixture was centrifuged at 4,000 g for 5 min at room temperature. The supernatant obtained was mixed with buffer (Tris HCl 60 mM, pH 6.8 containing 2% SDS and 25% glycerol) at a ratio of 1:1 (v/v) and containing 10% β-mercaptoethanol (β-ME). The mixture was heated in boiling water for 2 minutes. A 5μL sample was inserted into a polyacrylamide gel comprising 7.5% running gel and 3% stacking gel and electrophoresis at a constant current of 15 mA/gel for 3 hours. After the electrophoresis was complete, the gel was stained with 0.05% (w/v) coomassie blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid for 3 hours, then the destaining sample with 30% (v/v) methanol and 10% (v/v) acetic acid for 2 hours. The sample protein molecular weight was estimated based on the molecular weight of the marker. The markers used were Pre-stained Protein Markers (Broad Range) for SDS-PAGE from Nacalai Tesque with molecular weight of 8.8 KDa to 192 KDa.

3. Result and discussion

3.1. *Papain soluble collagen extract of skin of redbelly yellowtail fusilier*

Enzymes are proteins that have the activity of a catalyst to decrease the activation energy of a reaction so that the conversion of the substrate into the product can take place more rapidly. The papain enzyme (EC 3.4.22.2) consists of 212 amino acid residues arranged in a single polypeptide chain. The papain enzyme is a class of endopeptidases that breaks the peptide bond at the center of the protein chain [15]. The activity of the papain catalyst proceeds on the active sides of the papain consisting of histidine and cysteine groups [16].

This study used a commercial enzyme with an activity unit of 30,000 U/mg. This activity shows that 1 mg of papain enzyme protein capable of converting 30,000 μmol protein substrate. The extraction process using this papain enzyme is a mixture between the use of acids and enzymes. Song et al. [17] in his study stated that the maximum pH in the extraction of collagen using papain enzyme is 3. At a higher pH close to neutral, the yield of the collagen is low. The results of the papain enzyme concentration from 0–25,000 U/mg/g skin showed that the addition of papain enzyme concentration above 5,000 U/mg/g skin had no significant effect (p> 0.05) on the dissolved collagen product (figure 1). Therefore, the treated papain concentration was reduced to 0 - 9,000 U/mg/g (figure 2). The results showed that enzyme activity of 5000 U/mg/g of skin yielded the highest and most significant dissolved collagen (p <0.05) on the activity of enzyme with concentration 0; 1,000; 3,000 U/mg/g of skin while the addition of enzyme at 7,000 and 9,000 U/mg/g skin concentration was not significant (p> 0.05) to the result of dissolved collagen. The more enzyme papain enzyme molecules will increase the chances of hydrolysis substrate reactions by papain enzymes to the point where increased enzyme concentration has no significant effect. Song et al. [17] in his study stated that collagen extraction results will increase with increasing papain enzyme concentration to the maximum point and will decrease when the concentration of enzyme continues to be added.

Papain enzyme is a very powerful enzyme. The addition of papain enzyme concentration of 20,000 U/g of skin will damage the collagen peptide bonds so that not only the telopeptide part will be disconnected but also the tropocollagen part. Solving this part of the tropocollagen will cause the protein to have a low molecular weight and loss of β structure [12]. The proper use of enzyme concentration will only break the cross-linked part of the collagen telopeptide without causing damage to its molecular structure thus increasing the amount of collagen dissolved [18]. PaSC produced using papain with 5000 U/mg/grams of skin activity has the highest dissolved collagen. Collagen was then characterized by yield, amino acid composition, and protein molecular weight.
3.2. Characteristics of soluble collagen extract papain skin redbelly yellowtail fusilier
The characteristics observed in papain soluble collagen are amino acid composition and molecular weight estimation.

3.2.1. Yield of soluble collagen extract papain skin redbelly yellowtail fusilier. The higher the yield, the higher the economic value. Collagen extracted using acid had yield 18.4% (dry basis) [7]. The results showed that collagen extracted using papain enzyme can yield higher collagen yield (33.28% db) than collagen which only extracted using acid alone. The low ASC yield is due to the presence of cross-linked collagen telopeptide between aldehyde with lysine and hydroxylinine which causes difficult soluble collagen [18]. Papain enzyme is one of the non-specific proteolytic enzymes that can break down proteins. The enzyme is capable of breaking the crosslinking of the collagen telopeptide part and dissolving it so as to increase the collagen extraction results [18, 17]. Jamilah et al. [12] states that the collagen extracted from the skin of the snapper using papain enzyme has a much higher rendemen (43.9% dry base) than the collagen extracted with only the acid (8.3% db).

The yield of PaSC collagen of skin of redbelly yellowtail fusilier was 33.28 ± 2.74% (dry basis). Jamilah et al. [12] reported that the snapper skin extracted using papain enzyme had a yield of 43.9% (db). The yield of PaSC collagen skin of redbelly yellowtail fusilier was higher than PSC collagen from balloon fish (19.5%) (dry basis). Differences in yield results were due to skin character, extraction process, and different papain enzyme concentrations of 5,000 U/mg/g of skin on PaSC collagen skin of yellow tail fish and 20,000 U/g of skin on PSC collagen fish snapper [12].

The yield of PaSC collagen skin of yellow tail fish when calculated on the basis of wet is 10.48 ± 0.86% (wet basis or wb). The yield of PaSC collagen was larger than the collagen content extracted using pepsin soluble collagen (PSC) skin of chicken fish (8.48%), shark (7.68%), snapper (4.7%), snipers (3.68%), and pangasius (7.7%) [19,20,21,22,11]. This suggests that the enzyme papain is able to extract collagen well and can replace the use of pepsin enzymes in producing collagen.

3.2.2. Composition of amino acids of soluble collagen extract papain skin redbelly yellowtail fusilier. Collagen consists of three large and repeating polypeptide chains. The amino acid composition of collagen tends to be dominated by glycine, proline, hydroxyproline and alanine (60%) [23]. Collagen extracted with the addition of papain enzyme (PaSC) has a higher total amino acid content (3.80 ± 0.033%) than that extracted only with acid (ASC) of 2.80 ± 0.062% (w / v) [7]. The protein content of PaSC collagen and ASC skin redbelly yellowtail fusilier was higher than that of Atelo Helogen commercial collagen which had 1.05% (w / v) total protein and CLR Collagen 0.28% (w/v) but lower than Collasol commercial Collagen 4% (b / v) [24].
PaSC collagen from the skin redbelly yellowtail fusilier has a higher amino acid content than ASC collagen. The PaSC collagen contains 9.97 mg / g of glycine amino acid; proline 4.7 mg / g; and alanine 5.17 mg / g. Astiana et al. [7] states that ASC collagen has a glycine amino acid content of 7.11 mg / g; proline 3.45 mg / g; and alanine 3.89 mg / g (w / v). The amino acid chain associated with peptide bonds will form proteins with a variety of complex and distinctive structures. Papain is able to break the crosslinks on collagen telopeptides so that PaSC collagen has a higher amino acid content than ASC collagen.

The amino acid composition of skin redbelly yellowtail fusilier is either extracted using acid (ASC) as well as papain enzyme (PaSC) dominated by glycine, proline, and alanine. Collagen of ASC and PaSC from yellow tail skin has 25.43% glycine and 26.52% of total amino acid collagen. This shows that glycine is the most dominant amino acid in collagen and occupies about 1/3 of the total amino acid. This is in accordance with research Jongjaroenrak et al. [21], which states that collapsible skin collagen contains 1/3 of the total amino acid content of 25.5% in ASC and 23.5% in PSC (Pepsin Soluble Collagen).

Kittiphatanabawon et al. [22] also stated that glycine is the main amino acid to form collagen which accounts for 30% of the total amino acid. This is because glycine is present in every three amino acid residues in collagen and occupies a central position on the \( \alpha \) chain. Glycine also plays a role in the formation of \( \alpha \) triple helix chains in collagen [23]. Glycine is the simplest amino acid, since it does not have an optical isomer and has only a H group on the branch chain R [25].

The major amino acids of other collagen formers are proline and alanine. The percentage of proline and alanine content in ASC was 12.32% and 13.9% [7] while in PaSC was 12.5% and 13.7%. The content of proline and alanine collagen ASC and PaSC from yellow tail fish skin is not much different from collagen from the skin of snapper. Percentage of collagen proline and alanine content from skin of snapper were 13.1% and 14.3% in ASC and 13.5% and 14.2% in PSC [21]. Bae et al. [23] states that the content of collagen proline is 10–12% and alanine content of 10–13%.

Proline and hydroxyproline are the amino acids that function in improving collagen stability [26]. Tamilmozi et al. [27] states that proline is a unique amino acid in collagen because it plays a role in maintaining the structural integrity of collagen. Proline plays a role in the formation of triple helix chains in collagen. The pyrrolidine ring of proline keeps the stability of the protein polypeptide chain so it is not easily transformed into a secondary structure and helps in strengthening the triple helix chain (Bae et al. 2008). The average amino acid proline has 50% hydroxyl group residue and because of the prolyl-hydroxylation process with the help of prolyl 3-hydroxylase and prolyl 4-hydroxylase it will form hydroxyproline. Hydroxyproline is instrumental in intramolecular hydrogen bonding and helps in maintaining thermal stability in triple helix structures [28]. The amino acid of alanine belongs to the type of aliphatic amino acid with \( \text{CH}_3 \) as its R group. These amino acids play a great role in the synthesis of glucose [29].

3.2.3. Molecular Weight of papain soluble collagen (PaSc). The collagen molecular weight from the skin of the yellow tail fish was analyzed using SDS-Page. SDS-PAGE is a protein separation technique based on its molecular weight. Smaller proteins will move faster across the gel than large proteins so that low molecular weight proteins have longer (Rf) mileage than high molecular weight proteins [30]. Results of SDS-Page collagen skin of yellow tail fish extracted using acid and papain enzymes can be seen in figure 2.
Fish-derived collagen has an identical structure of α1 and α2 belonging to the type of collagen type I structure. The triple helix structure of type I collagen is formed over the heterotrimer of two α1 chains and one α2 chain [28]. The β (α chain dimers) and γ (α chain trimers) structures indicate the presence of covalent crosslinking of collagen molecules [31]. Collagen from skin redbelly yellowtail fusilier both extracted using acid and papain enzymes have a type I collagen structure containing identical structures α1, α2, β and γ.

The molecular weights of α1, α2, β, and γ in skin redbelly yellowtail fusilier PaSC are similar to those of catfish leather [11] and some marine fish [23]. This indicates that the resulting PaSC collagen has not changed into its derivative product of gelatin. Gelatin has a lower molecular weight range than collagen [32]. Hermanto et al. [33] also stated that pork gelatin has a protein with a molecular weight of 28.6 KDa and 36.2 KDa. Karim and Bhat [32] stated that gelatin contains a mixture of components with molecular weight ranging from 80 250 kDa. The structures of α1, α2 and β in ASC collagen from skin redbelly yellowtail fusilier have slightly higher molecular weight [7] than PaSC collagen extracted from skin redbelly yellowtail fusilier using papain 5000 unit/mg/g skin. This is possible because the enzyme papain is able to break some peptide bonds in collagen into smaller structures. Extraction of collagen with an enzyme concentration that is too high will damage the telopeptida and tropocollagen so it has a lower molecular weight than collagen in general. Jamilah et al. [12] states that collagen extraction with papain enzyme concentration of 20,000 units /g fish skin has a protein-molecular weight of 37–75 KDa. This suggests that the extraction of PaSC collagen from the skin redbelly yellowtail fusilier with a papain concentration of 5,000 U/mg/g of skin is capable of breaking the collagen molecule smaller than that of ASC collagen but can still retain the α1, α2, β, and γ collagen structures.

PaSC collagen from the skin redbelly yellowtail fusilier has a component with high molecular weight of the structure β and γ. The β and γ components show the presence of collagen molecules that are cross-linked from the α chains that form dimers and trimers. Singh et al. [11] explain that the thickness of the protein band intensity of the β structure shows the high number of collagen with cross linking. The structures of α1, α2, and β in PaSC have thicker bands, while the γ PaSC structure has thinner bands. PaSC. Jamilah et al. [12] states that collagen extracted with papain enzymes has a lower molecular weight because proteases hydrolyze peptide bands into bands that have lower molecular weights. This causes the structure of γ in PaSC to be thinner than ASC and hydrolyzes it into lower
molecular weight bands of the structures α1, α2, and β. Singh et al. [11] and Song et al. [17] states that with the addition of pepsin enzyme (PSC), the collagen 11-collagen-containing structure component becomes the α1 and α2 structures so that the intensity of the α1 and α2 bands is increased in the PSC. Singh et al. [11] suggest that cross-linking of intra- and inter-collagen molecules in ASC is higher than PSC.

4. Conclusion
The best concentration of papain enzymes used for extract PaSC collagen is 5,000 U/mg of skin. Collagen extracted using papain has high yield and amino acid composition. PaSc collagen from the skin of yellow tail fish has collagen structure α1, α2, β and γ.

References
[1] Friess W 1998 European Journal of Pharmaceutics and Biopharmaceutics. 45 113–36
[2] Nagai T, Suzuki N 2000 Food Chem. 68 277–81
[3] Sato K, Yoshinaka V, Itoh Y, Sato M 1989 Food Chem. 92 87–91
[4] Djailani F, Trilaksani W, Nurhayati T JPHPI. 19(2) 156–67
[5] Alhana, Suptijah, Tarman K 2016 JPHPI 18(2) 150–61
[6] Wulandari, Suptijah P, Tarman K 2015 JPHPI. 18(3) 288–302
[7] Astiana I, Nurjanah, Nurhayati T 2016 JPHPI. 19(1) 79-93
[8] Kittiphattanabawon P, Benjakul S, Visessanguan W, Kishimura H, Shahidi F 2010 Food Chem. 119 1519–26
[9] Guillen M C, Gomez J T, Fernandez M D, Ulmo N, Lizarbe MA, Montero P 2002 Food Hydrocolloids 16 25–34
[10] Wang L, An X, Yang F, Xin Z, Zhao L, Hu Q 2008 Food Chem. 108(2) 616–23
[11] Potoros T, Raksakulthai N, Runglerdkreangkrai J, Worawattanamateekul W 2009 Kasetsart Journal. 43(3) 584–93
[12] Singh P, Benjakul S, Maqsood S, Kishimura H 2011 Food Chem. 124 97–105
[13] Shyni K, Hema G S, Ninan G, Mathew S, Joshy C G, Lakshmanan PT 2014 Food Hydrocolloids. 39 69–76
[14] [AOAC] Association of Official Analytical Chemist. 1995. Official Methods of Analysis. Washington: The Association of Official Analytical Chemist
[15] Grzonka Z, Kasprzykowski F, Wiczek. 2007. Cysteine Proteases. Di dalam: Polaina J, MacCabe AP, editor. Industrial Enzymes: Structure, Function and Application. Netherlands: Springer
[16] Wong D W S. 1989. Mechanism and Theory in Food Chemistry. New York: Van Nostrand Reinhold
[17] Song W, Chen W, Yang Y, Li C, Qian G. 2014. Extraction optimization and characterization of collagen from the lung of soft-shelled turtle Pelodiscus
[18] Di Y, Feng C C, Bin W, Fang D G, Rui L Z 2014 Chinese J Nat.Med. 12(9) 712–20.
[19] Ahmad M, Benjakul S 2010 Food Chem. 120 817–24
[20] Hema GS, Shyni K, Mihew S, Ananda R, Ninan G, Lakshmanan PT 2013 Annals of Biological Research. 4(1) 271–78
[21] Jongjareonrak A, Benjakul S, Visessanguan W, Nagai T, Tanaka M. 2005 Food Chem. 93 475–84
[22] Kittiphattanabawon P, Soottawat Benjakul S, Visessanguan W, Nagai T, Tanaka M 2005 Food Chem. 89 363–72
[23] Bae I, Osatomi K, Yoshida A, Osako K, Yamaguchi A, Hara K 2008 Food Chem. 108 49–54
[24] Peng Y, Glattauer V, Werkmeister J, Ramshaw JAM 2004 J Cosmetic Sci. 55 327–41
[25] Shanmugam V, Ramasamy P, Subhapradha N, Sudharsan S, Seedevi P, Moovendhan M, Krishnamoorthy J, Shanmugam A, Srinivasan A 2012 African J Biotech. 11 (78) 14326–37
[26] Tamilmozh S, Veeruraj A, Arumugam M 2013 Food Res. Intl. 54 1499–1505
[28] Gelse K, Poschl E, Aigner T 2003 *Advanced Drug Delivery Reviews*. **55** 1531–46
[29] Mahan L K, Stump S E. 2008. *Krause’s Food and Nutrition Therapy. International Edition 12*. Missouri: Elsevier

[30] Bollag D M, Edelstein SJ. 1991. *Protein Methods*. New York: Wiley-Liss
[31] Chi C F, Cao Z H, Wang B, Hu F Y, Li Z R, Zhang B 2014 *Molecules*. **19** 11211–30
[32] Karim A A, Bhat R 2009 *Food Hydrocolloids*. **23** 563–76
[33] Hermanto S, Sumarlin L O, Fatimah W 2013 *J Food and Pharm. Sci*. **1** 68–73