Isolation and Allergenicity of Protein Collagen from Parang-Parang Fish Skin (Cirocentrus dorab)

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Abstract. Commercial collagen, either comes from cow or pigs, usually are susceptible to biological contamination exposure. Other sources of collagen that are safe and halal are obtained from the fish skin waste. Collagen from fish skin can potentially cause allergies because fish proteins are generally allergen. The aims of this study is to isolation, characterization and testing the allergenic properties of protein collagen from Parang-Parang fish skin. Collagen analysis are done using UPLC and FTIR. Allergens properties assay done by using SDS PAGE electrophoresis and Immunoblotting. Collagen was isolate using 0.1 M NaOH in 12 hours and hydrolyse using 0.5 M acetic acid before experiment. The result shows non-collagen protein content is 0.2163 mg/mL, with 1.915% yield. FTIR analyst shows that chemical characteristic of the collagen has: some amide groups, which is amide A (3419.79), B (2924.09), I (1656.85), II (1558.48), dan III (1246.02) (cm\textsuperscript{-1}); content of amino acid collagen constituent proline (13.19), alanine (10.733), arginine (10.148), glutamic acid (8.216)(%); and molecular collagen weight α1 (129), α2 (119), dan β (244) (kDa). Immunoblotting test results showed that there were no collagen protein binding sites with antibodies on nitrocellulose membranes which showed that collagen was not allergen.

1. INTRODUCTION
Collagen, the main extracellular matrixes protein that achieves 30% of whole body compound proteins of mammals and animals. Collagen protein has triple helixes structure, consist of three polypeptide chains with repeated glycine-X-Y sequences, which much of X and Y are proline and hydroxyproline [1]. The special collagen composition characterized by high glycine and proline, also contain no cysteine [2]. Collagen basic molecule formers are three units of spiralized α polypeptide chains, formed triple helixes structure known as tropocollagen [3].

For this time, collagen has become precious compound and used for many industries as pharmacy, biomedical, and beauty sectors. It concerned of collagen multification as improve strength and elasticity skin, differentiation of spinal cord cell, chemotaxis, proliferation, antioxidant, angiotensin inhibitor, and thrombocyte aggregation [4]. The functions supported by collagen characteristics such absorbable, nontoxic, biodegradable, and stable relatively, in proportion to rapidly developed of collagen utilization in industries [5].

The raw materials of collagen in industry used commercial collagen comes from cows or pigs skin, but it’s unsuitable utilizing concerned of religion and biological contamination like BSE (Bovine Spongiform Encephalopathy), TSE (Transmissible Spongiform Encephalopathy), and FMD (Foot and
Mouth Disease). Epidemic disease such bovine spongiform encephalopathy caused by collagen from cows and pigs, effected disquietude in collagen consumer society [6]. In this way, it’s required a secure and allowed alternative collagen source. According to Widowati [7], Parang-Parang fish as one of marine life has potential be a collagen source. Parang-Parang fish found easily in Indonesia and used a lot as raw material of processed food, so it will produce a lot of skin wastes. Utilizing of Parang-Parang fish skin as collagen raw material becomes a prospective alternate to solve the waste problem. Fish skin also an acceptable collagen source for all consumer societies.

Fish-produced collagen potentially caused a few side-effects like allergies because of protein-contained in fish. Collagen identified as the 2nd new allergen after parvalbumin in processed seafood [8]. Allergen, an allergic-trigger substance, represents an immunity respond to other substances in difference sensitivity of each individual. A susceptible of seafood products allergy also be a focus to examine allergen protein collagen of isolated Parang-Parang fish skin.

Widowati [7] researched isolation and characterization collagen of Parang-Parang fish skin used ASC method (Acid Soluble Collagen), as well Rachmawati [9] formulated moisturizing gel-based Parang-Parang fish skin collagen (Chirocentrus dorab). However, the collagen allergenic characterize study has not been done. Therefore, it needs the Parang-Parang fish skin collagen allergenic characterize study.

The study intends to isolate, characterize, and examine the Parang-Parang fish skin collagen protein allergenic characteristic (Chirocentrus dorab). Identification of isolated collagen protein used UPLC and FTIR. The allergenic test used SDS-PAGE electrophoresis and Immunoblotting. Principle of the allergic test in this study based on antigen bond site formulation of serum antibody sample will result color in nitrocellulose after adding the substrate. The study expected will give information of collagen allergic characteristic result of Parang-Parang fish skin using acid soluble collagen method. The benefit of this study expected will raise the commercial value of Parang-Parang fish skin waste and providing the resulted collagen allergic characteristic information, so it would support the further development related to collagen application.

2. Material and methods

2.1. Materials

The sample using Parang-Parang fish skin obtained from Pasar Anyar, Bogor, West Java. The materials are NaOH (Merck), aquades, asetic acid (CH₃COOH) (merck kGaA 6427 Darmstadt for analysis), potassium bromide (KBr), standard solution of Bovine Serum Albumin (BSA) (Merck), reagent Bradford (Merck), HCl (Merck), tris base (nzytech), Sodium Dodecil Sulfate (SDS) (Nivatis Biochemical), glysine (Phyto Technology Laboratories), TEMED (N, N, N’, N’-tetramethyl-ethylenediamine) (Himedia REF MB026-100 mL), tris base (nzytech), glycerol (MP Biomeddicals LLC), merkaptopetanol, bromfenol 5%, Coomassie blue R250, tricloroasetic acid, Dual Color New Broghter Marker 10-250 kDa, dan metanol 95% (merck kGaA 6427 Darmstadt for analysis), phosphat acid 85% (merck kGaA 6427 Darmstadt for analysis), ammonium persulfate (APS) (MP Biomedical LLC), acrilamide (BIO-RAD Laboratories), 2-merkaptopetanol (Himedia MB041-100 mL), bromfenol blue (SIGMA-ALDRICH), tween 20 (SIGMA-ALDRICH).

2.2. Method

2.2.1. Raw material characterization. Moisture content referred to BSN [10] and Crude protein content referred to BSN[11].

2.2.2. Parang-parang fish skin collagen isolation. Pre-treatment [12]. Collagen was extracted from Parang-Parang fish skin. Firstly, the fish skin soaked in 0.1 M NaOH solution at 4°C. NaOH solution used to remove the non collagen protein on the sample. The fish skin soaked in 0.1 M NaOH solution with a ratio of 1:10 (w/v) for 12 hours. NaOH solution was changed every 2 hours then examined the
protein content with Bradford method quantitatively. The soaked-NaOH sample neutralized with distilled water until pH 7 was obtained. Isolation with Acid [13]. After pre-treatment and neutralizing, the fish skin soaked in 0.5 M acetic acid with a ratio of 1:10 (w/v) for 48 hours at 4°C. Next, filtering it used calico. The fluid filtrate was earned and added NaCl until the collagen concentrate 1 M. Left the filtrate out for 12 hours furthermore centrifuged it at 1000 G for 30 minutes. Separated the pellet from supernatant and dissolved using acetic acid 0.5 M. Put the fluid pellet in dialysis bag and dialyzed with distilled water for 12 hours at 4°C. Distilled water was changed every 4 hour. Collagen in dialysis membrane was centrifuged at 1000 G every 1 hour then freeze-dried.

**Analyzing of Non Collagen Proteins with Bradford Method** according to Bradford [14].

**Analyzing of the collagen yield** [15]. The collagen yield calculated from proportion of dry weight with fish skin weight. The yield calculated with the formula:

$$\% \text{ Collagen Yield} = \frac{\text{collagen dry weight}}{\text{raw material weight}} \times 100\%$$  \hspace{1cm} (1)

2.2.3. **Functional group identification** [16]. Analyzing the functional group of collagen used FTIR. 100 mg KBr pounded till fine then put in pellet cup to be pressed became a thin layer. KBr disc then tested using spectrophotometer FTIR at wavelength from 4000 to 400 cm$^{-1}$. The result became a blank. 100 mg KBr and 10 mg test sample were mixed then pounded till fine and mixed equally on agate mortar. The test sample measured with the same method of blank measurement. The resulted spectra FTIR showed the wavelength absorption peaks of test sample. The functional group of test sample determined based on the detected wavelength absorption peaks with absorption area for protein functional group.

2.2.4. **Amino acid profile** [17]. Amino acid analyzed by using Ultra Performance Liquid Chromatography (UPLC). 0.1 g sample were added 5 ml of 6 N HCl then put on vortex. Sample was hydrolyzed for 22 hours at 110°C. Cooling the sample down and put into laboratory flask then diluted till 50 mL with distilled water. Filtering sample using Millipore paper 0.45 µm. 500 µL of the filtrate were added 40 µL AABA (alpha amino butyric acid) and 460 µL aquabidest. Taking 10 µL of solution using pipette and added 70 µL ACCQ boric flour and 20 µL flour A reagent. Put on the vortex and left out for 1 minute. Sample was incubated for 10 minutes at 55°C then loaded onto UPLC. Chromatography condition used ACCQ-Tag Ultra C18 column, at 49°C, gradient elution system, 0.7 mL per minutes of flow rate, wavelength 260 nm of PDA detector, and 1 µL of injection volume. The concentration of amino acid calculated with formula:

$$\text{Amino acid concentration} = \frac{\text{sample area} \times C \times \text{FP} \times \text{volume (ml)}}{\text{standard area} \times \text{sample weight (g)}} \times 100\%$$  \hspace{1cm} (2)

Note:
- C : amino acid standard concentration (ug/mL)
- FP : dilution factor

2.2.5. **Hypothesis of molecular weight** [18]. **Reagent Preparation.** Acrylamide : bis acrylamide (30% : 0.8 %) made by mixed 87.6 gram acrylamide and 2.4 gram bis-metilen acrylamide with 300 mL distilled water. 1.5 M Tris-HCl pH 8.8 made by dissolving 9.08 gram basa tris with ± 20 mL distilled water, then adjusted to pH 8.8 precisely with 1 N HCl, furthermore diluted with distilled water till 50 mL of solution volume. 0.5 M Tris-HCl pH 6.8 made by dissolving 3.02 gram basa tris with ± 60 mL distilled water till 20 mL of the solution volume, then adjusted to pH 6.8 precisely with 1 N HCl,
furthermore diluted with distilled water till 50 mL of the solution volume. 10% SDS made by dissolving 10 gram SDS in 100 mL distilled water. Sample buffer made by dissolving the following substances: 4 mL distilled water, 1 mL of 0.5 M Tris-HCl pH 6.8, 0.8 mL glycerol, 1.6 mL of 10%SDS, 0.4 mL of mercaptoethanol, 0.2 mL of 5% bromphenol blue. Resolving buffer made by mixing 30.3 gram basa tris, 144 gram glycine, 10 gram SDS, dan 1 L distilled water. The staining solution made by dissolving 1.25 gram Coommasie blue R-250 and 100 gram trichloroacetic acid in 500 mL distilled water. The distaining solution made by mixing 70 mL concentrated acetic acid, 250 mL of 95% methanol and distillated water till 1 L of solution volume. Separating Gel. Mini slab used as gel mold, arranged according to equipment instructions. 3.4 mL bis acrylamide 30% : 0.8%, 3 mL of 1.5 M Tris-HCl pH 8.8, 120 µL of 10% SDS and 5.42 mL aquabidest mixed in beaker then stirred slowly by shaking the beaker. Next, 60 µL APS 10% and 5 µL TEMED added into the solution and re-stirred slowly. Poured solution in mini slab without incurring air bubbles till 1 cm on the plate. Filled the unfilled-gel part with distilled water then let the gel polymerized for 140 minutes. Stacking Gel. Water exhausted from above the separating gel and dried with tissue paper. 1.33 mL bis acrylamide 30% : 0.8%, 2.5 mL of 1.5 M Tris-HCl pH 6.8, 100 µL of 10% SDS and 6 mL aquabidest mixed in beaker then stirred slowly by shaking the beaker. Furthermore, 50 µL APS 10% and 5 µL TEMED added into solution and re-stirred slowly. Poured solution in mini slab without incurring air bubbles. Then put the rake quickly to make well. Let stacking gel undergo polymerization for 30 minutes. After the polymerization gel, the rake lifted from over the gel slowly and slab placed in electrophoresis vessel. Electrophoresis buffer put in the electrophoresis vessel inside and outside in order to soak the gel. Preparation and sample injection. Before took the sample into gel, 20 mg of sample diluted in 1 mL of SDS 10% then incubated at 85°C for 1 hour. Sample was centrifuged at 4000 rpm for 5 minutes, next 40 µL of sample mixed with 10 µL of sample buffer which contained mercaptoethanol and SDS. Boiling sample in boiled water 100°C for 5 minutes to reduce the negative-contained protein in sample then cooled it down at room temperature. 10 µL of sample injected to well and standard protein (marker) injected to the first well. 10 µL of marker boiled for 15 minutes then injected to gel well. Running the SDS-PAGE. Electrode valve was installed with current flows to anode. Electric source switched on and kept constant at 400 mA and 90 V. The process was run for 125 minutes. The electric current was shut down after the standard protein dye (bromphenol blue) reached end, approximately 1 cm from gel end. After finishing, the electric current was shut down and the electrode valve was released, then the gel plat moved from electrode. Staining gel and distaining color. Staining was performed by soaking gel in dye solution for 15 minutes and stirred with magnetic stirrer at 50-100 rpm. After that, the gel washed with distilled water 5 times for 5 minutes each. Then the gel soaked with distaining solution. The distaining solution replaced till achieved the clear gel. Estimation of protein molecular weight. The molecular weight was estimated by measuring the migration length of protein bands. Determination of Rf and BM value analyzed with GelAnalyzer 2010a software (http://www.gelanalyzer.com/).

Detection of allergen protein [18]. Unstained gel electrophoresis transferred to nitrocellulose membrane (0.45 µm). The gel and nitrocellulose membrane arranged in transblotting devices, then filled with transfer buffer. Blotting performed at 90 V for 90 minutes. After blotting, membrane released from the set of devices and soaked or fixation with 50% methanol for 2 minutes, then blocked with skim milk 5% in PBST for 1 hour at room temperature. The membrane washed with PBST 3 times for 5 minutes each. After that, added serum of allergic suspect through the membrane with dilution ratio 1:10 in Phosphate Buffered Saline Tween 20 (PBST), next incubated for 2 hours at room temperature. Re-washed with PBST 3 times for 5 minutes each then added HRP conjugated monoclonal mouse anti-human IgE antibody (dilution ratio 1:3000 in PBST) and incubated for 1 hour while shaking. Furthermore, re-washed with PBST 3 times for 5 minutes each and added substrat 3,3'-diaminobenzidine (DAB), which dissolved in Tris Buffer saline till pH 7.5. The detection result proved the present of allergen protein complex with serum positively, marked by formation of brown band on nitrocellulose membrane. Marker membrane stained using black amido for 15 minutes then washed with distilled water and distaining solution.
3. Results and Discussion

3.1. Raw material characterization
The moisture content of Parang-Parang fish skin earned 77.29% and crude protein was 15.87% of wet weight and 68.39% of dry weight.

![Figure 1. Raw material of parang-parang fish skin](image1)

![Figure 2. characteristic of fish skin after pre-treatment step](image2)

3.2. Parang-parang fish skin collagen isolation
Firstly, collagen isolation passed pre-treatment step using NaOH solution 0.1 M for 12 hours. This step aims to eliminate pollutes, lipids, and non-collagen protein in Parang-Parang fish skin. Fig. 2 shown characteristic of fish skin after pre-treatment step. During pre-treatment step, fish skin and pore dilated so looked crumble. The result of dissolved-protein by NaOH-bath for every 2 hours appeared on fig. 3.

![Figure 3. The non-collagen protein concentration of NaOH-bath by soaking time](image3)

The non-collagen protein concentration of NaOH-bath measured using Bradford method. Protein reacted with Bradford reactor and producing blue color which its concentrate equivalent to protein concentrate. Fig. 3 shown the non-collagen protein concentration of NaOH-bath declined along with increasing soaking time.

This result indicated that the non-collagen protein concentration of sample released excessively during pre-treatment step occurred. The first two hours, protein concentration in high level 1.5808 mg/mL. The level continued to decline until 0.2163 mg/mL at 12th hour. Alhana et al. [3] processed...
pre-treatment using NaOH 0.1 M for 48 hours until obtained 0.2 mg/mL of non-collagen protein concentration. The declining of dissolved protein concentration at 2\textsuperscript{nd}, 4\textsuperscript{th}, and 6\textsuperscript{th} hour occurred enough significant. However after 8\textsuperscript{th} to 12\textsuperscript{th} hour, the dissolved-NaOH protein concentration has not different significantly. Thereby, the 6\textsuperscript{th} hour as the optimum time in pre-treatment of Parang-Parang fish skin using NaOH solution.

Collagen isolation of Parang-Parang fish skin used Acid Soluble Collagen. Isolation result, salt precipitation, and freeze dry of collagen protein showed on fig. 4. Collagen yield of Parang-Parang fish skin using ASC method were 1.915 % of wet weight and 8.3695% of dry weight. The collagen yield also could be counted by comparing the collagen weight after freeze dry to protein concentration of raw material. According to the calculation, 12.068% of the yield resulted.

![Collagen extraction](image1)
![Collagen precipitate](image2)
![Collagen freeze dry](image3)

**Figure 4.** (a) Collagen extract of isolation by acid solution; (b) collagen precipitate using NaCl; (c) collagen Freeze dry result

### 3.3. Parang-Parang fish skin collagen functional group identification

Analysis result of collagen functional group shown collagen protein of isolation owned five functional group identified with wave number of amida A 3419.79 cm\(^{-1}\); amida B 2924.09 cm\(^{-1}\); amida I 1656.85 cm\(^{-1}\); amida II 1558.48 cm\(^{-1}\); and amida III 1246.02 cm\(^{-1}\). The uptake of amida A area showed collagen characteristic functional group as NH\textit{stretching}, in amida B area as CH\textsubscript{2} asimetri \textit{stretching}, in amida I area as C=O \textit{stretching}, in amida II area as NH \textit{bending} CN \textit{stretching} and in amida III area as NH \textit{bending}. Collagen uptake area based on literature and calculating result of Parang-Parang fish skin collagen uptake also standard collagen appeared on Table 1.

| Type of Amida | Uptake peak of wave number (cm\(^{-1}\)) | characteristics |
|---------------|------------------------------------------|------------------|
| Amida A       | 3440-3400\(^1\)                         | 3419.79          | NH\textit{stretching} |
|               | 3400-3440\(^2\)                         | 3332.99          |                 |
| Amida B       | 2940-2922\(^3\)                         | 2924.09          | CH\textsubscript{2} asimetri \textit{stretching} |
|               | 2924-2940\(^4\)                         | 2924.09          |                 |
| Amida I       | 1690-1625\(^5\)                         | 1656.85          | C=O \textit{stretching} |
|               | 1656.85-1690\(^6\)                      | 1647.21          |                 |
| Amida II      | 1560-1540\(^7\)                         | 1558.48          | NH\textit{bending}, CN\textit{stretching} |
|               | 1540-1560\(^8\)                         | 1546.91          |                 |
| Amida III     | 1350-1220\(^9\)                         | 1246.02          | NH \textit{bending} |
|               | 1246.02-1350\(^10\)                     | 1334.74          |                 |

**Note:** \(^1\)Kaewdang \textit{et al.} \[19\], \(^2\)Huang \textit{et al.} \[20\], \(^3\)Duan \textit{et al.} \[21\], \(^4\)Sagita \[22\], \(^5\)Ahmad \textit{et al.} \[23\], \(^6\)Barzideh \textit{et al.}\[1\].
Analysis result of amino acid concentration of Parang-Parang fish skin collagen presented in graphic data of percentage (y) to amino acid type (x) (fig. 5). Percentage calculation processed according to the amount of amino acid concentration with total of whole 15 standard amino acids.

![Figure 5. Function group spectrum of Parang-Parang fish skin collagen](image-url)

3.4. Amino acid profile on Parangparang fish skin collagen

Collagen protein characteristic has dominant level of glysine amino acid. Analysis result showed amino acid composition of Parang-Parang fish skin collagen dominated by glysine (26.687%) followed by proline (13.19%), alanine (10.733%), arginine (10.148%) and glutamic acid (8.216%). The lowest amino acid in Parang-Parang fish skin collagen i.e tyrosine (0.798%), histidine (1.093%) dan isoleucine (1.385%). The result of amino acid composition indicated the hydrophobic amino acid in the amount of 62% and hydrophilic amino acid in the amount of 38%. It showed that collagen sample of Parang-Parang fish skin owned collagen protein characteristic because of the dominant glysine quantity and tend to hydrophobic.

![Figure 6. Amino acid composition of Parang-Parang fish skin collagen](image-url)

3.5. Parangparang fish skin collagen molecular weight

Electropherogram of collagen protein sample (fig. 7) presented three clear main ribbons and other unclear ribbons at bottom. The result described that sample in the study must be re-purified because of the existence of other protein in very low concentration.
SDS-PAGE gel result of collagen protein showed three dominant ribbons i.e two ribbons of $\alpha$ ($\alpha_1$ and $\alpha_2$) and one ribbon of $\beta$ and four ribbons of others. The molecule weights of $\alpha_1$, $\alpha_2$, and $\beta$ of Parang-Parang fish skin collagen successively 129, 113 dan 244 kDa. Other proteins owned molecule weight successively 72, 63, 25 dan 22 kDa (fig. 7). The result indicated compatibility of the committed-purifying process. Rf value of seven ribbons from up to down successively 0.062 cm, 0.17 cm, 0.197 cm, 0.283 cm, 0.313 cm, 0.675 cm, dan 0.899 cm.

**Figure 7.** Electropherogram of protein marker (M) and protein collagen (K)

Immunoblotting analysis result showed one protein positively as allergen protein. It marked by formulating brownish ribbon in nitrocellulose membrane (fig. 8). Analyzing membrane used software gel analyzer 10a and gained allergen protein molecule weight 17 kDa. The molecule weight of allergen protein appeared on membrane not the characteristic molecule weight of allergen protein as showed at fig. 7, but other proteins still in collagen sample. Collagen owned molecule weight of $\alpha_1$, $\alpha_2$, and $\beta$ successively 129, 113 and 244 kDa. Nevertheless, the result explained that immunoblotting process succeeded and the collagen protein of Parang-Parang fish skin known as non-allergenic.

**Figure 8.** Immunoblotting of protein marker (M) and collagen protein of Parang-Parang fish skin (K)

4. Conclusions

Collagen isolation of Parang-Parang fish skin using acid method succeeded with collagen yield in the amount of 1.915%. The existence of collagen shown by composition of dominant amino acid glysine, proline, and alanine also existence of functional group of amida A, B, amida I,II dan III. Collagen of this study result classified as type I collagen with molecule weight of $\alpha_1$ 129 kDa, $\alpha_2$ 119 kDa and $\beta$ 244 kDa, and unclassified as allergen protein.

The suggestion of this study needed a further purifying process in order to produce collagen uncontained-other proteins. Examination of allergen using collagen standard and examination of bioactive compound in collagen are needed.

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