Antioxidant activity of collagen from skin of parang-parang fish (*Chirocentrus dorab*) using DPPH and CUPRAC methods

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Abstract. Gross Collagen is fibrous protein that becomes the main component of connective tissues. An alternative source of collagen derived from marine animals as a source of antioxidants is needed. Utilization waste from skin of parang-parang fish (*Chirocentrus dorab*) as a source of collagen can increase the sale value of waste. The purpose of this research were to extract the collagen from skin of parang-parang fish with acid soluble collagen method include salting out and dialysis, also to test the antioxidant activity of collagen with DPPH and CUPRAC method. Collagen extraction steps consisted of pretreatmented with 0.1 M NaOH for 12 hours, hydrolysed with CH₃COOH 0.5 M for 48 hours, salting out with 1 M NaCl for 12 hours, and dialysed with distilled water for 12 hours. Concentration of dissolved protein for 12 hours pretreatment was 0.791 ppm. The yield of collagen was 0.9%. The characteristic of collagen analysed by FTIR spectra showed amide A (3425), B (2924.09), I (1647.21), II (1543.05), and III (1246.02) (cm⁻¹). The yield amino acid of collagen was glycine (26.69%), proline (12.24%), glutamic acid (11.72%), arginine (9.81%), alanine (9.51%). IC50 from DPPH method was 926.25 ppm. The antioxidant capacity of 100 ppm collagen was 104.14 µmol trolox / g extract.

1. Introduction

*Anguilla* Collagen is a fibrous protein that is a major component of connective tissue, such as the bones, tendons, skin, blood vessels and cornea of the eyes [27]. Collagen has a range of strength, special structure, and contains hydroxilisin and hydroxyproline [20]. The human body is naturally capable of producing collagen, but the amount decreases with age, so the addition of collagen from commercial collagen is needed. Collagen in the non-food industry is used as a health and beauty product. According to Schmidt et al. [36] collagen provides elastic properties to the skin and can reduce wrinkles due to aging. Commercial collagen is mostly sourced from cows and pigs that have a risk of disease transmission, ethnic barriers, and beliefs [25] so an alternative source of collagen is needed.

Kittiphathanabawon et al. [22] stated that alternative sources of collagen can be derived from the skin and bone parts of fish which are waste from processing results. Fish skin has complex structural proteins to maintain the flexibility strength of skin, ligaments, bones, joints, muscles, tendons, gums, eyes, blood vessels, nails, and hair [23]. Therefore, the utilization of Parang-Parang fish skin waste (*Chirocentrus dorab*) as a source of collagen has the potential to make fish waste as an alternative collagen source.
Parang-parang fish is one type of fish that can be found in the northern sea of Sumatra, the southern seas of West Sumatra, Java, southern Java, and the eastern sea of Indonesia [1]. In addition to being consumed directly, parang-parang fish are also used as ingredients for the manufacture of processed products such as crackers and pempek.

The role of collagen in cosmetics is as an active compound to increase skin moisture, maintain skin elasticity, and prevent signs of aging in the skin due to free radicals. Isolation collagen from parang-parang fish skin was done by Widowati [38] with an acid-soluble collagen (ASC) method and by Kurniawan [24] with a hydro-extraction method, which was formulated as a skin moisturizing gel. Collagen in skin moisturizing cosmetic products can act as antioxidants. Antioxidants are compounds that can inhibit oxidation reactions by binding to free radicals and highly reactive molecules, which can inhibit cell damage [35]. Several types of peptides have been shown to act as antioxidants [41]. Collagen is a protein composed of polypeptides. The use of collagen-based products is expected to be one of the efforts to reduce oxidative stress on the skin.

Antioxidant activity can be tested by several methods, including DPPH (1,1-Diphenyl-2-Picrylhydrazyl), FRAP (Ferric Reducing Antioxidant Power), and CUPRAC (Cupric Reducing Antioxidant Capacity). DPPH antioxidant activity is showed in percent inhibition of DPPH radicals or IC50 values [5] which mean the concentration of sample solutions needed to inhibit 50% DPPH free radicals. The antioxidant capacity of the CUPRAC method is expressed as the equivalent of trolox in millimolar trolox units per gram of sample [8]. The purpose of this study was to extract collagen from parang-parang fish skin using the acid-soluble collagen method followed by salting and dialysis, and to test the antioxidant activity of collagen using DPPH and CUPRAC methods.

2. Materials and Methods

The material used include parang-parang fish skin (*Chirocentrus dorab*), sodium hydroxide (NaOH) Merck®, distilled water, acetic acid (CH3COOH) Merck®, sodium chloride (NaCl), Bovine Serum Albumin (BSA), Bradford reagent, DMSO Merck®, ethanol Merck®, ammonium acetate Merck®, CuCl2.2H2O Merck®, neocuproin Sigma Aldrich®, trolox Sigma Aldrich®, ascorbic acid Sigma Aldrich®, and DPPH (1,1-Diphenyl-2-Picrylhydrazyl) Sigma Aldrich®.

2.1. Extraction of Collagen from Parang-Parang Fish Skin

Pre-treatment [38]. Fish skin soaked in 0.1 M NaOH solution with a ratio of 1:10 (b / v) for 12 hours at cold temperatures. NaOH solution was replaced every 2 hours. The remaining NaOH solution from the process was measured for protein content by Bradford test and BSA as a standard to determine the non-collagen protein content. Then the fish skin was neutralized with distilled water.

Extraction with Acid [21]. Fish skin soaked in 0.5 M acetic acid solution with a ratio of 1:10 (b / v) for 48 hours at cold temperatures. NaCl added into filtrate until 1 M and stored for 12 hours at cold temperatures. The results centrifuged with speed of 10000g for 30 minutes. The residu dissolved in 0.5 M acetic acid, then dialyzed in distilled water for 12 hours by replacing the distilled water for 4 hours. The dialysis collagen centrifuged again with the same speed and duration. The residu then dried by freeze dry method.

2.2. Rendemen Analysis [6]

The yield is obtained by the formula:

\[
\text{Collagen yield (\%)} = \frac{\text{Dry weight of collagen}}{\text{Weight of skin raw material}} \times 100\%
\]

2.3. Analysis of Protein Contain [11]

100 µL of BSA solution (0, 200, 400, 600, 800, 1000, 1200, and 1400 ppm) was added to 5 mL Bradford reagent, then incubated for 5 minutes. The absorbance read by spectrophotometer 595 nm. Absorbance data used to create a standard curve. The measurement of protein content in NaOH was done according with the same procedure.
2.4. DPPH Method Antioxidant Activity

Collagen solutions (100, 200, 400, 600, 800, and 1000 ppm) was put into a microplate well of 160 µL, then DPPH (0.3 mg / mL) 40 µL was added. The sample blank was made by 160 µL of methanol and 40 µL of DPPH. Negative control was made by 160 µL of collagen solution and 40 µL of methanol. As blank used methanol which was put into the well as much as 200 µL. Ascorbic acid was used as positive control with concentration 2, 4, 6, 8, and 10 ppm. Mixture then incubated for 30 minutes and read by microplate reader at 517 nm. Percent of inhibitory activity (% inhibition) calculated by the formula:

\[
\text{% Inhibition} = \frac{(A-B)-(C-D)}{(A-B)} \times 100\%
\]

Information:
A: Absorption of negative controls (sample + methanol)
B: absorbance of blank (methanol)
C: absorbance of the sample (sample + methanol + DPPH)
D: absorbance of the blank sample (methanol + DPPH)

2.5. CUPRAC Method Antioxidant Activity (Öztürk et al. [32] with modifications)

50 µL of CuCl₂.2H₂O 0.01 M, 50 µL of 7.5 x 10⁻³ M neocuproin, 50 µL of ammonium acetate buffer pH 7, and 50 µL of collagen solution with concentration series of 100, 200, 400, 600, 800, and 1000 ppm was added into the microplate well. Standard curves for measuring antioxidant capacity used trolox with concentrations of 10, 20, 30, 40, and 50 µM. As a blank, the mixture used by replacing collagen with a solvent. The mixture incubated for 30 minutes and measured by microplate reader at 450 nm.

2.6. Data Analysis (Steel and Torrie [37])

The design used for the results of CUPRAC method was a Completely Randomized Design (CRD). The treatment was a combination of collagen concentration with antioxidant capacity carried out 3 times. The data obtained were analysed by analysis of variance (ANOVA) at a 95% confidence level and if there were significant differences (reject H0 if the P-value was smaller than the alpha value (0.05)), then the statistical analysis was followed by further testing. The further test used in the analysis of the results is the Tukey test. Hypothesis of the effect of collagen concentration:

H₀ = The administration of different collagen concentrations in the CUPRAC antioxidant test has no significant effect on antioxidant capacity.
H₁ = There is a minimum of 1 treatment giving collagen concentration in the CUPRAC antioxidant test which gives a significant difference in antioxidant capacity.

3. Results and Discussion

3.1. Non-Collagen Protein Contain and Rendemen

The process of making collagen begins with pre-treatment to remove non-collagen proteins and other components from the sample, such as fats, minerals, pigments, and odors. During the pre-treatment process, fish skin expands with time. Non-collagen protein levels dissolved in NaOH were measured spectrophotometrically at a wavelength of 595 nm using the Bradford method with the standard Bovine Serum Albumin (BSA). Figure 1 shows that the highest levels of non-collagen protein were obtained at the first 2 hours of immersion, which was 13.009 ppm. The measurement results show that the levels of non-collagen protein contained in fish skin have been widely released with NaOH solvent in the first 2 hours of soaking, so the amount of non-collagen protein in fish skin is getting reduced. This is evidenced by the smaller concentration of protein in NaOH solution soaking the skin at the next observation.
Figure 1. The concentration of non-collagen protein dissolved during soaking time.

Pre-treatment is the initial stage of collagen extraction carried out to remove contaminants (fats, minerals, and pigments) and non-collagen proteins [36]. NaOH concentrations of 0.05 and 0.1 M can dissolve non-collagen proteins without removing collagen content, but if the concentration of NaOH used exceeds 0.1 M, the excess OH can dissolve collagen due to the partial breakdown of covalent bonds [26]. Soaking fish skin in NaOH is also able to convert the tropocollagen to procollagen, cause skin swelling, and facilitate the entry of water into the fish skin, so that the release of contaminants and non-collagen proteins in the matrix can occur [18].

Fish skin then neutralized with distilled water to remove contaminants and does not affect in pH during the extraction process. Collagen extraction was done by soaking fish skin in acetic acid to change the structure of the fiber and dissolve collagen. During the hydrolysis process, the fish skin expands and forms a clear colored layer beneath it. The type of acid used was acetic acid because it was capable of dissolving cross-linked or not collagen [26]. The acetic acid used was 0.5 M, higher than the acid concentration in the hydro-extraction method. The higher concentration of acetic acid makes swelling that occurs on the skin more faster. Acetic acid can increase H⁺, so that water is easier to enter into collagen fibers and form hydrogen bonds between non-polar collagen groups to disrupt non-covalent bonds in collagen fibers and facilitate the dissolution process of collagen [18]. The yield obtained from the collagen purification process is 0.9%. It caused by purification process to remove components other than collagen protein after hydrolysis.

The purification process consists of salting out, centrifugation, and dialysis. When a solution contains high concentrated salt, the protein will tend to gather and settle. As salt concentration increases in solution, the charged side of the surface of the protein will interact with salt, so that hydrophobic interactions on the surface of the protein cause protein to settle [10]. The purpose of the dialysis process is to reduce salt levels in collagen. Dialysis causes water and salt molecules diffuse out of the bag towards lower concentrations. Removing water every 4 hours can reduce the concentration of salt, so that the obtained collagen protein is purer. In addition, the skin of machete fish has a moisture content of 75.04%, fat content of 8.22%, and protein content of 11.79% [24]. Protein levels in fish skin determine the amount of collagen contained in skin tissue [4]. The difference in collagen yield value produced can be caused by differences in extraction methods, solution concentrations in removing non-collagen proteins, types of materials, and temperature and time of extraction [33].

3.2. Characteristics of Collagen
**Table 1.** Characteristics of collagen functional groups.

| Amide Type | Number of Wave Absorption Peaks (cm⁻¹) | Characteristics |
|------------|----------------------------------------|------------------|
|            | Absorption Area                        |                  |
|            | Collagen of Skin Parang-Parang         |                  |
|            | Standard Collagen                      |                  |
| Amide A    | 3440 – 3400 ¹                         | 3425.58          |
| Amide B    | 2940 – 2922 ²                         | 2924.09          |
| Amide I    | 1690 – 1625 ³                         | 1647.21          |
| Amide II   | 1560 – 1540 ⁴                         | 1543.05          |
| Amide III  | 1350 – 1220 ⁵                         | 1246.02          |

Information: ¹ Kaewdang *et al.* [19], ² Huang *et al.* [17], ³ Duan *et al.* [13], ⁴ Ahmad *et al.* [3] ⁵ Barzideh *et al.* [9], ⁶ Sagita [34]

Functional groups and amino acid analysis used to characterized collagen. Functional group analysis was performed spectroscopically with Fourier Transform Infrared (FTIR) at 400 - 4000 cm⁻¹. Amino acid analysed with Ultra Performance Liquid Chromatography (UPLC) using 15 standard amino acids. The five typical collagen functional groups were identified through FTIR spectra with amide A (3425 cm⁻¹), amide B (2924.09 cm⁻¹), amide I (1647.21 cm⁻¹), amide II (1543.05 cm⁻¹), and amide III (1246.02 cm⁻¹). The ratio value between the absorption peak at wave number 1450 cm⁻¹ with the absorption peak of amida III is 1.13, indicated that collagen has a triple helix structure [28].

![Figure 2. Amino acid composition of collagen from skin parang-parang.](image)

The amino acid composition of collagen skin of parang-parang fish was dominated by glycine (26.69%), proline (12.24%), glutamic acid (11.72%), arginine (9.81%), alanine (9.51%). Glycine composes almost one third of the structure of collagen [12]. This caused by glycine which is present in every three amino acid residues in collagen and plays a role in the formation of triple helical chains in collagen. The majority of amino acids in collagen are dominated by non-polar amino acids (glycine, proline and alanine), thus affecting the physical properties of collagen which is difficult to dissolve in water. Collagen has a low level of tyrosine and histidine (0.7% and 1.08%) and doesn’t contain tryptophan and cysteine [2]. Based on the characterization of functional groups and amino acids, it can be proved that the compounds extracted in this study were collagen.

### 3.3. Collagen Antioxidant Activity DPPH Method
Collagen antioxidant activity was measured using DPPH (1,1-Diphenyl-2-Picrylhydrazyl) method because this is the most commonly used method in measuring antioxidant activity in vitro. Based on the results, the value of IC\textsubscript{50} collagen was 926.25 ppm and classified as a very weak antioxidant [31]. The antioxidant activity of collagen was much lower than the positive control (vitamin C) with IC\textsubscript{50} was 9.09 ppm.

**Table 2. Antioxidant Activity of Collagen with DPPH method**

| [Collagen] (ppm) | % Inhibition DPPH | IC\textsubscript{50} (ppm) |
|------------------|-------------------|-----------------------------|
| 1000             | 53.48             |                             |
| 800              | 43.14             |                             |
| 600              | 35.36             | 926.25                      |
| 400              | 21.60             |                             |
| 200              | 14.17             |                             |
| 100              | 7.34              |                             |

Collagen antioxidant activity influenced by the composition, structure and hydrophobicity of amino acids, and the weight of collagen molecules [29]. Three of the dominant amino acids in these collagen were non-polar amino acids, so collagen tends to be hydrophobic. The hydrophobicity caused the measurement of collagen activity with the DPPH method does not effective. The DPPH method is more effective for measuring antioxidant activity in polar samples because the DPPH method is less sensitive for measuring antioxidant activity than phenol compounds [7]. Therefore, collagen of parang-parang skin showed the very high IC\textsubscript{50}.

3.4. Collagen Antioxidant Activity The CUPRAC method

Antioxidant activity should be analyzed using several methods because antioxidant compounds have specific molecules that can inhibit the formation of certain radical chains. Therefore, antioxidant activity also analysed by CUPRAC method. The standard used in the CUPRAC method was trolox to make regression curve. The effect of collagen concentration on antioxidant capacity in the CUPRAC method was then analyzed statistically by variance test (ANOVA) and Tukey test as a further test. Table 3 shows that the highest antioxidant capacity values with CUPRAC method were collagen at concentrations of 1000 ppm. Although collagen with concentrations of 100 and 200 ppm has a very low percent inhibition value on DPPH radicals, the antioxidant capacity of the CUPRAC method is quite good with a value of 109.54 and 104.14 mol of trolox / g extract.

**Table 3. Antioxidant Activity of Collagen with CUPRAC method.**

| [Collagen] (ppm) | Antioxidant Capacity (\(\mu\)mol trolox/g extract) |
|------------------|--------------------------------------------------|
| 1000             | 121.68 \textsuperscript{a}                      |
| 200              | 109.54 \textsuperscript{ab}                     |
| 100              | 104.14 \textsuperscript{ab}                     |

The Tukey test showed that giving 1000 ppm of collagen resulted in significantly different antioxidant capacity compared to other concentrations. The antioxidant capacity of collagen at 100 and 200 ppm were not significantly different. Collagen is a protein composed of three polypeptide chains with a triple-helix structure and has three recurrent residues in the form of Gly-X-Y. Glycine is the
dominant amino acid found in collagen, while position X is proline and Y is hydroxyproline [14]. The composition and position of amino acids in peptides play an important role in determining the antioxidant activity of collagen. The inhibition of chain reactions to free radicals caused by the high content of proline and glycine in the sequence of collagen amino acids which act as contributors to protons against free radicals [39]. Another possibility is the presence of -OH and -NH₂ groups in collagen peptides that are capable of binding to free radicals [42].

Amino acid collagen of skin parang-parang dominated by glycine, proline, glutamic acid, alanine, and arginine. Total Hydrophobic Amino Acid (THAA) has a high level of solubility in fat and increases antioxidant activity (Zhang et al. 2010). N- or C- peptide endings in hydrophobic amino acids can interact with lipid molecules and donate protons to free radicals [15]. In addition, Mendis et al. [30] stated the relationship between antioxidants and molecular weights, which is peptides that have antioxidant activity generally consist of 2-20 amino acids with molecular weights less than 3000 Da. The dialysis bag used in this research has pore size of 12-14 kDa, so the collagen produced has less powerful antioxidant activity.

The difference results of antioxidant activity in the DPPH and CUPRAC methods caused by differences in the mechanism of free radical reduction. Free radicals can be suppressed by hydrogen capture reactions, electron donations, and shared electron use [16]. The DPPH method dampens free radicals with hydrogen capture reactions, while the CUPRAC method with electron donations. The redox reaction that occurs in the CUPRAC method caused by a redox potential which is the energy needed to carry out a redox reaction. The smaller of redox potential makes the faster the reaction. According to Apak et al. (2004), Cu²⁺ has a small metal redox potential value (0.337 V), so the CUPRAC method is more selective for non-oxidized compounds in DPPH. Based on the results in this study, the measurement of the antioxidant activity of collagen from skin parang-parang fish was more effective using the CUPRAC method than DPPH.

4. Conclusions
Collagen of skin parang-parang fish was successfully extracted by acetic acid with salting-out and dialysis procedures. The collagen yield of the skin parang-parang was 0.9%. Collagen of skin parang-parang fish had an IC₅₀ 926.25 ppm against DPPH and antioxidant capacity value of 104.14 µmol trolox/g extract at a concentration of 100 ppm with CUPRAC method.

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