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Characteristics of Collagen from Parrotfish (*Chlorurus Sordidus*), Tiger Grouper (*Epinephelus Fuscoguttatus*) and Pink Ear Emperor (*Lethrinus Lentjan*): Effect of Acetic Acid Concentration and Extraction Time

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Abstract: The use of marine fish's skin waste such as parrotfish (*Chlorurus sordidus*), tiger grouper (*Epinephelus fuscoguttatus*), and pink ear emperor (*Lethrinus lentjan*) needs improvement to increase its economic value and sustainability. Therefore, this study aims to investigate the effect of fish skin to acetic acid ratio with different extraction times on the yield and characteristics of skin collagen obtained from three-marine fishes (parrotfish, tiger grouper, and pink ear emperor). The skin samples were divided into 4 groups (A, B, C and D) and treated with different sample to acetic acid ratios and extraction time of A. [ratio 1:10 (w/v) with 24 h]; B. [ratio 1:10 (w/v) with 36 h]; C. [ratio 1:20 (w/v) with 24 h]; and D. [ratio 1:20 (w/v) with 36 h]. Furthermore, characterization of collagen functional groups was carried out using FTIR while the peptide pattern analysis was carried out with SDS-polyacrylamide gel electrophoresis. The results showed that the collagen yield from the groups was different and the longer the extraction time, the higher the yield. Collagens from the three fishes in the treatment groups had wave spectra that were consistent with the standard collagen amide band while the peptide patterns showed α_1 and α_2 chains with molecular weights of 93-106 kDa and the dominant amino acids were glycine, proline, and alanine. They were also identified as collagen type 1 with similar protein patterns and FTIR spectra.

Keywords: Acetic Acid, Collagen, Marine Fish Ratio

Introduction

Collagen is a widely used biomaterial that plays an important role in the biomedical, pharmaceutical, food, and cosmetics industries (Sionkowska *et al*., 2020). It also has a biological function in the formation of tissues and organs as well as in cell division, defense, and differentiation, hence, it is widely used, specifically in medicine, such as in the pharmaceutical industry for implantation, cancer treatment, and drug delivery (Song *et al*., 2018). Collagen is an early anti-aging treatment in the cosmetics industry (Ramos-e-Silva *et al*., 2013) which is found in many skin care products and makeup in form of lotions, gels, and powders (Sionkowska *et al*., 2020).

Generally, it is obtained from cow and pig skins (Park *et al*., 2021), but the outbreaks of some animal diseases such as bovine spongiform encephalopathy, transmissible spongiform encephalopathy, and foot and mouth diseases pose risks to human health (Tang and Saito, 2015). Moreover, pig products are forbidden for Muslims (haram) (Rakhmanova *et al*., 2018). This has led to the use of collagen from other sources with no risk of disease transmission and religious prohibition such as fish waste which is widely available and has a high biological value with guaranteed halal. Indonesia is dominated by Muslims, hence, concomitant products derived from halal raw materials such as fish are needed.

Fish skin contributes 20% of the total waste in the processing industry and it is valuable as a raw material for

collagen production. Wastes commonly produced by fish processing companies in Indonesia are from parrotfish (*Chlorurus sordidus*), tiger grouper (*Epinephelus fuscoguttatus*), and pink ear emperor (*Lethrinus lentjan*) which have thick skin texture and they are a promising source of collagen. Furthermore, these fishes live in tropical and subtropical marine waters associated with coral reefs (Sugama *et al*., 2014; Pane *et al*., 2020).

Enzymes or pepsin soluble collagen is used in the extraction process which involves hydrolysis of the sample using acid or Acid Soluble Collagen (Liu *et al*., 2015). However, extraction with pepsin has the disadvantage that it comes from pigs and cannot be used by Muslims (haram). Generally, collagen dissolves in acidic solvents but when exposed to a very acidic pH, the solubility decreases. This leads to collagen extraction being carried out using weak acids such as acetic acid with a carboxyl group (–COOH) that binds to an amine group (-NH) on collagen protein to facilitate the collagen extraction process (Liu *et al*., 2015).

Furthermore, studies on collagen extraction from fish skin using acetic acid showed that the yield of soluble acetic acid collagen and the optimum conditions required vary with the type of raw material (Kiew and Don, 2013; Arumugam *et al*., 2018; Chinh *et al*., 2019; Jafari *et al*., 2020). Therefore, this study aims to investigate the effect of fish skin to acetic acid ratio with different extraction times on the yield and characteristics of skin collagen from parrotfish (*Chlorurus sordidus*), pink ear emperor (*Lethrinus lentjan*), and tiger grouper (*Epinephelus fuscoguttatus*).

Materials and Methods

Samples and Materials

Parrotfish (*Chlorurus sordidus*), pink ear emperor *(Lethrinus lentjan*), and tiger grouper (*Epinephelus fuscoguttatus*) wastes were obtained from a fish processing company named Alam Jaya Seafood Co. located in Surabaya, Indonesia. The fillet skins were separated from the meat, packaged in plastic, and immediately brought to the laboratory at a temperature of -10°C. Furthermore, all materials such as tris-base, HCl, acrylamide, N'N'-bis-methylene-acrylamide, glycine, SDS, *commasie brilliant blue* R-250, methanol, acetic acid, butanol, β-mercaptoethanol, 10% APS and TEMED were purchased from *Sigma*-Aldrich Co (USA).

Sample Preparation

The fish skins were thoroughly washed in running tap water and frozen at -0°C until it was needed. It was then thawed in running water, cut into small pieces (0.5 × 0.5 cm^2) and the non-collagen protein was removed by immersing 100 kg of fish skin in a 0.1 m NaOH (1:10) (w/v) solution at 4°C for 6 h and the solution was changed every three hours. Subsequently, they were washed with tap water to obtain a neutral pH and immersed in a 10% (1:10) (w/v) butyl alcohol solution at 4°C for 18 h. This solution was replaced every 6 h to remove fat and the skin was finally rewashed to a neutral pH.

Collagen Extraction

The fish skins were divided into 4 groups (A, B, C and D) and extracted with 0.5 m acetic acid with different sample to acetic acid ratio and extraction time, namely A. [ratio 1:10 (w/v) with 24 h]; B. [ratio 1:10 (w/v) with 36 h; C. [ratio 1:20 (w/v) with 24 h]; and D. [ratio 1:20 (w/v) with 36 h]. All extractions were carried out at 4°C and filtered with two layers of cotton cloth to obtain the filtrate. The extraction results were precipitated by adding 0.9 m NaCl at 4°C, centrifuged at a speed of 3500 rpm for 30 min and the supernatant was removed while the residue was obtained with 0.5 m acetic acid and washed. Subsequently, the residue was dialyzed in 0.1 m acetic acid solution for 12 h and dialyzed again with distilled water for 24 h.

Yield Analysis

The yield was determined by calculating the percentage of yield obtained by multiplying the ratio of final weight to initial weight by 100 (Sani *et al*., 2014).

$$\%yield = \frac{W1(g)}{W0(g)} \times 100\%$$

where *W*1 was the weight of extracted collagen while *W0* was the weight of raw material.

Fourier-Transform Infrared Spectrum (FTIR) Analysis

Collagen functional groups were characterized using FTIR (Fourier-Transform Infrared) to determine the pattern of cross-linking that occurs which helps in studying changes in the secondary structure of collagen. The collagen samples were placed in crystal cells which were clamped to an FTIR spectrometer for spectrum analysis. Then, spectra within the range of 400-4000 cm^{-1} with automatic signal amplification were collected in 32 scans at 4 cm^{-1} resolutions and finally compared with background spectra recorded from the blank at 25°C (Singh *et al*., 2011).

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis

The sample was dissolved in a buffer containing β-mercaptoethanol heated for five minutes at 100°C and then applied in a 12% separating gel and 4% stacking gel. After electrophoresis, the gel was stained with 0.04% Coomassie blue in 25% (v/v) ethanol and 8% (v/v) acetic acid for two hours and the excess stain was removed by washing with a destaining solvent (25% v/v ethanol, 8% v/v acetic acid) (Blanco *et al*., 2019).

Amino Acid Analysis

The amino acid analysis was carried out using Ultra Performance Liquid Chromatography (UPLC). The samples were hydrolyzed in 6 m HCl at 110°C for 22 h and α-Aminobutyric Acid (AABA) was used as a standard reagent for amino acid derivatization with the Acq Fluor reagent kit. The UPLC conditions used were the ACCQ-Tag Ultra C-18 column, the temperature of 49°C, water rate of 0.5 mL per minute, photodiode array detector, wavelength 260 nm, and injection volume of 1 µL while the elution system was carried out in a gradient with a specific composition.

Statistical Analysis

The study data is the average of three independent treatments and the statistical analysis was carried out with the SPSS 25 program using analysis of variance at a 95% confidence interval and continued with Duncan's difference test.

Results

Yield

Yield is one of the important parameters used in collagen production and a high value indicates that the treatment given during the process is efficient and effective. The results showed that the sample to volume of acetic acid ratio and the extraction time are significant (P<0.05) to the yield of liquid collagen in parrotfish (*C. sordidus*), pink ear emperor (*L. lentjan*) and tiger grouper (*E. fuscoguttatus*), Fig. 1. Aside from the tiger group, the results also showed that the higher the solvent ratio and the longer the extraction time, the higher the yield of liquid collagen.

Furthermore, parrotfish, pink ear emperor, and tiger grouper had the highest yield of liquid collagen in the A2B2 treatment (1:20 for 36 h) with a value of 51.80±0.53, 43.06±1.36 and 45.7±1.12%, respectively. Meanwhile, all fishes had the lowest yield in the A1B1 treatment, where the yields produced by parrotfish, pink ear emperor, and tiger grouper were 29.83±0.35, 29.89±1.23, 36.53±1.75%, respectively.

Fourier-Transform Infrared Spectrum (FTIR)

The collagen structure was analyzed using FTIR to ensure that the resulting compound is collagen-based with its constituent functional groups. Figure 2 shows the result of FTIR analysis of collagen extraction from the skin of parrotfish, pink ear, emperor fish, and tiger grouper.

The FTIR spectrum of the four treatments on the skin of parrotfish, pink ear emperor, and tiger grouper showed peak absorption of amide A, B, I, II, and III which are typical spectra of collagen.

Pattern

The collagen patterns obtained from SDS-PAGE are similar. This is caused by the amino acid composition, which has little difference (relative molecular weight) among the treatments (Putra *et al*., 2013). The extracts have a peptide pattern of α1 and α2, as shown in Fig. 3. Parrotfish skin collagen has an electrophoresis pattern that indicates the presence of α1 and α2 proteins with a molecular weight of 93-106 kDa, while pink ear emperor collagen indicates that α1 and α2 are the main components with molecular weights ranging from 100.32-113.34 kDa. 24 h; A2B2: Treatment with ratio 1:20 (w/v) with 36 h.

Amino Acid Analysis

Amino acids are essential parameters used to determine the nature of collagen because it contributes to the stability of the helix structure (Simpson *et al*., 2012). The primary collagen molecule is formed from three polypeptide chains that twist to form a triple helix structure with a unique arrangement of amino acids. The amino acid composition is expressed as a percentage and shown in Table 1.

Table 1: Amino acid composition of collagens

No	AA	Parrot Fish*	Pink Ear Emperor fish*	Tiger grouper*	Mackerel#	Bovine$
1	Ser	5.47	4.91	4.53	3.26	3.39
2	Glu	9.28	9.15	11.30	6.72	11.53
3	Phe	4.20	3.70	2.47	1.53	4.38
4	Iso	1.63	1.09	1.16	1.34	1.87
5	Val	3.29	2.29	2.45	2.38	2.45
6	Ala	11.32	10.25	11.78	13.50	9.51
7	Arg	3.19	11.02	8.57	5.07	7.30
8	Gly	33.27	28.19	27.51	34.16	19.22
9	Lys	1.58	2.81	3.76	2.08	3.11
10	Asp	4.65	5.14	6.33	5.01	7.49
11	Leu	3.34	2.72	2.41	2.35	3.67
12	Tyr	2.27	1.38	1.03	0.34	2.02
13	Pro	12.21	12.01	13.46	10.84	13.92
14	Thr	2.86	3.75	3.24	1.95	2.19
15	His	1.43	1.58	0.00	0.70	2.25
16	Hyp				6.87	8.15

Note: *# Li *et al*. (2013); $ Gauza-Włodarczyk *et al*. (2017)

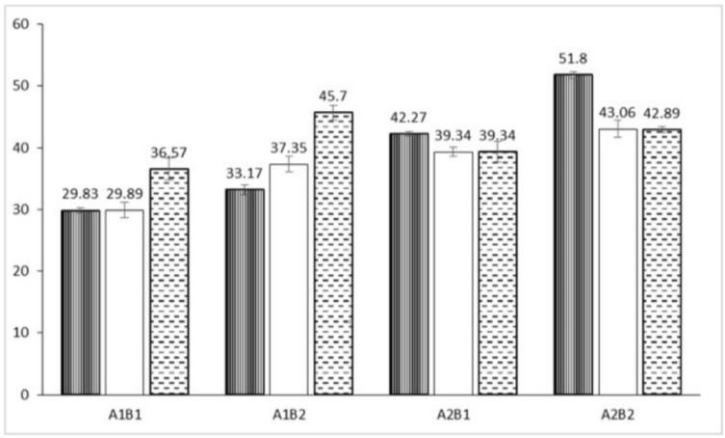
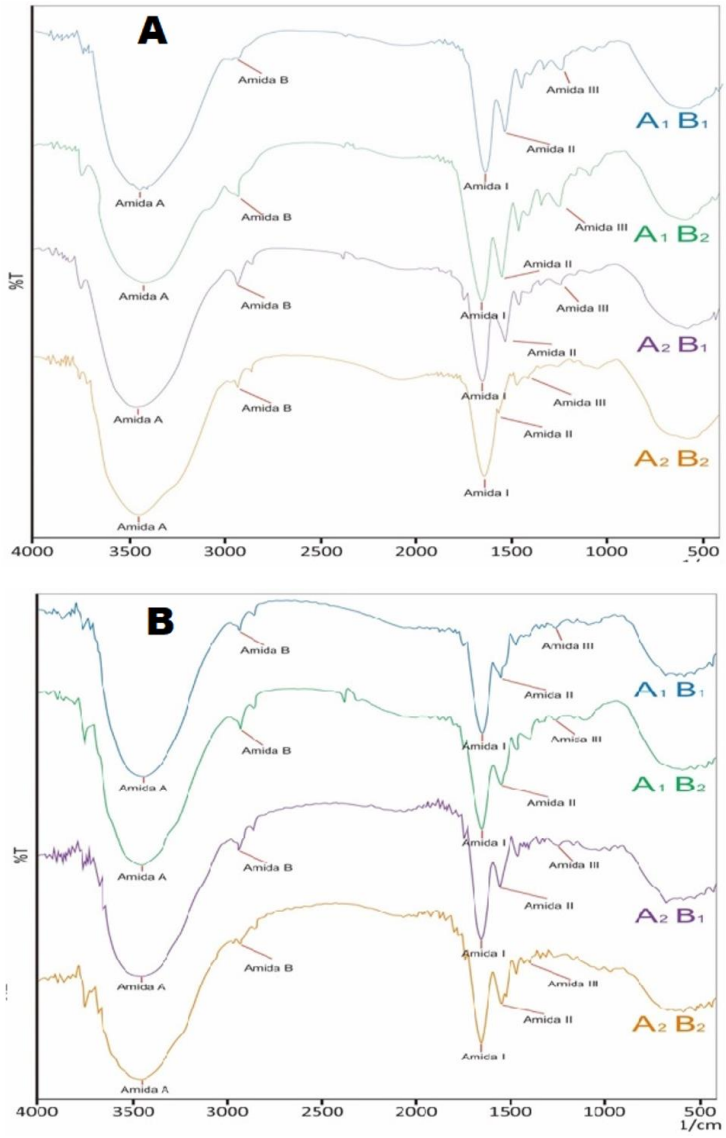


Fig. 1: Collagen yield. The straight line is collagen from Parrotfish (*C. sordidus*); the white is collagen from Pink Ear Emperor (*L. lentjan*); The dotted lines are tiger grouper (*E. fuscogutt*atus). A1B1: Treatment with ratio 1:10 (w/v) with 24 h; A1B2: Treatment with ratio 1:10 (w/v) with 36 h; A2B1: Treatment with ratio 1:20 (w/v) with 24 h; A2B2: Treatment with ratio 1:20 (w/v) with 36 h



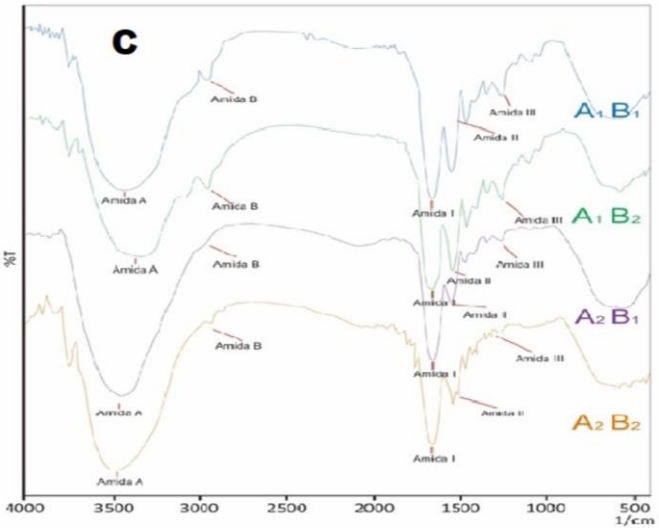
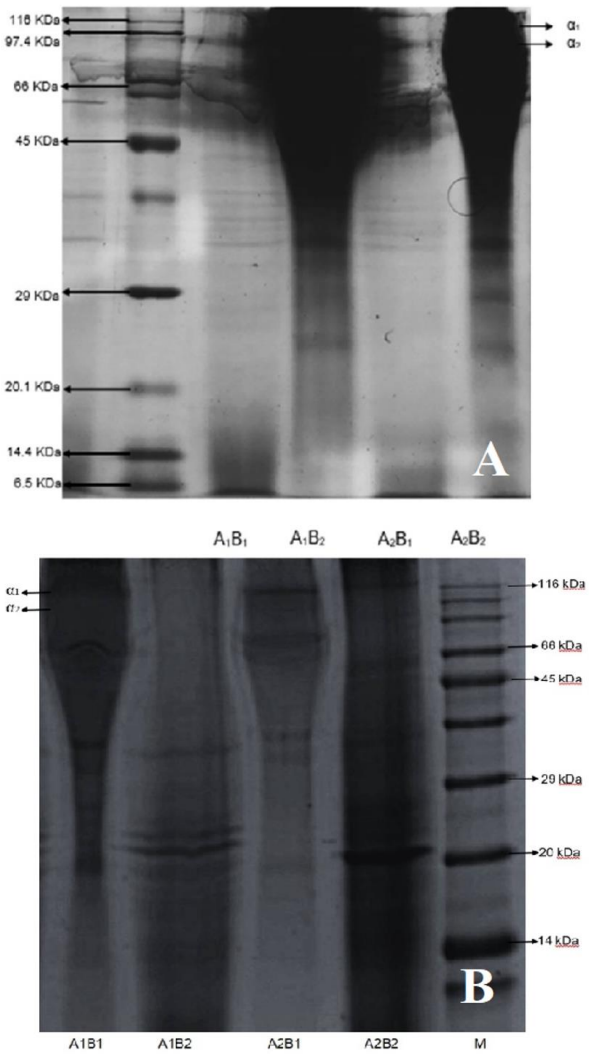


Fig. 2: FTIR profile of fish skin collagen. A. Parrotfish (*C. sordidus*); B. Pink Ear Emperor (*L. lentjan*); C. Tiger grouper (*E. fuscoguttatus*). A1B1: Treatment with ratio 1:10 (w/v) with 24 h; A1B2: Treatment with ratio 1:10 (w/v) with 36 h; A2B1: Treatment with ratio 1:20 (w/v) with 24 h; A2B2: Treatment with ratio 1:20 (w/v) with 36 h



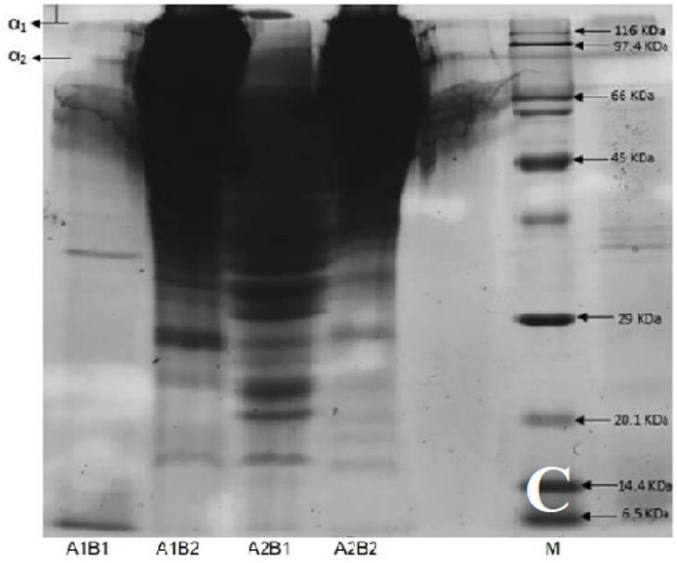


Fig. 3: SDS-PAGE patterns of Collagen. A. Parrotfish (*C. sordidus*); B. Pink Ear Emperor (*L. lentjan*); C. Tiger grouper (*E. fuscoguttatus*). A1B1:Treatment with ratio 1:10 (w/v) with 24 h; A1B2: Treatment with ratio 1:10 (w/v) with 36 h; A2B1: Treatment with ratio 1:20 (w/v) with 24 h; A2B2: Treatment with ratio 1:20 (w/v) with 36 h

Table 1 shows that the collagen of parrotfish, pink ear emperor, and tiger grouper contain more glycine, proline, and alanine than other amino acids. They are present in parrotfish with 33.27, 12.21 and 11.32%, respectively. Conversely, the lowest glycine content was found in tiger grouper collagen with 27.51% while the lowest content of proline and alanine was found in pink ear emperor with 12.01 and 10.25%, respectively.

Discussion

This study shows that the solvent ratio and the length of extraction time significantly affect fish collagen extraction and the solvent ratio is an essential variable that affects extraction efficiency. An increased ratio of acetic acid produces a higher yield (Kiew and Don, 2012; Yu *et al*., 2018) and collagen yields also increase with a longer extraction time (Arumugam *et al*., 2018; Song *et al*., 2014; Jafari *et al*., 2020). Different extraction processes can produce collagen with different characteristics based on solubility.

Collagen yield from fish skins generally ranges from 5 to 42.36% (Tan and Chang, 2018), and this is consistent with the study on leather jacket (*Odonus niger*) skin with a value of 50.24% (Muralidharan *et al*., 2013). Besides, collagen extracted from carp scales using citric acid for 25 h resulted in a yield of 74.34%. (Suo-Lian *et al*., 2017). Fish skin usually contains type I collagen with a high level of purity (about 70%) depending on the species' age and season (Jafari *et al*., 2020). This result is similar to the study on leather jacket (*Odonus niger*) skin with a value of 50.24% (Muralidharan *et al*., 2013). The collagen yield of the extracted Atlantic cod (*Gadus morhua*) skin had a value of 13.8% (Sousa *et al*., 2020). Whereas striped catfish (*Pangasianodon hypophthalmus*) with a value of 5.1% (Singh *et al*., 2011).

Furthermore, amide A is associated with NH frequencies that range from 3400-3440 cm^{-1} (Bhagwat and Dandge, 2016). When the NH peptide group is involved in hydrogen bonds, it shifts to lower frequencies usually around 3300 cm^{-1} (Singh *et al*., 2011). In parrotfish, collagen A1B1, A1B2, A2B1, and A2B2 spectra were obtained with a frequency value of 3446.56, 3390.63, 3448.49, 3423.41 cm^{-1}, respectively, indicating that hydrogen bonds are more damaged in the treatment of A1B1 and A2B1 during the extraction process. The collagen extracted from all the fishes shows a pattern that is similar to the structure of amide A.

Meanwhile, the amide B wave number is formed due to the asymmetrical stretching of the CH_2 group (Zaelani *et al*., 2019) and the amide B group with a wave absorption range of 2915-2935 cm^{-1} also correlates with this stretching (Singh *et al*., 2011). In all treatment groups, the three collagens extracted have several spectrum values higher than the standard uptake and this was due to an increase in the NH-NH_3 + group free of lysine residues in N-terminals (Liao *et al*., 2018). Therefore, the small wave number in collagen indicates that it has a lower amount of lysine (Barzideh *et al*., 2014).

Amide I, II, and III bands are produced from the C = O, N-H, and C-H of the peptide which is responsible for the level of molecular order, and they are involved in the triple helix collagen structure (de Campos Vidal and Mello, 2011). Furthermore, amide I occurs at wave

numbers between 1600 and 1700 cm^{-1} and is related to the vibration stretching of C = O or H-bonds that join with COO- (Chuaychan, 2016). Collagen obtained from the treatment of A1B1, A1B2, A2B1, and A2B2 spectra were included in the standard absorption range and amide I in A2B2 has a lower wavenumber which indicates a higher C = O interaction with adjacent chains and the C = O is associated with higher compactness (Ali *et al*., 2018).

Amide I contain four structural components of secondary protein, namely α-helical, β-sheet, β-turn, and random coil and each of these components has a different absorption area. The α-helical component is shown in the region of wave number absorption range of 1656-1662 cm^{-1}, β-sheet at 1616-1637 cm^{-1}, β-turn at 1663-1696 cm^{-1}, and random coil at 1638-1655 cm^{-1}. Based on the results of the amide I wave number, parrotfish showed that the collagen produced had a random coil structure which indicates that the molecular compounds produced from the extraction process are collagen that has not been degraded to gelatin. However, the denaturation of collagen due to the heating process causes the triple-helical chain to completely transform into a single α-helical (gelatin) chain.

Amida II bonds indicate the presence of C-N stretching and N-H bending with an absorption area of 1575-1480 cm^{-1} and lower absorption shows that the NH group is involved in bonds with α chains, a complete triple helix of collagen and hydrogen bonds (Zaelani *et al*., 2019). Meanwhile, Amida III has an absorption area of 1229-1301 $cm^{-1,}$ which shows C-N stretching and N-H bending (Kong and Yu, 2007) while the uptake area of collagen from these three fish species indicates the presence of amide III. Peak Amide III has an intermolecular interaction with collagen (Chuaychan, 2016).

Amide A, B, I, II, and III peaks have an absorption area of 3433, 2926, 1641, 1549, and 1240 cm^{-1} and this was reported on mackerel fish skin collagen (Li *et al*., 2013). This is similar to the results of collagen from all the fishes, specifically parrotfish (*C. sordidus*) while the results of the analysis using FTIR showed that the overall treatment does not have a significant effect on the functional groups of the collagen formed.

The molecular weight of the α_2 collagen from pink ear emperor is slightly lower than the collagen of the sole fish skin (*Aseraggodes umbratilis*) which ranges from 116-118 kDa (Arumugam *et al*., 2018). This difference depends on the extraction method, amino acid composition, species, and habitat source of collagen (Liu *et al*., 2008).

Furthermore, the presence of two identical α chains indicates that the collagen extracted belongs to type I (Ogawa *et al*., 2004) which contains two identical chains α1 connected by hydrogen bonds to each other and to the third chain with different amino acid sequences called α_2.

Most species studied exhibit the characteristics of type I collagen namely, two α chains of around 100 kDa (Sotelo *et al*., 2016). The molecular weight obtained from this study is similar to pooled collagen bubble striped catfish (*P. hypophthalmus*) of 98-100 kDa (Vijayan *et al*., 2018) and catfish skin collagen of 94 and 98 kDa, but lower in the collagen present the skin of adjoining fish of 116-118 kDa (Arumugam *et al*., 2018). Generally, type I collagen contains three polypeptide chains of 94 kDa, two α1 chains, and one α2 chain with an average molecular weight of 290 kDa. These three polypeptides are known as α chains and they encircle other chains such as strings to form a triple helix structure.

The amount of alanine and proline present in collagen obtained from parrotfish is similar to the yield from mackerel skin with 13 and 10% proline (Li *et al*., 2013). Furthermore, the glutamic acid and aspartic acid compositions were moderately high while histidine and isoleucine were low. The glycine yield value of this study is consistent with the yield obtained from Skate (*Raja kenojei*) skin which has 33.4% glycine (Shon *et al*., 2011), rowing skin with 34.9% and this value is higher than Brownstripe Red snapper (*Lutjanus vita*) skin and white snapper bark with 23-25% (Jongjareonrak *et al*., 2005) and 18% (Jamilah *et al*., 2013), respectively. A similar trend pattern was observed in the collagen from other fish species such as carp fish and red snapper (Wang *et al*., 2014; Nurilmala *et al*., 2021), and high glycine, proline, and alanine content are characteristic of collagen. It has a high level of proline, alanine, glutamic acid, and aspartic acid, but a low level of histidine, tyrosine, and isoleucine (Jamilah *et al*., 2013).

Glycine is commonly found in the central regions of the collagen α chain, followed by the amino acids proline (Li *et al*., 2018) and alanine which is present in carp skin at 11.8 and 11.4%, respectively (Duan *et al*., 2009). The tri-peptide sequence is dominated by proline and hydroxyproline which are responsible for the stability of the collagen helices. Differences in amino acid content among animals are related to differences in the temperature of their habitat and the temperature used for collagen denaturation, the higher the amino acid, the higher the temperature of collagen denaturation (Muralidharan *et al*., 2013). However, the helical collagen is more stable with a higher amino acid content because the molecular structure is maintained by changes in the secondary structure of the polypeptide chain. It is also maintained by the ability of hydrogen bonds in the hydroxyproline group (Li *et al*., 2013).

Conclusion

Based on the results, the longer the extraction time, the higher the collagen yield, and the collagen obtained from the three fishes [parrotfish (*C. sordidus*), pink ear emperor (*L. lentjan*), and tiger grouper (*E. fuscoguttatus*)] were all identified as collagen type I showing similar protein pattern and FTIR spectra. High content of glysin, proline, and alanine is proof of the success of the extraction.

Hence, all samples are a good source of collagen for food and other applications.

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Author's Contribution

Asep Awaludin Prihanto: Conception and design, analysis and interpretation of data, drafting the article, final approval of the manuscript.

Abdul Aziz Jaziri: Acquisition of data, analysis, and interpretation of data, drafting the article, final approval of the manuscript.

Mohammad D. Pratomo, Sabrina E. Putri and Cahyaning Fajriati: Acquisition of data, analysis, and interpretation of data.

Rahmi Nurdiani and Muhamad Firdaus: Interpretation of data, drafting the article, final approval of the manuscript.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and that no ethical issues are involved.

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