Isolation and Characterization of Collagen from Salmon (*Salmo salar)* Skin Using Acid Method

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Abstract

Microalgae are gaining interest as a potential renewable energy resource for biodiesel Collagen is a type of protein that is plentiful and has a wide variety of applications. Collagen is often derived from porcine and bovine. However, both sources are considered to transmit disease, particularly porcine sources, which haram to Moslem. There are some methods for collagen extraction, one of them using the acid method. This study aimed to obtain the best extraction method and analyze collagen from alternative sources, specifically salmon skin, which is abundant. This study included two stages, i.e., pretreatment with sodium hydroxide (0.01 M, 0.03 M, 0.05 M, 0.1 M) and time (2, 4, 6, 8, 10, and 12 h) for eliminating non-collagen proteins prior from salmon skin. Moreover, the extraction was done with acetic acid (0.1 M, 0.3 M, 0.5 M) and time (24 h, 48 h and 72 h) for collagen extraction. The results indicated that the extract was collagen. It was indicated by high glycine, alanine, and proline amino acid content. Based on electrophoretic pattern, salmon skin collagen has two α chains (α1 and α2) and one α chain. The propeptides of type I procollagen have a similar composition. The most effective method for eliminating non-collagen proteins prior to extracting collagen from salmon skin was pretreatment with a 0.05 M NaOH solution for ten h. Collagen extraction using acetic acid at a concentration of 0.5 M for 48 h yielded 12.79%.

Keywords: acetic soluble collagen, collagen, salmon skin, yield

Introduction

Collagen is a fibrous protein critical for the biomedical and cosmetic industries due to its skin advantages, biocompatibility, bioactivity, and low immunogenicity (Rodríguez et al., 2018). Collagen's primary structure comprises amino acids, primarily glycine, proline, and hydroxyproline. As of now, 29 different types of collagens have been identified (Leon-Lopez et al., 2019). Collagen is widely applied in the cosmetic, pharmaceutical, medicine, and food industries. This extensive application is due to its physicochemical properties, excellent bio-compatibility, nontoxicity, and biodegradability (Bhagwat & Dandge, 2016).

Collagen can be extracted from various animal species and is frequently obtained as a by-product. Collagen is mostly found in skin, tendons, cartilage, and bones. Another source is the by-product of porcine, bovine, and poultry slaughter (Jia et al. 2010; Silva & Penna 2012; Moraes & Cunha 2013; Hashim et al., 2014; Nagai et al., 2014; Munasinghe et al., 2015; Suparno & Prasetyo 2019). Collagen products derived from bovine and poultry have downsides, including the danger of developing diseases associated with transmission risks, such as prion bovine spongiform encephalopathy (BSE), foot and mouth disease (FMD) (Schmidt et al., 2016), and bird flu (Alves et al., 2017). Meanwhile, porcine collagen extract is associated with halal status in the Islamic religion.

Furthermore, numerous studies are currently being conducted on alternate collagen sources, including those derived from fish processing by-products (Wang et al., 2014). According to Kolodziejska et al. (2008), fish skin is a good raw material for isolating collagen because collagen composes 80% of fish skin protein. One of the potential fish skins is salmon skin. Salmon (*Salmo salar*) is one of the fish used to make skin-off fillets. From 2012 to 2017, the value of salmon imports climbed by an average of 47.83% yearly (KKP, 2018). The import value of salmon increased by 46.43% per year based on the import value of fishery products from 2012 to 2017 (KKP, 2018). Salmon skin contains 45.11% protein (Zhang et al., 2022). According to

Nurjanah (2010), the protein content contained in fish ranges from 15-25%.

Collagen can be extracted using various methods, including acid extraction, hydro extraction, enzymes, and combinations (C. Huang et al., 2016; Kolanus et al., 2019; Schmidt et al., 2016; Wang et al., 2014). Organic acids such as acetic acid, citric acid, lactic acid, and inorganic acids such as hydrochloric acid can be used for acid extraction. Organic acids, on the other hand, are more efficient than inorganic acids (Kiew & Don, 2013). Due to its effectiveness, acetic acid is more frequently used to extract collagen than other acids. According to Liu et al. (2015), acetic acid dissolves non-crosslinked and crosslinked collagen more effectively. Therefore, the research aimed to obtain the best treatment based on the acetic acid concentration and immersion time in the collagen extraction process. In addition, the collagen produced will be analyzed based on its molecular weight, group function, and amino acid content.

Materials and Methods

This research was conducted from January 2020 to August 2020. The research was conducted in two stages, namely pretreatment and extraction. The materials used were salmon skin obtained from the Center for Coastal and Ocean Resources Studies (PKSPL) IPB, NaOH (Merck), acetic acid (Merck), 96% ethanol (Merck), NaCl, and distilled water. The equipment used was a cooling chamber, vacuum pump (Chemker 300), pH-meter, UV-vis spectrophotometer (GENESYS 50 UV-Vis Spectrophotometers Thermo Scientific), magnetic stirrer with hot plate stirrer (Corning Inc), water bath (DAIHAN Scientific Indonesia), centrifuge (Himac Technologies Co., Ltd.), freeze dryer (Martin christ Alpha 1-2 LDplus), and cut-off dialysis bag 12 KDa – 14 KDa.

Pretreatment

With slight modification, the pretreatment method followed Liu et al. (2015). Non-collagen proteins were removed from salmon skin after 24 hours of immersion in 1:10 alcohol and replaced after 12 hours at 10°C. The skin was then immersed for 12 hours in an alkaline NaOH solution (0.01, 0.03, 0.05, and 0.1 M) at a 1:10 (w/v) ratio, with observation times of 2, 4, 6, 8, and 10 h at 10°C. Every two hours, the alkaline solution was replaced. The samples were neutralized with cold distilled water by soaking the sample until pH neutral. The dissolved protein content was analyzed on the remaining NaOH immersion water to determine the concentration of alkali and the best immersion time. The sample was analyzed as follows (Bradford, 1976).

Acid Soluble Collagen (ASC) Extraction

The collagen extraction was conducted, as mentioned by Liu et al. (2015). Fish skin from the pretreatment stage was extracted using acetic acid at a solid-to-solvent ratio of 1:10 (w/v) for a specified period at 10°C. The extract was filtered using a calico cloth to obtain a collagen filtrate. The filtrate was coagulated using 2.6 M NaCl for 24 hours and centrifuged at 10,000 rpm for 30 minutes. The precipitate was dialyzed with 0.5 M (1:2) acetic acid against distilled water for 12 hours. Collagen was dried using freeze-drying. This stage used a factorial, completely randomized design with two factors: the concentration of CH_3COOH (0.1 M, 0.3 M, 0.5 M) and immersion time (24 h, 48 h, 72 h). The analyses carried out were yield (%) (AOAC, 2019), molecular weight (Cordeiro *et al*., 2020), functional groups (Kiew & Don, 2013), and amino acids (Waters, 2012).

Analysis and Characterization

Non-collagen protein

Bovine Serum Albumin (BSA) is the standard. The Bradford test measured the concentration of non-collagen proteins in the leftover NaOH immersion water. Bradford's solution was prepared by mixing 10 mg of Coomassie Brilliant Blue (CBB) with 5 mL of 95% ethanol, then adding 10 mL of 85% phosphoric acid solution and distilled water until the volume reached 500 mL. The Bradford solution was then filtered using filter paper. Determination of dissolved protein by the Bradford method was carried out using a spectrophotometer. A 0.01 mL sample was mixed with 1.99 mL of distilled water and vortexed. The material was vortexed after 5 mL of Bradford's solution was added. The absorbance of the sample was measured with a spectrophotometer at 595 nm (Bradford 1976).

Yield

The collagen yield (%) was calculated by comparing the dry weight of collagen to the weight of raw skin before extraction (AOAC, 2019).

The Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed by the method of Laemmli (1970). Separating gel was prepared to harden in the glass for 60 minutes, and stacking gel was added to harden. In the preparation of stacking gel, wells were prepared for sample injection using a well comb. SDS-PAGE analysis of collagen requires pretreatment by dissolving 2 mg of collagen in 1 mL

of 5% SDS and heating in a water bath of 85°C for 1 hour. Collagen in the pretreatment was then centrifuged at 8000 rpm for 5 minutes. The supernatant was taken, added with buffer in a 1:1 (v/v) ratio, and heated in a water bath of 85°C for 10 minutes. 10 L of the sample and 5 L of the marker were injected into the electrophoresis well. The marker was a protein marker with a molecular weight of 10-250 KDa. Electrophoresis was run constantly at a current of 13 mA and 100 volts for 2 hours. The staining and destaining processes were carried out after removing the gel from the glass slab. The staining process with Coomasie Blue was carried out for 1 hour and destaining for approximately 2 hours until the protein bands were visible. Photocapt software was used to determine the molecular weight.

Fourier transform infrared (FTIR) Spectroscopy

The analysis of specific functional groups in collagen was carried out using the Fourier Transform Infrared Spectrophotometer (FTIR) principle. The collagen to be examined was pelletized using a potassium bromide solution (KBr). 2 mg collagen and 100 mg KBr were pulverized to a fine powder. The collagen mixture was molded in a pellet mold. The pellets were molded using a hydraulic pump to minimize the water content in the mixture. After removing the pellet from the mold, it was placed on a tablet holder for FTIR analysis. Infrared rays were fired from the infrared spectrophotometer IR-408 nm onto pelletized collagen. The frequency of the collagen wavenumber was in the range of 500-4000 cm^{-1}. Functional group detection was generated on a monitor that displayed wavenumber absorption peaks ((Muyonga et al., 2004).

Amino Acid

The Amino Acid Analysis was determined by ultra-performance liquid chromatography (UPLC). Prior to operation for 2-3 hours, the UPLC device was cleaned with eluent. Before usage, the syringe was washed with distilled water. UPLC consists of two stages: preparation of sample solution and preparation of the standard solution or standard solution (Nollet, 2004; Waters, 2012).

Statistical Analysis

All the experiments were performed in triplicate, and a completely randomized design was used. Data was presented in means±standard deviation. Analysis of variance was performed, and Duncan's multiple range test did mean comparison with a 95% significant level. The analysis was performed using SPSS 16 software.

Results and Discussion

Non-collagen protein (Pretreatment)

The decrease in non-collagen proteins during the pretreatment stage is depicted in Table 1. The Table indicates that the longer the pretreatment time, the lower the non-collagen protein content, but the non-collagen protein content increases after a certain time. The levels of non-collagen protein in all treatments decreased until the 6th hour. After that, there was an increase in the concentration of 0.01 M. The analysis showed that the NaOH concentration and immersion time significantly affected the concentration of non-collagen protein ($P<0.05$).

At specific hours, as shown in Table 1, there was an increase in non-collagen protein levels in the sample. Skin caused it, and the NaOH solution was too long to interact. The interaction of skin and NaOH solution causes collagen protein to dissolve, and collagen will be hydrolyzed. According to Liu et al. (2015), numerous variables, including raw materials, time, temperature, and NaOH concentration, influenced the pretreatment stage. Depolymerization and deacetylation may occur due to the duration and ratio of alkaline immersion. NaOH-based solution has a role in the separation of strands from collagen fibers. Excessive OH leads to the partial breakage of covalent bonds in collagen's tertiary structure (Jaswir et al., 2011).

The best treatment at this stage is soaking for ten hours with a concentration of 0.05 M NaOH. This is because the lowest concentration of non-collagenous protein occurs in this condition. Reduced concentrations, according to Huang et al. (2016), may reduce the risk of collagen component degradation. Meanwhile, the prolonged soaking in NaOH results in the hydrolysis of collagen proteins (Liu et al., 2015). As a result, yields are declining. This optimal treatment will be a foundation for preparing the skin before collagen extraction.

Collagen Extraction

Yield

The analysis of variance (ANOVA) revealed a significant effect of acetic acid concentration (0.1M, 0.3M, and 0.5M) and immersion period (1, 2, and 3 days) on soluble collagen ($p>0.05$) (Table 2). It reveals that increasing the duration of the immersion increases

Table 1. Non-collagen protein content with NaOH concentration treatment and immersion time

Treatment	dissolved proteins (mg/ml)
0.01 M 2h	0.88 ± 0.11^{e}
0.01 M 4h	0.55 ± 0.00^{d}
0.01 M 6h	0.44 ± 0.02^{c}
0.01 M 8h	0.45 ± 0.01^{c}
0.01 M 10h	0.43 ± 0.00^{c}
0.01 M 12h	0.35 ± 0.01^{ab}
0.03 M 2h	0.87 ± 0.04^{e}
0.03 M 4h	0.56 ± 0.01^{d}
0.03 M 6h	0.44 ± 0.03^{c}
0.03 M 8h	0.43 ± 0.04^{c}
0.03 M 10h	0.42 ± 0.01^{bc}
0.03 M 12h	0.39 ± 0.02^{abc}
0.05 M 2h	1.11 ± 0.00^{f}
0.05 M 4h	0.54 ± 0.05^{d}
0.05 M 6h	0.44 ± 0.01^{c}
0.05 M 8h	0.41 ± 0.01^{bc}
0.05 M 10h	0.32 ± 0.01^{a}
0.05 M 12h	0.39 ± 0.01^{abc}
0.1 M 2h	1.15 ± 0.03^{f}
0.1 M 4h	0.54 ± 0.03^{d}
0.1 M 6h	0.43 ± 0.02^{c}
0.1 M 8h	0.39 ± 0.02^{abc}
0.1 M 10h	0.42 ± 0.01^{bc}
0.1 M 12h	0.43 ± 0.03^{c}

Note: Means in the same column with different superscripts differ significantly

the amount of water absorbed by the skin, allowing the collagen fibers to be separated and extracted more easily. Two days was the optimal time since it was capable of dissolving the most amount of collagen. Acetic acid concentrations of 0.1, 0.3, and 0.5 M significantly affected soluble collagen ($p>0.05$). Collagen extraction using acetic acid at a concentration of 0.5 M for 48 h yielded the highest yield of 12.79%. Thus, the concentration of 0.5 M was chosen as the most efficient concentration in extracting collagen from salmon skin. The best treatment yield in this research was bigger than Taberastani *et al*. (2012). The result was that 9.45% (rainbow trout skin) used acid extraction. Meanwhile, it was less than with the enzyme extraction method that used big snapper skin (18.74% db) and balloon skin fish (19.5% db) (Huang *et al*., 2011; Nalinanon et al., 2007).

According to Table 2, the highest yields were obtained using a two-day immersion treatment and 0.5 M of CH_3COOH. It was caused by the immersion time, which will dissolve collagen optimally. Then, the 0.5 M NaOH could be caused by the immersion of collagen for two days, which can dissolve collagen optimally. In addition, using a concentration of 0.5 M

Table 2. The yield of collagen from salmon skin with acetic acid concentration treatment and soaking time

Treatment	Yield (%w/w)
0.1 M 1d	3.41 ± 0.24^{a}
0.1 M 2d	8.63 ± 0.51^{c}
0.1 M 3d	5.49 ± 0.03^{b}
0.3 M 1d	10.63 ± 0.03^{de}
0.3 M 2d	10.17 ± 0.79^{de}
0.3 M 3d	9.76 ± 0.49^{d}
0.5 M 1d	11.09 ± 0.57^{e}
0.5 M 2d	12.79 ± 0.65^{f}
0.5 M 3d	12.37 ± 0.22^{f}

Note: Means in the same column with different superscripts differ significantly

can effectively break down the protein structure in fish skin. Rahmayanti (2014) discovered that immersing the skin in acetic acid for 24 hours did not entirely degrade the collagen in fish skin. Collagen has a low solubility in acids due to the crosslinking of aldehyde groups in the telopeptide region via covalent bonding (Wang et al., 2014). This telopeptide region ends a triple helix series of open (non-helix) collagen that will form intermolecularly and intramolecularly on type I collagen from skin or other soft tissues (Kalamajski et al., 2014).

Liu et al. (2015) stated that adding acetic acid above 0.5 M did not affect collagen yield. According

to Nurhayati et al. (2013), acetic acid concentrations of more than 0.5 M reduce the amino acid content of the collagen produced. A higher acid concentration may cause the substitution of negative ions in the salt by positive ions in the acid, thus disrupting the protein structure. The extraction in 0.5 M 48 hours is the best treatment for isolating collagen from salmon skin. Then, the yield was characterized by its amino acid component, functional group, and molecule weight.

Amino acids in salmon skin collagen

Amino acids are a collection of peptides that compose proteins. Based on their structure, amino acids consist of an amino group (NH_2), a carboxyl group (COOH), a hydrogen atom (H), and a radical group (R) bonded to a C atom known as carbon (Voet et al., 2013). Table 3 shows that collagen is composed of different types of amino acids. The highest amino acid content in collagen is glycine (27.98%), proline (12.07%), arginine (10.14%), and alanine (8.76%). According to Hema et al. (2013), glycine, proline, hydroxyproline, and alanine dominated the amino acid composition. Based on the research result, glycine percentage is like glycine in black ruff skin (collagen) (Bhuimbar et al., 2019) and the skin of clown featherback (Kittiphattanabawon et al., 2015).

Amino acids contribute to the stability of the helical structure of collagen. Amino acid analysis was performed to determine the characteristics of collagen. Table 3 presents the percentages of amino acids in collagen from salmon skin. The research revealed that the collagen extract from salmon skin was identified as collagen because the biggest amino acids were glycine and proline. According to Hema et al. (2013), the amino acid composition of collagen tends to be dominated by glycine, proline, hydroxyproline, and alanine. According to Nalinanon et al. (2011), type I collagen contains a high glycine concentration, alanine, and proline. This study may demonstrate that the protein extract obtained from salmon skin was collagen due to its high glycine, alanine, and proline amino acid content.

Proline is necessary for the collagen structure to remain intact. In this study, proline content was lower than in Caesionidae skin (Astiana et al., 2016)—high proline content in collagen protein results in increased heat stability. Proline is a unique amino acid in collagen because it maintains the protein's structural integrity. According to Huang et al. (2011), the pyrrolidin rings of the amino acid proline and hydroxyproline constrain the conformation of the polypeptide chain and contribute to the triple helix's heat stability.

Functional groups and molecular weight of salmon skin collagen

Collagen extracted from salmon skin has five infrared absorption bands: Amide A, Amide B, Amide I, Amide II, and Amide III (Figure 1). Table 4 summarizes the wave absorption area. The collagen absorption area shows the character of each functional group. Salmon skin collagen exhibits a complying amide absorption area to the absorption region standard.

Table 3. Amino acid percentage in salmon skin collagen

Amino acid	ASC from salmon (%)[1]	ASC from Caesionidae (%)[2]
L-Methionine	1.83	1.34
L-Cystine	0.35	-
L-Histidine	1.93	-
L-Threonine	2.91	3.41
L-Proline	12.07	12.15
L-Tyrosine	0.44	-
L-Leucini	2.60	2.87
L-Aspartic acid	5.69	5.95
L-Lysine	3.41	5.9
L-Glycine	27.98	25.09
L-Arginine	10.14	10.12
L-Alanine	8.76	13.71
L-Valine	1.92	1.48
L-Isoleucine	1.32	0.86
L-Phenylalanine	2.77	1.73
L-Glutamic acid	10.23	12.49
L-Serine	5.61	2.84

[1]Research results of salmon skin, Research results from [2]Astiana et al. (2016)

Collagen comprises Amides A, B, I, II, and III. According to Liu et al. (2012), the absorption peak of Amide A demonstrates the N-H stretching vibration. Hydrogen bonding could shift the N-H peptide to a lower frequency (Hashim et al., 2014). According to Joeng et al. (2013), Amide B is one of the bands that determine the characteristics of collagen. The wavenumber indicating Amide B absorption is created by the asymmetric stretch of CH_2 (Chen et al., 2015). Absorption of wavelengths on the Amide I group shows stretching vibrations of C- or O- (C=O bonds) along the polypeptide chain (Hashim et al., 2014). Amide I consists of four components of the secondary protein structure: helix, -sheet, -turn, and random coils that overlap each other (Kong & Yu, 2007). The Amide II group contains N-H bending and C-N stretching groups. The amide group III is associated with N-H bending and C-N stretching (Kong & Yu, 2007). According to Lin & Liu (2006), the wave spectrum between 1200-

1300 cm^{-1} is the signature of the collagen molecule, which is related to specific tripeptides (Gly-Pro-Hyp). The triple helix structure of collagen could be seen from the Amide III wavenumber. The intensity ratio between the Amida III and 1450 cm-1 bands was 1.16. This ratio shows a value close to 1, indicating a triple helix structure (Matmaroh et al., 2011).

The molecular bands of salmon skin collagen are presented in Figure 2. The analysis showed that α, α1, and α2 bands were found in the samples with molecular weights of 179.92 kDa, 58.11 kDa, and 45.61 kDa, respectively. The basic structure of collagen consists of a triple helix with twin chains α1 and α2 (Silvipriya et al., 2015). According to Chi et al.

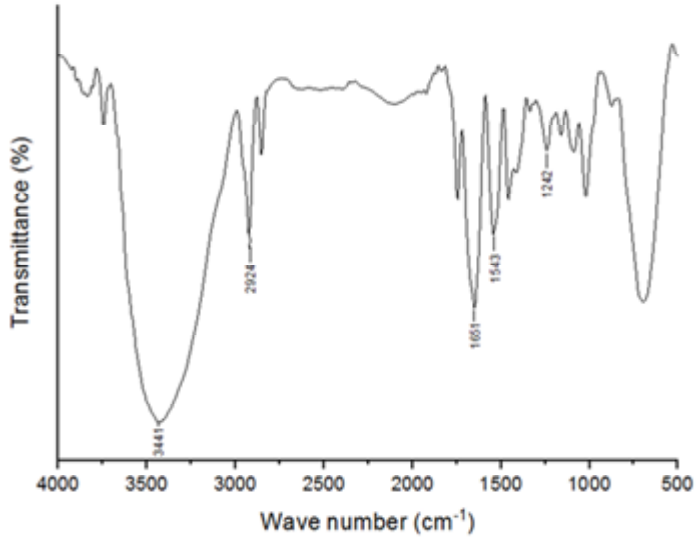

Figure 1 Infrared spectra of salmon skin collagen extracted from acetic acid.

Table 4. The wave absorption of the functional group

Absorption area	Wave number at the absorption peak (cm^{-1})	Standard
Amida A	3441	3490-3430*
Amida B	2924	2935-2915*
Amida I	1651	1690-1600**
Amida II	1543	1575-1480**
Amida III	1242	1301-1229**

(2014), the structure of α (α chain dimers) indicates the presence of covalent crosslinks in the collagen molecule. Lin and Liu (2006) reported electrophoretic patterns of collagen with molecular weight distribution in the 25–150 kDa range. The result had similar characteristics with albacore tuna (Hema et al., 2013), dog shark (Hema et al., 2013), rohu (Hema et al., 2013) and black ruff (Bhuimbar et al., 2019) that showed collagen α1, α2 and α band.

Salmon skin collagen was categorized as type I collagen based on molecular weight analysis using bands α1 and α2. It is consistent with Huang et al. (2016) that type I collagen has two chains in the form of [α1 (I) α2 (I)], which is a distinguishing trait. In addition, Riaz et al. (2018) reported that the presence of Amide A at a higher peak intensity of 3350 cm”1 could be pleasing with Col-I. The peak intensity of 1632 cm”1 shows the arrangement of high-order collagen structures, which refers to the -sheet and triple-helix structures. Type I collagen is an extracellular protein matrix with the characteristics of increasing cell proliferation, directly affecting cell physiology and morphology (Cardoso et al., 2014). Col-1 can be applied in tissue engineering, medical devices, the pharmaceutical industry, cosmeceutical, and the food industry. Col-1 has a high potency to use in several sectors. It caused high cell affinity, biodegradability, weak antigenicity and high biocompatibility (Naomi et al., 2021).

Salmon skin collagen analysis revealed the presence of α1 and α 2, indicating that the structure was a triple helix. It follows the statement of Silvipriya et al. (2015) that the basic structure of collagen consists of a triple helix with α1 and α2 twin chains. In addition, salmon skin collagen contains a structure (α chain dimers) that indicates the presence of covalent crosslinks in the collagen molecule (Chi et al., 2014).

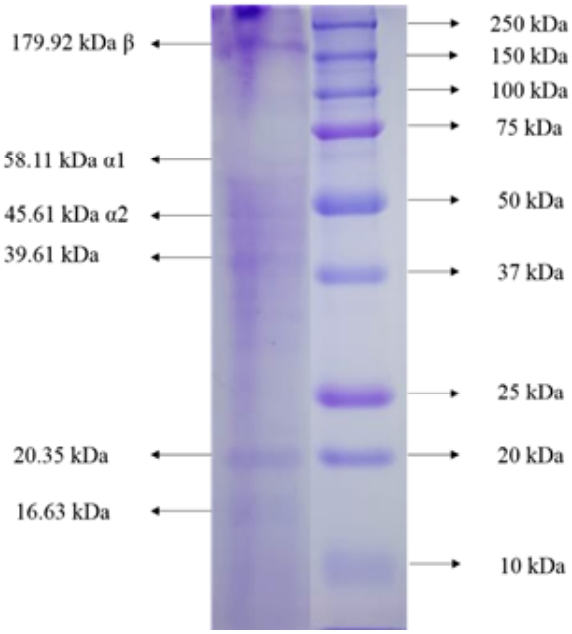

Figure 2. The pattern of collagen protein bands from salmon skin extracted with acetic acid (A) and (M) marker.

Conclusion

The collagen was extracted by acetic acid with 0.5 M for 48 h as the best treatment. The yield was 12.79% from salmon skin. The amino acid composition consists of high glycine, alanine, and proline. SDS-Page shows α1, α2, and α band, and FTIR shows absorption peaks in amides A, B, I, II, and III, indicating that the extracted collagen was collagen type I. Then, Pretreatment with 0.05 M NaOH for ten h was the most effective method for eliminating non-collagen proteins prior to extracting collagen from salmon skin. The high collagen produced an alternative for reducing or eliminating porcine collagen in foods, beverages, and others. This potency also supports halal policies in Indonesia.

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Supplementary Materials

Supplementary materials is not available for this article.

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