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ISOLATION AND CHARACTERIZATION OF COLLAGEN FROM SALMON (*Salmo salar*) SKIN USING PAPAIN ENZYME

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Abstract

Salmon skin is a fish byproduct that is rich in collagen. There are several methods to extract collagen from the skin, one of which involves combining chemical (acid) and enzymatic (protease) processes. Papain soluble collagen (PaSC) refers to the collagen that is obtained through the use of papain. This collagen can be classified as halal. The objective of this study was to ascertain the optimal duration and concentration for extracting collagen from salmon skin, taking into account both the yield of collagen and its chemical properties. The skin was obtained by treating it with a mixture of acetic acid (0.5 M) and papain enzyme (500, 1,000, and 1,500 U/mg/g skin) for 1, 2, and 3 hours. The parameters examined encompassed heavy metal content, collagen yield, amino acid composition, functional group analysis, and molecular weight determination. The maximum collagen solubility was achieved by combining acetic acid at a concentration of 0.5 M with papain enzyme at a concentration of 1,000 U/mg/g skin for a duration of 2 hours. The percentage of PaSC extracted from salmon skin was 15.38% (dry basis). The dominant amino acids in PaSC were proline, glycine, and arginine. The molecular weight distribution range of the collagen was approximately 20–142 kDa. According to the FTIR spectrum, the PaSC extraction did not alter the triple helix structure.

Keyword: amino acid, byproduct, extraction, PaSC

Isolasi dan Karakterisasi Kolagen dari Kulit Ikan Salmon (*Salmo salar*) menggunakan Enzim Papain

Abstract

Kulit ikan salmon merupakan salah satu hasil samping yang dapat diolah menjadi kolagen. Kolagen larut papain (PaSC) adalah istilah yang digunakan untuk menggambarkan kolagen yang diekstraksi menggunakan kombinasi asam dan enzim papain. PaSC berpotensi menjadi sumber kolagen halal. Penelitian ini bertujuan untuk menentukan waktu ekstraksi dan konsentrasi enzim papain terbaik dalam menghasilkan kolagen kulit ikan salmon berdasarkan persentase rendemen dan sifat kimia. Kulit ikan salmon diekstrak menggunakan kombinasi asam asetat (0,5 M) dan enzim papain (500; 1.000; 1.500 U/mg/g kulit) selama 1; 2; dan 3 jam. Parameter yang dianalisis dalam penelitian ini meliputi logam berat, rendemen, asam amino,

gugus fungsional, dan berat molekul. Hasil penelitian menunjukkan bahwa perlakuan kombinasi asam asetat konsentrasi 0,5 M dan enzim papain 1.000 U/mg/g selama 2 jam menghasilkan kelarutan kolagen yang maksimal dengan rendemen sebesar 15,38% (bk). Kolagen PaSC kulit ikan salmon terdeteksi memiliki asam amino prolina, glisina, dan arginina. Distribusi berat molekul kolagen berada pada rentang 20-142 kDa. Struktur triple helix kolagen tidak mengalami perubahan selama proses ekstraksi berdasarkan analisis FTIR.

Kata kunci: asam amino, ekstraksi, hasil samping, PaSC

INTRODUCTION

The fish processing business generates byproducts. Byproducts from fishery processing accounted for 70% of the total fish weight. The byproducts consist of fish bones and scales, which make up 14-20% of the total amount (Boronat *et al*., 2023). Unprocessed fish-cutting waste is frequently discarded, which leads to environmental contamination. Some byproducts are converted into fish meal (Lestari *et al*., 2024) and oil (Suseno *et al*., 2023; Suseno *et al.*, 2020), but these processes do not add much value. The byproducts retain high concentrations of collagen, minerals, vitamins, and other beneficial substances (Subhan *et al*., 2021).

Salmon meat is rich in critical amino acids, which greatly enhance protein quality. The salmon-processing business generates significant quantities of by-products, particularly skins, which account for approximately 7% of the total volume (Aryee *et al*., 2012). The raw ingredients for gelatin and collagen production are sourced from bioresources that are not fully used (Jafari *et al*., 2020; Tylingo & Mania, 2016). Utilizing fish collagen can boost the producer's income and serve as a substitute biomaterial for various purposes.

The fish-processing business yielded skin with a significant concentration of collagen. The byproduct generated during fish processing has the potential to be a cost-effective source of collagen (Bhagwat & Dandge, 2016). The processing byproducts of fish include the highest concentration of collagen observed in fish skin. The collagen content of fish skin ranges from 5% to 30% (Afifah *et al*., 2019). Terzi *et al*. (2020) stated that collagen is mostly derived from skin and tendons. Collagen is a non-soluble fibrous protein present in the connective tissue of animals and constitutes the extracellular matrix. This particular protein has found extensive applications in several industries, such as leather and gelatin, food, cosmetics, photography, and the pharmaceutical sector (Ghomi *et al*., 2021; Schmidt *et al*., 2016). Collagen possesses various exceptional characteristics including mechanical qualities, biodegradability, and biocompatibility. Collagen possesses advantageous characteristics that render it valuable for various medicinal purposes such as wound healing, tissue engineering, skin replenishment, and photography (Ghomi *et al*., 2021).

Collagen is derived from diverse animal sources including pigs, cattle, and poultry. Multiple sources of collagen must be assessed for various reasons, including the risk of spongiform encephalopathy in cows and avian influenza in chickens. Furthermore, the utilization of pig-derived collagen is limited because of its non-compliance with the halal requirements of the Muslim community. Utilizing fish skin to produce collagen is a viable alternative for mitigating these issues. Several techniques can be used to extract collagen from fish skin, including acid-(Kittiphattanabawon *et al*., 2005; Matmaroh *et al*., 2011; Singh *et al*., 2011) and enzyme-based methods (Jamilah *et al*., 2013; Kittiphattanabawon *et al*., 2010; Singh *et al*., 2011). Enzyme extraction is more efficient than water and acid extraction because of the presence of cross-links in the collagen molecule. Collagen can be extracted using proteolytic enzymes, specifically pepsin, pancreatin, and trypsin (Matinong *et al.*, 2022). Zhang *et al*. (2011) found that porcine pepsin can break down molecules called telopeptides that are bound together in certain locations. Nevertheless, it is important to consider the tolerance of the Muslim community to the ingestion of porcine pepsin. Plant-derived proteases, such as papain, may serve as feasible substitutes for extracting collagen

from salmon skin. Furthermore, knowledge regarding collagen that is capable of dissolving in papain is scarce. The findings of this study revealed a novel source of collagen obtained from aquatic creatures that can be used as a substitute for collagen sourced from bovines and pigs. This study also introduced a cost-effective extraction process employing an enzyme derived from plants (Ramli *et al*., 2020).

This study employed a combination of the acid technique and papain enzymes. Papain, classified as EC 3.4.22.2, is composed of a single polypeptide chain of 212 amino acid residues. Papain is an endopeptidase that belongs to a group of enzymes that break peptide bonds within the protein chain at certain locations (Grzonka *et al*., 2007). The activity of the papain enzyme is characterized by the decomposition of substrates into products by the action of the amino acids histidine and cysteine located in the enzyme's active site (Sumarlin *et al*., 2012). Papain is a commercially available enzyme with an activity of 30,000 U/mg. The primary objective of this study was to extract collagen from salmon skin by using a combination of acid and papain enzymes. Hence, the objective of this study was to ascertain the ideal duration and strength of extraction to obtain the maximum amount of collagen from salmon skin and analyze its chemical properties.

MATERIALS AND METHODS

Papain Soluble Collagen (PaSC) Extraction (Modification Astiana *et al.,* 2016a; Jamilah *et al.,* 2013; Kittiphattanabawon *et al.,* 2019)

The initial step in the extraction of collagen involved the processing of salmon skin using a combination of acetic acid and papain. Salmon skin was acquired from the Center for Coastal and Ocean Resources Studies (PKSPL) at IPB University. The purpose of this pretreatment was to eliminate non-collagen proteins, lipids, and pigments. The salmon skin was immersed in alcohol (Merck) at a ratio of 1:10 for 24 h. The solution was then replaced twice every 12 h. Next, the fish skin was thoroughly cleansed until the scent of alcohol dissipated. The skin was subsequently submerged in a 0.05 M NaOH (Merck) solution with a weight-to-volume ratio of 1:10 for 12 h at a temperature of 10°C. The samples were neutralized by immersion in cold distilled water until the pH reached a neutral level. The pretreatment stage involved extracting the samples using acetic acid (Merck) at a ratio of 1:10 (weight/volume) at a concentration of 0.5 M. The enzyme papain (Himedia) was introduced at treatment concentrations of 500, 1,000 U/mg/g skin, and 1,500 U/mg/g skin (factor 1). The extraction procedure was conducted at a temperature of 40°C with soaking durations of 1, 2, and 3 h (a factor of 2). The skin and filtrate were isolated using a vacuum pump (Chemker 300), and thereafter, NaCl 1.8 M was introduced to the filtrate. The solution was precipitated for 24 h, followed by centrifugation at 10,000 rpm for 15 min. Collagen was supplemented with a 0.1 M solution of acetic acid (Merck) at a concentration ratio of 1:1 (weight/volume). Furthermore, the sample was introduced into the dialysis bag with a molecular weight cut-off of 12 kDa-14 kDa. Subsequently, the sample was dialyzed against distilled water for 24 h at 10°C. The samples were subsequently subjected to freeze-drying using a Martin Christ Alpha 1-2 LDplus freeze dryer to obtain collagen in a dry state.

Heavy Metal Analysis

An atomic absorption spectrophotometer (ASS) was used to analyze the heavy metal concentration in salmon skin. The analysis method relied on the standards SNI 01-2354.5-2006 for cadmium (Cd) (BSN, 2006a), SNI 01-2354.6-2016 for mercury (Hg) (BSN, 2016), SNI 01-2354.7-2006 for lead (Pb) (BSN, 2006b), and SNI 01-4866-1998 for arsenic (As) (BSN, 1998). The procedure involved the destruction of samples, followed by the reading and calculation of the heavy metal content.

Salmon Skin Characterization

Salmon skin was characterized by proximate analysis. The ash content followed SNI 01-2354.1-2006 (BSN, 2006c), SNI 01-2354.2-2006 for moisture content (BSN, 2006d), SNI 01-2354.3-2006 for lipid content

(BSN, 2006e), SNI 01-2354.4-2006 to protein content (BSN, 2006f), carbohydrate by different, and fiber content (SNI 01- 2891-1992) (BSN, 1992).

Collagen Yield

Collagen yield was determined by comparing the weight of collagen after drying to the weight of raw skin prior to extraction, according to the AOAC (2019) method.

Amino Acid Analysis

Amino acid analysis was performed using ultra-performance liquid chromatography (UPLC). The UPLC device was cleaned with an eluent for a period–2-3 hours before the operation. Prior to use, the syringe was cleansed with distilled water. UPLC involves two stages: the production of the sample solution and the preparation of the standard solution or standard solution (Nollet, 2004; Waters, 2012).

Functional Group Analysis

Identification of particular functional groups in collagen was conducted using Fourier transform infrared spectroscopy (FTIR). FTIR spectroscopy quantifies the wavelength at which a sample absorbs IR radiation of a specific intensity. FTIR spectroscopy is a widely used analytical technique for identifying the functional groups in a molecule. Each light frequency, including infrared, corresponds to a distinct wavenumber. When a specific infrared (IR) frequency is absorbed by an organic substance, it causes the compound to vibrate (Nagarajan *et al*., 2012).

Collagen was condensed into pellets using potassium bromide solution (KBr). Collagen and KBr were ground into fine powders with masses of 2 mg and 100 mg, respectively. The collagen mixture was shaped using a pellet mold. The pellets were shaped using a hydraulic pump to reduce moisture levels in the mixture. Upon extraction of the pellet from the mold, it was positioned on a tablet holder for FTIR examination. Infrared spectrophotometer IR-408 nm emitted infrared photons onto the pelletized collagen. Collagen wavenumbers ranged from 500 to 4,000 cm^{-1}. Functional group detection was performed using a monitor that indicated absorption peaks in the wavenumber (Muyonga *et al*., 2004).

The Molecular Weight Analysis with SDS-PAGE

The Laemmli method (1970) was used to conduct sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After a 60-minute period of enabling the separating gel to set in the glass, the stacking gel was introduced. During production of the stacking gel, wells were created for sample injection using a sterile comb. To prepare collagen for SDS-PAGE analysis, 2 mg of collagen was dissolved in 1 mL of 5% SDS solution and then heated it for one hour in a water bath at a temperature of 85°C. Subsequently, the pretreated collagen was subjected to centrifugation at 8, 000 rpm for 5 min. Following collection of the supernatant, a buffer was introduced in a 1:1 (v/v) proportion, and the resulting mixture was heated at 85°C for a duration of ten minutes using a water bath. Subsequently, 5 liters of the marker and 10 liters of the sample were introduced into the electrophoresis wells. A protein marker with a molecular weight ranging from 10 to 250 KDa was used. Electrophoresis was conducted for a duration of two hours, with a constant voltage of 100 volts and a current of 13 mA. The gel was extracted from the glass slab, and staining and destaining processes were performed. The Coomassie blue staining method required one hour for the protein bands to become visible, whereas the destaining procedure took approximately two hours. The molecular weight was determined using the Photocapt program.

Statistical Analysis

The study employed a fully randomized design for all experiments, and each experiment was conducted three times. The data are presented as the mean ±standard deviation. The data were subjected to two-way Analysis of Variance (ANOVA), and means were compared using Duncan's multiple range test at a significance level of 95%. The analysis was conducted using SPSS 16.

RESULT AND DISCUSSION

Characterization of Salmon Skin

Heavy metals

According to SNI 8076:2014, salmon skin has levels of Hg, Pb, As, and Cd that are considered acceptable for fish and its processed products, as shown in Table 1. Fish can accumulate heavy metals in their bodies, which may pose a concern if they are swallowed. Food contains heavy metals, such as mercury (Hg), lead (Pb), arsenic (As), and cadmium. An analysis is conducted to determine the concentration of heavy metals in salmon skin in order to confirm that collagen products are free from heavy metal contamination.

Chemical composition

Prior to collagen extraction, salmon skin quality was assessed. The chemical makeup of raw materials may be used to ascertain the utilization of the material. Salmon skin was subjected to proximate analysis. Table 2 displays the chemical makeup found in the salmon skin.

Table 1 displays the chemical composition of the salmon skin. As shown in Table 1, the protein level in salmon skin was lower than that in the other samples. Furthermore, the fat and carbohydrate contents were higher than those of the other components. This has an impact on the collagen extraction procedure.

Fish are rich in protein. Mahboob (2015) found that as fish size and weight increased, protein and fat levels increased. Nagai and Suzuki (2000) demonstrated that type I collagen can be extracted from skin, bones, and fins. The majority of the protein is located in the skin, whereas scales and fins contain only a small quantity of protein. According to Widyanto *et al.* (2022) and Mahboob (2015), the protein content in the Lattice Monocle Bream Scale is only 20.33% (wb), whereas Catla Catla fin has a protein content of 18.37% (wb).

Elevated protein content enhances the significance of collagen extraction and may be linked to alterations in the extraction procedure (Zamorano-Apodaca *et al.*, 2020).

Table 1 Heavy metal content in salmon skin

Tabel 1 Logam berat kulit ikan salmon

Parameter (mg/kg)	Result	BSN standard (2014)
Mercury (Hg)	<0.001	max. 0.5
Lead (Pb)	<0.03	max. 0.4
Arsenic (As)	<0.01	max. 0.1
Cadmium (Cd)	<0.005	max. 0.1

Table 2 Chemical composition in salmon skin and other type fish skin

Tabel 2 Komposisi kimia kulit ikan salmon dan jenis ikan lainnya

Parameter (% wb)	Salmon	Lattice monocle bream[a]	Tilapia[b]	Yellowfin tuna[c]	Pangas catfish[d]
Moisture	58.42	65.10	61.38	57.42	67.22
Protein	20.76	28,55	35.70	37.45	27.19
Ash	2.02	1.05	0.11	0.49	0.16
Lipid	8.33	4.44	2.02	3.80	3.39
Carbohydrate	10.47	0.87	0.79	0.84	2.04
Crude fiber	0.33	-	-	-	-

[a]Widyanto *et al.* (2022); [b]Prastyo *et al.* (2020); [c]Nurjanah *et al.* (2021); [d]Yanti *et al.* (2022)

The fat content of salmon skin is higher than that of yellowtail fish (1.17%), sharks (0.16%), rohu (2.93%), and tilapia (6.8%) (Astiana *et al.*, 2016; Hema *et al.*, 2013). Pretreatment is necessary to remove contaminants such as minerals and lipids that are present in fish skin.

Characterization of Salmon Collagen

Yield

Collagen was extracted from salmon skin that was pretreated with 10% alcohol, followed by immersion in 0.05 M NaOH for 8 h to eliminate non-collagen components such as non-collagen proteins, lipids, minerals, and fragrance. In addition, the skin was immersed in a solution containing 0.5 M acetic acid, and further treated by immersing it in a solution of papain enzyme.

ANOVA analysis showed that varying concentrations of papain enzyme (500 U/mg/g skin, 1,000 U/mg/g skin, and 1,500 U/mg/g skin) and immersion durations (1 h, 2 h, and 3 h) had a statistically significant impact ($p<0.05$) on the dissolution of collagen. The investigation revealed that concentrations of 500 U/mg/g skin, 1,000 U/mg/g skin, and 1,500 U/mg/g skin had a substantial impact on collagen solubility. Collagen yield was positively correlated with soaking time. An enzyme concentration of 1,000 U/mg/g skin resulted in the maximum yield, but the yield was considerably affected by enzyme concentrations of 500 U/mg/g skin and 1,500 U/mg/g skin. The collagen yield was lower when the enzyme concentration was 1,500 U/mg/g skin than when the enzyme concentration was 500 U/mg/g skin. The optimal time for collagen production was 2 h, which resulted in the highest yield. This time point was significantly different from both the 3-hour and 1-hour immersion times (Figure 1).

As shown in Figure 1, treatment with a concentration of 1,000 U/mg/g applied to the skin for 2 h yielded the most favorable results compared to the other treatments. The yield was 15.38±0.29%. The maximum collagen yield, 15.58±0.59%, was obtained after treating the skin with 1,000 U/mg/g for 3 h; however, this increase was not considered significant. An extraction duration of less than 3 h was more efficient and effective. On the other hand, when the skin was treated with a concentration of 1,500 U/mg/g for 1 h, the collagen yield was found to be the lowest at 8.38±0.5%. The papain activity was elevated. The extracted collagen underwent further hydrolysis to be transformed into a more simplified form.

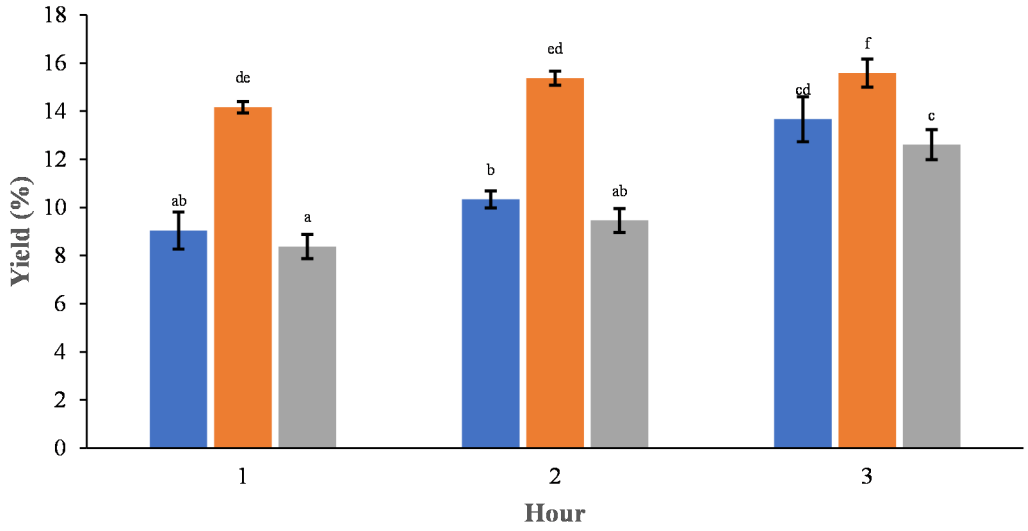

Figure 1 Collagen yield resulting from immersion in papain 500 U/mg/g kin (■), 1,000 U/mg/g skin (■), and 1,500 U/mg/g skin (■) for 1 hour, 2 hours, and 3 hours (n=3 repetitions)

Gambar 1 Rendemen kolagen dari ekstraksi enzim papain 500 U/mg/g kulit (■),1.000 U/mg/g kulit (■), dan 1.500 U/mg/g kulit (■) selama 1 jam, 2 jam dan 3 jam (3 ulangan)

Enzymatic collagen separation offers benefits over acid-based collagen isolation, as it yields a higher amount of collagen products (Nurhayati *et al*., 2018). The yield obtained in this work is greater than the yield obtained from isolating collagen from horse mackerel skin using the pepsin enzyme, which is reported to be 13.68% (wb) (Kumar & Nazeer, 2013). A separate investigation showed that the pepsin enzyme yielded only 7.26% (wb) collagen after isolating it from the skin of golden carp fish (Ali *et al*., 2018). Furthermore, the swim bladder, another organ, had a collagen content of 7.01±0.4% (wb) when subjected to papain extraction, as reported by Cahyono *et al*. (2022). These factors, including variations in enzyme concentrations, fish sources, and extraction procedures, could potentially be the origin of this phenomenon.

Amino acid composition

Amino acid analysis was conducted using the most effective treatment, which yielded 1,000 U/mg/g of skin over a period of 2 h. The amino acid content in salmon skin collagen was evaluated using ultra-performance liquid chromatography (UPLC). The amino acids examined in this investigation included the conventional 15 amino acids, L-cysteine and L-methionine. The percentage computation was derived from the total of all 15 amino acid concentrations, including L-cysteine and L-methionine. Table 3 illustrates the proportion of amino acids obtained from salmon skin collagen using acids and enzymes.

Collagen retrieved using the papain enzyme method contained the following amino acids in the highest proportions: glycine (27.77%), proline (11.34%), arginine (11.73%), and alanine (7.96%). The study results demonstrate that the collagen extract obtained from salmon skin using the enzyme technique can be categorized as collagen according to its predominant amino acid composition of glycine and proline. Hema *et al*. (2013)

Table 3 Amino acid percentage of salmon skin collagen

Tabel 3 Persentase asam amino kolagen kulit ikan salmon

Amino acid	Resulted PaSC (%)	Yellow tail skin PaSC (%)[a]
L-Glycine	27.77	26.18
L-Arginine	11.73	10.28
L-Proline	11.34	12.35
L-Glutamic acid	9.23	11.52
L-Alanine	7.96	13.56
L-Serine	6.20	2.93
L-Aspartic acid	5.41	5.25
L-Threonine	3.34	3.70
L-Phenylalanine	3.17	1.95
L-Lysine	3.01	5.64
L-Leucine	2.84	2.93
L-Histidine	2.07	-
L-Valin	2.00	1.56
L-Isoleucine	1.58	0.83
L-Methionine	1.46	1.33
L-Tyrosine	0.78	-
L-Cystine	0.13	-

[a]Nurhayati *et al.* (2018)

reported that glycine, proline, hydroxyproline, and alanine are the primary amino acids found in collagen. Muyonga *et al.* (2004) confirmed that glycine is the primary amino acid found in collagen. Kittiphattanabawon *et al.* (2015) also affirm that glycine is the primary amino acid responsible for the formation of collagen in the skin, accounting for one-third of all amino acids. These findings align with previous data, indicating that glycine is the predominant amino acid in collagen recovered using enzyme-based techniques. The results obtained are consistent with those of previous studies, which found that red snapper skin collagen contains 25.2% glycine (Jongjareonrak *et al.*, 2005), yellowfin tuna skin collagen contains 25.1% glycine (Woo *et al.*, 2008), and pepsin soluble collagen from Nile tilapia fish skin contains 34% glycine (Zeng *et al.*, 2009). Glycine inside collagen contributes to the assembly of three α-helix chains into a superhelical structure (Regenstein & Zhou, 2006).

Amino acids, which are peptides, are fundamental units that unite to form bonds and form proteins. A protein is a large molecule comprising several amino acids connected by peptide bonds (Sumandiarsa *et al.*, 2020). Amino acids play an important role in maintaining the stability of the helical structure of collagen. Amino acid analysis was performed to determine the properties of the collagen. The main collagen molecule consists of three polypeptide chains that coil to form a triple helix structure called Gly-X-Y. In this structure, X represents proline and Y represents hydroxyproline, which is responsible for the construction of the triple helix (Friess, 1998). Glycine decreases steric hindrance and initiates the formation of hydrogen bonds in helical structures (Fontaine-Vive *et al.*, 2009). Proline plays a crucial role in preserving the structural integrity of collagen and is a common amino acid in collagen (Tamilmozhi *et al.*, 2013).

Proline, an amino acid found in collagen, plays a crucial role in preserving its structural integrity. Proline is an amino acid that is found in significant amounts in collagen. The proline content of red snapper skin collagen is 12.96%, while that of white snapper skin collagen is 13.1% (Hardiyanti, 2017). Additionally, the proline content of yellowfin tuna skin collagen is 12.5% (Woo *et al.*, 2008), and that of Nile tilapia skin collagen is 10.2% (Zeng *et al.*, 2009). The findings of this investigation indicate that the proportion of proline amino acid content is almost the same as that previously reported in the literature, specifically at 12.07% and 11.3%, respectively. Elevated levels of proline in collagen protein enhance its resistance to temperature changes. Proline is a distinctive amino acid found in collagen because of its crucial role in preserving its structural integrity. Huang *et al.* (2011) found that the pyrrolidine rings of proline and hydroxyproline amino acids limit the conformation of the polypeptide chain and enhance the thermal stability of the triple helix.

Alanine is a nonpolar amino acid with an aliphatic R group comparable to the R group of the amino acid glycine. Alanine fulfils the same function as glycine, which contributes to the formation of three alpha helix chains. The alpha helix conformation requires lower energy expenditure to maintain the fundamental structure, yielding a protein that is more stable. Katili (2009) stated that collagen contains a significant amount of alanine, an amino acid.

Collagen extracted using acid and enzymes contained significant amounts of glutamic and aspartic acids, with concentrations of 10.23% and 9.22%, respectively. The study conducted by Cui *et al.* (2007) demonstrated that PSC has a high glutamic acid concentration of 10.39%. Saito *et al.* (2002) also reported that PSC from *Stichopus japonicus* had a high glutamic acid content of 10.90%. The abundant presence of glutamic acid imparts an umami flavor to salmon. Saito *et al.* (2002) demonstrated that the comparatively high levels of glycine, hydroxyproline, and hydroxylysine amino acids, along with the abundant presence of glutamic acid, indicate the dominance of collagen as the principal protein component. Additionally, high glutamic acid concentration contributed to the umami flavor. Nalinanon *et al.* (2011) said that type I collagen is rich in the amino acids glycine, alanine, and proline. This

work has verified that the protein extract from salmon skin is indeed collagen, as it possesses a significant abundance of the amino acids glycine, alanine, and proline.

Functional groups

The collagen functional groups were detected using Fourier Transform Infrared (FTIR) spectroscopy. The triple helix structure was verified by functional group analysis using FTIR. This structure is identified by the absorption ratio of amide III, which has an absorption peak at 1,450 cm^{-1} that is close to 1. According to Kong & Yu (2007), polypeptide and protein units produce nine characteristic IR absorption bands: Amide A, Amide B, and I-VII. The FTIR analysis in this work was undertaken to validate that the resulting molecule was collagen based on its functional groups. Figure 2 depicts the functional groups of salmon skin collagen extracted with the papain enzyme.

The papain enzyme-soluble collagen extracted from salmon skin exhibits five distinct infrared absorption bands, namely Amide A, Amide B, Amide I, Amide II, and Amide III. The collagen structure is composed of Amide A, Amide I, Amide II, and Amide III, as described by Muyonga *et al.* (2004). Joeng et al. (2013) identified Amide B as one of the bands that contribute to the defining features of collagen. Each of the five bands possesses a distinct absorption region. The collagen absorption regions provide information on the distinctive features of each functional group. The collagen absorption regions of salmon fish are both acid- and papain-enzyme-soluble. The standard absorption areas are listed in Table 4.

Liu *et al.* (2012) found that the absorption peak of Amide A corresponds to the stretching N-H vibration. Hydrogen bonds can cause a shift in the N-H peptide towards a lower frequency, as demonstrated by Hashim *et al.* in 2014. The wave number corresponding to the absorption of Amide B was generated by asymmetrical stretching of CH2. The amide I group exhibits wavelength absorption that corresponds to the stretching vibration of the C- or O- (C=O bond) along the polypeptide chain (Hashim *et al.*, 2014). The Amide I region encompasses four elements of the protein secondary structure: α-helix, β-sheet, β-turn, and random coil, which exhibit overlapping

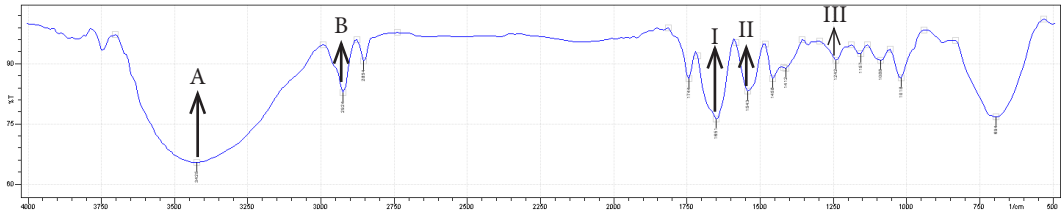

Figure 2 Infrared spectra of collagen extracted from a combination of acetic acid and papain
Gambar 2 Spektrum inframerah collagen yang diekstraksi kombinasi asam asetat dan papain

Table 4 Wave absorption PaSC from salmon skin
Tabel 4 Absorpsi gelombang PaSC dari kulit salmon

Absorption area	Peak absorption wave number (cm^{-1})	
	PaSC	Standard
Amide A	3,425	3,400-3,440[a]
Amide B	2,924	2,935-2,915[b]
Amide I	1,651	1,690-1,600[c]
Amide II	1,543	1,575-1,480[c]
Amide III	1,242	1,301-1,229[c]

[a]Doyle *et al.* (1975); [b]Gómez-Guillén & Montero (2001); [c]Kong & Yu (2007)

characteristics (Kong & Yu, 2007). The amide II group exhibited the existence–H bending and C-N stretching functionalities. Amide group III is associated with the bending of the N-H bond and stretching of the C-N bond (Kong & Yu, 2007). According to Lin and Liu (2006), the wave spectrum ranging from 1,200 to 1,300 cm^{-1} is distinct from that of the collagen molecule that is linked to a specific tripeptide (Gly-Pro-Hyp).

The amide absorption area of the papain-soluble collagen conformed to the normal absorption area. The absorption peak wave numbers on papain-soluble collagen were nearly identical in the absorption regions of Amide B, Amide I, Amide II, and Amide III. Table 3 presents the soluble absorption areas of salmon fish collagen for papain enzymes, as well as the standard absorption areas. The collagen absorption area demonstrates the characteristics of each functional group. PaSC exhibits amide absorption regions that adhere to the absorption area requirements. The peak wavenumbers of the PaSC absorption exhibited identical values in the Amide B, Amide I, Amide II, and Amide III absorption regions. This was a result of the significant elongation of NH bonds during this process.

The arrangement of the collagen triple helix, determined by its functional groups, is observable through the Amide III wavenumber. The wavenumbers were compared to 1,450 cm^{-1} wavenumbers to assess their level of similarity. The acid treatment and papain enzyme exhibited the same intensity ratio between the amide III bands and the 1,450 cm-1 band, which was measured to be 1.16. This ratio indicates a value close to 1. According to Matmaroh *et al.* (2011), the structure exhibits a triple-helix configuration when collagen integrity is approximately 1. This suggests that collagen PaSC has not undergone degradation into gelatin, as it still has a triple helix shape. These results suggest that the acid-enzyme papain, when used at a temperature of 40°C, produces a molecular product that is collagen and has not undergone degradation into a gelatinous state. Silvipriya *et al.* (2015) found that heating leads to the denaturation of collagen, resulting in the complete transformation of collagen triple helix chains into a single α-helix chain, known as gelatin. FTIR analysis detected the presence of amide A, amide B, amide I, amide II, and amide III groups, which provided information about the physical properties of the resulting collagen. The compound formed is collagen, as evidenced by the presence of triple helix structures in amides I and III.

The molecular weight of papain soluble collagen (PaSC)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze the molecular weight of the proteins. Electrophoresis is a technique that can be used to separate proteins by considering their molecular size and interaction with electric charge. Polyacrylamide gel is used as a support in electrophoresis to enhance the stability of the protein bands (Kryndushkin *et al.*, 2003). Figure 3 displays the molecular bands of the acid-soluble collagen and papain enzymes.

Collagen is fundamentally composed of a triple helix comprising two chains, α1 and α2 (Chi *et al.*, 2014). Gelse *et al.* (2003) found that the β-(α-chain dimer) structure of collagen suggests the existence of covalent crosslinks inside the molecule. The electrophoretic pattern of collagen, which exhibits a dispersion of molecular weights ranging from 25 to 150 kDa, was described by Lin and Liu in 2006. The molecular bands present in PaSC consisted of α1 (141.22 kDa) and α2 (100.21 kDa). Jamilah *et al.* (2013) found that the usage of the papain enzyme resulted in a reduction of protein molecular weight by up to 50 kDa. This was due to the ability of papain to break down molecules in the telopeptide and tropocollagen. This could potentially render the β-band in PaSC unidentifiable. Furthermore, Singh *et al.* (2011) and Huang *et al.* (2016) reported that pepsin breaks down the telopeptide-containing γ structure of collagen, resulting in the formation of α1 and α2 structures. This leads to an increase in the intensity of the α1 and α2 bands in the pepsin-soluble collagen. A similar phenomenon may have been observed with papain-soluble collagen in the present study.

Based on the molecular weight studies, PaSCs exhibited α1 and α2 bands. According to

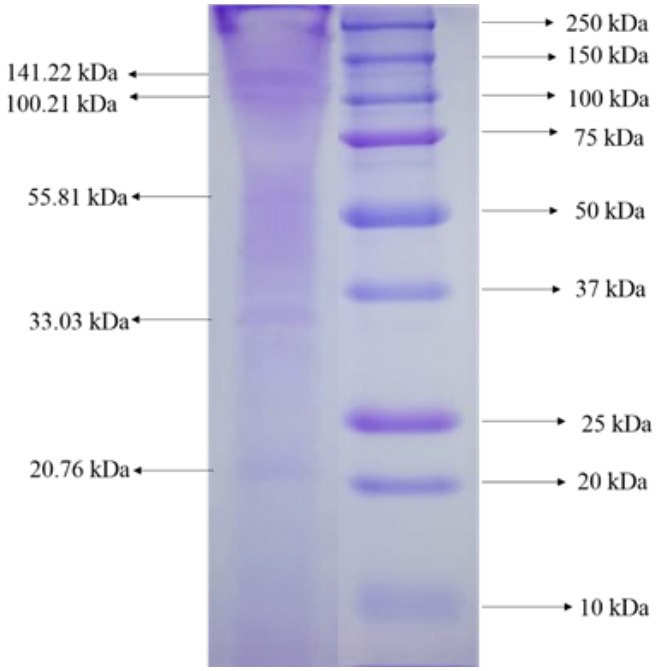

Figure 3 Band pattern of collagen protein extracted using papain enzymes, (A) PaSC, and (M) marker in kilodalton unit (kDa) enzymes

Gambar 3 Pola pita protein kolagen yang diekstrak menggunakan enzim papain, (A) PaSC, dan (M) penanda dengan satuan kilodalton (kDa)

Cardoso *et al.* (2014), the statement indicates that salmon skin collagen can be categorized as a type I collagen. Type I collagen is distinguished by the presence of heterologous α1 and α2 chains, which combine to form a triple helix. Type I collagen is characterized by the presence of two α-chains arranged as [α1 (I) α2 (I)]. Type I collagen, an extracellular matrix protein, plays a role in enhancing cell proliferation, leading to significant effects on cell physiology and morphology (Cardoso *et al.*, 2014). Type I collagen is present in large quantities in the form of fibrin, a protein with a fibrous structure. This protein has several applications in the pharmaceutical, biomedical, and cosmetics sectors (Gómez-Guillén & Montero, 2001). The extraction process yielded high-purity collagen, as confirmed by SDS-PAGE and FTIR analysis, which showed a characteristic profile consistent with that of type I collagen. Furthermore, as shown in Figure 3, the sample contained not only collagen type I but also additional constituents, such as collagen hydrolysate. The molecular weight of the collagen hydrolysate was determined to be 33.03 and 20.76 kDa.

CONCLUSION

Collagen from salmon skin can be extracted using papain. The optimal treatment involves utilizing the skin papain enzyme at a concentration of 1,000 U/mg/g and soaking for 2 h, resulting in the highest yield. The sample was identified as collagen, with a molecular weight ranging from 25 to 150 kDa, containing the amino acids glycine and proline, as well as other functional groups. Papain enzymes can enhance collagen production without causing any harm to the triple helix structure of collagen. Salmon skin can serve as a substitute source of collagen.

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