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Kulit Ikan Patin (*Pangasius hypophthalmus*) sebagai Sumber Nanokolagen: Pengaruh Waktu Ekstraksi dan Konsentrasi Asam Asetat dengan Metode Berbantuan Ultrasonik

Catfish (*Pangasius hypophthalmus*) Skin as a Nanocollagen Source: Impact of Extraction Time and Acetic Acid Concentration with Ultrasonication Assistance

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Abstrak

Ekstraksi kolagen dengan asam asetat yang tinggi dapat mempengaruhi karakterisasi dan merusak struktur kolagen. Selain itu juga berpengaruh terhadap tingkat stabilitas suhu pada kolagen, maka perlu metode kombinasi untuk mengurangi penggunaan asam asetat dalam ekstraksi kolagen. Ekstraksi kolagen dengan metode kombinasi asam asetat dan ultrasonikasi lebih efisien dan dapat membantu permasalahan tersebut. Ekstraksi kolagen kulit ikan patin menggunakan kombinasi konsentrasi asam asetat dan lama waktu ultrasonikasi dapat mempercepat ekstraksi kolagen, mengurangi penggunaan asam asetat, dan meningkatkan struktur kolagen. Penelitian ini bertujuan untuk melihat pengaruh potensi kulit ikan patin (*Pangasius hypophthalmus*) dengan kombinasi lama waktu esktraksi dan perbedaan konsentrasi asam asetat dengan ultrasonik sebagai sediaan nanokolagen. Penelitian ini menggunakan rancangan acak lengkap (RAL). Preparasi pembuatan kolagen kulit ikan patin menggunakan pelarut asam asetat konsentrasi 0.1 M dan 0.2 M dengan lama waktu ultrasonikasi 150 menit dan 200 menit dalam 4 perlakuan dengan tiga kali pengulangan. Analisa data menggunakan *Analysis of Variance* (ANOVA) yang dilanjutkan dengan uji Tukey. Hasil penelitian ini menunjukan bahwa kombinasi konsentrasi asam asetat dan lama waktu ultrasonikasi yang berbeda berpengaruh nyata ($P<0.05$) pada parameter rendemen kolagen kulit ikan patin. Rendemen tertinggi yaitu perlakuan 0.2 M asam asetat dan 200 menit waktu ekstraksi dengan nilai rendemen basah kolagen kulit ikan patin yaitu 85,18% dan rendemen kolagen setelah pengeringan yaitu 1.75%. Kandungan asam amino glisin yaitu

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sebesar 23,96 % dan prolin sebesar 17.40 %. Hal ini menunjukan bahwa kulit ikan patin (*P. hypophthalmus*) berpotensi untuk dikembangkan sebagai bahan sediaan nanokolagen dengan variasi waktu dan konsentrasi asam asetat dengan metode ultrasonik.
Kata kunci: ikan patin, kolagen, kulit, perikanan, rendemen

Abstract

Collagen extraction with high acetic acid can affect the characterization and damage the collagen structure, which then affects the level of temperature stability in collagen, so a combination method is needed to reduce the use of acetic acid in collagen extraction. Collagen extraction with a combination method with ultrasonication is more efficient and can help with these problems. Extraction of catfish skin collagen using a combination of acetic acid concentration and ultrasonication time can accelerate collagen extraction, reduce the use of acetic acid, and improve collagen structure. The aim of this study is to assess the potential of catfish (*Pangasius hypophthalmus*) skin as a nano collagen source through the combination of extraction time variation and acetic acid concentration under ultrasonication treatment. This study used a completely randomized design (CRD). Preparation of catfish skin collagen using acetic acid solvents with concentrations of 0.1 M and 0.2 M with ultrasonication times of 150 minutes and 200 minutes in four treatments with three repetitions. Analysis of statistical test data using Analysis of Variance (ANOVA) followed by Tukey's test. The results of this study indicate that the combination of varying acetic acid concentrations and ultrasonication times has a significant effect ($P<0.05$) on the collagen yield parameters from catfish skin. The highest yield was obtained with the treatment of 0.2 M acetic acid and 200 minutes of extraction time, resulting in a wet collagen yield of 85.18% and a dried collagen yield of 1.75%. The amino acid content consisted of 23.96% glycine and 17.40% proline. These findings demonstrate that catfish skin (*P. hypophthalmus*) has the potential to be developed as a raw material for nanocollagen development through variations in extraction time and acetic acid concentration using the ultrasonication method.

Keywords: catfish, collagen, skin, fisheries, yield

1. Introduction

Pangasius catfish (*Pangasius hypophthalmus*) is widely cultivated in Indonesia. In 2019, its production reached 1,149,400 tons (KKP, 2016; Rumimpunu *et al.,* 2017). Processing this fish into fillets generates skin waste, which accounts for approximately 4% of the total fish weight (KKP, 2021). According to Sadi and Yoga (2021), the skin of Pangasius catfish contains protein ranging from 21.03% to 24.71%. The collagen layer in the fish skin has a thickness of about 0.48 to 0.68 mm, and this thickness affects the degree of damage to the skin during the extraction process. Moreover, the skin thickness also plays a role in the fish's resistance to environmental toxins, as the epidermis serves as a protective barrier.

Pangasius catfish skin contains type I collagen, ranging from 28.12 ng/g to 29.69 ng/g. Given this potential, Pangasius skin serves as a promising raw material for collagen production. To ensure high-quality collagen output, appropriate technology is needed-one such approach involves combining acetic acid extraction with ultrasonication.

Conventionally, collagen is extracted from fish skin using 0.5 M acetic acid for approximately 72 hours at 4°C (Wang *et al.,* 2014; Nagai *et al.,* 2015; Kaewdang *et al.,* 2014). However, this lengthy process is inefficient for large-scale production and may reduce collagen quality. Excessively high acetic acid concentrations may disrupt the collagen's structural integrity and influence its thermal stability. It can also impair the gel-forming capacity and molecular weight of the extracted collagen (Schmidt *et al.,* 2016; Gao, 2021). To address these issues, combining acetic acid extraction with ultrasonication offers a more efficient alternative.

Ultrasonication has been reported to improve collagen extraction by reducing the required concentration of acetic acid, shortening processing time, and enhancing the quality of collagen as demonstrated in studies on seabass (*Lateolabrax japonicus*) by Kim *et al.*

(2012, 2013), Ran and Wang (2014), and Tu *et al.* (2015). Zou *et al.* (2017) found that ultrasound-assisted extraction and purification (UASC) increased collagen yield by 16.3% in soft-shelled turtles, from 436.2±3.3 g/kg to 507.5±4.1 g/kg compared to standard acid-soluble collagen (ASC) methods.

A comparison also showed that collagen extracted from Pangasius skin using 0.05 M acetic acid for 72 hours resulted in glycine and proline contents of 23.43% and 10.94%, respectively. Meanwhile, a combination of acetic acid concentration and ultrasonication time using flatfish skin and 0.05 M acetic acid for only 3 hours produced higher glycine (24.51%) and proline (18.80%) levels (Girsang *et al.,* 2020; Song *et al.,* 2018).

The optimum ultrasonication time is reported to be around 200 minutes; exceeding this duration leads to a decline in collagen extraction efficiency. Ultrasonication that is too brief results in incomplete extraction, while excessive duration can degrade the collagen and reduce extraction yield (Gao, 2021). Thus, optimizing both acetic acid concentration and ultrasonication duration is critical to producing high-quality collagen.

Combining these two parameters acetic acid concentration and ultrasonication time can accelerate the extraction process, reduce acetic acid usage, and preserve collagen structure. This improvement is attributed to the mechanical energy generated by ultrasonication, particularly through cavitation, which can break covalent bonds and alter the chemical characteristics of the collagen sample (Akram and Zhang, 2020; Kim *et al.,* 2012).

Producing high-quality collagen in accordance with national standards (SNI) is essential for the development of high-grade nanocollagen. Good fiber and structural properties in collagen support the formation of uniformly distributed nanoparticles (Lo and Fauzi*,* 2021). According to Trilaksani *et al.* (2020), nanocollagen ranges in size from 1 to 1000 nm. While structurally different from regular collagen, nanocollagen exhibits a porous network resembling fish tissue, which allows for optimal absorption, making it highly suitable for pharmaceutical and cosmetic applications (Yang and Wright, 2020). Therefore, this study aims to evaluate the potential of Pangasius catfish skin (*P. hypophthalmus*) for nanocollagen production by exploring the effects of varying extraction times and acetic acid concentrations combined with ultrasonication.

2. Material and Methods

The preparation of acid-soluble collagen using ultrasonic-assisted extraction, proximate analysis, and freeze-drying was conducted at the Chemical Analysis Laboratory, Faculty of Fisheries and Marine Science, Universitas Airlangga, Surabaya. Amino acid analysis was performed at Saraswanti Indo Genetech (SIG), Surabaya.

The equipment used in this study included: Ultrasonic Cleaner (40 kHz, GT Sonic 3L, China), Centrifuge (Hettich Merck Rotanta 460), Freeze Dryer (Merck Buchi Lyovapor L-200), Analytical Balance (Merck PIONEER PX224/E), Erlenmeyer Flasks (Iwaki, Germany), Measuring Cylinders (Merck Herma), Volumetric Flasks (Merck Iwaki), Hot Plate Stirrer (Merck Thermo Scientific Cimarec), Moisture Analyzer (BEL i-Thermo 163L, India), Soxhlet Extractor Apparatus (250 ml, Merck Duran), Oven (Merck Thermo Scientific), Micropipette (Merck Thermo Scientific), FTIR Spectrometer (Merck Bruker ALPHA II), Amino Acid Analyzer (Acquity UPLC), SDS-PAGE System (Bis-Tris Gel, Merck Thermo Fisher Scientific), Refrigerator (Merck GEA Type Expo-350/Phar), Freezer (Merck Modena).

The raw material used for collagen extraction was Pangasius catfish (*P. hypophthalmus*) skin, obtained as filleting waste from PT. Dimas Reiza Perwira, Surabaya, with an average fish weight of 500 grams (consumption-grade fish). The skin samples were frozen and subjected to proximate analysis based on Suptijah *et al.* (2018), Yanti *et al.* (2022), and Nurilmala *et al.* (2019). According to these references, suitable fish skin should

contain 19.48–30.28% protein, 63.92–67.22% moisture, 0.14–0.39% ash, and 2.33–8.29% fat. The skins must be clean, intact, and free from holes to avoid contamination (Gomez et al., 2012; Mahboob, 2015). Chemicals were used Acetic acid (Fulltime, China), Absolute ethanol (SmartLab, Indonesia), Sodium hydroxide (Merck, Germany), Sodium chloride (Merck, Germany), Tris(hydroxymethyl)aminomethane (Vivintis, Malaysia), n-Hexane (Merck 104367, USA), Kjeldahl tablets (MERCK 1.10958.0250 Kjeld, Germany), Sulfuric acid (Merck, Germany), Hydrochloric acid (Merck Pro Analys, Germany), AccQ-Fluor Borate Reagent (Merck, USA).

This research design used a Completely Randomized Design (CRD) with four treatments and three repetitions. The selection of acetic acid concentration in this study refers to the research of Song *et al.,* (2018) and the duration of ultrasonication in this study is based on Gao (2021). Based on this study, the best concentration of acetic acid is 0.2 M and the best duration of ultrasonication is 200 minutes. This study used acetic acid concentrations of 0.1 M and 0.2 M with ultrasonication times of 150 minutes and 200 minutes, to see changes in results when using 0.1 M acetic acid and ultrasonication of 150 and 200 minutes, the following are the methods used in this study:

P1: Acetic Acid concentration of 0.1 M with ultrasonication time of 150 minutes
P2: Acetic Acid concentration of 0.1 M with ultrasonication time of 200 minutes
P3: Acetic Acid concentration of 0.2 M with ultrasonication time of 150 minutes
P4: Acetic Acid concentration of 0.2 M with ultrasonication time of 200 minutes.

Cleaning Process of Raw material

The process of cleaning catfish skin samples (*P. hypophthalmus*) is based on research by Liu *et al.,* (2015) and Xu *et al.,* (2017). The catfish skin sample used as a material for making collagen was 3 kg, the sample was then separated from the remaining dirt and meat that was still attached using a knife. After that, the catfish skin sample was washed using distilled water 5 times and washed using distilled water 3 times. The cleaned catfish skin (*P. hypophthalmus*) was cut into small pieces measuring 0.5 x 0.5 cm and then washed with cold distilled water. The next process, the cut catfish skin sample was packed in polyethylene bags and stored at a temperature of -20°C until further use. All preparations were carried out at a temperature of 4°C (Hukmi and Sarbon, 2018).

Defatting with Ultrasonic-Assisted Extraction (*UAE*)

The pre-treatment process of fat removal was carried out based on the research of Xu *et al.* (2017). Before the fat removal process was carried out, the catfish skin sample was thawed first until the skin was not frozen. The thawed catfish skin sample was then put into an Erlenmeyer flask and 25% ethanol was added with a sample and ethanol solution ratio of 1:10. The sample was homogenized with a stirring rod for 1 minute, then the sample was ultrasonicated for 10 minutes at a chilling temperature (<10°C). The sample was then dried by airing to evaporate the solvent and then the bleaching process was carried out.

Bleaching with NaOH

The bleaching process was modified from the methods of Liu *et al.* (2019) and Singh and Benjakul (2017). The bleaching process was carried out by stable stirring using 80 grams of catfish skin sample with 0.1 M NaOH at a sample ratio and NaOH solution of 1:10, then put into a 1000 mL Erlenmeyer. The sample was then stirred with a magnetic stirrer at a speed of 500 rpm for 3 hours, where every 1 hour the solution was replaced by filtering the skin first and then putting it into an Erlenmeyer containing a new NaOH solution. The catfish skin that had gone through the bleaching process was neutralized using distilled water every 15 minutes for 5 times. The neutral catfish skin sample was then drained with a clean cloth and stored in the freezer for use in the next stage.

Acid-Soluble Collagen (ASC) Extraction with Ultrasonication

The collagen extraction process using acetic acid concentrations of 0.1 M and 0.2 M with an ultrasonication time of 150 minutes and 200 minutes, was carried out using catfish skin samples after the bleaching process. The extraction process was carried out at a chilling temperature (> 10°C). During the extraction process for a period of 150 minutes and 200 minutes, the ultrasonication device was stopped every 15 minutes, stopping for 15 minutes. This was done to keep the ultrasonication device cool and stable. After the extraction process using ultrasonication, the sample was filtered and the liquid was taken, then the liquid from the extraction results was calculated using a measuring cup. This was done for the acidification process by precipitating the collagen extract by adding NaCl until the acid solution reached a concentration of 2.5 M. The acidification process with NaCl was carried out by calculating the formula:

$$\text{NaCl (g)} = 2.5\ \text{M} \times \text{Volume of resulted of extraction: } 58.5\ \text{M}$$

After the acidification process using NaCl, the collagen extract was added with 0.05 M TRIS buffer to neutralize the pH. The addition of TRIS buffer will produce natant and supernatant. The precipitate produced from the saltingout process using Tris buffer was centrifuged using a cold centrifuge at a speed of 6000 rpm for 15 minutes, then the natant was taken. Furthermore, the collagen natant was dried using a freeze dryer for 24 hours at a temperature of -54°C. The collagen sample was then stored at chilling temperature for further analysis.

Collagen Yield Analysis

The collagen yield from Pangasius catfish skin is calculated based on the wet weight of the collagen sample. The yield calculation method refers to the study by Kusumawinahyu *et al.* (2022). The yield is measured per 160 grams of Pangasius catfish skin sample. The formulas used for calculating the wet and dry collagen yield from the fish skin are as follows:

$$\text{Yield (\%)} = \frac{\text{Initial weight of fish skin } (g)}{\text{Final weight of collagen } (g)} \times 100$$

Proximate Analysis of Catfish (Pangasius hypophthalmus) Skin Raw Material

The quality of Pangasius catfish (*P. hypophthalmus*) skin as raw material was analyzed using proximate analysis. This analysis aims to determine the compositional characteristics of the material. The proximate tests include moisture content, fat content, protein content, and ash content, following the guidelines outlined in AOAC (2005). Each test was performed on 0.5 grams of sample with three replications. The procedures are described below:

a. Moisture content analysis

The procedure begins by weighing 0.5 grams of sample into an aluminum foil dish, followed by activating the Moisture Analyzer by pressing the F2 button. The analysis proceeds for approximately 3 minutes until data acquisition is complete. The test is conducted at a temperature of 100°C with three replications per sample. The Moisture Analyzer used is based on the drying method, which integrates weighing and halogen heating processes. The analysis time using the HB43-S Moisture Analyzer ranges between 3–15 minutes per sample depending on the material type. Results are displayed on the monitor or directly printed. All weighing, drying, and calculation steps are automated within the instrument, reducing human error and improving result accuracy (Kumalasari, 2012).

b. Fat content analysis

The fat test involves preparing 0.5 grams of sample, 250 mL of n-hexane, a beaker glass, filter paper, wool thread, ice, gloves, a dish, and an oven. A 5 cm piece of filter paper is soaked in n-hexane for 10 minutes, oven-dried at 60–70°C for 30 minutes to remove residual solvent odor, and stored in a desiccator for 10 minutes. Once dried, the filter paper is weighed.
The sample is wrapped in the prepared filter paper and inserted into a Soxhlet distillation tube filled with n-hexane, heated to 90°C, and processed for five reflux cycles. The sample is then placed in a clean alcohol-washed dish, oven-dried at 105–110°C for 24 hours, and stored in a desiccator for 30 minutes before final weighing. Fat content is calculated using the following formula:

Fat Content (%) = (W3−W1): (W2×100)

Where:
W1 = Weight of empty fat flask (g)
W2 = Weight of sample (g)
W3 = Weight of flask plus extracted fat (g).

c. Protein content analysis

Protein analysis consists of three stages: digestion, distillation, and titration. Digestion: 0.5 g of sample is placed into a Kjeldahl flask with 40 mg HgO, 1.9 mg K_2SO_2, and 2 mL H_2SO_4. The flask is heated to 430°C in a fume hood for 1.5 hours until the solution becomes clear. The digested solution is cooled and diluted with 20 mL of distilled water. Distillation: Using a Kjeldahl system, the digested sample is transferred to the distillation unit. The flask is rinsed six times with 2 mL of 70% aquadest. A 125 mL Erlenmeyer flask containing 5 mL boric acid (H_3BO_3) solution and 2-4 drops of mixed indicator (0.2% methyl red in alcohol and 0.2% methylene blue in alcohol) is prepared. The condenser tip must be submerged in the boric acid solution. Add 10 mL of NaOH–$Na_2S_2O_3$ solution to the sample. Distillation continues until ~15 mL of distillate is collected. Rinse the condenser with aquadest and add the rinsing to the Erlenmeyer flask. Dilute the contents to approximately 50 mL. Titration: The distillate is titrated with 0.02 N HCl from a burette until the solution turns pink. The volume of HCl used is recorded. Protein content is calculated using the formulas below:

N(%) = (A−B)×NHCl×14×100: Sample weight (mg)

Protein Content (%) = %N×Conversion factor (6.25)

Where**:**
A = Volume of HCl used for sample (mL)
B = Volume of HCl used for blank (mL)
Mr N = Molar mass of nitrogen (14)
Conversion Factor = 6.25.

d. Ash content analysis

The primary purpose of ash content analysis is to determine the total ash present in a material, which is related to its mineral content. The procedure begins by cleaning a porcelain crucible, which is then dried in an oven at approximately 105°C for 2 hours. After drying, the crucible is placed in a desiccator for 30 minutes. A dry sample weighing 0.25 grams is then added to the crucible. The crucible containing the sample is placed in an electric muffle furnace at 600 °C and left for 3 hours until the sample is completely incinerated into ash. After incineration, the crucible is cooled and placed in a desiccator for another 30 minutes (Nurhidayah *et al.,* 2019). The sample is then weighed, and the ash content is calculated using the following formula:

Ash Content (%) = (B−A)×100: C

Where:
A = Weight of the empty crucible (grams)
B = Weight of the crucible with ash after incineration (grams)
C = Weight of the sample (grams)

e. Amino analysis of collagen from fish skin of catfish (P. hypophthalmus)

The amino acid analysis was carried out based on the laboratory method of SIG (Saraswanti Indo Genetech) in 2013, using the Ultra Performance Liquid Chromatography (UPLC) technique. The amino acid test was conducted on the collagen sample with the highest protein content from the proximate analysis. The amino acid analysis consisted of four stages. The first stage was the preparation of the protein hydrolysate. A total of 0.5 grams of extracted collagen was weighed, finely ground, and placed into a sealed reaction tube. The fine powder was then hydrolyzed using 6 N HCl in a 1:50 ratio and heated in an oven at 110°C for 22 hours. After hydrolysis, the sample was cooled to room temperature and transferred into a 500 mL volumetric flask, then filled with distilled water up to the maximum volume mark. The acid-soluble collagen from Pangasius skin was filtered using a 45 µL millipore filter. In the second stage, 10 µL of the filtrate was pipetted and mixed with 70 µL of AccQ-Fluor Borate Reagent, then vortexed. Next, 20 µL of Flour Adan reagent was added and vortexed again. The sample was allowed to stand for 1 minute and then incubated at 55 °C for 10 minutes.In the third stage, 1 µL of the incubated, reagent-treated collagen sample was injected into the UPLC system using an ACCQ-Tag Ultra C18 column at 49°C. The chromatographic system used a gradient mobile phase, PDA detector, gradient pump system, a flow rate of 0.7 µL/min, and a detection wavelength of 260 nm. The concentration of amino acids was calculated based on the ratio between the analyte area and the internal standard area using the following formula:

$$\text{Amino acid concentration (mg/kg)} = \frac{(\text{Sample ratio}:\text{Standard ratio}) \times (\text{C std}/106) \times \text{MW} \times \text{Va} \times \text{Fp}}{\text{Sample weight or volume}}$$

Where:
C std = Concentration of the amino acid standard solution (pmol/µL)
Fp = Dilution factor
Va = Final volume of the sample (mL)
BM = Molecular weight of each amino acid (g/mol)
W sample = Sample weight (g)
V sample = Sample volume (mL).

Statistical Analysis

Statistical evaluation was conducted using Microsoft Excel with two-way ANOVA to determine the significance and interaction effects between acetic acid concentration and ultrasonication duration on collagen yield and quality.

3. Results and Discussions

Results

Proximate Analysis Results of Pangasius Skin

The Pangasius catfish skin used for collagen extraction exhibited the following characteristics: protein content of 29.43%, moisture content of 63.88%, ash content of 0.35%, and fat content of 8.30% (as shown in Table 1). The Pangasius skin used in this study meets the requirements for collagen raw material as referenced in several published scientific articles.

Table 1. Proximate analysis results of Pangasius skin (*Pangasius hypophthalmus*)

Parameter	Research Data (%)
Protein content	29.43
Moisture content	63.88
Ash Content	0.35
Fat Content	8.3

Collagen Yield Analysis of Pangasius Skin

The collagen extracted from Pangasius catfish skin using different combinations of acetic acid concentrations and ultrasonication durations yielded the following results.

Table 2. Wet and dry yield of collagen from Pangasius skin (*Pangasius hypophthalmus*)

Perlakuan	(%) ± Stdv	
	Wet of collagen yield (wb)	Dry of collagen yield (db)
P1 (0.1 M dan 150 minute)	74.05±14.93^{a}	0.77±0.072^{d}
P2 (0.1 M dan 200 minute)	83.04±11.33^{ab}	1.38±0.062^{c}
P3 (0.2 M dan 150 minute)	78.76±14.71^{bc}	1.08±0.036^{b}
P4 (0.2 M dan 200 minute)	85.18±9.63^{c}	1.75±0.062^{a}

Keterangan : Huruf superskrip yang berbeda pada kolom yang sama menunjukkan bahwa terdapat perbedaan yang nyata pada setiap perlakuan ($p<0.05$)

Amino Acid Analysis Results of Pangasius Skin Collagen

The collagen extracted from Pangasius catfish skin was analyzed for its amino acid composition using the HPLC method. The analysis was conducted on the sample with the highest protein content, which was obtained using 0.2 M acetic acid and 200 minutes of ultrasonication (P4). The results showed that the main amino acids present in the collagen were glycine and proline. The collagen contained 23.96% glycine and 17.40% proline. Detailed results are presented in Table 3.

Table 3. Amino acid composition of collagen from Pangasius skin (*Pangasius hypophthalmus*)

Amino acic types	Results (%)
Glycine	23.96
Proline	17.40
Arginine	14.56
Alanine	12.18
Glutamic acid	8.72
Serine	5.14
Aspartic acid	4.06
Threonine	2.84
Lysine	2.57
Leucine	1.82
Phenylalanine	1.60
Valine	1.58
Isoleucine	1.22
Tyrosine	1.26
Histidine	1.08
Total	**100**

Discussions

Different combinations of acetic acid concentration and ultrasonication time during the collagen extraction process significantly influenced the yield, protein content, moisture content, and ash content of the resulting collagen. Acetic acid is used as a solvent in collagen extraction due to its better extraction efficiency and lower cost compared to other solvents (Fabella *et al.,* 2018; Kusumawinahyu *et al.,* 2022). Acetic acid facilitates collagen solubility by increasing H^{+} ion concentration, which causes swelling in dermal cells, thus aiding in

collagen extraction. However, using excessively high concentrations of acetic acid may lead to collagen denaturation due to the abundance of H^+ ions, thereby reducing collagen quality (Akram and Zhang, 2020; Hadfi and Sarbon, 2019).

Previous studies using 0.5 M acetic acid for collagen extraction from fish skin reported yields between 0.1–11.5% (Fabella *et al.,* 2018; Girsang *et al.,* 2020; Kusumawinahyu *et al.,* 2022; Abbas *et al*., 2022) and protein content ranging from 8.31–69.7% (Kusumawinahyu *et al.,* 2022; Abbas *et al.,* 2022; Suptijah *et al.,* 2018). Although higher concentrations of acetic acid are necessary to penetrate dermal cells, they can cause denaturation of collagen proteins. A solution to this issue is the combination of acetic acid with ultrasonication, which allows for lower acid concentrations while still achieving efficient collagen extraction (Di *et al.,* 2014; Kim *et al.,* 2012; Zou *et al.,* 2017; Akram and Zhang, 2020).

Ultrasonication facilitates acetic acid penetration into the dermal layers by creating microcavities in the cells, enabling even low concentrations of acetic acid to extract collagen. The combination of acetic acid and ultrasonication generates mechanical energy and cavitation bubbles that disrupt the fish skin matrix, allowing acetic acid to enter the dermal layer more easily and isolate collagen rapidly by breaking covalent bonds (Akram and Zhang, 2020; Tetti, 2014; Schmidt *et al.,* 2016; Jiang *et al.,* 2016; Zou *et al.,* 2017; Shaik *et al.,* 2021; Kim *et al.,* 2012). This method reduces the need for high acid concentrations and improves collagen quality, as lower acid concentrations reduce the risk of collagen denaturation (Li *et al*., 2009; Akram and Zhang, 2020; Kim *et al.,* 2012; Song *et al.,* 2018).

The combination of 0.2 M acetic acid and 200 minutes of ultrasonication produced the highest collagen yield: 85.18 ± 9.63% (wet basis) and 1.75 ± 0.062% (dry basis). This indicates that the combination can effectively isolate collagen while maintaining its structural integrity. This high yield correlates with a high protein content of 83.3 ± 0.030%, as protein is the major component of collagen (Akram and Zhang, 2020; Jiang *et al.,* 2016; Zou et al., 2017; Kim *et al.,* 2012).

Increasing ultrasonication time from 150 to 200 minutes reduced the moisture, fat, and ash content at both 0.1 M and 0.2 M acetic acid concentrations. Moisture content was influenced by the interaction of acetic acid and ultrasonication with the collagen matrix. Lower acetic acid concentrations resulted in higher moisture content due to reduced water expulsion from the collagen matrix (Muharja *et al.,* 2021). Lower moisture levels indicate higher collagen purity, as excessive moisture may hinder collagen fiber separation (Jaswir *et al.,* 2011; Nursyam, 2010; Nurjanah *et al.,* 2021).

The moisture content of Pangasius collagen is similar to that of collagen from tuna swim bladders and croaker fish, which contain 12.75% and 12.12% moisture, respectively (Hadinoto *et al.,* 2019; Gadi *et al.,* 2017). However, it is higher than that of tilapia skin and sea cucumber collagen, which contain 8.9% and 10.9% moisture, respectively (Li *et al.,* 2020). These differences may be attributed to species, age, breeding method, skin structure, and environmental conditions, although further studies are needed (Basuni *et al.,* 2018; Hamzah *et al.,* 2019).

The use of ethanol and NaOH in the pretreatment stage helped reduce the ash content in Pangasius skin collagen by removing impurities and minerals. The combination of acetic acid and ultrasonication further reduced mineral content due to the mechanical energy and cavitation effects, yielding higher-purity collagen (Hadinoto *et al.,* 2019; Desmelati *et al.,* 2020). The ash content in this study aligns with results from Suptijah *et al.* (2018) at 1.80%, sea cucumber collagen at 6.70%, and yellowfin tuna skin collagen at 2.25% (Desmelati *et al.,* 2020; Nurilmala *et al.,* 2019).

According to Atef *et al.* (2020), Hadinoto *et al.* (2019), Hu *et al.* (2017), Liao *et al.* (2018), and Nurilmala *et al.* (2019), the primary amino acids in collagen are glycine and proline. The collagen extracted from Pangasius skin

using 0.2 M acetic acid and 200 minutes of ultrasonication contained 15 amino acids, dominated by glycine and proline, which together comprised 40% of the total amino acids (Table 3). This suggests that Pangasius skin collagen is classified as Type I collagen (Girsang *et al.,* 2020; Liao *et al.,* 2018; Nurmalia *et al.,* 2019). Its composition is comparable to that of collagen from catfish, tilapia, and flatfish skins (Vijayan *et al.,* 2018; Girsang *et al.,* 2020; Fabella *et al.,* 2018; Hu *et al.,* 2017; Song *et al.,* 2018).

Collagen amino acid chains are characterized by repeating tripeptides of glycine (Gly), proline (X), and hydroxyproline (Y), which are critical for the formation of the triple helix (Vijayan *et al.,* 2018; Asaduzzaman *et al.,* 2020; Girsang *et al.,* 2020). Glycine contributes to triple helix formation due to its small molecular size, allowing the collagen chain to bend and form a stable helix more easily. Glycine is located at the center of the collagen triple helix, where it provides space for proline and hydroxyproline to form strong cross-links within the collagen structure (Atef *et al.,* 2020; Fabella *et al.,* 2018; Liu *et al.,* 2017; Chen *et al.,* 2016; Gorres and Raines, 2010). Proline contributes to thermal stability through its pyrrolidine ring, which forms hydrogen bonds with water molecules, helping maintain structural integrity at varying temperatures (Ghosh *et al.,* 2021; Asaduzzaman *et al.,* 2020; Atef *et al.,* 2020; Vijayan *et al.,* 2018).

Previous studies using 0.5 M acetic acid for collagen extraction reported glycine contents ranging from 20.92–24.03% and proline contents from 10.06–17.40% (Vijayan *et al.,* 2018; Girsang *et al.,* 2020; Hu *et al.,* 2017; Song *et al.,* 2018). In this study, Pangasius collagen also contained 14.57% arginine, which plays a role in thermal stability due to its positively charged guanidinium group. This group forms salt bridges with negatively charged residues in the collagen triple helix, enhancing structural stability (Huang *et al.,* 2018; Srichana and Aramwit, 2013; Kadler *et al.,* 2007).

The histidine content was found to be 1.08%, indicating minimal metal presence in the collagen matrix. Low histidine levels suggest low metal contamination, which aligns with the low ash content. This may be attributed to effective pretreatment procedures (Song e*t al.,* 2018).

4. Conclusion

The combination of different acetic acid concentrations and ultrasonication durations significantly affected both the wet and dry yields of the extracted collagen. The optimal condition was observed with 0.2 M acetic acid and 200 minutes of ultrasonication, resulting in a wet yield of 85.18% and a dry yield of 1.75%. The collagen extracted under these conditions exhibited the characteristics of type I collagen with a triple-helix structure. This type I collagen was characterized by a glycine content of 23.96% and a proline content of 17.40%.

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Author Contributions

All authors contributed to the final manuscript. Contributions are as follows: Money Carattri Kusuma Werdani and Ahmad Shofy Mubarak: conceptualization, methodology, formal analysis, original draft preparation, writing-review and editing.Laksmi Sulmartiwi: writing-review and editing. All authors have read and approved the final version of the manuscript.

Conflict of Interest

The authors declare no financial or commercial conflict of interest.

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