

Characteristics of collagen from swim bladder of catfish (*Pangasius* sp.) under different ultrasound exposure times

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Abstract. The swim bladder is fishery by-product whose main components are collagen protein. Collagen is the main structural component of connective tissue. This study aimed to determine the chemical composition, structure of catfish swim bladder tissue, extraction process, and collagen hydrolysis of catfish swim bladder with different sonication times (0, 10, 15, and 20 minutes). The swim bladder is composed of wavy fibers, branches and spaces between fibers. The best treatment for catfish swim bladder collagen was determined using ANOVA and Duncan's test. The best quality collagen hydrolysate was obtained from a sonication treatment time of 15 minutes. Collagen contained α_1 , α_2 , and β chains. Collagen hydrolysate contained functional groups with peak absorption of amide A, amide B, amide I, amide II, and amide III, with a pH value of 6.67 ± 0.15 , viscosity value of 4.00 ± 1.92 , and antioxidant activity with an IC_{50} value of 44.09 ± 0.48 .

1 Introduction

Market demand for catfish meat *fillets* has increased significantly from year to year. Data shows that in 2022 catfish production increased to 340,444 tons [1]. The increase in production of catfish *fillets* is directly proportional to the increase in byproducts, one of which is swim bladder. Swim bladder are widely used as traditional or conventional medicine. Several countries in Southeast Asia, China and Hong Kong have widely used fish swim bladder for the treatment of amnesia, insomnia and several other treatments [2]. A new innovation applied in the utilization of fish swim bladder is to make them as raw materials for making glycosaminoglycans [3]. The swim bladder has a proportion of the total weight of the fish which is 2% with a protein content of 93.39% [4]. The main component of swim bladder is collagen which is of high quality and very well used as raw material for collagen extraction [5]. Swim bladder have the potential as collagen but in Indonesia, the utilization is still not optimal.

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Collagen is the main structural component of connective tissue, accounting for nearly 30% of total body protein. Collagen in cosmetics is the largest segmentation in the industrial sector, reaching 12.5% [6]. The Ministry of Industry designated the cosmetics industry into the mainstay industrial sector from 2015 to 2035 with growth reaching 20%. Collagen market share based on CAGR in 2030 is expected to increase by 10%, dominated by North America. This shows that collagen in the future has promising prospects. Collagen in the cosmetic field can be used into *dermal fillers* and skin care products [7].

Exactitude Consultancy 2024 data shows that the collagen market is still dominated by terrestrial animals where the three groups of terrestrial animals occupy the top 3 ranks as collagen producers, namely cattle, pigs and poultry [8]. According to Nurilmala [9], the problems that arise from these three groups of terrestrial animals as a source of collagen are still against religion and disease concerns. This is evident due to outbreaks of bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE) and foot and mouth disease (FMD). The use of collagen sources from aquatic animals is an alternative to solve the problem, especially for Muslims. Bi [10] reported several advantages of collagen from fish, namely halal, diverse available sources, safe from terrestrial animal diseases, and high biocompatibility.

The quality of collagen is influenced by several factors, one of which is the extraction method. Selection of appropriate extraction method treatment needs to be done to obtain optimal characteristics. Kartika [4] reported that collagen extraction method can cause differences in protein content produced. Collagen extraction methods that are often used are acid dissolution with or without the addition of enzymes [11]. In terms of bioactivity, the extraction method still does not provide optimal results. Nurilmala [12] reported in their research that collagen hydrolysate has antioxidant activity that is significantly different from collagen antioxidant activity. Collagen hydrolysate has an IC_{50} value of 66 ppm, while collagen has an IC_{50} value of 300 ppm. The lower the molecular weight, the stronger the antioxidant activity. Nakchum [13] reported that collagen hydrolysate from squid skin has very strong antioxidant activity with its IC_{50} value of 1.3 ppm. Bioactivity can be achieved through hydrolysis process [14]. The hydrolysis process is able to optimize collagen bioactivity, for example optimal antioxidant activity with low molecular weight and nanoparticle size [15-17].

Hydrolysis can be done by several methods, including physical, chemical and enzymatic methods. Enzymatic methods are used because of the ability of a particular enzyme to cut the peptide chain. An example of an enzyme that is often used in the hydrolysis of collagen using enzymatic is pepsin. Pepsin has the ability to cut non-helix peptide chains. This is certainly useful to maintain the condition that the helix peptide chain is not broken so that the collagen does not change [18]. Behind these advantages, in fact, the enzymatic method has disadvantages. Arajuo in their research said that there are several disadvantages of the enzymatic method, namely having a fairly expensive price for purchasing enzyme ingredients and the process takes a long time [19].

Another method that can be used for both extraction and hydrolysis is the chemical method. This method has been done in several studies, such as Wang [20] who used saline solution for collagen extraction from sturgeon fish. Wang [21] and Safithri [22] used acetic acid as a solvent in extracting collagen from freshwater fisheries and gama sea cucumber. The method of using chemicals also has disadvantages, Ahmed [14] stated that the use of chemicals is basically difficult to use because collagen has low solubility so that the chemicals are quite limited. Departing from these problems, hydrolysis was carried out using a physical method, namely sonication.

The principle of sonication in the hydrolysis process is to utilize ultrasonic waves which will form electrical signals that are converted into physical vibrations so that they have a cavitation effect that causes the rupture of solution molecules [23]. The factors that

influence the success of sonication are the amplitude of the sonicator, the diameter of the *probe*, the frequency of sonication, and the length of sonication time [24]. Sonication treatment of tuna fish skin can increase the yield to 2.7 times compared to conventional hydrolysis methods such as chemical and enzymatic [17]. Previous studies using sonication as a collagen hydrolysis method with specific time variations have been conducted. Liu [25] stated that sonication treatment ≤ 200 W, $t \leq 15$ min had more homogeneous characteristics ($82.78 \pm 16.47 - 115.52 \pm 19.51$ nm), having a long D-periodicity ($62.1 \pm 2.9 - 66.5 \pm 1.8$ nm), can decrease the viscoelasticity index and gel strength, and increase thermal stability. This was also done by Wirayudha [56] where the swim bladder of reef fish (grouper) was subjected to sonication treatment to produce collagen in nano size of 404.1 nm. Hydrolysis of collagen by sonication method is expected to maintain the quality and stability of collagen. Different sonication time treatments are expected to determine the optimal collagen characteristics in an effort to utilize the by-products into value-added products.

2 Materials and methods

2.1 Tools and Materials

The equipment used for the preparation of collagen hydrolysate are waterbath shaker (Depolab, Seoul, Korea), ultrasonic probe (BRANSON, processor cell disruption mixer), dryer (oven) (Blue M, China). Tools used for the characterization of collagen hydrolysates are kjehdahl tube, furnace, porcelain cup, desiccator, distillator, biuret, Olympus CX41 light microscope, DP-21 camera, stream stant software, Fourier Transform Infrared (functional group analysis), SDS-Page (molecular weight distribution analysis), pH meter (acidity analysis), Brookfield TV-10 viscometer (viscosity analysis), and SPECTRO UV-Vis (RS spectrophotometer UV-2500).

The materials used in this study were swim bladder of catfish (*Pangasius sp.*) by-products of catfish fillet processing. Other materials used in this study include sodium hydroxide (NaOH) (Merck, New Jersey, USA), citric acid ($C_6H_8O_7$), distilled water (Toko Central Kimia, Bogor, Indonesia), stacking gel, separating gel, buffer solution, coomassie brilliant blue (CBB), azino bisethylbenzothiazoline-sulphonicacid (ABTS) solution (Sigma, Missouri, USA).

2.2 Work Procedure

This research consists of three stages of work. The first stage is sample preparation. The material used is a frozen swim bladder from the processing of catfish fillets. The second stage is pre-treatment, the process is carried out by immersion in NaOH and $C_6H_8O_7$ to remove non-collagen proteins and open collagen fibers. The third stage is hydrolysis using sonication method. The research flow of making patin (*Pangasius sp.*) swim bladder collagen hydrolysate is presented in Figure 1.

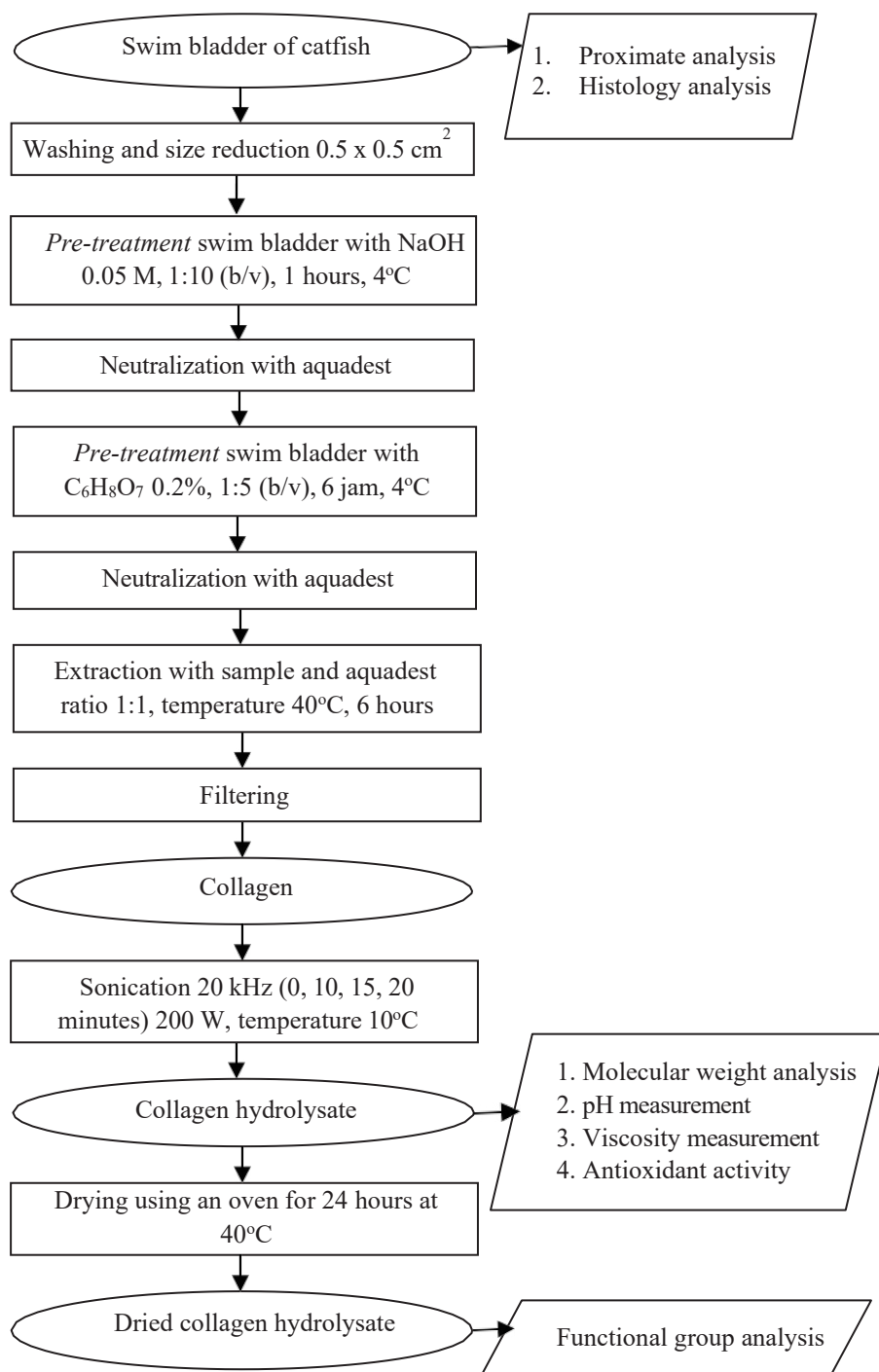


Fig. 1. Collagen hydrolysis procedure of swim bladder of catfish (*Pangasius sp.*)

2.2.1 Sample preparation

The swim bladder of catfish was obtained from the catfish fillet industry in a frozen state, then during the transportation process to the laboratory the swim bladder was arranged in a cool box and maintained at a temperature of -17 °C to 2 °C. In the next stage, the swim

bladder was stored in a freezer at -20°C . Swim bladder was prepared by starting with the thawing process, then washing to remove dirt and fat that are still attached. Swim bladder was cut into $0.5 \times 0.5 \text{ cm}^2$.

2.2.2 Pre-treatment with NaOH immersion and $\text{C}_6\text{H}_8\text{O}_7$

Prepared swim bladder was pre-treated by immersing them in 0.05 M NaOH solution for 1 hour to remove non collagenous proteins. The ratio between swim bladder and NaOH solution was 1:10 (b/v), then swim bladder stored at 4°C . Swim bladder was neutralized with distilled water until the pH was neutral. The swim bladder was pre-treated by immersing them in 0.2% $\text{C}_6\text{H}_8\text{O}_7$ for 6 hours with the ratio between swim bladder and $\text{C}_6\text{H}_8\text{O}_7$ being 1:5 (w/v) and stored at 4°C . Pre-treatment with $\text{C}_6\text{H}_8\text{O}_7$ aims to open the collagen fibers in the swim bladder so that in the extraction process the collagen will be extracted easily.

2.2.3 Collagen extraction

The extraction method by Devita [44] was modified and carried out using distilled water and carried out at a certain temperature. Samples that have been soaked are then extracted using a waterbath shaker. The ratio between the sample and distilled water was 1:1 (b/v). The extraction was carried out at 40°C for 6 hours and then filtered.

2.2.4 Collagen hydrolysis

The extracted liquid collagen was hydrolyzed using sonication at 20 kHz, 50% output power, and temperature maintained at 10°C . The sonication time was 0 min, 10 min, 15 min, and 20 min.

2.3 Analysis procedure

The analysis carried out in this study includes proximate analysis, functional group analysis, molecular weight analysis, color and whiteness degree analysis, pH measurement, particle size, and antioxidant activity.

2.3.1 Proximate analysis [48]

a) Moisture

The analysis of water content begins with the porcelain cup being washed and then dried in an oven at 105°C for 30 minutes. The porcelain cup was put into a desiccator for 15 minutes and then weighed. A 1 g sample was put into a cup and then oven dried at 105°C for 5 hours or until the weight was constant. The cup was cooled in a desiccator for 30 minutes and then the final weight was weighed. Presentation of moisture content can be calculated by the formula:

$$\text{Moisture content (\%)} = \frac{B - C}{B - A} \times 100\%$$

Description:

A = weight of empty cup (g)

B = weight of cup filled with sample (g)

C = weight of cup with dried sample (g)

b) Protein

Analysis of protein content includes three stages, namely deconstruction, distillation, and titration. The deconstruction stage was carried out with a 0.5 g sample put into the deconstruction flask, then added 1 Kjeldahl tablet and 10 mL of concentrated sulfuric acid (H₂SO₄). The sample was deconstructed at 400°C until the solution became yellowish green-clear and then cooled for 15 minutes. The distillation stage was carried out by adding 50 mL of distilled water to the solution and then putting the solution into a distiller and adding 20 mL of 20% sodium hydroxide (NaOH) and a few drops of phenolphthalein indicator. The solution was distilled in a distiller at 100° C for 10 minutes. The distillation results were collected in a 125 mL erlenmeyer flask containing 25 mL of 2% boric acid (H₃BO₃) containing bromcherosol green and methyl red indicators. The distillation process is stopped after the solution is bluish green in color. The distillate is then titrated with 0.09 N hydrochloric acid (HCl) until the color of the solution turns pink. The titrant volume is read and recorded. The blank solution is then analyzed as an example. Protein content can be calculated using the following formula:

$$\text{Nitrogen} = \frac{\text{HCl volume (mL)} - \text{blanko volume (mL)} \times 14.007}{B - A} \times 100\%$$

Protein content = %N×6.25

c) Fat

The procedure for the soxhlet method is that the soxhlet flask is dried in an oven at 110°C for one night. A 5 g sample (W1) was put into a filter paper and then put into a fat flask that had been weighed to a fixed weight (W2) and connected to a soxhlet tube. The fat flask was inserted into the extractor chamber of the soxhlet tube and flushed with n-hexane solvent. The extraction tube was attached to the soxhlet distillation apparatus, then heated at 40°C using an electric heater for 16 hours. The n-hexane solvent in the fat flask is distilled until it evaporates, then the solvent is removed and the fat flask is dried in an oven at 105°C, then the flask is cooled in a desiccator until its weight is constant (W3). Fat content can be calculated by the formula:

$$\text{Fat content} = \frac{W3 - W2}{W1} \times 100\%$$

Description:

W1 = sample weight (g)

W2 = weight of empty flask (g)

W3 = weight of fat flask with fat (g)

d) Ash

Analysis of ash content is that the cup is dried in the oven for 1 hour at a temperature of 105°C. The cup was then cooled in a desiccator and weighed. A 5 g sample was put into an ignition cup and ignited over a bunsen flame until it was not smoking. The cup was then

placed in a furnace at 600°C for 7 hours. The cup was placed in a desiccator and weighed until a constant weight was obtained. Ash content can be calculated by the formula:

$$\text{Ash content (\%)} = \frac{B - C}{B - A} \times 100\%$$

Description:

A = weight of the empty blank porcelain cup (g)

B = weight of cup with sample (g)

C = weight of cup with sample after drying (g)

2.3.2 Histology analysis [29]

Histological observation of the swim bladder of catfish begins with making preparations and then taking pictures of objects using a microscope. Preparation of catfish swim bladder is done by paraffin method. The paraffin method has several stages, namely fixation, dehydration, clearing, impregnation, embedding, blocking, trimming, tissue cutting, staining, and tissue gluing using a mounting agent.

Fixation is done in BNF (Buffer Normal Formalin) solution for more than 24 hours (3 days), after which the fixation solution is discarded. After the fixation was discarded, dehydration was carried out by immersing the tissue in bottles containing 80%, 90%, 95% alcohol, each for 2 hours and 100% alcohol for 12 hours. Immersion is done at room temperature.

The next stage is *clearing* which begins with soaking the sample in a clearing agent. The tissue was immersed in alcohol:xylol (1:1) for 30 minutes, followed by impregnation and embedding stages. The impregnation stage is the immersion of tissue into xylol:paraffin (1:1) for 45 minutes in a glass cup. The embedding stage is the immersion of tissue in liquid paraffin, namely paraffin I, paraffin II, and paraffin III for 45 minutes each. Impregnation and embedding processes took place in an oven at 60°C.

The embedded tissue is then blocked (molded for easy cutting) with liquid paraffin and then frozen. Liquid paraffin is poured into the mold to fill about 1/8 of the mold and left to freeze slightly. The next process, the tissue is arranged in a mold and poured with liquid paraffin until the tissue material is submerged and then let it freeze for 24 hours at room temperature. After the paraffin is frozen with the paraffin block was removed from the mold and trimmed using a razor blade to fit the block into the cutting tool. Tissue cutting was performed using a 14 µm thick rotary microtome. The results of cutting the tissue were taken with a needle and placed on the surface of warm water with a temperature of 45° C-50° C and glued to glass object and left to dry. Tissue preparations are stained hematoxylin-eosin which begins with immersion of the object glass into xylol I and xylol II for 2 minutes each. The next stage, immersed in absolute alcohol 100%, 95%, 90%, 80%, 70%, and 50% each for 2 minutes then the object is put into hemotoxylin dye for 7 minutes and washed with running water to remove excess dye that cannot be absorbed. The object was immersed again in eosin for 3 minutes and washed again with distilled water. Tissue preparations were immersed in 50%, 70%, 85%, 90%, 100% alcohol, xylol I, xylol II for 2 minutes each.

The next process is closing the object glass by applying mounting agent to the object glass and then covered with a glass cover and dried for 24 hours. Observation of the preserved preparations was carried out with an Olympus CX41 light microscope with a magnification of 200x. Object images were taken with a DP21 camera. Furthermore, the images obtained were optimized using image analyzer software.

2.3.3 Analysis of functional groups [30]

Functional group analysis of collagen and collagen hydrolysate was analyzed using Fourier Transform Infrared (FT-IR) spectrophotometry instrument. This functional group analysis aims to determine the 2 mg sample and 100 mg KBr (potassium bromide) crystal were mixed and crushed using a mortar. The sample was placed on a disk printer. The printed disk is then inserted into the Fourier Transform Infrared (FT-IR) spectrophotometry and measured at wave numbers 4000 to 450 cm^{-1} . The FT-IR spectra shows the absorption peaks of the wave number of the sample.

2.3.4 Molecular weight analysis (SDS) [31]

Molecular weight analysis on collagen hydrolysate using SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) method. This analysis used 4% stacking gel, 10% separating gel for collagen sample, and 15% separating gel for collagen hydrolysate sample. Collagen and collagen hydrolysate samples were used as much as 50 μL which was added to buffer solution in a ratio of 1:1 (v/v), then heated at 95°C for 10 minutes. Samples of collagen and collagen hydrolysate were taken as 20 μL and then inserted into the polyacrylamide gel.

The next stage is gel staining using a staining solution for 1 hour. The solution was made from a mixture of 0.05% coomassie brilliant blue (CBB), 40% methanol, 10% acetic acid, and 50% distilled water. The process after gel staining, which is color removal using destaining solution for ± 45 minutes, then soaked using 5% acetic acid for 10 minutes. The composition of the destaining solution is 40% methanol, 10% acetic acid, and 50% distilled water. Protein bands that have been seen are then scanned and measured using the photocapt software application.

2.3.5 Measurement of acidity (pH) [32]

Measurement of acidity (pH) was carried out using a pH meter based on the SNI 01-8076-2014 reference. The initial stage is that the electrode is calibrated with a solution of pH 4, 7, and 10. Each calibration process is carried out drying using tissue. The calibrated electrode was dipped directly into the liquid sample as much as ± 20 mL until the pH meter showed a stable number and recorded. Each electrode that has been dipped in the sample is calibrated using distilled water and dried using a tissue.

2.3.6 Viscosity measurement

Viscosity measurement was conducted to determine the viscosity of collagen and collagen hydrolysate resulting from hydrolysis using sonication. The viscosity measurement was performed using a Brookfield viscometer RV type with spindle number 3. The spindle was mounted on the viscometer and then dipped into a *beaker glass* containing collagen and collagen hydrolysate.

2.3.7 Antioxidant activity test using azino bisethylbenzothiazoline- sulphonic acid method (ABTS)

Antioxidant activity testing with ABTS method is done by mixing 1 mL sample in 2 mL ABTS. The samples used consisted of several concentrations, namely 62.5; 125; 250; 500; 1000 ppm. Mixing the sample with ABTS solution was done using a vortex, then incubated

for 10 minutes at room temperature. Absorbance measurements were taken using a spectrophotometer at a wavelength of 750 nm (Modification of [33]).

$$\text{Antioxidant activity (\%)} = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100\%$$

Description:

A blank: Absorbance of ABTS solution and distilled water.

A sample: The difference in absorbance between the sample and A blank.

2.4 Data analysis

The experimental design used in this study was a completely randomized design (CRD) with one factor, namely sonication duration (0, 10, 15, 20 minutes). Analysis of variance was conducted using ANOVA with 95% confidence interval ($\alpha = 0.05$), and Duncan's further test was conducted. The experimental design model was as follows:

$$Y_{ij} = \mu + A_i + \epsilon_{ij}$$

Description:

Y = Observation result of jth activity with i-th treatment
i = Difference in treatment used
j = Repeat of each treatment

μ = Common mean value

A_i = Effect of i-th method treatment

ϵ_{ij} = Effect of error

The test data was obtained with three replicates of each sample. The test hypothesis for the difference in sonication time treatment on the characteristics of the collagen produced is:

H0= The difference in sonication time does not influence the characteristics of the resulting collagen hydrolysate.

H1= Different sonication times influence the characteristics of the resulting collagen hydrolysate.

If the data analyzed were significantly different ($P < 0.5$) then Duncan's test was conducted. The confidence interval used is 95%. Duncan's test was conducted. The confidence interval used is 95%. Duncan's test formula is as follows:

$$R_p = (\sum p; \text{dbs}; a) \sqrt{\frac{KTS}{r}}$$

Description:

R_p = Critical value for compared treatments

P = Treatment

Dbs = Free degree

KTS = Sum of the mean squares

r = Number of replicates

3 Result and discussion

3.1 Characteristics of swim bladder of catfish

3.1.1 Chemical composition of swim bladder of catfish (*Pan gascius sp.*)

Raw materials are the main component to make a product. The chemical composition is an illustration that swim bladder has the potential to be utilized given that the main

component is collagen protein [34]. The high protein in the swim bladder of catfish indicates good quality to be used as a source of collagen. The chemical composition analysis of the raw material of the patin fish swim bladder was carried out by testing the moisture, protein, fat, ash, and carbohydrate content. The results of the chemical composition analysis of catfish swim bladder are presented in Table 1.

Table 1. Chemical composition of swim bladder of catfish

Content	Percentage (%)		
	Research result	Swim bladder of catfish (<i>Pangasius hypophthalmus</i>) [35]	Swim bladder of catfish (<i>Pangasius</i> sp.) [36]
Moisture	77.37 ±0.40	73.90±0.50	74.61±0.02
Protein	21.84±0.33	20.50±0.20	21.63±0.18
Lipid	0.39±0.14	3.77±0.10	0.55±0.11
Ash	0.16±0.00	0.89±0.10	0.50±0.11
Carbohydrate (by different)	0.24±0.07	0.16±0.00	2.70±0.02

The chemical composition of catfish swim bladder shown in Table 1 indicates that the content contained in catfish is 77.37%. The water content in fresh fish organs ranges from 70-80% [37]. Water content in swim bladder functions as a pH balancer, swim bladder texture, and maintains flexibility [38]. The swim bladder of catfish contains 21.84% protein. Protein is the main component found in swim bladder to be an alternative source of collagen [34]. Swim bladder with high protein content will also have a high purity level of collagen [39]. The fat and ash content of catfish swim bladder were 0.39% and 0.16%, respectively. Fat content and ash content are thought to affect the purity of the extraction results. This is supported by [40] who stated that the lower the ash content, the higher the purity level of a material. The presence of fat and other minerals will also interfere with the effectiveness of collagen in its application in various products [41].

3.1.2 Tissue structure of catfish swim bladder

Histology is a science that studies the structure and properties of tissues in detail using a microscope by cutting thin sections of tissue. Microscopic description of catfish swim bladder tissue begins with the preparation of histology preparations. Staining and observation of preparations histology aims to see the morphological description of the swim bladder organ of catfish [34]. The tissue structure of the swim bladder of catfish was analyzed using image analyzer software. The swim bladder consists of three main layers: tunica externa, submucosa, and mucosa. The tunica externa consists of a dense layer of connective tissue that is a potential source for collagen production [42]. The tissue structure of the swim bladder of catfish is presented in Figure 2.

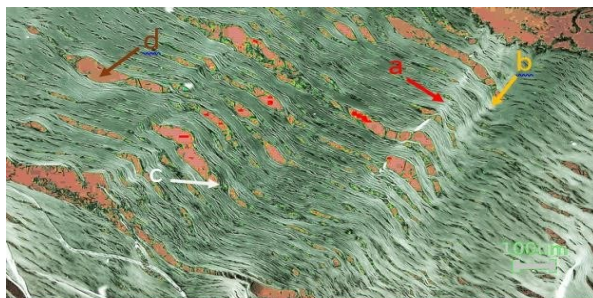


Fig. 2. Tissue structure of the tunica externa of the swim bladder of catfish: wave crest; (b) wave valley; (c) fiber branching; (d) inter-fiber space

The thin section preparation of the catfish swim bladder shows that the swim bladder is composed of wavy fibers that form wave peaks (a) and wave valleys (b). Some of the fibers show branching, which joins the neighboring fibers (c). Most of the fibers are not tightly packed, so there are spaces between fibers (d). The wave shape of the fibers allows them to contract and relax, given the swim bladder function of sucking and pumping air. The space between the fibers also allows the swim bladder to store water and oxygen. The branching between fibers also helps to keep the fibers from coming apart easily.

3.2 Characteristics of catfish swim bladder collagen hydrolysate

3.2.1 Functional groups of catfish swim bladder collagen hydrolysate

The functional groups of collagen hydrolysate are the main characteristics that distinguish collagen from other proteins. Functional group testing of collagen hydrolysate needs to be done to determine the absorption region of the constituent amides on catfish swim bladder collagen. Analysis of the functional groups of catfish was carried out using *Fourier Transform Infrared* (FTIR) spectroscopy technique. The principle of FTIR spectroscopy is to identify compounds and functional groups by measuring the wavelength and absorption intensity of infrared radiation. In general, a bond in a molecule can undergo various molecular vibrations, namely *stretching* (extension or shortening of the bond) and *bending* (enlargement or reduction of the bond angle) [43]. The results of FTIR spectroscopy functional group analysis are presented in Figure 3.

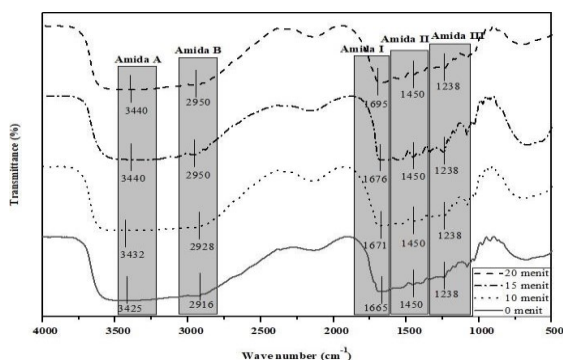


Fig. 3. Functional group spectra of collagen hydrolysates Functional group analysis; P0= 0 minutes sonication, P1= 10 minutes sonication, P2= 15 minutes sonication, P3= 20 minutes sonication

The result of functional group analysis of collagen hydrolysate of catfish swim bladder showed the peak of amide A, amide B, amide I, amide II, amide III absorption region. The wave number of amide A in catfish swim bladder was detected at the absorption peak of 3400-3440 cm^{-1} . Amide A is NH stretching associated with hydrogen bonding. The presence of amide B group in the swim bladder is found at a wave number of $\pm 2900 \text{ cm}^{-1}$. The absorption peak of amide I contained in the collagen hydrolysate of catfish swim bladder was detected at wave number 1600-1700 cm^{-1} . Amide II in the collagen hydrolysate of catfish swim bladder was detected at the absorption region of 1550-1600 cm^{-1} . Amide III in the collagen hydrolysate of catfish swim bladder was detected at the absorption region of 1200-1350 cm^{-1} . The wavelength of the FTIR spectrum corresponds to the amide of the collagen support functional group. [44] stated that collagen has typical characteristics of amide absorption A, B, I, II, and III. The amide absorption is involved with the *triple helix* structure of collagen, where amide I is a sensitive marker of secondary structure [45].

The absorption spectrum of amide A provides information about the strength of the ideal hydrogen bonds of the β -sheet structure. The absorption obtained from amide A of 3400-2440 indicates the presence of NH groups. NH groups at high wave numbers have weaker hydrogen bonds and are compatible with the α -helical structure. The wave number of amide B which is close to $\pm 2900 \text{ cm}^{-1}$ indicates that the absorption is formed from *asymmetrical stretching* of CH_2 [46]. The absorption spectrum of 1600-1700 cm^{-1} in the amide I group shows the presence of C-O *stretching* [48]. Amide II in the absorption spectrum of 1450-1575 cm^{-1} showed characteristic C-N *stretching* and N-H *bending* vibrations [47]. The absorption spectrum of amide III was shown at 1200-1350 cm^{-1} with characteristics in the form of N-H bending and C-H *stretching* [49]. The collagen hydrolysate of catfish swim bladder also has a spectrum number that is almost similar to that found in bovine bone collagen by sonication method, which has wave numbers 3400-3440 in amide A, 3400-2980 in amide B, 1600-1700 in amide I, and 1550-1600 in amide II [25]. The absorption regions and absorption peak numbers of collagen hydrolysates with different methods are presented in Table 2.

Table 2. FTIR spectroscopic functional group characteristics of collagen hydrolysates with different methods

Absorption area	The number of peak absorption groups (cm^{-1})				
	Absorption standard (cm^{-1})	P0 (0 minutes)	P1 (10 minutes)	P2 (15 minutes)	P3 (20 minutes)
Amide A	3400-3440 ¹	3425	3432	3440	3440
Amide B	± 2900 ¹	2916	2928	2950	2950
Amide I	1600-1700 ²	1665	1671	1676	1695
Amide II	1450-1575 ³	1550	1550	1550	1695
Amide III	1200-1350 ⁴	1238	1238	1238	1238

¹[46]; ²[48]; ³[47]; ⁴[49]

3.2.2 Molecular weight of catfish swim bladder collagen hydrolysate

The principle of SDS-PAGE analysis is to separate proteins based on molecular weight. The low molecular weight of collagen hydrolysate indicates the presence of biological activity. The molecular weights of collagen hydrolysates are presented in Figure 4.

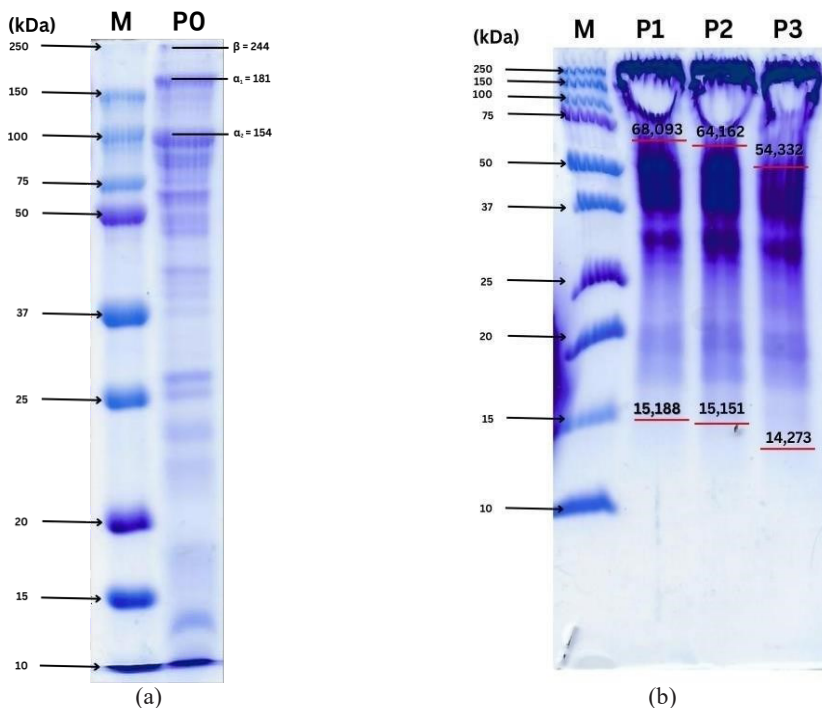


Fig. 4. Molecular weight of collagen hydrolysate; (a) molecular weight of collagen (control) (b) molecular weight of collagen hydrolysate (M=marker, P0=sonication 0 minutes, P1=sonication 10 minutes, P2=sonication 15 minutes, P3=sonication 20 minutes).

The results of molecular weight analysis on collagen (control) sample (a) and collagen hydrolysate (b) showed different values. The molecular weight of collagen hydrolysate (a) shows the presence of α_1 , α_2 and β bands with values of 181 kDa, 154 kDa, and 244 kDa, respectively. Orgel [50] suggested that proteins with a molecular weight range of 135-245 kDa are beta and α (alpha) and β (beta) structures. The two α chains formed show the characteristics of collagen so that the collagen produced is predicted to be type I collagen [51]. Type I collagen is collagen with constituent amino acids dominated by glycine, proline, alanine, and glutamic acid [52]. The presence of β chain indicates high cross linking in collagen [53].

The hydrolysis process with different sonication times resulted in varying molecular weights. The lowest molecular weight was obtained from the sonication method hydrolysis process with a sonication time of 20 minutes. Hydrolysis with a sonication time of 20 minutes resulted in molecular weights ranging from 54,322 to 14,273 kDa, lower than sonication time 10 ranging from 68,093 to 15,188 kDa and sonication time 15 minutes ranging from 64,162 to 15,151 kDa. The results show that the longer the sonication time, the lower the molecular weight and the more the color fades. The treatment of probe type sonication with sonication time >15 minutes has a fading β -chain. This may reduce the structural and thermal stability of the collagen hydrolysate [25]. The altered protein structure due to sonication will affect the surface hydrophobicity, free sulfhydryl groups and disulfide bonds. The molecular weight value of the collagen hydrolysate can influence the activity of the biological activity produced [54]. This is in line with Ketnawa [16] who stated that the lower the molecular weight produced, the higher the antioxidant activity obtained. Low molecular weight is also found in products that have high antioxidant activity with disconnected oligopeptide chains [55].

3.2.3 The degree of acidity (pH) of patin fish swim bladder

Collagen hydrolysate the degree of acidity (pH) is a parameter to measure the level of acidity of a sample. The pH measurement was conducted based on SNI 01- 8076-2014 related to crude collagen from scales. The results of acidity (pH) can be seen in Figure 5.

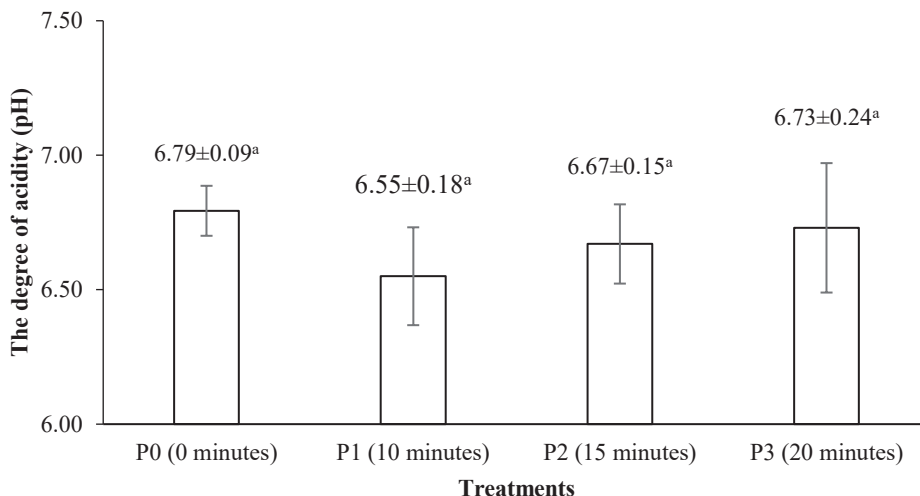


Fig. 5. The degree of acidity (pH) of collagen hydrolysate of catfish swim bladder

The measurement results of the degree of acidity (pH) in Figure 5 are normally distributed ($p > 0.05$) based on Shapiro-Wilk analysis and homogeneous ($p > 0.05$) based on Levene analysis. The results of analysis of variance (ANOVA) showed that the hydrolysis time treatment did not significantly affect the acidity (pH) value of collagen hydrolysate ($p > 0.05$). Sonication treatments P0 (0 minutes), P1 (10 minutes), P2 (15 minutes), P3 (20 minutes) did not significantly affect the acidity (pH). The degree of acidity (pH) of collagen hydrolysate P0, P1, P2, P3 were 6.79 ± 0.09 , 6.55 ± 0.18 , 6.67 ± 0.15 , and 6.73 ± 0.24 , respectively. High and low pH can also be caused by the neutralization process during collagen extraction using acid [56]. A perfect neutralization process can reduce the acid residue, so the collagen has a neutral pH. The collagen hydrolysate of patin fish swim bladder has met the standard of SNI 01-8076-2014, namely 6.5-8.

3.2.4 Viscosity of catfish swim bladder collagen hydrolysate

Viscosity measurement aims to determine the viscosity of a collagen hydrolysate. Collagen hydrolysate has characteristics such as water soluble, colorless, low allergenicity, and low [57]. The result of viscosity measurement can be seen in Figure 6.

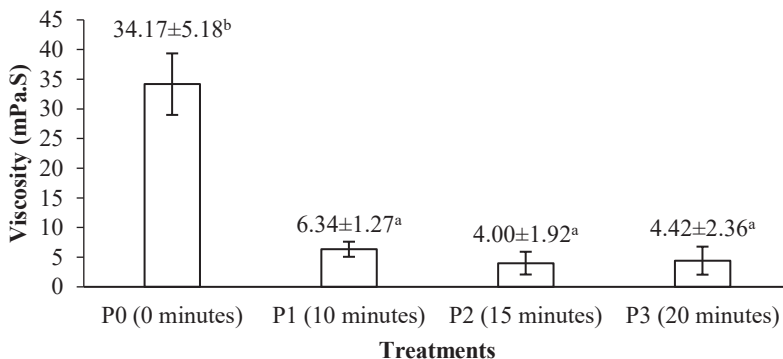


Fig. 6. Viscosity of catfish swim bladder collagen hydrolysate

The viscosity test results in Figure 6 are normally distributed ($p > 0.05$) based on Shapiro-wilk analysis and homogeneous ($p > 0.05$) based on Levene analysis. The results of *analysis of variance* (ANOVA) showed that hydrolysis time treatment significantly affected the viscosity value of collagen hydrolysate ($p < 0.05$), so Duncan's further test was conducted. The P0 treatment was significantly different from the P1, P2, and P3 treatments. The treatment of P1, P2, P3 did not significantly affect each other, but significantly affected the treatment of P0. The highest viscosity value was found in the P0 treatment, namely 34.17 ± 5.18 mPa.S, while the lowest IC₅₀ value was found in the P2 treatment, namely 4.00 ± 1.92 mPa.S.

Hydrolysis treatment using sonication resulted in a significant decrease in viscosity value compared to the control this is thought to be due to the fact that sonication treatment will produce excess heat, which will cause the molecules in the liquid to move quickly, resulting in weak interactions between molecules and decreased viscosity [17]. The viscosity value in sonication causes the water content to be lower. The decrease in moisture content causes the ability to bind water, so that the resulting collagen hydrolysate solution will be uniform [15]. Viscosity is a physicochemical characteristic of collagen that is closely related to molecular weight [58]. The particle size of collagen decreases with increasing sonication time which is likely due to the cavitation of particles under ultrasonication [65]. Sonication can also improve particle aggregation via the formation of various types of covalent bonds [17].

3.2.5 Antioxidant activity of catfish swim bladder collagen

Antioxidant is one of the biological activities of a compound. Antioxidants can illustrate that a compound has the ability to inhibit free radicals. Collagen is a compound that is thought to have good antioxidant activity, but basically the testing method can also affect the results. Various methods can be used to test antioxidant activity, such as DPPH, FRAP, and ABTS. The DPPH antioxidant activity testing method focuses on providing hydrogen electrons to stabilize the electron-deficient DPPH compound, which is a potential source of free radicals. Another method is FRAP where in this method, the antioxidant activity of collagen is measured from its ability to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). ABTS can also be used for antioxidant testing by applying a similar principle as DPPH, but the material used is different, namely the ABTS radical cation.

DPPH is one method that is quite easy. Zulfa [59] in their research explained the weaknesses found in several antioxidant methods. Behind the ease of the method, it turns out that DPPH has several limitations in measuring antioxidant activity where this method cannot give good results on lipid-soluble samples so that its sensitivity to these samples is

low compared to ABTS. FRAP method with its principle of reducing metal ions, in fact, has a weakness. This is because the FRAP method cannot determine other antioxidant mechanisms, only focusing on the ability of compounds to reduce metal ions. This is the basis or reason why in testing the antioxidant activity of collagen hydrolysates using the ABTS method.

The principle of the ABTS method is that the antioxidant will change into a non-radical form if reduced and the loss of ABTS cation color. ABTS has a characteristic blue color [60]. The sonication method used in the collagen hydrolysis process can be a factor that determines the antioxidant capacity [55]. Antioxidant capacity can be determined by IC_{50} value. The lower IC_{50} value, the ability to inhibit the oxidation process of 50%, so that the antioxidant activity produced is very strong and able to counteract free radicals well [61]. The results of the ABTS method antioxidant test are presented in Figure 7.

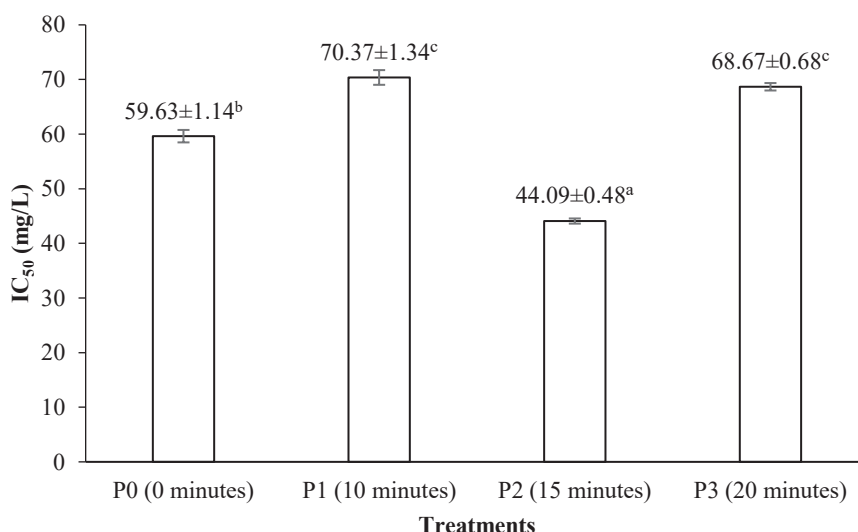


Fig. 7. Antioxidant activity of catfish swim bladder collagen hydrolysate

The results of the ABTS method antioxidant activity test in Figure 7 are normally distributed ($p > 0.05$) based on Shapiro-wilk analysis and homogeneous ($p > 0.05$) based on Levene analysis. The results of analysis of variance (ANOVA) showed that hydrolysis time treatment significantly affected the IC_{50} value of collagen hydrolysate ($p < 0.05$), so Duncan's further test was conducted. The P0 treatment was significantly different from the P1, P2, and P3 treatments. P1 treatment was significantly different from P0, P2, but not significantly different from P3. The P2 treatment is significantly different from the P0, P1, and P3 treatments. P3 treatment is significantly different from the treatment of P0, P2, but not significantly different from the treatment of P1. The highest IC_{50} value was found in P1 treatment 70.37 ± 1.34 ppm, while the lowest IC_{50} value was found in P2 treatment 44.09 ± 0.48 ppm.

The results showed that sonication time of 15 minutes was the best time in the process of collagen hydrolysis of catfish swim bladder. The IC_{50} value of P2 treatment had the lowest IC_{50} value compared to P0, P1, and P3 treatments. The low IC_{50} value indicates that the P2 treatment (15 minutes) has very strong antioxidant activity in counteracting free radicals. Srihari [61] stated that samples that have IC_{50} values < 50 ppm are classified into a very strong category, IC_{50} values of 50-100 ppm are classified into a strong category, IC_{50} values of 100-150 ppm are classified into a moderate category, IC_{50} values of 150-200 ppm

are classified into a weak category, and IC_{50} values >50 ppm are classified into a very weak category.

The P2 treatment gave very strong antioxidant activity compared to the other treatments. This is thought to be the influence of the molecular weight produced. Molecular weight can affect the biological activity of collagen hydrolysate [16]. Biological activities such as antioxidants can be known from the molecular weight. Amino acid number and sequence can also affect the antioxidant activity of collagen hydrolysate [62]. The difference between the antioxidant values in this study may also be due to differences in the number and sequence of amino acids so that the lower molecular weight (P3) does not necessarily have low antioxidant activity. This is in line with the research of Nurilmala [12] which revealed that collagen peptides with weights <3 kDa have weaker antioxidant activity compared to collagen peptides with weights of 3-10 kDa. On the other hand, sonication time and temperature are also thought to influence the antioxidant activity. Agustina [64] stated that sonication time will generate excess heat resulting in an increase in temperature. The increase in temperature can cause biological activity to increase. Indriana [23] also stated that sonication method can stabilize the total antioxidant content of an ingredient.

The high antioxidant activity of a compound is one of the advantages of the compound to be applied. Agustina [64] in their research revealed that the application of collagen hydrolysate can be used as an additional ingredient for herbal soup. This is because collagen hydrolysate has good antioxidant activity seen from the test results using ABTS and DPPH. Antioxidant activity in fact can not only be applied to food, but can be applied to pharmaceuticals and cosmesetics. The high antioxidant activity in collagen hydrolysate can be utilized for the manufacture of supplements, especially oral supplements. Antioxidants can also inhibit the induction of pigmentation by UV-B [65].

4 Conclusion

The best sonication time was obtained in treatment P2 (15 minutes). This can be seen in the physicochemical characteristics, namely low molecular weight of 14,273 kDa, pH value of 6.67 ± 0.15 , and viscosity value of 4.00 ± 1.92 . Collagen hydrolysate has a characteristic triple helix structure with absorption peaks of amide A, amide B, amide I, amide II, and amide III. Antioxidant activity showed the best treatment was P2 (15 minutes) with IC_{50} value of 44.09 ppm.

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