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ENZYMATIC HYDROLYSIS OF COLLAGEN FROM YELLOWFIN TUNA BONES AND ITS POTENTIAL AS ANTIBACTERIAL AGENT

H. Natsir[1,✉], S. Dali[1], Sartika[2], Leliani[2] and A.R. Arif[1]

[1]Department of Chemistry, Hasanuddin University, Makassar-90245, South Sulawesi, Indonesia
[2]Master Study Program of Chemistry, Department of Chemistry, Hasanuddin University, Makassar-90245, South Sulawesi, Indonesia
✉Corresponding Author: hasnahnatsir@gmail.com

ABSTRACT

There are several sources of collagen, one of which is the yellowfin tuna bones. Collagen is a bioactive peptide with specific biological characteristics so that it can be used widely. However, hydrolysis is carried out to produce collagen hydrolysate to increase the biological effectiveness of collagen and its application. This study aimed to produce collagen hydrolysate from yellowfin tuna bones using collagenase from *Bacillus* sp. 6-2 and evaluate its ability as an antibacterial agent. The Hydrolysis process was optimized by varying the concentration of substrate, enzyme and hydrolysis time. To determine the effectiveness of the hydrolysis process in inhibiting bacterial growth, the hydrolysis degree and antibacterial activity of collagen hydrolyzate have been investigated. Results showed that hydrolysis at a substrate concentration of 10 %, enzyme of 10 % and hydrolysis time of 4 h was the optimum conditions to obtain the highest degree of hydrolysis (22.48 %). All collagen hydrolysates obtained have antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* with the highest inhibition zones of 11.50 mm and 12.60 mm respectively. Collagen hydrolysate from yellowfin tuna bones is bactericidal so it is a very good candidate to be developed as an antibacterial agent.

Keywords: Yellowfin Tuna, Collagen Hydrolysate, Collagenase, Antibacterial Activity.

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INTRODUCTION

The Yellowfin tuna is a species of tuna that is widely produced in Indonesia. Total production of tuna in Indonesia reached 1.352.802 tons during the period 2005-2012. Around 72 percent of the total production of large tuna groups is dominated by yellowfin tuna.[1] Yellowfin tuna is widely exported to several countries, including the United States, Japan, and the European Union. Export demand is not only in the form of fresh or frozen products but also processed (fillets). The processing of yellowfin tuna produces by-products in large quantities, reaching 56.6 % of total fish weight.[2] By-products especially bone have not been fully utilized, whereas bones contain large amounts of collagen which can be processed into high-value economic products by converting it to collagen hydrolysate.[3,4]

Collagen hydrolyzed or more commonly known as collagen hydrolysate consists of free amino acids and collagen peptides with low molecular weight, causing high solubility, very easily digested, absorbed and distributed to various body tissues. These properties make it a suitable component to be applied in various industries and used in food, drinks, dietary supplements, and cosmetics.[5,6]

A variety of attractive bioactivities are collagen hydrolysate, including antimicrobial, antioxidant, antitumor, anti-inflammatory, antihypertensive, antidiabetic, accelerating wound healing, and effective in osteoporosis and osteoarthritis prevention and treatment.[7,8] Therefore, they are widely used as supplements that promote health effects such as treating bone and joint diseases, digestive disorders and improving the condition of skin, nail, and hair tissues.[9]

Collagen hydrolysate can be produced through enzymatic or chemical hydrolysis. Enzymatic hydrolysis is the most popular method with offers several advantages.[10] The selection of enzymes in this process is very important due to it affects the functional properties and bioactivity of the products.[11,12] Several studies have

confirmed that collagenase from bacteria is a suitable enzyme to produce collagen hydrolysate due to it is proven to be able to release various bioactive peptides from fish collagen polypeptide chains and efficiently hydrolyze marine collagen produce small peptides with good bioactivity and a high degree of hydrolysis.[13-15] Bacterial collagenases have successfully produced bioactive collagen hydrolysate from fishery wastes, for example, collagen hydrolysate with anticancer activity, ACE inhibitors, and antioxidants.[11,16]
Based on the literature search, the production of collagen hydrolysate with antibacterial activity from fish bones has not been reported. Due to broad-spectrum activity, low biodeposition rates in body tissues, various structures, very specific to targets with a lower risk of side effects, several studies show that antibacterial peptides isolated from marine sources are excellent candidates as antibacterial agents.[17]
The production of bioactive collagen hydrolysate depends on many factors.[7,14] Hydrolysis conditions such as enzyme-substrate ratio, substrate concentration, pH, temperature, and hydrolysis time are important factors due to their effects characteristics and bioactivity of the final product.[18] Therefore, optimization of hydrolysis conditions makes it possible to produce collagen hydrolysate with desirable bioactivity. This research was carried out to produce antibacterial collagen hydrolysate from yellowfin tuna (*Thunnus albacares*) bone using collagenase from *Bacillus* sp. 6-2 by optimization of hydrolysis conditions.

EXPERIMENTAL

Material

Yellowfin tuna (Thunnus albacares) bone was obtained from a local market in Makassar, Bacillus sp. 6-2 (isolate from fish liquid waste), Collagen powder from tilapia (food and medical grade), NaOH, CH_3COOH, Trichloroacetic Acid (TCA), Bovine Serum Albumin (BSA) and aquadest.

Preparation of Fish Bone

The bones were separated from the remains of meat then washed several times with cold water. After that, they were manually cut into small pieces using a scissor and crushed with a hammer. Finally, they were packed in a plastic bag (100 g per unit) and stored in the freezer until it will be used.

Extraction of Collagen

Collagen extraction follows the Baehaki *et al*[15] methods with modification. The samples (100 g) were soaked in 0.1 N NaOH with a sample-solvent ratio of 1:10 (w/v) while stirring for 6 h using a magnetic stirrer. The solvent was replaced every 2 h then neutralized with cold aquadest. After that, they were soaked in 1.5 % CH_3COOH with a sample-solvent ratio of 1: 2 (w/v) for 24 h and neutralized again with cold aquadest. The last process was extraction using aquadest with a sample-solvent ratio of 2:1 (w/v) for 3 h at 45 ^oC. Next, the amino acid composition of the collagen extract was analyzed using UPLC following the procedure described by Huang *et.al*.[19]

Production of Collagenase

Bacillus sp. 6-2 was cultured in an inoculum medium for 18 h at 37 ^oC with a shaking speed of 180 rpm. Cell culture (10%) was transferred aseptically to fermentation medium and produced for 30 h at 37 ^oC and 180 rpm. Medium containing cells was centrifuged at 3500 rpm, 4 ^oC for 30 min to separate filtrate and cell debris. The filtrate obtained was a crude extract enzyme (collagenase). Inoculums medium and fermentation medium contained the same composition. [20]

Production of Collagen Hydrolysate

Collagen solution was hydrolyzed by collagenase from Bacillus sp. 6-2 with the presence of 1 mM $CaCl_2$ for 30 min at pH 7.0 and 40 ^oC. The mixture was placed in boiling water for 5 min to stop the reaction, then centrifuged (10.000 rpm) for 10 min at 4 ^oC. The hydrolysis process was designed by varying substrate concentration (5 %; 10 %; 20 %; 30 %), enzyme concentration (5%; 10%; 15%; 2 %) and hydrolysis time (0.5; 1.0; 2.0; 3.0; 4.0; 5.0; 6.0 h) to determine the optimum hydrolysis conditions in production of collagen hydrolysate.

Determination of the Degree of Hydrolysis (DH)

DH was determined based on the method of Hoyle and Merritt[21] with little change. A total of 0.5 mL of sample was mixed with 0.5 mL of 20 % TCA to obtain soluble protein. The mixture was left for 30 min then centrifuged (3500 rpm) for 20 min at 10 °C. The filtrate was collected and expressed as a soluble protein. The total protein concentration in the sample and soluble protein were analyzed using the Lowry method[22], with BSA as a standard. DH was calculated using a formula:

$$\text{DH (\%)} = \frac{\text{Soluble protein concentration in 10 \% TCA}}{\text{total protein concentration in sample}} \text{ x } 100$$

Determination of Antibacterial Activity

The antibacterial activity of the samples was determined through an inhibition test against Escherichia coli and Staphylococcus aureus using the agar diffusion method.[23,24] The test was carried out based on the modification of the Ramssel method.[25] The paper disk (6.0 mm) was dipped into the sample and then aseptically placed on the surface of MHA medium containing the bacterial suspension. The test plate was incubated for 48 h at 37 °C and observed every 24 h. The inhibition zone obtained was used to determine the properties of the sample as antibacterial.

- Inhibition zone (mm) decreases, 48 h < 24 h indicating bacteriostatic.
- Inhibition zone (mm) increases, 48 h > 24 h indicating bactericidal.

RESULTS AND DISCUSSION

The results of the analysis of the collagen amino composition using UPLC are shown in Table-1. The content of non-essential amino acids was higher than the essential amino acid group, especially glycine, proline, alanine, and glutamic acid were found in high amounts of (6614.41 mg/kg), (2906.86 mg/kg), (2850.03 mg/kg) and (2843.68 mg/kg) respectively. This result is similar to collagen from other fish species, which contain major amino acids such as glycine, proline, alanine, and glutamic acid.[26,27]

Table-1: Amino Acid Content of Collagen Extracted from Yellowfin Tuna Bones.

Groups	Amino acids	Concentration (mg/kg)
Essential amino acids	Lysine (Lys)	1830.70
	Phenylalanine (Phe)	500.81
	Leucine (Leu)	1186.16
	Isoleucine (Ile)	351.38
	Valine (Val)	679.05
	Arginine (Arg)	2221.17
	Threonine (Thr)	1154.16
	Histidine (His)	185.29
Non-essential amino acids	Proline (Pro)	2906.86
	Glycine (Gly)	6614.41
	Alanine (Ala)	2850.03
	Glutamic acid (Glu)	2843.68
	Aspartic acid (Asp)	1412.18
	Tyrosine (Tyr)	<222.88
	Serine (Ser)	1159.64

In this study, the hydrolysis process for the production of collagen hydrolysate was optimized to obtain high DH. DH is a parameter that is used to determine the effectivity of the process of enzymatic hydrolysis. DH is represented by the number of peptide bonds degraded during the hydrolysis process and is directly related to the size, functional characteristics, and bioactivity of peptides.[28] The high DH indicates that smaller peptides that may have strong bioactivity and have a high level of protein hydrolysis.

Figure 1 shows the effect of substrate concentration on DH, with a collagen solution without enzyme treatment as control. Substrate concentration is one of the factors that greatly affect DH. The increased

concentration of substrates tends to decrease DH.[29] This is in line with the data in Fig.-1, which showed a decrease in DH with a substrate concentration increase of more than 10 percent. This condition occurs because of the higher concentration of the substrate that causes saturation of the enzyme's active site so that the hydrolysis reaction decreases. The optimal substrate concentration was 10% with DH of 9.15%, which indicated that 10% of substrate concentration is the amount of effective collagen to be converted into the product during the hydrolysis process.

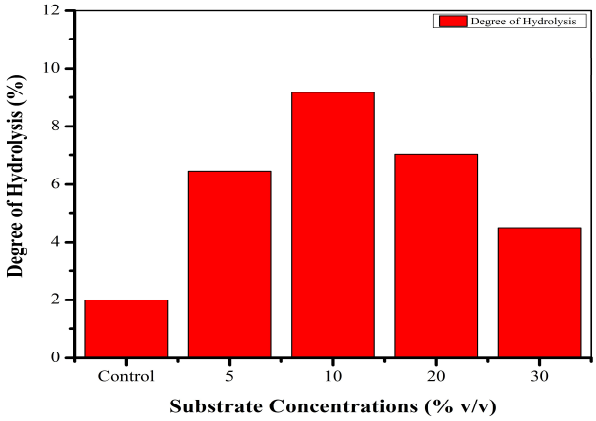

Fig.-1: The Effect of Substrate Concentration on The Degree of Hydrolysis

The concentration of the enzyme also significantly influences DH. Increasing the number of the enzyme in the hydrolysis process will increase DH due to more enzymes can bind the substrate to produce more products.[29] To determine how much enzyme is required so that the hydrolysis reaction runs optimally, information about the optimal enzyme concentration is very important. With increasing enzyme concentration, DH increases and DH tends to be constant with the addition of certain concentrations. The highest DH was achieved by the addition of 10% enzyme with a value of 14.64% (Fig.-2). With the addition of enzymes above 10 percent, DH decreased slowly. A decrease in DH can be caused by several factors, according to Guérard *et al*[28], such as a decreased number of peptide bonds susceptible to enzymes, decreased activity of the enzyme, and the formation of a product that can block the reaction. Under those conditions, the rate of hydrolysis reactions can no longer be increased by an increase in enzyme concentration.

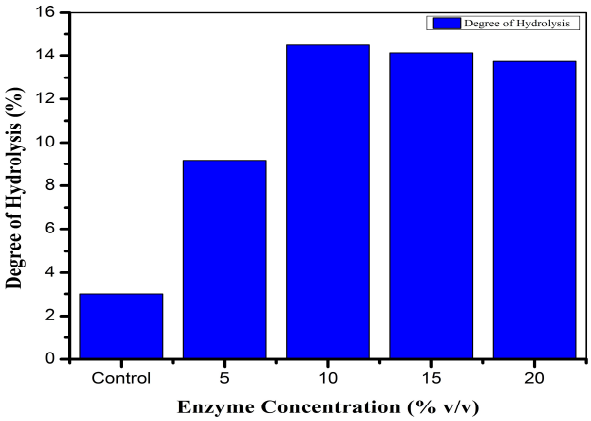

Fig.-2: The Effect of Enzyme Concentration on The Degree of Hydrolysis

The hydrolysis process with variations in hydrolysis time aims to determine the optimum hydrolysis time required by the enzyme to break down the peptide bonds in collagen. The effect of hydrolysis time on DH is shown in Fig.-3. The highest DH was obtained after 4 h hydrolysis with a value of 22.48%. DH showed

an increase since the first 0.5 h but above 4 h DH tends to be constant. This is similar to the results of Bousopha *et al*[14], who reported that DH of cuttlefish skin collagen hydrolysate produced by collagenase *Clostridium histolyticum* increases with increasing hydrolysis time and after that tends to be constant when no clear hydrolysis occurs. The highest DH at 4 h hydrolysis indicated the high level of breakdown of peptide bonds in collagen to produce peptides and free amino acids, while the constant conditions in the hydrolysis reaction are suspected due to the reduced number of peptide bonds that are susceptible to enzymes and the formation of product which can block the reaction.[30]

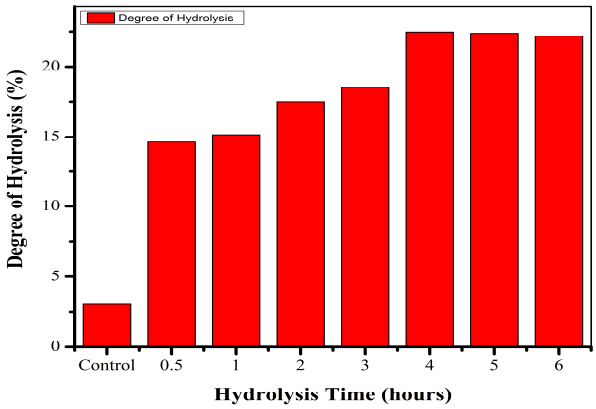

Fig.-3: The Effect of Hydrolysis Time on The Degree of Hydrolysis

The value of DH obtained in this study is different from some previous studies. Hydrolysis of cuttlefish skin collagen using collagenase *Clostridium histolyticum* produced DH of 35%[14], and koan fish skin of 10.7%.[16] Whereas hydrolysis of base fish skin collagen using protamex, alcalase, trypsin dan neutrase generally results in a lower DH of 12.29%, 17.40%, 15.02%, and 13.81% respectively.[31] Based on the results of these studies, collagenase from *Bacillus* sp. 6-2 is quite effective in hydrolyzing yellowfin tuna collagen with DH obtained of 22.48 %.

All collagen hydrolysate obtained at different hydrolysis time (HC0.5-HC6) were evaluated for antibacterial activity. The results of measurements of inhibition zones against *E.coli* and *S.aureus* are shown in Table-2.

Table-2: Antibacterial Activity of Collagen Hydrolysate

Sample	Inhibition Zone (mm)			
	Escherichia coli		*Staphylococcus aureus*	
	24 h	48 h	24 h	48 h
Collagen	7.00	8.70	8.80	9.20
HC0.5	7.20	8.90	8.90	9.60
HC1	7.20	9.20	8.90	10.50
HC2	7.60	9.90	9.30	11.00
HC3	7.90	10.50	9.90	11.40
HC4	8.30	10.80	9.90	11.80
HC5	8.30	10.90	10.10	12.10
HC6	8.90	11.50	10.80	12.60
Control (+)	27.20	32.20	25.70	26.20

Based on the data in Table-2, all collagen hydrolysate was active against *E. coli* and *S. aureus* with a higher inhibition zone than samples without enzyme treatment (collagen). The results showed that the peptides produced from the hydrolysis of yellowfin tuna bone collagen with collagenase from Bacillus sp. 6-2 are effective in producing peptides that have antibacterial properties. Antibacterial activity increased with increasing hydrolysis time and DH. An increase in DH value indicates that the size of the peptide produced is getting smaller. Thus, small peptide molecules contribute to the increased bioactivity of collagen

hydrolysate as antibacterial. The reduced peptide size, according to Najafian and Babji[32], leads to better exposure and structural acquisition of amino acid residues and their charges that promote interaction with bacterial membranes. In our study, the activity continued to increase until hydrolysis of 6 h even though DH value decreased, which showed that activity not only depends on the size of the peptide but also the composition and sequence of amino acid peptides.

The higher activity of antibacterial agents against *E. coli* and *S. aureus* was obtained after 6 h of hydrolysis with 11.50 mm and 12.60 mm inhibition zones. The activity increased after 48 h incubation. The results indicate that the antibacterial peptides produced are bactericidal, which means they can kill bacteria. The mechanism of bactericidal peptides against pathogens is unclear, but several studies have revealed the mechanism of antibacterial peptides. Mechanism of peptides generally starts with interactions on cell membranes and can subsequently show different actions such as the formation of channels in a lipid bilayer, carpet formation on the surface of the membrane, dissolving membranes, and the entry of peptides into the cell without damaging the membrane. The mechanism of collagen peptide follows the carpet model.[33] Peptides accumulate on the surface of the membrane to reach threshold concentrations to cover the surface forming the carpet. This interaction will affect the integrity of the membrane, resulting in cell lysis..[34]

CONCLUSION

Collagen hydrolysate with antibacterial activity successfully produced from yellowfin tuna (*Thunnus albacares*) bone using collagenase from *Bacillus* sp. 6-2. Collagen hydrolysate obtained is bactericidal so it is a very good candidate to be developed as an antibacterial agent.

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