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

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

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ABSTRACT

Fish bones can be processed into more useful materials and high economic value by converting of its constituent collagen to collagen hydrolysate. The objective of this study was to produce collagen hydrolysate from yellowfin tuna bones using collagenase from *Bacillus* sp. 6-2 and evaluate its ability as antibacterial agent. The Hydrolysis process was optimized by varying the concentration of substrate, enzyme and hydrolysis time. The degree of hydrolysis and antibacterial activity of the collagen hydrolysate were investigated. Results showed that hydrolysis at substrate concentration of 10 %, enzyme of 10 % and hydrolysis time of 4 h were the optimum conditions to obtain the highest degree of hydrolysis (22.48 %). All collagen hydrolysates obtained have antibacterial activity against to *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: Fish bone, Yellowfin tuna, Collagen hydrolysate, Collagenase, Degree of hydrolysis, Antibacterial activity.

INTRODUCTION

Yellowfin tuna is a tuna species with large production in Indonesia. During the period 2005-2012, total tuna production in Indonesia reached 1.352.802 tons. Yellowfin tuna dominates around 72 % of total big tuna group production.[1] Yellowfin tuna is widely exported to several countries including U.S, Japan, and European Union. Export demand is not only in the form of fresh or frozen products but also processed (fillets). 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]

Collagen hydrolysate has shown a variety of attractive bioactivity, including antimicrobial, antioxidant, antitumor, anti-inflammatory, antihypertensive, antidiabetic, accelerating wound healing, and efficacious in the prevention and treatment of osteoporosis and osteoarthritis.[7,8] Therefore, they are widely used as supplements which 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 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. Studies show that antibacterial peptides isolated from marine sources are good candidates as antibacterial agents because of broad-spectrum activity, low biodeposition rates in body tissues, diverse structures, very specific to targets with a lower risk of side effects.[17]

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 factor due to it affects 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.

EXPERIMENITAL

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 from bone follows the Baehaki et al[15] method with little change. 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 65 °C. 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 °C with a shaking speed of 100 rpm. Cell culture (10 %) was transferred aseptically to fermentation medium and produced for 30 h at 37 °C and 180 rpm. Medium containing cells was centrifuged at 3500 rpm, 4 °C 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, consisting of 2 % collagen substrate, peptone, yeast extract, NaCl and ammonium sulfate.[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 °C. The mixture was placed in boiling water for 5 min to stop the reaction, then centrifuged (10.000 rpm) for 10 min at 4 °C. The hydrolysis process was designed by varying substrate concentration (5 %; 10 %; 20 %; 30 %), enzyme concentration (5 %; 10 %; 15 %; 20 %) and hydrolysis time (0.5; 1; 2; 3; 4; 5; 6 h) to determine the optimum hydrolysis conditions in production of collagen hydrolysate.

Determination of 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 was analyzed using Lowry method[22], with BSA as a standard. DH was calculated using a formula:

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

Determination of Antibacterial Activity

Antibacterial Activity of samples was determined by inhibition test against Escherichia coli and Staphylococcus aureus using agar diffusion method. The test was carried out based on the modification of Ramssel method.[23] The paper disk (6.0 mm) was dipped in the sample then placed aseptically on the surface of MHA medium containing 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.[24,25]

Table-1: Amino acid content of collagen extracted from yellowfin tuna bones.

Groups	Amino acids	(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
	Arginin (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 hydrolysis parameter that is commonly used to monitor enzymatic hydrolysis. DH represents the number of peptide bonds degraded during the hydrolysis process and it is directly related to the size of the peptide, functional characteristics and bioactivity.[26] The high DH indicates a high level of protein hydrolysis to smaller size peptides which may have strong bioactivity.

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. Increasing substrate concentration tends to reduce DH.[27] This is consistent with the data in Figure 1, which showed a

decrease in DH with an increase in substrate concentration above 10 %. This condition occurs due to the higher substrate concentration that causes the active site of the enzyme to saturate 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 product during the hydrolysis process.

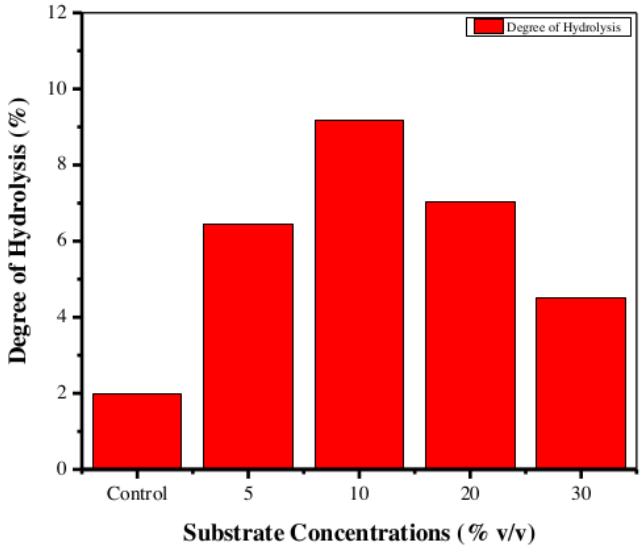

Fig.-1. The effect of substrate concentration on DH at pH 7.0 and 40 °C, with enzyme concentration of 5 % and hydrolysis time of 30 min

The enzyme concentration also greatly affects DH. Increasing the number of enzyme in the hydrolysis process will increase DH due to more enzymes can bind the substrate to produce more products.[27] Information about the optimal concentration of enzyme is very important to determine how much enzyme is needed so that the hydrolysis reaction runs optimally. DH increases with increasing concentration of enzyme and at the addition of certain concentrations DH tends to be constant. The highest DH was achieved by the addition of 10 % enzyme with a value of 14.64 % (Figure 2). DH decreased slowly with the addition of enzyme above 10 %. According to Guérard et al[28], a decrease in DH can be caused by several factors such as a reduced number of peptide bonds that are susceptible to enzymes, decreased enzyme activity and the formation of a product which can block the reaction. Under these condition, an increase in enzyme concentration can no longer increase the rate of hydrolysis reactions.

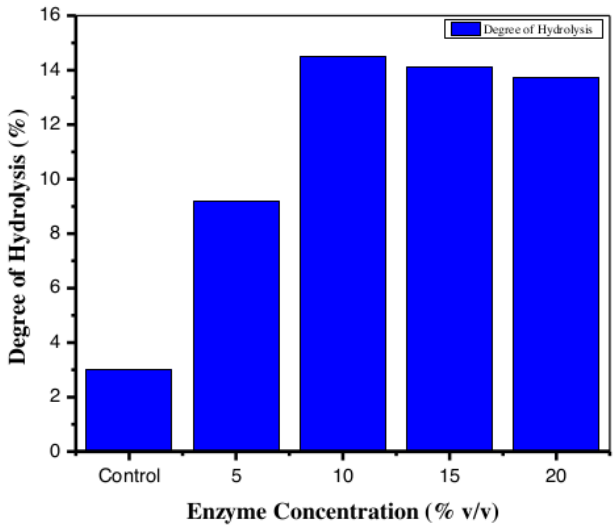

Fig.-2. The effect of enzyme concentration on DH at pH 7.0 and 40 °C, with substrate concentration of 10 % and hydrolysis time of 30 min

Hydrolysis process with variations in hydrolysis time aims to determine the optimum hydrolysis time required by enzyme to break down the peptide bonds in collagen. The effect of hydrolysis time on DH is shown in Figure 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.[28]

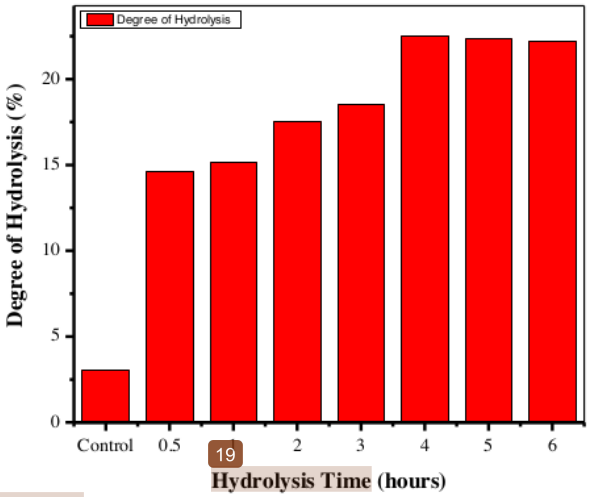

Fig.-3. The effect of hydrolysis time on DH at pH 7.0 and 40 °C, with substrate concentration of 10 % and enzyme concentration of 10 %.

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.[29] 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 show that collagenase from *Bacillus* sp. 6-2 successfully releases antibacterial peptides from yellowfin tuna bone collagen. 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 increased bioactivity of collagen hydrolysate as antibacterial. According to Najafian and Babji[30], the reduced size of peptide leads to better exposure of amino acid residues and their charges, and structure acquisition which support 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 peptide, but also the composition and sequence of amino acid peptides.

The higher antibacterial activity against E. *coli* and *S. aureus* was obtained after 6 h hydrolysis with inhibition zones of 11.50 mm and 12.60 mm respectively. 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 a number of studies have revealed the mechanism of antibacterial peptides. Mechanism of peptides generally starts with interactions on cell membranes and can subsequently show different action 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.[31] Peptides accumulate on the surface of the membrane to reach threshold concentrations to cover the surface forming the carpet. These interactions will affect membrane integrity and cause cell lysis.[32].

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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