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Antibacterial Activity of Mackerel Fish Bone Collagen Hydrolysate (Scomberomus sp.) Against Staphylococcus aureus Bacteria

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ABSTRACT

Background: Staphylococcus aureus is a normal microflora that can become a pathogen in the oral cavity. An unbalanced presence of S. aureus bacteria is often involved in various oral infections, ranging from minor infections to infections to severe one. Long-term use of synthetic antibacterial agents can cause side effects. Natural ingredients from hydrolysed mackerel fish bones can be used as an alternative antibacterial agent. Hydrolysed collagen from spanish mackerel fish bones contains antimicrobial peptides that can inhibit bacterial growth.

Objective: To determine the antibacterial activity of hydrolysed collagen from spanish mackerel fish bones (Scomberomorus sp.) against Staphylococcus aureus.

Method: An in vitro laboratory experimental research with a post-test only control group study design. The test groups consisted of hydrolysed collagen from spanish mackerel fish bones with different hydrolysis times, namely 60 minutes, 90 minutes, and 120 minutes using bromelain enzymes, 0,2% chlorhexidine gluconate was used as a positive control, and distilled water (aquades) as a negative control. The antibacterial activity test of the hydrolysed collagen from spanish mackerel fish bone was tested using the Kirby-Bauer disc diffusion method to determine the value of the inhibition zone. The inhibition zone values were than analyzed statistically using one-way ANOVA and Bonferroni tests.

Results: Hydrolysed collagen from spanish mackerel fish bones had an average inhibition zone at hydrolysis time of 120 minutes (14.45 mm), but smaller than 0.2% chlorhexidine gluconate.

Conclusion: The hydrolysed collagen from spanish mackerel fish bones (Scomberomorus sp.) had antibacterial activity against Staphylococcus aureus.

Keywords : Antibacterial, hydrolysed collagen, staphylococcus aureus, spanish mackerel, fish bone.

INTRODUCTION

The oral cavity is one of the main gateways for the entry of various kinds of microorganisms and is a complex environment that can serve as an ideal habitat for communities of fungi, bacteria, protozoa, archaea, and viruses.[1] Bacteria are the most commonly found type of microorganism in the oral cavity, numbering around 500 to 700 species, some of which include *Streptococcus* spp., *Staphylococcus* spp., *Neisseria* spp., and *Lactobacillus* spp.[2,3] These bacteria can be found on the teeth, buccal mucosa, palate, dorsum of the tongue, and other soft tissues that form a heterogeneous, species-rich ecological system, such as *Staphylococcus aureus*.[3] This microorganism plays an important role in physiological development, such as aiding in host defense and preventing colonization by exogenous organisms; however, it can become

pathogenic if quantitative changes occur in an unbalanced environment.[2,4] *S. aureus* bacteria are classified as normal microflora that can live in the oral cavity as well as in the skin and mucosal layers.

Based on its characteristics, *S. aureus* is categorized as a facultative anaerobic Gram-positive, non-motile, non-spore-forming, and spherical bacterium that forms grape-like clusters.[5] The ability of *S. aureus* to multiply rapidly, spread widely in tissues, and produce extracellular substances in the form of toxins and enzymes can lead to infections.[4] *S. aureus* infections are known to be frequently found in the bloodstream (bacteremia) and can also occur in the skin and mucosal layers, presenting as abscesses, cellulitis, and impetigo. An unbalanced presence of *S. aureus* bacteria is often involved in various oral cavity infections, ranging from minor infections to major severe ones. This is evidenced by several studies showing that the prevalence of *S. aureus* bacterial infections is found in cases of periodontal abscesses (44%), angular cheilitis (33.3%), denture stomatitis (49.5%), and Ludwig's angina (26.5%).[7-10] Other studies also report that the involvement of *S. aureus* is found in mucositis, gingivitis, periodontitis, peri-implantitis, parotitis, endodontic infections, and even dental caries.[11,12]

In dentistry, one of the efforts that can be made to eliminate *S. aureus* bacteria is using antibacterial agents such as chlorhexidine. Chlorhexidine is a broad-spectrum antimicrobial agent that can inhibit and kill both Gram-positive and Gram-negative bacteria, as well as fungi. However, long-term use of chlorhexidine can also cause side effects, such as tooth discoloration, a burning sensation, and triggering supragingival calculus formation.[4,13] Utilizing natural materials from plants and animals can serve as an alternative antibacterial compound to minimize these side effects. Collagen is the main protein in the extracellular matrix of animal bodies and is widely utilized in the biomedical industry.[14] Currently, marine animal waste in the form of fish skin, scales, and bones is considered a more promising source of collagen compared to mammals, including cows and pigs.[15,16]

Based on data from the Ministry of Maritime Affairs and Fisheries in 2022, marine fisheries production increased to more than 7 million tons per year. Mackerel is a primary fish commodity in Indonesia, with its meat and skin widely processed into various food items, especially in the city of Palembang.[17] Processed food products from Palembang that are frequently made from mackerel include *pempek*, *model*, meatballs, *otak-otak*, and *kemplang*. In the production process of these various foods, fish bone waste is left unutilized due to its hard texture, making it difficult to grind. Mackerel is known to be a bony fish with a collagen content reaching 15–17 g per 100 g of fish weight. Type 1 collagen is the most common type found in

bones.[14,17] The available collagen from mackerel fish bones can be produced in the form of hydrolysate.[14,15] Collagen hydrolysate is known to possess bioactivity that can inhibit and kill bacteria due to its active antimicrobial peptide content, making it a potential alternative antibacterial agent.[16,19]

Ennaas et al. reported that collagen hydrolysate from Atlantic mackerel produces antimicrobial peptides that act as antibacterial agents against *Staphylococcus aureus*.[19] Another study by Natsir et al. showed that yellowfin tuna bone collagen hydrolyzed enzymatically was able to inhibit *E. coli* and *S. aureus* bacteria.[16] This is associated with the presence of antimicrobial peptides (AMPs), which can cause damage to cell membranes, thereby inhibiting bacterial activity.[20,21] The resulting collagen hydrolysate is also influenced by several factors, such as the enzyme-substrate ratio, substance concentration, pH, temperature, and hydrolysis time.[16] A study conducted by Hartina et al. indicated that the type of enzyme and the hydrolysis time used affect the effectiveness of the collagen hydrolysis product.[22] Based on the described background, the researchers aimed to test the antibacterial activity of enzymatically hydrolyzed mackerel fish bone collagen against *Staphylococcus aureus* bacteria.

METHODS

The type of research conducted is an in vitro laboratory experimental study with a post-test only control group design to determine the antibacterial activity of mackerel fish bone collagen hydrolysate (*Scomberomorus* sp.) against *Staphylococcus aureus*. The study was conducted from March 18 to April 8, 2024, at the Laboratory of Chemical Engineering, Politeknik Negeri Sriwijaya, Palembang, and the Microbiology Laboratory Research Center, Faculty of Dentistry, Universitas Airlangga, Surabaya, East Java.

The object of the study was mackerel fish bone collagen hydrolysate. The sample and control groups were categorized into 5 groups, namely mackerel fish bone collagen hydrolysate with hydrolysis times of 60 minutes, 90 minutes, and 120 minutes, a positive control with 0.2% chlorhexidine gluconate, and a negative control with sterile distilled water (aquades). The number of replications for each treatment was 5 times, resulting in a total of 25 samples: 15 samples of mackerel fish bone collagen hydrolysate, 5 positive control samples, and 5 negative control samples. **Inclusion criteria:** fresh mackerel fish obtained from the Sungailiat Fishing Port, Bangka Belitung, weighing 3–4 kg/fish, which had been selected based on morphological characteristics.

Sample Preparation

The mackerel fish bones (*Scomberomorus* sp.) used in this study were obtained from the Sungailiat Fishing Port, Bangka Belitung, weighing 3–4 kg/fish in fresh condition. The mackerel was cleaned, and the bones were separated from the skin and meat. The head and tail bones were removed, and the ribs and fin rays were cut into small pieces. The mackerel fish bones were then soaked sequentially in 0.25 mol/L NaOH solution, 0.2% sulfuric acid, and 0.3 M acetic acid (each with a ratio of 1:7 w/v for 40 minutes), with rinsing until a neutral pH was reached between each soaking step. After that, the fish bones were filtered, ground using a chopper, and weighed to be used in the collagen hydrolysate preparation stage.[22]

Preparation of Collagen Hydrolysate

The preparation of mackerel fish bone collagen hydrolysate was carried out by soaking the ground fish bones in a 0.1 M NaH_2PO_4 and Na_2HPO_4 buffer solution at pH 6 with a ratio of 2:1 (w/v). Then, 1% bromelain enzyme was added (enzyme/substrate ratio 1:100) and the mixture was placed in a water bath at a temperature of 50°C with different hydrolysis times, namely 60, 90, and 120 minutes.

After the hydrolysis process was complete, the samples were heated at 100°C for 10 minutes to stop the enzyme reaction, then filtered and centrifuged at 10,000 g for 30 minutes at 4°C. Next, the pH of the hydrolysate was neutralized by adding NaOH or HCl, filtered again using Whatman filter paper No. 4, and stored in a closed, contaminant-free container.[22]

Preparation of Growth Media

The growth media was prepared by dissolving 2.3 grams of nutrient agar into 100 ml of distilled water in an Erlenmeyer flask. The mixture was then heated on a hot plate until homogeneous and boiled for ±40 minutes. The prepared agar media was then sterilized using an autoclave at 121°C for 15 minutes.[83,84]

Bacterial Rejuvenation

Bacterial rejuvenation was performed before the antibacterial test by streaking one inoculating loop (*ose*) of *Staphylococcus aureus* bacterial culture onto fresh nutrient agar slants, which were then incubated at 37°C for 24 hours in an incubator.[79,84]

Preparation of Bacterial Suspension

The bacterial suspension was prepared by taking a 24-hour-old bacterial culture from the agar slant using a sterile inoculating loop. The test bacterial colonies were then suspended in a test tube containing 10 ml of 0.9% NaCl until a turbidity matching the McFarland standard (10^8 CFU/ml) was achieved.[79,84]

Antibacterial Activity Test of Collagen Hydrolysate

Antibacterial activity testing was carried out using the disc diffusion method (Kirby-Bauer) with paper discs. A total of 15 ml of nutrient agar medium was poured into each petri dish and allowed to solidify. Then, a sterile cotton swab dipped into the bacterial suspension was wiped evenly across the entire surface of the medium. Paper discs impregnated with sample solutions from groups A, B, C, D (0.2% chlorhexidine gluconate), and E (distilled water) were placed on the surface of the nutrient agar. The petri dishes were then incubated for 24 hours at 37°C. The clear zone formed around the disc indicated inhibition of bacterial growth, and its vertical and horizontal diameters were measured using a vernier caliper and expressed in millimeters.[80,83,85]

The collected data were analyzed using SPSS, starting with the Shapiro-Wilk normality test and Levene's homogeneity test. If the data were normally distributed ($p > 0.05$) and homogeneous ($p > 0.05$), data analysis proceeded with the One-Way ANOVA test to determine differences in the average inhibition zone of *Staphylococcus aureus* between treatment groups, followed by the Bonferroni test to determine the significance of differences in average inhibition zone diameters for each pair of treatment groups.

RESULTS

Antibacterial Activity Test Results of Mackerel Fish Bone Collagen Hydrolysate Inhibition Zone Test Results

Measurement results showed that the average diameter of the inhibition zone increased along with the length of hydrolysis time, namely 11.28 mm at 60 minutes, 12.86 mm at 90 minutes, and 14.45 mm at 120 minutes. Meanwhile, 0.2% chlorhexidine gluconate produced the largest inhibition zone (20.48 mm), and distilled water showed no inhibition zone at all (Table 1).

Table 1. Inhibition zone formed in each group

Treatment	Repetition					Average Inhibition Zone Diameter (mm)
	1	2	3	4	5	
Mackerel bone collagen hydrolysate 60 mins	11.40	11.20	11.05	11.35	11.40	11.28
Mackerel bone collagen hydrolysate 90 mins	12.80	12.95	12.75	12.80	13.00	12.86
Mackerel bone collagen hydrolysate 120 mins	14.55	14.60	14.20	14.55	14.55	14.45
Chlorhexidine gluconate	20.15	20.40	20.60	21.05	21.05	20.48

0.2%					
Distilled water	0	0	0	0	0

The results of the Shapiro-Wilk normality test and Levene's homogeneity test showed that data from all groups were normally distributed and homogeneous ($p > 0.05$). The One-Way ANOVA test results showed a significant difference between the treatment and control groups ($pp < 0.05$) (Table 2), and further analysis with the Bonferroni test indicated that each pair of groups had a significant difference in average inhibition zone diameter ($p < 0.05$).

Table 2. One-Way ANOVA Test Results for the Inhibition Zone of Mackerel Fish Bone Collagen Hydrolysate (*Scomberomorus sp*.) against *Staphylococcus aureus* Bacteria

Group	**Mean ±SD**	**p-*value***
60 Minutes	11,28±0,15	0,000*
90 Minutes	12,86±0,11	
120 Minutes	14,45±0,17	
Chlorhexidine	20,48±0,37	
Distilled Water	0	

Data analysis was followed by the Bonferroni test to identify groups with significant differences by comparing the average inhibition zone diameters between treatment groups and control groups. The Bonferroni test results (Table 3) showed that each pair of groups had a significant difference in average inhibition zone diameter ($p < 0,05$).

Table 3. Results of Multiple Comparison Analysis with Bonferroni Test on the Inhibition Zone Diameter of Mackerel Fish Bone Collagen Hydrolysate (*Scomberomorus sp*.) against *Staphylococcus aureus* Bacteria

Group Pair	**60 minutes**	**90 minutes**	**120 minutes**	**Chlorhexidine**	**Distilled Water**
60 minutes		0.000*	0.000*	0.000*	0.000*
90 minutes	0.000*		0.000*	0.000*	0.000*
120 minutes	0.000*	0.000*		0.000*	0.000*
Chlorhexidine	0.000*	0.000*	0.000*		0.000*
Distilled Water	0.000*	0.000*	0.000*	0.000*	

DISCUSSIONS

Testing the antibacterial activity through the measurement of inhibition zones in this study showed that mackerel fish bone collagen hydrolysate was able to produce a clear zone around the paper disc, proving the existence of antibacterial activity against *S. aureus*. This is in line with the study by Natsir et al., which reported that hydrolyzed tuna bone collagen extract was also able to inhibit *S. aureus* bacteria.[16]

Based on the inhibition zone categories by Davis and Stout, mackerel fish bone collagen hydrolysate with hydrolysis times of 60, 90, and 120 minutes falls into the strong inhibition category.[80] The largest mean inhibition zone was obtained at a hydrolysis time of 120 minutes (14.45 mm), followed by 90 minutes (12.86 mm) and 60 minutes (11.28 mm), indicating that a longer hydrolysis time results in a larger inhibition zone diameter. This finding is consistent with a study by Shaibani et al. on rocky shore crab shell hydrolysate, which also demonstrated an increase in the inhibition zone along with progressing hydrolysis time.[87]

Hydrolysis time is the period required by enzymes to break down peptides into low molecular weights, making it essential to find the optimum hydrolysis time to enhance antibacterial activity without sacrificing the nutritional content and physicochemical characteristics of the substrate.[88] However, the collagen hydrolysate with a 120-minute hydrolysis time was still unable to match 0.2% chlorhexidine gluconate as the positive control (20.48 mm).

This difference is suspected to be due to the substantivity property of chlorhexidine, which is its ability to release antibacterial effects continuously and gradually, thereby producing a prolonged antibacterial effect.[89] The quality of the hydrolysate in this study could potentially be improved to approach the effectiveness of chlorhexidine, one way being by increasing the concentration of the bromelain enzyme, as reported by Sagita et al. that increasing the concentration of bromelain enzyme can influence the size of the inhibition zone against *S. aureus*.[90]

A study by Sila et al. using a different substrate, namely hydrolyzed barbel fish muscle, also reported antibacterial activity against *S. aureus*, indicating that the hydrolysis process generally can break large molecular weight collagen molecules (300 kDa) into low molecular weight peptides (3–6 kDa) with higher bioavailability.[57,91] Mackerel fish bone collagen hydrolysate is known to contain antimicrobial peptides (AMPs) that act as antibacterials.

According to the literature by Baco et al., AMPs possess a mechanism of action that disrupts cell membrane integrity through interactions with the peptidoglycan layer and lipoteichoic acid, thereby increasing the membrane permeability of *S. aureus* bacterial cells, leading to cell death.[63,64] In addition, AMPs can also inhibit DNA, RNA, and protein synthesis, as well as inhibit the activity of intracellular bacterial enzymes such as protease enzymes secreted by *S. aureus*, as supported by the findings of Tao et al. on catfish hydrolysate, which also produced AMPs with the ability to inhibit *S. aureus*.[65,66,92]

This study has limitations as it did not identify or analyze the amino acids contained

within the mackerel fish bone collagen hydrolysate, for example, through high-performance liquid chromatography (HPLC); thus, it is not yet known specifically which amino acids play the most prominent role as antibacterials. Based on this, this research can be further developed to determine the specific mechanisms of the antimicrobial peptides produced by mackerel fish bone collagen hydrolysate.[16]

CONCLUSION

Based on the results of this study, it can be concluded that mackerel fish bone collagen hydrolysate (*Scomberomorus* sp.) possesses antibacterial activity against *Staphylococcus aureus* with a strong inhibition category at hydrolysis times of 60 minutes (11.28 mm), 90 minutes (12.86 mm), and 120 minutes (14.45 mm). A longer hydrolysis time results in a larger inhibition zone diameter; however, its effectiveness is still not comparable to 0.2% chlorhexidine gluconate as a positive control. Therefore, further research is required regarding variations in enzyme types and concentrations, amino acid identification of antimicrobial peptides, as well as tests against other types of pathogenic oral microorganisms.

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