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DEVELOPMENT OF RAW COLLAGEN FROM WASTE MILKFISH (*Chanos chanos*) SCALES AS A PRESERVATIVE AND EMULSIFYING AGENTS

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ABSTRACT

Collagen is an essential protein in connective tissue, widely used as a bioactive. Raw collagen is obtained from cattle, buffalo, and pigs with problems spreading infectious animal diseases. Collagen can be obtained from waste milkfish (Chanos chanos) scales as an alternative source of mammal raw materials. This study aims to determine the acetic acid's most effective concentration and maceration time in isolating collagen from milkfish scales based on the preservative and emulsifying determination. This research is quasi-experimental design by testing the isolated collagen against collagen yield, proximate analysis of collagen, pH test, preservative test, and analysis of emulsifier substances. The results of the isolation of collagen in all treatment groups had emulsifying and preservative abilities, where a concentration of 0.5 M acetic acid with a maceration time of 72 hours produced the most effective collagen as an emulsifier based on the results of the emulsion stability test and emulsion layer boundary test, as well as at the concentration of 0, acetic acid 5 M with variations in maceration time of 48 hours and 72 hours produced the most effective collagen as a preservative.

Keywords**:** *Collagen, emulsifying agent, milkfish scales, preservative*

INTRODUCTION

Collagen is an essential connective tissue protein widely used as a food additive. Collagen raw materials are primarily from cattle, buffalo, and pigs which have problems spreading infectious animal diseases[1]. About 70% of the country's protein sources are estimated to come from fish[2]. Domestic production of collagen itself is still not optimal. In 2003, Indonesia still imported as much as 6,200 tons of collagen[3]. Recently, the collagen produced by fish scales can be used as an alternative to replace the raw material for collagen from bovines, one of which is milkfish scales.

Karawang Regency is a milkfish production center, a supply chain for fresh milkfish for Bekasi, Bandung, and Jakarta[4]. National milkfish production in 2012 was 515,527 tonnes, of which the milkfish presto business had an average production of 1,000-2,000 packs per day. The results of this production activity are hard organic waste in the form of fish scales, skin and bones, which have the

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potential to pollute the environment, so it is necessary to process these wastes into products with selling value, including as a raw material for collagen[5]. Utilization of fish scale waste can be used as raw materials for chips, crackers, chitosan, collagen, and others[6,7].

Milkfish (*Chanos chanos*) processing waste in the form of scales can be used to produce collagen, which can be an alternative to replace the raw material for collagen from bovines, which is derived from cow, buffalo, and pig bones. Besides the high price of collagen from bovines, collagen from these animals has a risk of the spread of infectious diseases Bovine Spongiform Encephalopathy (BSE), Transmissible Spongiform Encephalopathy (TSE)[8], and Foot and Mouth Disease (FMD)[9]. Milkfish belonging to the *Canidae* family is one of the only fishery commodities in Indonesia. Milkfish protein content ranges from 20-24%, glutamic amino acid 1.39%, unsaturated fatty acids 31-32% and contains minerals[10].

Type I collagen on the scales consists of three polypeptide α chains[11]. In a study of collagen isolation from milkfish scales, milkfish scale collagen was 0.3%. In the proximate analysis, the water content was 24.50%, the protein content was 49.16%, the ash content was 21.90%, the fat content was 2.03%, and the carbohydrate content was 2.42%[3]. Collagen is a protein that can provide strength and flexibility to bone tissue and other body parts, such as skin and tendons, and is the main constituent of the body's extracellular matrix[12]. Collagen has bioactivity, including those for skin and bones[13].

Several studies have examined the potential of collagen as a preservative[14-16] and emulsifier agent [17-19]. Collagen isolation from milkfish scales can be performed by extraction using acetic acid[20–22]. Isolation of collagen in milkfish scales can be done by extraction method using acetic acid with a particular concentration[10-12]. Previous methods rarely determine the combination of acetic acid levels and maceration time in the extraction process, so an in-depth study is needed regarding the optimal combination in extracting collagen peptides made from milkfish scales.

We are seeing the abundance of available resources in Karawang Regency, namely the results of the home industry of processed milkfish in the downstream region of Karawang Regency. It is necessary to utilize milkfish waste as raw material for collagen and prove its potential as a preservative and emulsifier agent. This study applied a basic design in the form of a completely randomized design (CRD) by comparing the concentration of acetic acid and maceration time to the results of collagen yield and collagen proximate analysis (water content test, ash content test, protein content test, and fat content test), pH test, preservative test, and emulsifying agent analysis including emulsion viscosity test, emulsion stability test, and layer boundary test.

METHODS

Ingredient

Milkfish scale waste, Aquadest (Rendys Chemical®), NaOH (Brataco®), Acetic acid (Glacial), NaCl (Brataco®), Pepsin (Brataco®), Bromocresol green (Merck®), concentrated HCl (Merck®), Bovine serum albumin, Sodium sulfate (Merck®), *Staphylococcus aureus* bacteria, *Escherichia coli* bacteria, Agar media (Merck®), Diethyl ether (Merck®).

Tools

Whatman Filter Paper, Tissue, Electric Stove (Maspion®), Analytical Scale (Mettler Toledo®), Refrigerator (Sharp®), pH Meter (Hanna®), Brookfield Viscometer (Amatex DVI Digital), Ultra Turrax T25, Incubator, Furnace (Muffle Furnace), Desiccator (Duran®), Autoclave, Freeze Dryer, UV-Vis Spectrophotometry, and Oven (IKA®).

Raw Material Preparation

Samples of milkfish scales were taken by purposive sampling in the form of fresh fish scales obtained around the home industry of processed milkfish in the downstream area of Karawang Regency. Fish scales were cleaned after cleaning, and fish scales were dried using sunlight.

Fish Scales Pretreatment

Pretreatment of fish scales before extraction was carried out in three stages: deproteinization, demineralization, and hydrolysis. Deproteinization using 1,000 mL of 1 M NaOH, then fish scales are separated from the NaOH solution and washed using distilled water until the pH is neutral. Demineralization of fish scales was performed using 0.5 M Na_2EDTA with a ratio of 10% (w/v) for 24 hours at 4°C. The demineralized fish scales were continued with the hydrolysis process by immersing them in a 0.5 M acetic acid solution with a ratio of 10% (w/v) for 48 hours at 4°C. Fish scales from acetic acid immersion were washed with running water until they reached a neutral pH.

Extract Preparation

Fish scales were macerated at four °C with a ratio of 0.25 M acetic acid; 0.5 M; and 0.75 M with a ratio of the weight of fish scales and volume of acetic acid to 1:8 (w/v). The comparison of maceration time is for 48 hours and 72 hours, after which the extract solution is separated from the residue. The extract solution obtained was added with 1 g of pepsin. Then the solution was centrifuged until homogeneous and white lumps formed in the solution, filtered using filter paper. The precipitate formed was wet collagen and then dried using a freeze dryer.

Proximate Analysis

The dried collagen was then analyzed proximately to determine the chemical composition, including analysis of water content, ash, protein, pH, and fat content referring to the AOAC 2005.

Preservative Test

The tool is sterilized using an autoclave. Nutrient agar media is made by dissolving 14 g of nutrient agar media in 500 ml of aquadest, stirring and heating until homogeneous and sterilized in an autoclave at 121°C for 15 minutes, incubated at 37°C for 24 hours. The bacteria *Staphylococcus aureus* and *Escherichia coli* were each diluted by taking one loop of bacterial suspension and put into a test tube containing NaCl solution, then homogenized using a vortex, and the turbidity was standardized using 0.5 Mc Farland. Then the standardized bacterial solution was applied to the nutrient agar growth medium. Each nutrient agar medium was perforated using a well, and six samples of collagen were inserted using a micropipette in each nutrient agar medium hole, then incubated at 37°C for 24 hours. The diameter of the inhibition zone formed around the hole was observed and measured using a caliper.

Emulsifying Agent Analysis

The preparation of the emulsion sample was started by weighing 1 g of collagen, then dissolving it in 50 ml of distilled water and then homogenized while being heated to a temperature of 60°C and, held for 10 minutes, then cooled to a temperature of 30°C. The collagen solution was then homogenized using Ultra Turrax T25 at a speed of 8,000 rpm for 1 minute. Then 50 ml of palm oil was added. After adding oil, the homogenization speed was increased to 14,000 rpm for 3 minutes. We performed centrifugal method analysis to measure the emulsion's stability by measuring the creaming index (CI), viscosity test, and the boundaries of separate layers[23-25].

RESULTS AND DISCUSSION

Collagen Yield Results

Collagen yield was obtained from the comparison of the dry weight of collagen with the weight of the material or initial sample. The highest yield was found in the extraction of 0.5 M acetic acid concentration with a maceration time variation of 48 hours, namely 8.5%. The lowest at 0.75 M acetic acid concentration with a maceration time variation of 72 hours is 2.05%.

The high collagen isolation gain was caused by the pepsin enzyme's use in the extraction process. Pepsin is used to extract collagen peptides, this enzyme acts on the telopeptide region in the collagen molecule, increasing its solubility in acidic media, and hydrolysis is carried out by the action of proteolytic enzymes[18]. Collagen was isolated more effectively by enzymatic extraction[26]. The combination of acid and enzymatic treatment results in a higher and more efficient collagen extraction process.

Collagen Moisture Test

Moisture content is stated based on wet weight or based on dry weight. The collagen moisture content test was carried out by inserting 1 g of collagen sample into an empty cup and in an oven for 5 hours at a temperature of 105°C until the weight was constant. The results show that the average water content ranges from 0.07% - 0.31%, and the lowest water content is acetic acid concentrations of 0.5 M and 0.25 M with time variations of 48 hours and 72 hours (0.07%), the highest concentration of 0.5 M acetic acid with a time variation of 72 hours (0.31%). The collagen moisture test results meet the collagen quality requirements set by the National Standardization Agency (2014), which is a maximum of 12%. The higher the water content in collagen, the worse the quality of the collagen, while the lower the water content, the better the quality of the collagen[27]. Another study found that collagen samples from fish scales had an average water content of 7.85%[20]. A study conducted by Pamungkas et al. (2018) had an average water content of 10.87%[28].

Collagen Ash Level Test

The ash content test aims to determine the mineral content contained in fish scale collagen. The test was carried out by inserting 1 g of collagen sample into a porcelain dish, then the sample was put into a furnace at a temperature of 600°C for 1 hour, and the ash content was weighed to determine the ash content. The average ash content ranges from 0.01% - 0.3%, and the lowest ash content is owned by acetic acid concentrations of 0.5 M and 0.25 M with a time variation of 48 hours and 72 hours of 0.01%. The highest concentration of 0.5 M acetic acid with a time variation of 72 hours is 0.3%.

From the collagen ash test results, all values meet the collagen quality requirements set by the National Standardization Agency (2014), which is a maximum of 1%[29]. The low ash content of collagen indicates that the demineralization process can remove about 98% of the inorganic components from milkfish scales. The Collagen ash content of milkfish scales in the study of Pamungkas et al. (2018) has an average ash content of 40.35%, so the collagen ash content carried out in this study is better, with a moderate ash content of 0.01%[28].

Collagen Fat Level Test

The fat content test was carried out by drying the fat flask in an oven at 110°C, then put in a desiccator for 15 minutes and weighed. After that, 1g of collagen sample was put into the soxhlet apparatus, which already contained diethyl ether, and carried out the reflux process until the solution and solvent in the flask were clear. The average fat content ranges from 0.14% - 0.46%, the lowest fat content is owned by 0.5 M acetic acid concentration with a time variation of 72 hours, and the highest concentration of acetic acid 0.75 M with a time variation of 72 hours. The high-fat content of

milkfish scales indicates the need for optimization of the pretreatment process to remove fat in fish scales to improve the quality of the collagen produced.

Collagen Protein Level Test

In making the standard series of bovine serum albumin using UV-VIS spectrophotometry at a wavelength of 595 nm. The lowest average protein content is an acetic acid concentration of 0.5 M at 72 hours at 68.62 % and the highest at 0.75 M acetic acid concentration with a 72-hour time variation of 83.25 %. High protein and low water content in collagen indicate that the extraction method effectively extracts collagen from milkfish scales. The results of the test of collagen protein levels at a concentration of 0.75 M acetic acid with a time variation of 72 hours of 83.25% have met the collagen quality requirements set by the National Standardization Agency (2014), which is 75%.

The most widely known collagen type I collagen consists of three polypeptide chains. Type I collagen is most abundant in soft body parts such as skin and tendons and hard body parts such as bones, teeth, and connective tissue[11]. Protein is in the collagen content, where glycine and proline are essential components of collagen[30]. The protein content obtained in this study was higher than the collagen made from milkfish scales in the study of Nurhidayah et al. (2019), which has an average protein content of 49%[3], in addition to the research of Pamungkas et al. (2018) which has a moderate protein content of 69%[28].

Emulsion pH Test

The pH measurement is carried out to determine whether the collagen emulsion is acidic or alkaline. The pH test results were obtained by dissolving 1 g of the sample in 50 mL of distilled water and stirring it until homogeneous. Then the pH of the solution was measured using a pH meter. The results show that the pH ranges from 2.8 to 5.48. The lowest pH is 0.75 M acetic acid concentration with a time variation of 72 hours, and the highest is 0.75 M acetic acid concentration with a time variation of 48 hours. The pH test results above do not follow the collagen quality requirements set by the National Standardization Agency (2014) which are 6.5 - 8, because the extraction process uses acetic acid, so the pH becomes acidic. Differences in the pH value of collagen can also be caused by differences in the type and concentration of acid or base used during hydrolysis[31].

Emulsion Viscosity Test

The results of the emulsion using fish scale collagen have then measured the viscosity using a Brookfield viscometer. Select the appropriate rotation spindle at a speed of 100 rpm to achieve measurement stability. Based on the results of statistical processing, there was no significant difference in viscosity for each group. ($p \geq 0,05$). The results of the Average Value of Emulsion Viscosity can be seen in the figure below.

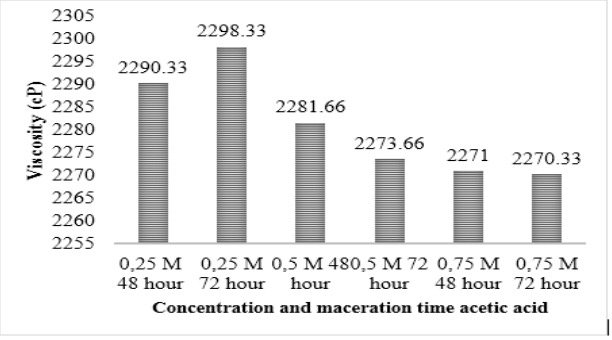

Figure 1. Average value of emulsion viscosity

Based on the bar chart above, the test results range from 2270.33 to 2298.33 cP. The highest 2298.3 cP is owned by a concentration of 0, 25 with a time variation of 72 hours. The viscosity in the previous study had an average viscosity value of 418-1030 cP[32]. Changes in emulsion viscosity indicate a change in intermolecular interactions in the milkfish scale collagen emulsion. A decrease in viscosity indicates a thinner preparation. The dispersed phase will move more quickly in the outer phase. Generally, the emulsion will become more watery at high temperatures and more viscous if left at room temperature[33].

Emulsion Stability Test

The emulsion stability test used a sample of 14 mL put into a centrifuge tube, and then the centrifugation was carried out at a speed of 4,500 rpm for 5 minutes. Centrifugation was carried out at room temperature, then the volume of cream formed was measured. The results of the obtained emulsion stability can be seen in figur below.

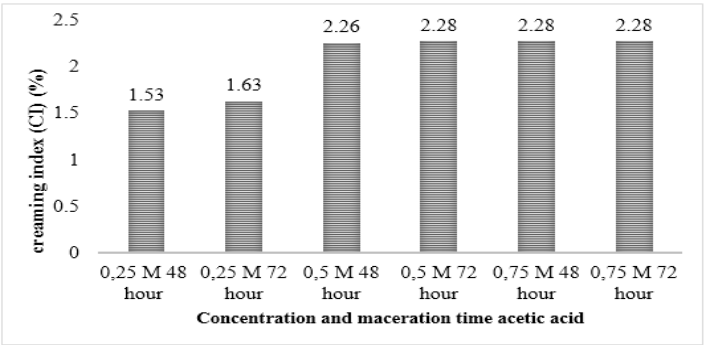

Figure 2. Average emulsion stability

Based on the table and diagram above, the results of the creaming index ranged from 1.53% - 2.28%. The highest was at a concentration of 0.5 M with a time variation 72 hours is 2.28%, the concentration is 0.75 M with a time variation of 48 hours is 2.28%. The concentration is 0.75 M with a time variation of 72 hours is 2.28%. Based on statistical processing, there were significant differences in emulsion stability for each group ($p \leq 0.05$). Creaming is a sign of the coalescence and purification phase, which can be determined using the creaming index. The creaming index provides

insight into the extent of droplet aggregation. The higher the index, the more agglomerated droplets[34]. The emulsion stability test aims to determine the presence of emulsion phase separation. An emulsion can be stable if it can return to its initial state or redispersed with regular shaking after the temperature has been intervened. Based on this, the emulsion that has experienced precipitation and phase separation can be said to be stable if it can be redispersed after shaking[33].

Emulsion Layer Boundary Test

In the emulsion layer boundary test, the 10 mL measuring cup is filled with the emulsion sample up to 10 mL of the volume of the measuring cup. The measuring cup is closed and placed in the room. On each day of observation, the height limit of the visible clear and cream solution was measured. The obtained emulsion layer boundaries were as follows.

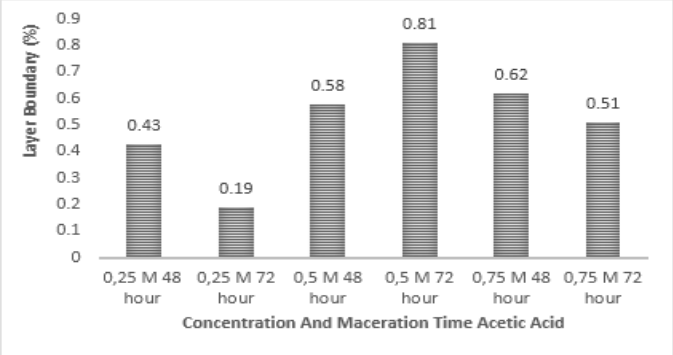

Figure 3. Emulsion layer boundary

The emulsion layer boundaries range from 0.19% to 0.81%. The highest is a concentration of 0.5 M with a time variation of 72 hours of 0.81%. Based on the homogeneity processing and normal distribution, the data obtained on the boundary of the emulsion layer was not normal ($p \geq 0.05$), so it was continued to the Kruskal-Wallis test. Statistical processing showed a significant difference in the emulsion layer boundary for each group ($p \leq 0.05$). Emulsions are thermodynamically unstable in that they tend to break down over time by various physicochemical mechanisms, including gravity processing, flocculation, coalescence, particle coalescence, Ostwald ripening, and phase breakdown[35]. Collagen is a protein that forms a triple helix of three polypeptide chains in the extracellular matrix, where it can be used as an emulsifier[16]. Emulsifier is the most crucial stabilizer in emulsion formulation. This emulsifier is used to improve long-term stability[36]. The emulsion layer boundary test aims to determine the presence of the boundary layer of the emulsion phase. The emulsion layer boundary can show the quality of the emulsion. This test can indicate the presence of the same velocity profile and nonlinear effects when compared to Newtonian fluids[33,37].

Preservative Test

Each nutrient agar medium was perforated in testing the preservatives using a borehole formed around the hole using a caliper. A stock of 6 concentrations of collagen was inserted using a

micropipette in each nutrient agar medium hole, then incubated at 37°C for 24 hours, observed, and measured the diameter of the inhibition zone. The following is the average result of *Staphylococcus aureus* bacteria.

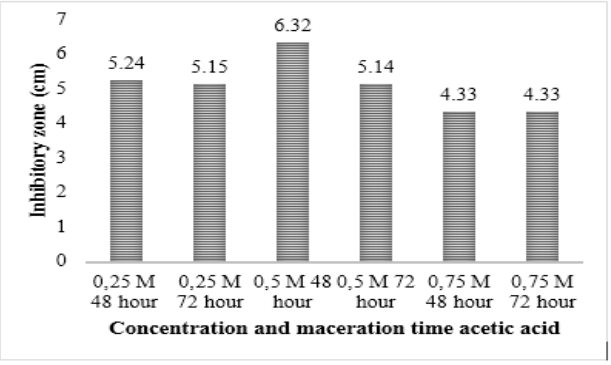

Figure 4. Inhibitory zone diagram of *Staphylococcus aureus*

Based on the table and graph diagram above shows the highest inhibition zone at 0.5 M acetic acid concentration with a time variation of 48 hours of 6.32 cm, then the lowest inhibition zone at 0.75 M acetic acid concentration with a time variation of 48 hours and 72 hours of 4.33 cm, where each group had a significant difference ($p < 0.05$). The results obtained from the preservative test on *Escherichia coli* bacteria can be seen in the figure below.

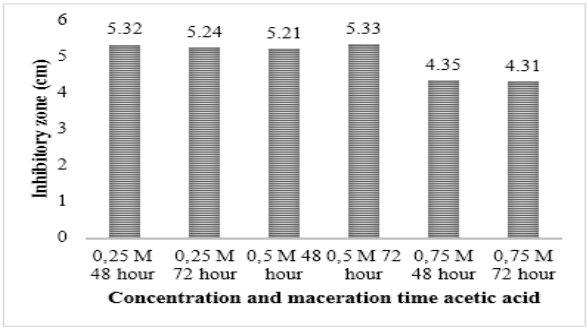

Figure 5. Inhibitory zone diagram of *Escherichia coli* bacteria

The highest inhibition zone at 0.5 M acetic acid concentration with a time variation of 72 hours at 5.33 cm, then the lowest inhibition zone at 0.75 M acetic acid concentration with a time variation of 72 hours at 4.31 cm where each group had a significant difference ($p < 0.05$). Differences in extract concentration, incubation temperature, time of making the wells, and the antimicrobial wells' distance affect the inhibition zone's size[38]. A preservative is effective for preserving certain foods but not adequate for preserving other foods because each food has different properties, so the spoilage of microbes that will be inhibited will also differ. Organic preservatives are more widely used than inorganic ones because these materials are easier to make[39]. It is still rare to research collagen from milkfish scales as a preservative using *Escherichia coli*, but studies show that this collagen is effective as an antifungal[40]. However, suppose it is associated with the provisions of the inhibition activity criteria formed. In that case, 5-10 cm is stated to have moderate inhibitory activity, and 5 cm is

declared weak inhibitory activity. The requirements for inhibitory activity at a concentration of 0.75 M acetic acid for 72 hours are considered to have weak inhibitory activity because the resulting inhibition zone is 5 cm. Concentrations of 0.5 M 48 hours, 0.5 M 72 hours, 0.25 M 48 hours, and 0.25 M 72 hours were considered moderate inhibitory activity because the resulting inhibition zone was 5-10 cm.

CONCLUSION

The results of the isolation of collagen in all treatment groups had emulsifying and preservative abilities, where a concentration of 0,5 M acetic acid with a maceration time of 72 hours produced the most effective collagen as an emulsifier based on the results of the emulsion stability test and emulsion layer boundary test, as well as at the concentration of acetic acid 0,5 M with maceration time of 48 hours and 72 hours produced the most effective collagen as a preservative.

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