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EFFECTIVENESS OF KLUWEK SEEDS (*Pangium edule*) AS NATURAL PRESERVATIVES MATERIAL IN SQUID (*Loligo* sp.) VIEWED FROM PROTEIN PROFILE BASED ON SDS-PAGE

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Abstract: Squid is a high source of protein that quickly decomposes, so efforts are needed to preserve it. One of the natural preservatives used is kluwek seeds. Kluwek contains flavonoid compounds that have antibacterial activity. This study aimed to determine the effectiveness of kluwek seeds as a natural preservative for squid in terms of protein profiles based on SDS-PAGE. The procedure begins with soaking the squid in kluwek seed suspension with concentrations of 10%, 15%, and 20% and then storing them for 24 hours. Protein content was calculated using the Bradford method and protein profiles were analyzed using the SDS-PAGE method. The molecular weight of protein bands is calculated by application *GelAnalyzer* 19.1. The data obtained were analyzed descriptively. The results showed that the total protein content of squid was highest at a concentration of 20% (17.86 µgµL^{-1}), then the concentration of 15% (17.03 µgµL^{-1}), and the lowest was the concentration of 10% (14.64 µgµL^{-1}). Based on its protein profile, squid soaked with 20% concentration of kluwek seed suspension was the most effective for natural preservatives. This is indicated by the composition of the protein bands which are almost the same as fresh squid and protein weights ranging from 30-100 kDa. The conclusion of the research is kluwek seed suspension concentration of 20% is effective as a natural preservative for squid.

Keywords: *Loligo* sp., *Pangium edule*, protein profile, preservatives

Introduction

Squid (*Loligo* sp.) is a marine commodity in Indonesia with great potential. Squid is susceptible to quality degradation due to high water content and has excellent nutritional value to be used as a growth medium for spoilage bacteria, protozoa contamination, fungi, and worms. Damage caused by microbes causes decay, discoloration, changes in shape, and chemical content in squid. Therefore, food must be preserved to maintain its freshness (Sahubawa, 2014; Patriani et al., 20201).

Preservatives are needed so that the squid is not contaminated with bacteria. Preservatives are divided into 2 types, namely synthetic and natural preservatives. Synthetic preservatives such as formalin. Natural preservatives come from plants such as beluntas leaves, ginger, turmeric, galangal, and kluwek (Paramitasari, 2020). Kluwek (*Pangium edule*) contains flavonoid compounds such as cyanide acid, hydrocarpic acid, khaulmograte acid, gorl acid, and tannins. Tannin compounds can be used as antimicrobials and can be used as microbial inhibitors in the process of preserving chicken eggs

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(Manuhutu, 2011). Kluwek seed extract can inhibit the growth of microbes (*Salmonella* sp., *Staphylococcus aureus, Bacillus cereus, Pseudomonas aeruginosa*, and *Escherichia coli* because they contain antimicrobial compounds (Mamuaja & Lumoindong, 2017; Kasim & David, 2013; Makagansa et al., 2015).

Decreasing the quality of squid is a problem that is often encountered when handling these foodstuffs. Improper storage and preservation will cause rapid decay. Preservation with natural methods is an easy, cheap, and inexpensive way to solve the problem. One of the natural ingredients that can be used as a natural preservative is kluwek, a local spice in Indonesia (Patriani et al., 20202).

Paramitasari's research (2020) found that there were differences in protein profiles in shrimp samples before and after treatment with the addition of kluwek seed powder suspension. Changes in protein profiles can occur due to protein denaturation. Protein denaturation is a change in the structure of the protein molecule without breaking the covalent bonds. The characteristics of the protein profile can be known by measuring the protein concentration with SDS-PAGE. SDS-PAGE (Sodium Dodecyl Sulphate-Polyacrilamide Gel Electrophoresis) is a method used to separate protein molecules based on their molecular weight (Bintang, 2010). Research on the effect of kluwek seeds as a preservative on squid has never been reported. The purpose of this study was to determine the effectiveness of kluwek seeds (*Pangium edule*) as a natural preservative in squid (*Loligo* sp.) in terms of the protein profile based on SDS-PAGE.

Materials and Methods

The study was conducted from April to March 2022 at the Molecular Biology Laboratory, Faculty of Nursing and Health Sciece, Universitas Muhammadiyah Semarang. The design of the research is experimental with 2 repetitions. The tools used are SDS-PAGE (Atto) toolset, cabinet dryer, pH meter (HANNA edge), mesh filter, Visible spectrophotometer (Genesys 20), analytical balance, vortex mixer (VM-300). Centrifuge (Hermle Z326K), conical tube, microtube, micropipette (Biorad 10-1000 µL), tip, Digital dry bath, rotator (Daihan Scientific SHO-1D). The ingredients used are cooked kluwek seeds, squid, H2O (aquadest) sterile, aluminum foil, H2O sterile, 30% polyacrylamide, TEMED, 10% APS, 10% SDS, 1.5 M Tris pH 8.8 and 6.8, staining 0.1%, Coomassie Brilliant Blue (BCB) R-250, destaining, acid 10% glacial acetate, butanol, 70% alcohol, 1x running buffer, bio rad assay, PBS pH 7.4, sample buffer, and protein markers.

Preparation of kluwek seeds

The flesh of the kluwek seeds is removed by breaking the kluwek seeds and then drying them with a Cabinet Dryer for one day until the water content in the kluwek seeds disappears. After the kluwek seed meat is dry, grind it with a blender and sift it through an 80-mesh sieve to get a fine kluwek seed powder.

Preparation of kluwek seed powder suspension with various concentrations

The prepared kluwek seed powder was then made into a suspension of kluwek seeds with various concentrations of 10%, 15% and 20% w/v. Suspension with concentrations of 10%, 15%, and 20%

w/v was prepared by weighing 10 g, 15 g, and 20 g of kluwek seed powder into a beaker glass and then adding 100 mL of distilled water at each concentration.

Soaking squid with kluwek seed powder suspension

A total of 10 g of squid was soaked in a volume of 50 mL of each kluwek seed suspension solution with various concentrations of 10%, 15% and 20% w/v for 30 minutes. Then the squid is removed and drained. After that, it was stored at room temperature for one day. In addition, there was a treatment of 10 g of fresh squid without soaking the kluwek seed suspension directly isolated from protein, and 10 g of squid samples without soaking and stored at room temperature for one day.

Making a standard curve of protein concentration

The protein standard curve was prepared using a BSA (Bovine serum albumin) solution according to the following table.

Table 1. Manufacturing of BSA (Bovine Serum Albumin) for Protein Standard Curves

BSA concentration (µg/µl)	dH2O (µl)	Biorad (BPA µl)
0	800	200
0,5	799,5	200
1,0	799,0	200
2,0	798,0	200
3,0	797,0	200
4,0	796,0	200
5,0	795,0	200
6,0	794,0	200
7,0	793,0	200
8,0	792,0	200
9,0	791,0	200
10	790,0	200

Calculation of Squid Protein Concentration

Squid before and after the immersion treatment, 3g of each meat was taken, then mortared until smooth. Then, approximately 7 mL of PBS 1X pH 7.4 was added, and homogenized. The mixture was put into a conical tube and rotary at 3000 rpm for 15 minutes at 4°C. After that, the supernatant was taken and put into a microtube, and stored as a stock sample to be examined.

Calculation of protein concentration using the Bradford method. Preparation of 1000 µL blanks was carried out using 800 µL of aquadest added 200 µL bio rad reagent. The 1000 µL sample was prepared using 2 µL of the sample added with 798 µL aquadest and 200 µL bio rad reagent. Protein absorbance was read using a spectrophotometer with a wavelength of 595 nm. After that, the protein

concentration can be known by using the equation formula of the protein standard curve that has been made.

SDS-PAGE sampling

Sampling was prepared by calculating PBS 1X + protein sample + sample buffer (4 µL) = 20 µL. First, pipette the calculated sample into the microtube, pipette 4 µL sample buffer, add PBS 1X pH 7.4 according to calculations, and homogenize. Then heated in a dry bath at 100°C for 2 minutes. After that, it was removed and put into the ice box.

Manufacturing of Polyacrylamide Gel

Protein separation using SDS-PAGE according to Laemmli's method (1970) prepared glass plate, comb, and spacer that had been cleaned for gel printing. Before inserting the gel, a leak test was first carried out using aquadest.

After the tool is ready to use, enter the separating gel that has been made into the gel printer, and add butanol to remove bubbles. wait for polymerization. After that, put the stacking gel on top of the separating gel quickly, then insert the comb above it, and wait for polymerization to occur. Lift the comb from the top of the stacking gel vertically and slowly. Spraying aquadest using a micropipette to remove the foam.

SDS-PAGE electrophoresis process

The chamber is filled with running buffer, then the tray and glass plate containing polyacrylamide gel is inserted into the apparatus. Pipette 20µl of the sample (containing PBS 1X, supernatant, and loading buffer) and enter the sample into the wells provided.

The ATTO electrophoresis device is connected to an electric current with a voltage of 100 volts and 90 mA. After the bromophenol blue reaches the bottom of the separating gel, the electricity is turned off. The gel was removed from the printer slowly, then put in a 0.2% Commasie Brilliant Blue dye solution for 30-60 minutes above the rotator until the bands were colored.

Furthermore, to remove the color in the gel that does not contain protein, it is given a destaining solution. The destaining solution is replaced 3-4 times until the gel looks clean. When the gel is clean, the washing is stopped and the destaining is replaced with 10% glacial acetic acid solution, then pressed using plastic.

Determination of the desired protein molecular weight is calculated using the Rf of each protein band with the following formula:

$$\text{Rf} = \frac{\text{The distance from the initial movement to the first band}}{\text{band start to finish}}$$

Calculating the Molecular Weight (BM) of known Rf values is plotted on a logarithmic graph with a known MW (marker) value (Feri, 2017).

Results and Discussion

Standard Curve for Protein Concentration

Standard curve of the protein concentration standard curve is used to determine the absorbance of BSA with a known concentration. Measurements with a spectrophotometer visible with a wavelength of 595 nm. The results of standard curve measurements are presented in table 2. Based on figure 2, a linear regression equation is obtained which states the relationship between the concentration of the standard solution and the absorbance, it is Y = 0.0324x + 0.0045 with R2 = 0.9883, where Y is the absorbance, X is the concentration in µg/µL. R value2 must be close to 1 because it shows the relationship between the two variables is getting stronger regardless of whether it is positive or negative (Subandriyo, 2020). The standard curve can be seen in Figure 1.

Table 1. Absorbance of standard solutions at various concentrations

Standard concentration (µl)	Absorbance
0	0
1	0,039
2	0,046
3	0,122
4	0,13
5	0,178
6	0,211
7	0,231
8	0,263
9	0,295
10	0,319

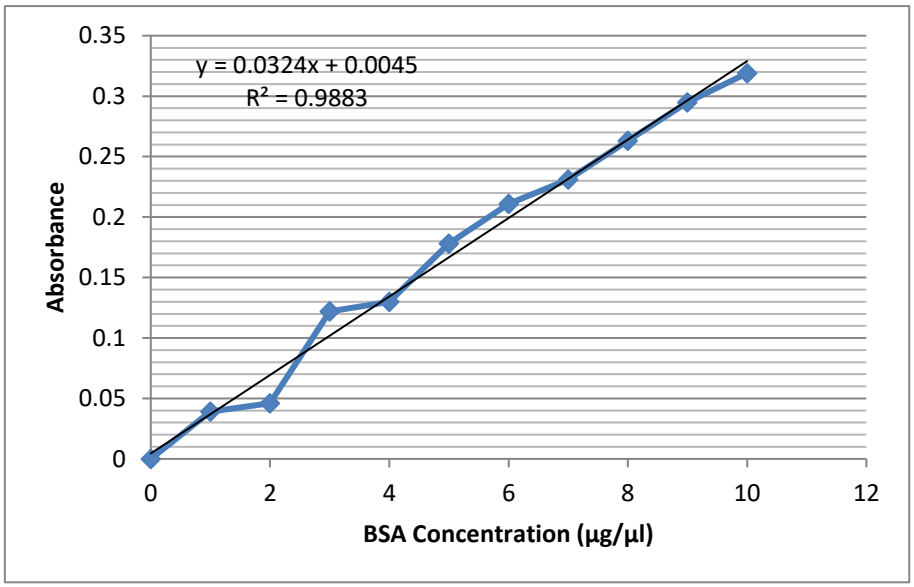

Figure 1. Standard curve based on the relationship between BSA concentration and absorbance

Analysis of the total protein concentration of the Squid

Total protein analysis in this study was calculated using the equation formula obtained from the standard curve. The total protein results can be seen in Table 3.

Table 3. Squid Total Protein

Concentration of kluwek seed powder (% w/v)	Total Protein (µg/µl)
0 (fresh)	19,67
0 (incubation 1x24 hours)	15,25
10	14,64
15	17,03
20	17,86

The highest concentration of total protein was fresh squid is 19.67 µg/µl. Squid with 20% w/v suspension of kluwek soaking treatment was the squid with the highest concentration 17.86 µg/µl. Squid treated with a lower concentration of kluwek seed suspension experienced a significant reduction in the total protein concentration. The addition of kluwek seeds has an effect on maintaining the total protein concentration because they contain antibacterial compounds (Patriani et al., 20201; Patriani et al., 20202). The higher suspended content of kluwek seeds is able to bind to the bacterial cell wall so that it can prevent bacterial growth and suppress enzyme activity (Asmaul, 2022).

The decrease in the concentration of stored squid is because squid easily decomposes due to microorganisms and enzyme activity in their own bodies (Sahubawa, 2014). When the squid is alive, enzymes can help digest food, after death it will turn around to damage the flesh or body of the squid. Enzymes can damage the body by breaking down proteins which are called autolysis. When the squid dies, the temperature rises so that it can become a breeding ground for bacteria, so it needs to be preserved (Widowati, 2014). Autolysis is the change in shape and smell of H2S and NH3 compounds

as the end result of the breakdown of proteins and fats. Increasing the number of microorganisms in squid, the more visible the autolysis process will be (Ramadhani, 2013).

- **Results of SDS-PAGE method of protein profile analysis**

Analysis of the protein profile of squid before and after treatment with the addition of kluwek seed suspension with various concentrations of 10%, 15% and 20% using the SDS-PAGE method is shown in Figure 2.

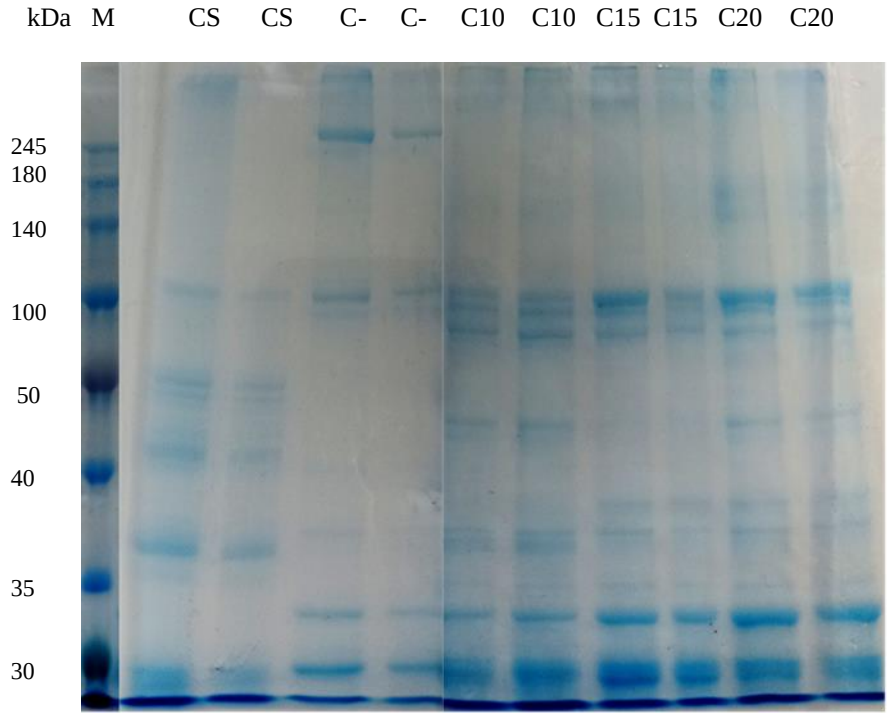

Figure 2. Results of SDS-PAGE electrophoresis on squid samples

Information:
CS : Fresh squid without treatment
C- : Squid without soaking treatment of kluwek seed suspension which was stored for 1x24 hours
M : Protein markers
C10: Squid soaking treatment of kluwek seed suspension with a concentration of 10% stored for 1x24 hours
C15: Squid soaking treatment of kluwek seed suspension with a concentration of 15% stored for 1x24 hours
C20: Squid soaking treatment of kluwek seed suspension with a concentration of 20% stored for 1x24 hours
kDa: kilodalton

Protein band analysis by comparing fresh squid and treated squid. Based on the figure, the C20 treatment has more protein bands in common compared to CS than C-, C10, and C15. The C20 protein band is not reduced much or denatured compared to the others. Major protein bands are still present at C20 the same as CS even though they have different protein weights. The appearance of protein bands occurs as shown in the figure because the lower the concentration of the kluwek seed suspension used the more denatured protein. In the figure, there are many protein bands that are reduced or missing. This is due to the overhaul of proteins by bacteria during storage due to the

content of tannin, khoulmograt acid, and gloric acid as antibacterials at low concentrations which have not been able to inhibit the growth of bacteria in squid. Protein will be degraded by bacteria into amino acids and then degraded again into CO2, H2O, and NH3 as the end result of the protein overhaul process (Hastuti, 2017). Another factor causing spoilage is the temperature during the incubation treatment of squid using room temperature which triggers the growth of bacteria (Asmaul, 2022).

- **Squid Molecular Weight Analysis**

Untreated squid and treated with immersion treatment of kluwek seed powder suspension with various concentrations of 10%, 15%, and 20% were analyzed for protein molecular weight using Gel Analyzer 19.1 software to obtain Molecular Weight (MW) results in Table 4.

Table 4. Molecule Weight Result (kDA) squid sample

Sample Code	Protein Band		Molekul Weight (kDa)	
	Major	Minor	Major	Minor
CS	2	8	101, 45	91, 69, 65, 57, 55, 44, 34, 30
C-	-	6	-	261, 101, 92, 42, 31, 30
C10	1	8	40	80, 59, 48, 36, 31, 30
C15	2	7	96, 40	89, 60, 74, 38, 36, 31, 30
C20	2	10	40, 99	187, 163, 84, 75, 60, 49, 38, 36, 31, 30

Based on the results of molecular weight calculations, CS still has 2 major bands with a molecular weight of 101 kDa and 45 kDa, while other protein bands such as C15 and C20 also have 2 major bands with a molecular weight of 96 kDa, 40 kDa, 40 kDa, respectively. and 99 kDa. The difference in concentration and protein bands between C15 and C20 is not too much, but C20 has a higher concentration of total protein so the higher the concentration of the kluwek suspension, the better its effectiveness as a preservative.

In addition to natural preservatives, the protein found in squid can also inhibit microorganisms. Peptides found in animals can also affect microorganisms, for example, protegrin, and porcine deficiency which can fight Escherichia coli, Listeria monocytogenes, and Candida albicans (Coutinho et al., 2008). Zhang et al. (2022) stated that the antibacterial protein CB-6 has antimicrobial activity that is effective against gram-positive bacteria (in particular, the MIC for MRSA was 16 μg/mL), thermostability, low toxicity, and an additive effect with conventionally used antibiotics. CB-6 protein antibacterial has a molecular weight of 31.405 kDa. In this study, the protein bands of each sample had an average protein weight of 30-100 kDa. The major protein bands have a molecular weight of 30 kDa and 31 kDa so that this sample still contains the antimicrobial protein CB-6.

Conclusion

Based on research that has been done, 20% suspension of kluwek seeds is the best concentration for preserving squid, as seen from the concentration of protein and protein bands compared to fresh squid. The higher the concentration of the kluwek seed suspension the stronger it suppresses the activity of microorganisms.

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Declaration of Interest Statement

The authors declare that they have no conflict of interests.

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