Extending Shelf Life of Indonesian Soft Milk Cheese (Dangke) by Lactoperoxidase System and Lysozyme

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ABSTRACT

Dangke is a traditional dairy product in South Sulawesi, Indonesia, a type of fresh soft cheese made of bovine or buffalo milk. As dangke was made from fresh milk using conventional method, it is easily damaged the quality by storage. This research was done to utilize lactoperoxidase system and lysozyme as preservative agents to suppress the growth of bacteria in dangke. Fresh bovine milk was treated with local papaya’s latex to precipitate the casein. Lactoperoxidase and lysozyme was purified from fresh bovine milk and duck egg white using SP Sepharose Fast Flow and their purity was confirmed. The pH value, total
microbe, and hardness of dangke have been determined to confirm the quality of dangke after immersion in the solution containing lactoperoxidase system (LPOS) or lysozyme, and a combination of them (LPOS+lysozyme). LPOS and lysozyme individually showed slight suppression of total bacteria in dangke. The LPOS+lysozyme remarkably suppressed total microbe in dangke from 7.78±0.67 to 5.30±0.42 Log CFU/ml during 8 hours storage. The immersion dangke using LPOS+lysozyme maintained pH value during storage and enhanced hardness of dangke. This research may open the knowledge on utilization the new method to preserve the quality and to extend shelf-life of dangke.

Keywords: dangke, lactoperoxidase system, lysozyme, quality.

INTRODUCTION

Dangke is an traditional dairy product, a type of fresh soft cheese from Enrekang regency, South Sulawesi province, Indonesian. Dangke contains 55% water and has high nutrients of 23.8% protein, 14.8% fat, and 2.1% mineral [1]. Dangke was produced by heating of fresh milk then adding papaya latex to precipitate casein. The utilization papaya latex was commonly applied in various food including milk processing since it was found in Malaysia and Indonesia with ease [2]. Traditionally, curd and whey are separated using a coconut shell, this is process for shaping stage of making dangke. After the shaping process, dangke is packed with banana leaf and ready to be consumed. Dangke is a conventionally produced with less respect to food hygiene standards resulted in the high possibility for contamination of bacteria. Dangke is usually preserved using salt, though remaining problem of relatively short in its shelf life at room temperature of storage.

Nowadays, the production dangke has been increasing along with the increase in consumer demand [3]. The distribution of dangke has been reported to reach out of province
and it has been distributed to other countries such as Brunei Darussalam and Malaysia. As national distribution, dangke has already distributed in Java and Sumatra Island, in line with the increase in the number of tourism activity. Therefore, the preservation is important point to maintain the quality of dangke.

Lactoperoxidase (LPO) is a heme-containing glycoprotein of 608 amino acids with a molecular mass of 78 kDa and has already been known as natural enzyme found in plants, animal, and human. Lactoperoxidase found in milk, saliva, and tear glands abundantly [4-6] and can serve as natural antimicrobics by the combination work of thiocyanate (SCN⁻) and hydrogen peroxide (H₂O₂), which has called as lactoperoxidase system (LPOS) [6-8]. Lactoperoxidase catalyzes the oxidation of thiocyanate (SCN⁻) by hydrogen peroxide (H₂O₂) resulting in the production of hypothiocyanite (OSCN⁻) [44]. OSCN⁻ is a compound that is responsible for killing bacteria, fungi, and viruses by destroying the sulphydryl groups (SH groups) of the cell membrane, resulting in damage to vital cell membrane, which leads to cell death [4, 9-14]. LPOS has been applied as a natural preservative in some foods, such as milk [15,16], fruit, chicken, and vegetable [17]. Lactoperoxidase system is effective on suppressing the growth of S. pseudomonas, E. coli, and S. typhimurium on cottage cheese [18].

Lysozyme (1,4-beta-N-acetylmuramidase, 14.4 kDa), is a hydrophilic protein that has been widely used as a natural preservative. Lysozyme is naturally found in egg white and milk [19,20]. Lysozyme hydrolyzes 1,4-beta-linkages between N-acetylmuramic acid and N-acetylglucosamine present in the peptidoglycan. Gram-positive bacteria are highly susceptible to lysozyme because gram-positive bacterial cell wall made of peptidoglycan, but lysozyme was not effective in killing gram-negative bacteria [21-23], therefore the combination with other compound is suggested. Lysozyme has been used as an antimicrobial and antiviral in the food and pharmaceutical industries [24] which caused inhibition of
pathogenic bacteria’s growth, thus able to extend the shelf life of food. Lysozyme has been used to preserve fruits, vegetables, beans, tofu, curd, meat, sausages, salads, and the type of semi-hard cheese such as Edam, Gouda, and some Italian cheese. Lysozyme has been explored to have the protection against pathogenic bacteria Bacillus cereus in cheese [25]. On the other hand, lysozyme was also added to infant formulas to achieve the similarity of infant formula to human milk [43].

Since the pathogenic bacteria in a complex strain might exist in dangke, the strong antimicrobial activity agent is required. Previous research showed the weak inhibition of lactoperoxidase system in dangke resulting in the extension time of shelf life for only 6 hour at room temperature [3]. Therefore the synergistic effect of LPOS to inhibit bacteria may useful to end this problem. Thus, this research used the addition of lysozyme to extend the shelf life of dangke. This experiment might provide benefit to open the new method for extending the shelf life of dangke using natural LPOS and lysozyme.

MATERIALS AND METHODS

Materials

Fresh bovine milks were provided by a campus farm. Fresh duck eggs were purchase from a local farm. Latex from young papaya was used to obtain papain enzyme to precipitate the protein. SP-Sepharose Fast Flow (SP-FF) (Lot No. 10072021) was used for lysozyme purification. LPO from bovine whey was obtained from Chemical and Food Nutrition Laboratory, Food Technology Department, Faculty of Animal and Agricultural Sciences, Diponegoro University. H$_2$O$_2$ and KSCN were used as an LPO’s substrate. Syringe filter 0.2 μm was used to sterilize the enzyme.

Lysozyme Purification
Duck lysozyme was purified using the method performed by previous researcher [26]. Duck egg white was mixed with 3-fold volume sodium acetate buffer (0.05 M, pH 5.0). The mixture was centrifuged at 6000 rpm for 15 minute to separate the supernatant, then the supernatant has been applied in a SP-FF column for lysozyme purification. A five hundreds milliliter of sodium acetate buffer (0.05 M, pH 5.0) was subsequently eluted through column. Lysozyme was obtained using serial dilution with 300 ml of 0.1, 0.3, and 0.5 M NaCl in sodium phosphate buffer (0.05 M, pH 9.0). The eluate was then collected 10 ml each tube. The purity of eluate was determined using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Manufacture of Dangke

The manufacture of dangke was adopted from traditional method that has been done by the local people in South Sulawesi. Fresh bovine milk was heated at 75°C for 20 minute then latex from young papaya (0.3% w/v) was subsequently added to fresh bovine milk. Curd was then formed and separated using clean filter cloth. The curd was then pressed to produce dangke. Through this method, one liter of milk was able to produce 680 g dangke.

Manufacture of LPOS solution

Lactoperoxidase system (LPOS) solution was made using the mixture of 300 μL of LPO, 300 μL of 0.9 mM H2O2, and 300 μL of 0.9 mM KSCN. Prior to application, this mixture was filtered using a 0.2 μm syringe filter and placed in micro tube and let stand for 1 hour at 30°C.

Applications of Dangke Preservation
One gram of dangke used for evaluation of total microbe and pH value while for the evaluation of hardness, dangke has been cut to the shape 2.5 x 1 x 1 cm. Dangke was then stored at 30°C for 0, 8, and 18 hour for calculation of total microbe, while the dangke with storage at 30°C for 0, 1, 2, 3, 4, 5, 6, 7, 8 hour was used to analyze pH value and hardness. Prior to evaluation, dangke was immersed in various preservation solution (LPOS, lysozyme, LPOS+lysozyme) at 30°C for 4 hour. The immersion in sterile pure water has been used as control.

Microbial count

3M Petrifilm Aerobic Count Plates (3M Microbiology, St. Paul, Minn., U.S.A.) was used to count the microbial count of dangke as the previous procedure that has been done by previous researcher (3) with a minor modification. Briefly, dangke was delivered to the serial dilutions of a sterile 0.88% NaCl solution to enumerate the bacteria. The diluted mixture (1000 µl) was spread onto plates. The plates were incubated at 37°C for 48 h. The CFU of microbes in the sample solution were counted on the plates.

Hardness measurement

Samples (2.5 x 1 x 1 cm) of dangke were analyzed for hardness. Texture analyses were conducted using Brookfield Texture Analyzer (CT3). Testing conditions were as follows. A ø12.7 mm ball probe was penetrated to a depth of 4 mm into the sample at a speed of 1 mm/s. The textural hardness was measured in triplicate and expressed as Newtons.

Statistical Analysis
Total microbe, pH value, and hardness were analyzed using ANOVA with 3 replications. Statistical analysis was performed using R software for Macintosh. Duncan’s multiple range test (P<0.05) was used to calculate the significance among values.

RESULT AND DISCUSSION

Purification of Lysozyme

Three steps dilution with various concentrations of NaCl were done to obtain lysozyme. Fig. 1 shows the absorbance at 280 nm of the elution from each step of dilution. Fraction number 1-10, 11-20, 21-30 were obtained from the elution against phosphate buffer pH 9.0 containing 0.1, 0.3, 0.5 M NaCl, respectively. A high peak of protein concentration activity was detected from fraction numbers 19 – 22 representing the high protein concentration. However, the elution from those fractions showed more than one band (Fig. 2), while fraction number 26 showed a single band representing pure lysozyme, with a molecular weight of 14 kDa. Thus, fraction number 26 has been used for the entire of research. The protein concentration of the fraction measured by the Lowry method was 0.10%. This value was comparable to that of the other researcher that determined the protein concentration from purified protein using similar method as much as 0.1% [26].

Total Microbe

Fig. 3 indicated total microbe in dangke immersed in sterile pure water, LPOS, lysozyme, and combination of LPOS+lysozyme for 0, 8, 18 hours at room temperature. Total microbe in dangke increased along with the increase in storage time. The immersing in sterile pure water at 0 hours resulted in the highest bacterial amount (4.15±0.21 CFU/ml) among those of other treatments. Total number of bacteria in dangke immersing in lysozyme was the fewest (2.07±0.32 log CFU/ml). The dangke immersing in LPOS and the combination of
LPOS+lysozyme exhibited the lesser bacterial number (2.95±0.91 and 2.39±0.54, respectively) than those in control. The total number of bacteria in all the dangke immersing in lysozyme, LPOS and a combination of lysozyme and LPOS showed lesser contamination (ranging from 5.30±0.42 to 7.00±0.00 log CFU/ml) than control dangke (7.78±0.67 log CFU/ml) after storing for 8 hours. The longer immersing time up to 18 hours resulted in the large increase in bacterial number ranging from 8.11±0.37 to 8.71±0.57 log CFU/ml (Fig. 3).

Immersing dangke in LPOS, lysozyme, and the combination of LPOS+lysozyme reduced the population of bacteria to 1/10-1/100 at zero hour of storage compared to those in control. Among the all treatments, lysozyme generated the strongest antibacterial activity.

The antibacterial activity of lactoperoxidase has been understood because of hyphothiocyanate (OSCN⁻) resulting from the enzymatic reaction between hydrogen peroxide and thiocyanate. OSCN⁻ is responsible for killing bacteria, fungi, and virus, by destructing sulfhidril (S-H) of the cell membrane [9,27,28], although OSCN⁻ is rapidly scavenged. Lysozyme has been known as antimicrobial agent against bacteria, fungi, protozoa, and virus by lowering the structural components on the cell walls of bacteria and fungi [29-31].

Lysozyme catalyses the β1-4 bond between N-acetylglucosamine and N-acetylmuramic acid in the peptidoglycan resulting in the bacterial death. Lysozyme are prone to kill gram positive bacteria, because gram positive bacteria have contains 90% peptidoglycan in cell wall, but peptidoglycan on gram negative bacteria only 5-10% [21; 32]. It has been well studied that several bacteria found in raw milk might be found in cheese due to the handling process prior to cheese making [33]. Among the those bacteria, the gram positive bacteria are the common bacteria found in milk such as Enterococcus, Pediococcus, Aerococcus, and Staphylococcus [33], Bacillus spp. [34]. The dominance of gram positive bacteria may provide the answer of the high antimicrobial activity of lysozyme in cheese.
All preservatives were unable to inhibit the growth bacteria in dangke stored at 18 hour, because of the high load of total microbe (from 8.11±0.37 to 8.71±0.57 log CFU/ml). This result is in line with the result of other researcher (35) that OSCN⁻ generated from limited amount of substrates (0.3 mM H₂O₂ and 0.3 mM SCN⁻) was able to kill total bacteria in milk if the initial population of bacteria was not exceed 8.00 log CFU/ml. Furthermore, previous researcher [3] stated that the lactoperoxidase system was unable to reduce total microbe in dangke stored 12 hour with total microbe 10¹⁰ CFU/ml.

The combination LPOS+lysozyme was unable to suppress the growth bacteria on dangke at the maximum storage, however the synergistic effect of this combination was able to be seen at 8-h storage of dangke resulting in the least of total bacteria of 5.30±0.42 CFU/ml among those of other treatments. Since Indonesian National Standard [36] stated the maximum allowed number of total bacteria in cheese was 6 log CFU/ml, the utilization of LPOS+lysozyme may be applied to fulfill the requirement of maximum allowed amount of total bacteria in cheese.

pH Value

The development of pH and texture is required to produce the preferred cheese by storage along period of time [37]. Based on the data shown in Table 1, the pH value of dangke stored at room temperature for 8 hours were varied from 6.22±0.30 to 6.77±0.02. Dangke immersed in sterile pure water showed considerable increase in pH value ranging from 6.22±0.30 to 6.54±0.05 while the immersing dangke in LPOS, lysozyme, and combination of LPOS+lysozyme showed less change in pH value.

It has been understood that the increase in pH value was due to the process of deamination of amino acids resulting in the production of NH₃ and the metabolism of lactic acid bacteria to produce CO₂ [38]. This reason was in line with the results of total bacteria.
shown in Fig. 3 that the total bacteria decreased along with the treatments in preservative solutions. The less of bacteria live contributed to the less of the production of CO₂ resulting in the less change in pH value.

Initial pH value of dangke was detected 6.22±0.30 while previous researcher [3] stated that initial pH value of dangke 7.17. Other researcher stated that the initial pH value was 6.40 [1]. It is recognized that the initial value of dangke was relatively similar to the pH of fresh milk. The variation in initial value of pH in dangke may be explained by the wide variation of pH in papaya latex. It has been documented that the pH of papaya latex were ranging from 6.00 to 8.75 [1,39], thus this may resulting in the alteration of initial pH of dangke from the initial pH value of fresh milk.

**Hardness**

Table 2 shows the results of the hardness of dangke immersed in sterile pure water, LPOS, lysozyme, and combination of LPOS+lysozyme at the 0 hour of storage time (initial) and 8 hour of storage time (final). Based on statistical analysis, Immersing in sterile pure water and combination of LPOS+lysozyme was not significantly affected to the hardness of dangke, whereas the hardness of dangke immersing in LPOS and LPOS+lysozyme showed high increasing of 72% and 37% at the final storage(3.62±0.90 N, 2.80±0.73 N). The significant decrease of hardness was found in the dangke immersed in lysozyme. Based on Table 2, the decrease of hardness of dangke immersing in lysozyme was 36% at the final storage (1.750±0.32 N). The elevation of hardness of dangke immersed in LPOS and LPOS+lysozyme can be explained by the generation of hypothiocyanite and hypothiocyanous acid. which can accelerate crosslinking of proteins in dangke. Previous researcher [40] stated that hypothiocyanite is anion and the conjugate base of the hypothiocyanous acid which is an organic compound as part of thio cyanate containing functional groups SCN⁻.
Hypothiocyanous acid is a fairly weak acid with acid dissociation constant of 5.3 [41]. It has been recognized that some factors including pH can affect the rheological properties. For instance, a decrease in pH of Gouda cheese resulted in an increase of hardness [42], and vice versa, which were similar to the present study.

The hardness is required to determine the quality of rheological properties. Since dangke is commonly consumed after deep frying process or served with other food product, hard-texture-dangke is commonly preferred. Therefore, based on this reason, the LPOS and LPOS+lysozyme treatment may be a good way to preserve the dangke and enhance texture.

CONCLUSIONS

The LPOS, lysozyme, and combination of LPOS+lysozyme were able to inhibit total microbe in dangke stored at 8 hour. The highest antimicrobial activity was found in the dangke preserved in the combination LPOS+lysozyme immersion. The change in pH value was also maintained by immersing dangke in all treatments. The hard-texture of dangke was found in the immersion dangke in LPOS and LPOS+lysozyme.

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Fig. 1: Elution pattern of duck lysozyme by SP Sepharose Fast Flow column (10 mL each tube). Fraction 1-10, 11-20, 21-30 was obtained from elution with 0.1, 0.3, and 0.5 M NaCl in sodium phosphate buffer (0.05 M, pH 9.0), respectively.

Fig. 2: SDS Polyacrylamide Gel Electrophoresis profile of the eluate through SP Sepharose Fast Flow. Lane from left to right: standard (std) using α-lactalbumin (a 14 kDa protein), fraction number 2, 5, 8, 10, 12, 14, 19, 22, 26. Fraction number 26 showed single band representing pure lysozyme was detected. Thus, this fraction was used for entire research.
Fig 3. Total microbe in Dangke after immersing in solution containing LPOS, lysozyme, and LPOS+Lysozyme for ten minutes. Dangke immersed in pure water has been used for control. Values are means from three replicates of experiment and error bars represents standard error.

Table 1. pH value of dangke immersed in pure water, LPOS, lysozyme and LPOS+lysozyme.

| Storage period (h) | Pure water | LPOS<sup>ns</sup> | LZ<sup>ns</sup> | LPOS+LZ<sup>ns</sup> |
|-------------------|------------|-------------------|----------------|---------------------|
| 0                 | 6.22±0.30<sup>b</sup> | 6.59±0.01 | 6.72±0.01 | 6.71±0.01 |
| 1                 | 6.46±0.13<sup>a</sup> | 6.48±0.09 | 6.54±0.04 | 6.62±0.01 |
| 2                 | 6.54±0.06<sup>a</sup> | 6.64±0.07 | 6.71±0.01 | 6.62±0.01 |
| 3                 | 6.52±0.08<sup>a</sup> | 6.68±0.10 | 6.72±0.05 | 6.64±0.05 |
| 4                 | 6.43±0.03<sup>ab</sup> | 6.56±0.11 | 6.71±0.03 | 6.64±0.08 |
| 5                 | 6.54±0.05<sup>a</sup> | 6.75±0.07 | 6.69±0.03 | 6.71±0.08 |
| 6                 | 6.53±0.04<sup>a</sup> | 6.64±0.05 | 6.73±0.09 | 6.68±0.03 |
| 7                 | 6.46±0.02<sup>a</sup> | 6.58±0.11 | 6.69±0.01 | 6.64±0.02 |
| 8                 | 6.54±0.05<sup>a</sup> | 6.66±0.01 | 6.77±0.02 | 6.70±0.03 |

The superscript means significantly different among storage period, while ns means not significant. Data were average from triplicate of experiment ± standard error.
Table 2. Hardness (N) of dangke after immersing in pure water, LPOS, lysozyme, and LPOS+lysozyme.

| Dangke | Pure water<sup>ns</sup> | LPOS      | LZ         | LPOS+LZ<sup>ns</sup> |
|--------|--------------------------|-----------|------------|-----------------------|
| Initial| 1.98±0.75                | 2.11±0.56<sup>b</sup> | 2.73±0.47<sup>a</sup> | 2.04±0.69              |
| Final  | 1.54±1.03                | 3.62±0.90<sup>a</sup> | 1.75±0.32<sup>b</sup> | 2.80±0.73              |

The superscript means significantly different among the storage, while ns means not significant. Final represents the value of hardness at 8 hour storage in 30°C.
Extending Shelf Life of Indonesian Soft Milk Cheese (Dangke) by Lactoperoxidase System and Lysozyme

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