Quality of fresh bovine milk after addition hyphothiocyanite-rich-solution

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~~COVER LETTER FOR SUBMISSION OF NEW MANUSCRIPTS~~

SOLUTION FROM LACTOPEROXIDASE SYSTEM

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ABSTRACT

Lactoperoxidase system (LPOS) has received high attention for milk preservation in the certain countries, because the system exerts hypothiocyanite ion to inhibit the growth of broad spectrum of bacteria. Since the system might be remained the substrates of thiocyanate ion (SCN^-) and hydrogen peroxide (H_2O_2) and the activity of LPO might be inhibited ~~by compounds~~ in milk, this research has been done for generating hyphothiocyanite-rich-solution to be added in fresh milk. The solution was obtained from the reaction solution of LPO, KSCN, and H_2O_2. The remaining substrates SCN^- and H_2O_2) in the reaction solution were detected spectrometrically. LPO was obtained from bovine whey using SP-Sepharose Fast Flow. Untreated bovine fresh milk (1 h after milking) was added with 1% (v/v) of hyphothiocyanite-rich-solution and store at 30°C until~~for~~ 6 h. The addition of sterile water has been used instead of the solution for control. It is concluded that hyphothiocyanite-rich-solution contained very less amount of residual ~~substrates~~SCN^- (±0.02 mM) and H_2O_2 (0.09 mM). Hyphothiocyanite-rich-solution remarkably ~~decreased~~ inhibited the growth of ~~the~~ total bacteria ~~count~~ in the fresh milk ~~at 6 h incubation~~. The solution was also kept ~~the~~ a stable pH value in fresh milk ~~during~~ until 6-h incubation. This result might open the new way of practical use of LPOS to preserve fresh milk using the hyphothiocyanite-rich-solution.

Key words: lactoperoxidase, hyphothiocyanite-rich-solution, total bacteria, pH value, fresh milk, residual substratese

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INTRODUCTION

Milk spoilage is a major problem to the dairy sector of tropical countries since the ambient temperature is preferable for the growth of bacteria (Seifu et al., 2005, Oghaiki et al. 2007). Bacterial contamination becomes critical for raw milk especially from the time between milking until it reaches to the consumers (Saad, 2008). The commonly used of milk preservation to maintain the quality is cooling method. Since this equipment is costly, the availability of cooling facilities in developing countries are still limited in number, therefore the use of another method to preserve the milk quality is required.

Cheese production from bovine milk in Indonesia has increased over the last one decade and projects aimed at promoting diversification of milk product (Directorate Generale of Animal Husbandry, 2011). As a consequent, whey as a by-product of cheese making has a huge number of production but very less of handling. Whey is source of biological and functional protein including ß-lactoglobulin, immunoglobulin, bovine serum albumin, and lactoferrin (Fee and Chand, 2006). It is well studied that whey generates antimicrobial activity from lactoperoxidase (LPO) (Madureira et al., 2007, Al-Baarri, 2011a). Lactoperoxidase activates the antimicrobial system named lactoperoxidase system (LPOS). This system will be active only in the presence of three components: LPO, thiocyanate (SCN^-), and hydrogen peroxide (H_2O_2) (Elliot et al., 2004, Boots and Floris, 2006). Lactoperoxidase catalyses the oxidation of thiocyanate by hydrogen peroxide and generates antibacterial agent of hyphothiocyanate or $OSCN^-$ (Seifu et al., 2005, Madureira et al., 2007). These products have a broad spectrum of antimicrobial effects against bacteria, fungi and viruses (Boots and Floris, 2006, Saad, 2008).

LPOS has been used to preserve raw milk quality in areas where it is not possible to use mechanical cooling unit for technical or economic reasons (FAO/WHO, 2005). Furthermore, FAO legally provides the method to prolong the quality of milk by LPOS. Many researcher studied the LPOS to preserve the quality of raw milk (Haddadin et al., 1996, Marks et al., 2001, Seifu et al., 2004, Oghaiki et al. 2007, Trujillo et al., 2007, Dajanta et al., 2008, Saad, 2008, Zhou and Lim, 2009). Common method for generating LPOS in raw milk is adding SCN^- and/or H_2O_2 separately into milk. However, since H_2O_2 maybe remained in the milk and the utilization of H_2O_2 is limited for certain country, the objective of this study is to use of hyphothiocyanite solution ($OSCN^-$) obtained from LPOS of H_2O_2 and SCN^- with less/no residue in the LPOS solution for keeping fresh milk quality.

The previous research was succeeded to control the growth of *Salmonella enteritidis* using hyphothiocyanate-rich-solution from LPOS using immobilized LPO onto resin, H_2O_2, and SCN^- indicating the potent mass production of antibacterial agent (Al-Baarri et al., 2010, Al-Baarri et al., 2012, Hayashi et al., 2012). In this study, the investigation is focused on the production of

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hyphothiocyanate from the reaction solution containing LPO, H_2O_2, and SCN^-, the remaining concentration of H_2O_2, and SCN^-, and the bacterial growth in fresh milk after addition of hyphothiocyanite-rich-solution.

MATERIALS AND METHODS

Materials

Fresh bovine's milk was provided by campus farm at Faculty of Animal and Agriculturale Sciences, Diponegoro University. H_2O_2, KSCN, 2,2-azino-bis(3-ethylbenzthia-zoline-6-sulfonic acid) (ABTS) and were purchased from Sigma. Rennet was purchased from Singapore. SP Sepharose Fast Flow (SPFF) was purchased from Amersham Pharmacia Biotech, Sweden. Unless otherwise specified, all other chemicals were reagent grade.

Purification of LPO

The LPO was purified using the method of Al-Baarri, 2011b with slight modification. Fresh bovine's milk was centrifugated at 8000 rpm and 10°C for 20 min. The whey was separated from skimmed milk after the addition of 0.02% (w/v) rennet and 2 ml lactic acid/l milk at 30°C for 30 min using a sterilized filter cloth. The obtained whey was dialyzed against a large volume (10 l) of 10 mM sodium phosphate buffer (PB, pH 6.8) overnight at 4°C and loaded into glass column containing 100 g SP-Sepharose FF (Amersham Pharmacia Bio-tech). For removal unwanted compound, the column was then washed with 500 ml of PB (pH 6.8) containing 0.1 M NaCl. LPO was eluted with 500 ml of PB containing 0.2 M NaCl. The purification was conducted in a refrigeration room. The eluate was collected (15 ml/tube), and the extinction coefficient at 280 nm of 1.5 cm^2 mg^{-1} for LPO was used to estimate the protein concentration. Each tube was spectrometrically checked for LPO activity using ABTS as substrate (Al-Baarri, 2011a). The highest LPO activity was collected and filtered through a 0.22-µm filter unit (Millipore, Bedford, USA). The purified LPO was stored at –20°C.

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Production of hyphothiocyanite-rich-solution

Hyphothiocyanite-rich-solution was generated from the enzymatic reaction of LPOS that was prepared by mixing 50 µl of LPO (2 U/ml), 25 µl of of 0.5 mM H_2O_2 and 25µl of 0.5 mM KSCN. After one minute storage in the room temperature, the reaction solution was analyzed for the remaining SCN^- and H_2O_2 concentration. This solution was prepared daily without preservation (Al-Baarri et al., 2010).

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Analysis of residual SCN^- concentration

Analysis for the remaining SCN^- in the hyphothiocyanate-rich-solution was conducted spectrometrically according to the method that was performed by Al-Baarri et al. 2011b with slight modification. Ten gram of $Fe(NO3)_3 \cdot 9H_2O$ was dissolved in 20 ml of concentrated nitric acid. Water was added to the solution to give the final volume of 200 ml. An aliquot of sample was added to nine volumes of the ferric nitrate solution. The absorbance of mixture was measured at 460 nm. The SCN^- concentration of sample was calculated from an established standard curve of KSCN solutions of known concentrations with the scale of 0,05 to 5 mM of KSCN.

Analysis of residual H_2O_2 concentration

Residual determination of H_2O_2 in hyphothiocyanite-rich-solution was measured using spectrophotometer according to the method that was performed by Al-Baarri et al. 2011b. Two hundred micro liter solution was made from 1,23 mM ABTS and LPO in 0,1 M Phosphate Buffer (pH 6,8). Enzymatic reaction was determined by adding 800 µl of hyphothiocyanite-rich-solution. Immediately after the enzyme addition, the absorbance of the mixture containing the enzyme was monitored at 412 nm at 25°C for 20 s. The absorbance change at 412 nm was used then to estimate H_2O_2 concentration, based on previously established standard curve of ABTS with the scale of 0,5 to 5 mM.

Microbial count

3M Petrifilm Aerobic Count Plates (3M Microbiology, St. Paul, Minn., U.S.A.) was used to count the microbial count of milk. The number of total bacteria in fresh milk in the presence of ~~LPOS~~ hyphothiocyanite-rich-solution was determined as follows: 1000 µl of the assay mixture containing 900 µl of fresh bovine's milk and 100 µl hyphothiocyanite-rich-solution (1% v/v) were incubated ~~for~~ until 6 h in water bath at 30˚C. The pure water was used instead of hyphothiocyanite-rich-solution as control. Subsequently, serial dilutions of the assay mixture were prepared with a sterile 0.88% NaCl solution to enumerate the bacteria. The diluted mixture (1000 µl) was spread onto plates. The plate were incubated at 37˚C for 48 h. The CFU of microbes in the sample solution were counted on the plates.

RESULTS AND DISCUSSION

Remaining substrates in hyphothiocyanite-rich-solution

It has been known that in the LPOS reaction, LPO catalyzes the oxidation of SCN^- by the presence of H_2O_2, which leads to the production of hyphothiocyanate as shown in Figure 1. Therefore, to generate the hyphothiocyanate-rich-solution, three compounds: LPO, KSCN, and H_2O_2 were mixed. This step was done for one minute at room temperature.

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Figure 2 shows the utilization of 0.1 – 0.5 mM of substrates (KSCN~~SCN~~ and H_2O_2) in the presence of LPO. After one minute reaction, a hundred microliter of sample from this solution was added to 900 µl ferric nitrate solution to ~~analyse~~ analyze the remaining concentration of SCN^- in the hyphothiocyanate-rich-solution. As shown in this figure, the residual concentration of SCN^- was stable at 0.2 mM along the increase in the applied substrate indicating there was no remarkable increase in SCN^- concentration. ~~t~~The residue of H_2O_2 in hyphothiocyanite-rich-solution was 0.04~~14~~ mM when ~~the maximum~~ concentration of employed H_2O_2 was 0.40 mM ~~employed~~ indicating almost all of employed H_2O_2 ~~(97.06%)~~ in LPOS was reducted into H_2O. However, the increase of employed H_2O_2 from 0.40 mM into 0.50 mM elevated the residual H_2O_2 concentration from 0.040 mM into 0.09 mM representing there was a lessening process of H_2O_2 reduction. The presence of H_2O_2 in the hyphothiocyanite-rich-solution might interfere the antibacterial activity of the solution since H_2O_2 has known as a preservative agent for inhibit the growth of bacteria. However, the detected residue of H_2O_2 in the solution was in very small amount if compare to the concentration H_2O_2 for preservative use (50 mM) (Silveira et al., 2008).

~~On the other hand,~~The detected residue of SCN^- in hyphothiocyanite-rich-solution was 0.09 mM when the maximum concentration of employed ~~0.5 mM~~ KSCN was ~~employed~~used (0.5mM). This indicate that 92% of SCN^- was oxidized into $OSCN^-$. We found that the increase of residual SCN^- was started when 0.6 mM KSCN was employed (data were not shown). The ~~remaining~~ increase ~~SCN^- was hig~~ ~~than the remaining H_2O_2,~~ in residual concentration may be explained ~~the solution~~ due to the ~~ion~~ binding of SCN^- to the heme of LPO resulting in the weakening of LPO activity. The study of crystal structure of LPO binding clearly concluded that the binding of the SCN ion at surface of helix protein H3 of LPO ~~presumably~~ might disturb the electrical charge of LPO resulting in the inactivation of LPO ~~which was inhibit reaction of forming $OSCN^-$~~ (Singh et al., 2008; Singh et al., 2009).

Concentration of $OSCN^-$ is the key for the antimicrobial activity of LPOS. This reserach used total concentration of 0.5 mM for SCN^- and H_2O_2, respectively. This amount of substrates should produce approximately 0.4 mM $OSCN^-$ in the reaction solution based on the our previous experiment on the production of $OSCN^-$ using immobilized LPO (Al-Baarri et al., 2010). The reaction solution containing 0.4 mM $OSCN^-$ was able to exert antimicrobial activity against *S. enteritidis* of approximately 5 log CFU/ml.

Total bacteria in fresh milk

This experiment used direct addition of sterile hyphothiocyanite-rich-solution into fresh bovine's milk. The hyphothiocyanite-rich-solution was filtered through a syringe fil[18] 0.22 µm, PTFE) for sterilization before the addition to the fresh bovine's milk. ~~Fresh milk was incubated for~~

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~~6 h at 30˚C. Prior to incubation, f~~Fresh milk was added with with the sterile hyphothiocyanite-rich-solution at every hour of incubation until 5 h. Fresh milk then was incubated ~~for~~until 6 h at 30˚C. The samples were collected hourly for enumeration of total bacteria. Fresh milk used in this experiment was also counted for total bacteria resulting number of 4.32±0.67 log CFU/ml (data were not presented). The result of total bacteria in fresh milk after addition of hyphothiocyanite-rich-solution and incubation until 6 hour is showed at Figure 3.

Figure 3 shows the inhibition of thiocyanite-rich-solution against total bacteria in fresh milk. The total bacteria of 8.00±0.80 CFU/ml has been detected in the sample with no addition of hyphothiocyanite-rich-solution while the total bacteria of 6.80±0.80 or less has been detected in the sample with the treatments indicating the suppresive effect of hyphothiocyanite-rich-solution to the bacterial growth. This result is also in line with the findings of Nigussie and Seifu, 2007 who reported that activation of the LPOS in the fresh milk resulted in supression of the growth of total bacteria ~~from~~ to 7.5 log CFU/ml from the initial count of 7.73 log CFU/ml. Based on these results, the hyphothiocyanite-rich-solution showed the higher suppresion effect than those of activation of LPOS in fresh milk. This can be explained that LPO might be inhibited by the existing lactose in milk (Al-Baarri et al. 2011b). This result are in agreement with the role of National Standardization Agency of Indonesia that was announced the maximum total bacteria number for fresh milk (6 log CFU/ml), representing the hyphothiocyanate-rich-solution was the potent preservatives for preserving fresh milk.

The inhibition effect of hyphothiocyanite-rich-solution to total bacterial count in fresh milk was detected on the sample ~~after~~ with one-hour addition of hyphothiocyanite-rich-solution. ~~For instance, w~~When the solution was added to fresh milk at first-hour incubation, the total bacteria reduced from 4.23±0.50 to 4.21±0.10 log CFU/ml. The addition of hyphothiocyanite-rich-solution ~~at~~ th~~e~~ fourth hour ~~was~~ ~~with~~ ~~the~~ ~~growth~~ ~~factor~~ ~~l~~ ~~level~~ ~~was~~ ~~not~~ ~~with~~ ~~the~~ ~~addition~~ ~~of~~ ~~the~~ ~~total~~ ~~number~~ ~~of~~ ~~bacteria~~ ~~known~~ ~~as~~ ~~the~~ ~~sample~~ ~~which~~ ~~the~~ ~~samples~~ ~~showed~~ ~~relevant~~ shown in the sample with fifth hour addition of hyphothiocyanite-rich-solution (data were not shown) ~~the~~ ~~inhibition~~ ~~effect~~ ~~to~~ ~~the~~ ~~growth~~ ~~of~~ ~~bacteria~~. This phenomenon could be explained that the population of bacteria is in line with the number of sulfhydryl group that should be oxidized by $OSCN^-$ (Al-Baarri et al., 2010, Hayashi et al., 2012). Therefore the high population of bacteria, the higher concentration of $OSCN^-$ might be required.

The direct addition of LPO's substrate i.e. KSCN and H_2O_2 to fresh milk has been guided by FAO for milk preservation in the area with less refrigeration facility (FAO/WHO, 2005). The addition of substrates has been proved to extend the shelf life of fresh milk stored at ambient temperature (Barrett et al., 1999, FSANZ, 2002, FAO/WHO, 2005, Oghaiki et al, 2007, Dajanta et al., 2008). However the substrates might be remained in the milk since the activity of LPO was depended on the storage, and substrates concentration (Boots and Floris, 2006, Trujillo et al., 2007,

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Singh et al., 2009). Therefore hyphothiocyanite-rich-solution might be applied to inhibit the growth of total bacteria in fresh milk without the issue of remaining substrate in fresh milk.

pH Value

Figure 4 shows the value of pH in fresh milk at sixth-hour incubation at 30˚C with and without addition of thiocyanite-rich-solution. The pH was analyzed at sixth hour incubation since this point is the critical value of fresh milk to reach the total bacteria of 6 log CFU/ml (Touch et al., 2004). As can be seen on this figure, hyphothiocyanite-rich-solution was able to maintain the pH of fresh milk into range from 6.66±0.12 to 6.71±0.02 at sixth-hour incubation while no addition of the solution decreased pH into 5.90±0.11. Prior to treatment, all fresh milk were detected on the pH value and resulted in the value of 6.76±0.080. The suppression of the decrease of pH value was in agreement with the previous result in the total bacteria. The addition of hyphothiocyanite-rich-solution was able to maintain the total bacteria into maximum of 6.80±0.8 CFU/ml while no addition of the solution increased total bacteria into from the initial count 6.76±0.08 CFU/ml into 8.00±0.80 CFU/ml ~~(Figure 3)~~.

The addition of compounds including KSCN and H_2O_2 directly to fresh milk to generate antibacterial effect of LPOS may be done to inhibit the growth of bacteria but since the LPO activity was easily inhibited in fresh milk there was a possibility of substrate to be remained in fresh milk. The application of hyphothiocyanite-rich-solution might provide benefit for inhibiting total bacteria in fresh milk with less an issue of remaining substrate in the fresh milk.

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CONCLUSION

The hyphothiocyanite-rich-solution could be obtained from the reaction solution of LPO, SCN^-, and H_2O_2. This solution contained very less amount of residual substrates. Addition of this solution into fresh milk remarkably inhibited the growth of total bacteria during 6 hour incubation at 30°C but did not change the pH value indicating the hyphothiocyanite-rich-solution had the potent ~~preservatives~~antibacterial agent for fresh milk. This result might be opened the new method of keeping the quality of fresh milk using LPOS.

ACKNOWLEDGEMENT

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Figure 1. Oxidation of thiocyanate by LPO catalysed reactions (Seifu et al., 2005).

$SCN^- + H_2O_2 \xrightarrow{LPO} OSCN^- + H_2O$ or

$2SCN^- + H_2O_2 + 2H^+ \xrightarrow{LPO} (SCN)_2 + 2H_2O$

$(SCN)_2 + H_2O \longrightarrow HOSCN + H^+ + SCN^-$

$HOSCN \longleftrightarrow H^+ + OSCN^-$ ~~$SCN^- + H_2O_2 \rightarrow OSCN^- + H_2O$~~

Figure 2. The remaining of reaction solution of LPOS that was consisted of LPO and two substrates: SCN^- (■) and H_2O_2 (◆) with concentration range from 0.1 to 0.5 mM and was kept in 30˚C for 1 hour to generates high concentration of hyphothiocyanite solution, named hyphothiocyanite-rich-solution. This solution was prepared daily. The data were obtained from 3 treatments. Error bars represent standard deviation of the mean

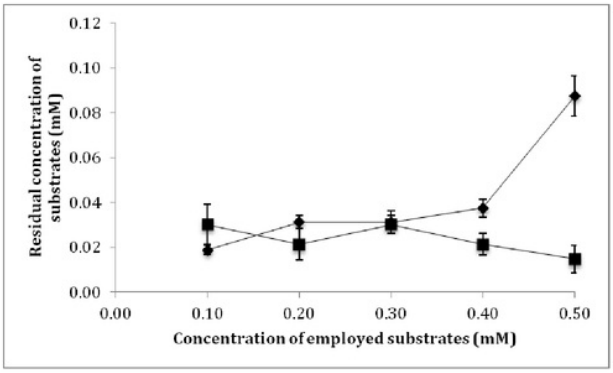

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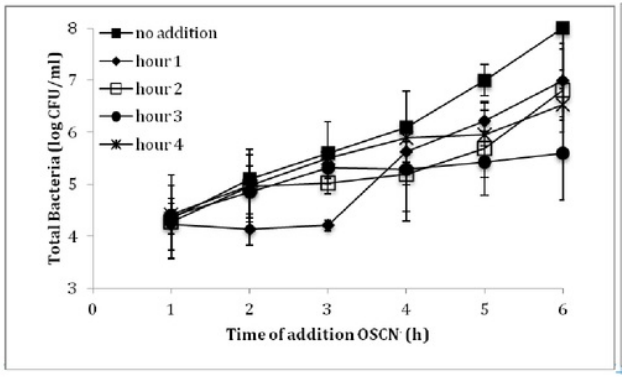
Figure 3. Effect of hyphothiocyanite-rich-solution to total bacterial ~~counts~~ in fresh milk during 6 hour incubation at 30˚C. Hyphothiocyanite-rich-solution (1% v/v) was added to the milk at 1st hour (◆), 2nd hour (□), 3rd hour (•), 4th hour (□) incubation. Sterile pure water was added to the fresh milk instead of hyphothiocyanite-rich-solution as control (■). Data were collected from three times of experiment. Error bars represent standard deviation of the mean.



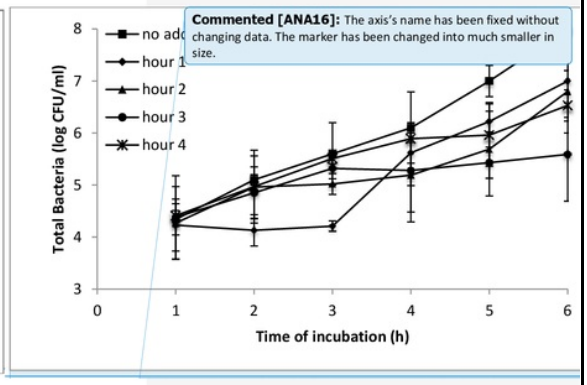


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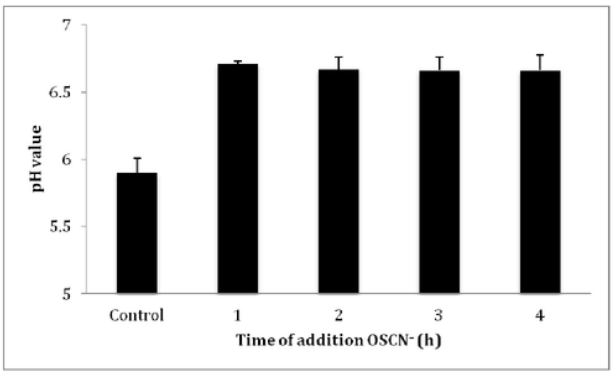
Figure 4. pH value of fresh milk with the addition of hyphothiocyanite-rich-solution after 6 hour incubation at 30°C. The addition was conducted hourly starting from first hour incubation until fourth hour incubation. Sterile pure water was added to the fresh milk instead of hyphothiocyanite-rich-solution as control. Data points are mean values based on three times experiments. Error bars represent standard deviation of the mean.



Quality of fresh bovine milk after addition hyphothiocyanite-rich-solution

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