International Journal of
Dairy Science

ISSN 1811-9743

Academic
Journals Inc.

www.academicjournals.com

International Journal of Dairy Science 9 (1): 24-31, 2014
ISSN 1811-9743 / DOI: 10.3923/ijds.2014.24.31

Quality of Fresh Bovine Milk after Addition of Hypothiocyanite-rich-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 (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 hypothiocyanite-rich-solution to be added in fresh milk. The solution was obtained from the reaction solution of LPO, SCN^- and H_2O_2. The remaining substrates 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 hypothiocyanite-rich-solution and store at 30°C for 6 h. The addition of sterile water has been used instead of the solution for control. It is concluded that hypothiocyanite-rich-solution contained very less amount of substrates. Hypothiocyanite-rich-solution remarkably decreased the total bacteria count in the fresh milk at 6-h incubation. The solution was also kept the pH value in fresh milk during 6-h incubation. This result might open the new way of practical use of LPOS to preserve fresh milk using the hypothiocyanite-rich-solution.

Key words: Lactoperoxidase, hypothiocyanite-rich-solution, total bacteria, pH value, fresh milk, residue

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 (DGAH, 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 *et al*., 2011). 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 hypothiocyanate 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 hypothiocyanite 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 hypothiocyanate-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, 2012; Hayashi *et al*., 2012). In this study, the investigation is focused on the production of hypothiocyanate 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 hypothiocyanite-rich-solution.

MATERIALS AND METHODS

Materials: Fresh bovine's milk was provided by campus farm at Faculty of Animal and Agriculture 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 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 *et al*. (2011) 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 $tube^{-1}$) 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 *et al.*, 2011). 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.

Production of hypothiocyanite-rich-solution: Hypothiocyanite-rich-solution was generated from the enzymatic reaction of LPOS that was prepared by mixing 50 μL of LPO (2 U mL^{-1}), 25 μL of of 0.5 mM H_2O_2 and 25 μL 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).

Analysis of residual SCN^- concentration: Analysis for the remaining SCN^- in the hypothiocyanate-rich-solution was conducted spectrometrically according to the method that was performed by Al-Baarri *et al.* (2011) with slight modification. Ten gram of $Fe(NO3)_3 \bullet 9H_2O$ 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 hypothiocyanite-rich-solution was measured using spectrophotometer according to the method that was performed by Al-Baarri *et al.* (2011). 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 hypothiocyanite-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 sec. 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: The 3M Petrifilm Aerobic Count Plates (3M Microbiology, St. Paul, Minn., USA) was used to count the total number of bacteria in milk. The number of total bacteria in fresh milk in the presence of LPOS was determined as follows: 1000 μL of the assay mixture containing 900 μL of fresh bovine's milk and 100 μL hypothiocyanite-rich-solution were incubated for 6 h in a water bath at 30°C. 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 hypothiocyanite-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 hypothiocyanate as shown in Fig. 1. Therefore, to generate the hypothiocyanate-rich-solution, LPO, KSCN and H_2O_2 were mixed for one minute at room temperature.

Figure 2 shows the utilization of 0.1-0.5 mM of substrates (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

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

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

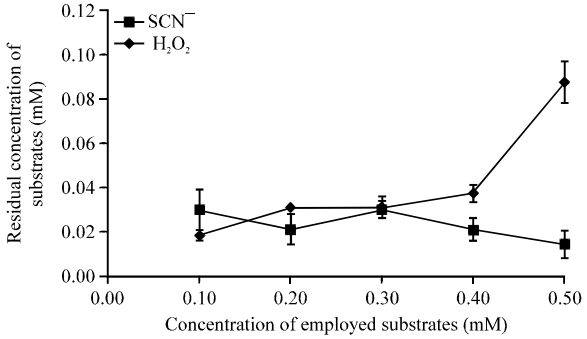

Fig. 2: Formation of hypothiocyanite solution (hypothiocyanite rich-solution) with LPOS, consists of LPO, SCN^- and H_2O_2. Reaction was carried out at 30°C for 1 h (Three concordant redings were taken. Error bars represent Mean±SD)

900 μL ferric nitrate solution to analyse the remaining concentration of SCN^- in the hypothiocyanate-rich-solution. As shown in this figure, the residue of H_2O_2 in hypothiocyanite-rich-solution was 0.014 mM when the maximum concentration of H_2O_2 was employed indicating almost all of employed H_2O_2 (97.06%) in LPOS was reducted to H_2O. The presence of H_2O_2 in the hypothiocyanite-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, detected residue of SCN^- in hypothiocyanite-rich-solution was 0.09 mM when 0.5 mM KSCN was employed. This indicate that 92% of SCN^- was oxidized into $OSCN^-$. The remaining SCN^- was higher than the remaining H_2O_2 in 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 disturb the electrical charge of LPO resulting in the inactivation of LPO which was inhibit reaction of forming $OSCN^-$ (Singh *et al.*, 2008, 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^{-1}.

Total bacteria in fresh milk: This experiment used direct addition of sterile hypothiocyanite-rich-solution into fresh bovine's milk. Fresh milk was incubated for 6 h at 30°C. Prior to incubation, fresh milk was added with with the sterile hypothiocyanite-rich-solution at

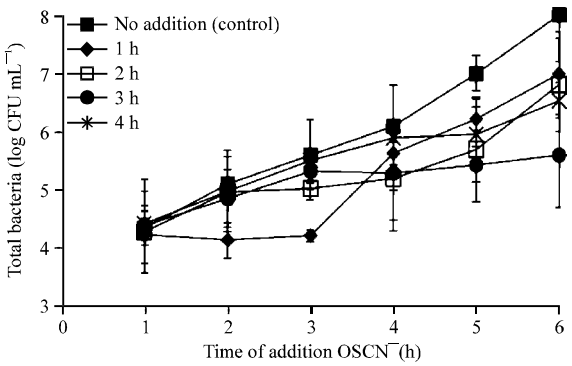

Fig. 3: Effect of hypothiocyanite-rich-solution to total bacterial counts in fresh milk during 6 h incubation at 30°C. Hypothiocyanite-rich-solution was added at different intervals. Sterile pure water was added to the fresh milk instead of hypothiocyanite-rich-solution as control (Three concordant readings were taken. Error bars represent Mean±SD)

every hour of incubation until 4 h. 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^{-1} (data were not presented). The result of total bacteria in fresh milk after addition of hypothiocyanite-rich-solution is showed at Fig. 3.

Figure 3 shows the inhibition of hypothiocyanite-rich-solution against total bacteria in fresh milk. The total bacteria of 8.00±0.80 CFU mL^{-1} has been detected in the sample with no addition of hypothiocyanite-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 hypothiocyanite-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 7.5 log CFU mL^{-1} from the initial count of 7.73 log CFU mL^{-1}. Based on these results, the hypothiocyanite-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.*, 2011). 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^{-1}), representing the hypothiocyanate-rich-solution was the potent preservatives for preserving fresh milk.

The inhibition effect of hypothiocyanite-rich-solution to total bacterial count in fresh milk was detected on the sample after one-hour addition. For instance, 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^{-1}. However the reduction of total bacterial count has only been shown at that sample while other samples showed 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 ie. 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).

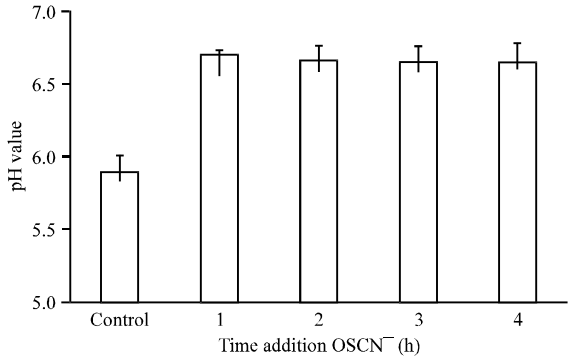

Fig. 4: pH value of fresh milk with the addition of hypothiocyanite-rich-solution after 6 h incubation at 30°C. The addition was conducted hourly. Sterile pure water was added to the fresh milk instead of hypothiocyanite-rich-solution as control (Three concordant readings were taken. Error bars represent Mean±SD)

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; Singh *et al.*, 2009).

pH value: Figure 4 shows the value of pH in fresh milk at sixth-hour incubation at 30°C with and without addition of hypothiocyanite-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^{-1} (Touch *et al.*, 2004). As can be seen on this figure, hypothiocyanite-rich-solution was able to maintain the pH of fresh milk into range from 6.66±0.12 to 6.71±0.02 at 6 h 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 hypothiocyanite-rich-solution was able to maintain the total bacteria into maximum of 6.80±0.8 CFU mL^{-1} while no addition of the solution increased total bacteria into from the initial count 6.76±0.08 into 8.00±0.80 CFU mL^{-1} (Fig. 3).

CONCLUSION

The hypothiocyanite-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 h incubation at 30°C but did not change the pH value indicating the hypothiocyanite-rich-solution had the potent preservatives. This result might be opened the new method of keeping the quality of fresh milk using LPOS.

ACKNOWLEDGMENT

This material is fully based upon work supported by grants from the Indonesian Directorate General of Higher Education (DIKTI). Authors also would like to acknowledge the material support from Professor Shigeru Hayakawa, Professor Masahiro Ogawa and Professor Hirotoshi Tamura (Faculty of Agriculture, Kagawa University, Japan).

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