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Alternative strategies for activation of the natural lactoperoxidase system in cows' milk: trials in Tanzania

Leonard WT Fweja, Michael J Lewis and Alistair S Grandison*

Department of Food Biosciences, The University of Reading, P O Box 226, Whiteknights, Reading, RG6 6AP, UK

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Thiocyanate content and lactoperoxidase activity of individual cow's milk of different breeds were determined, and the effects of different lactoperoxidase system (LP-s) activation strategies were compared. Lactoperoxidase activity varied significantly between Friesian and both Ayrshire and Tanzania Short Horn Zebu (TSHZ), but differences between Ayrshire and TSHZ were not significant. There was no significant variation in SCN^- content between breeds. The LP-s was activated using three strategies based on SCN^-: namely; equal concentrations of SCN^- and H_2O_2 (7:7, 10:10, 15:15 mg/l), excess SCN^- concentrations (15:10, 20:10, 25:10 mg SCN^-:H_2O_2/l), and excess H_2O_2 concentrations (10:15, 10:20, 10:25 mg SCN^-:H_2O_2/l), plus a fourth strategy based on I^- (15:15 mg I^-:H_2O_2/l). The keeping quality (KQ) was assessed using pH, titratable acidity, clot on boiling and alcohol stability tests. All activation strategies enhanced the shelf life of milk (typically increasing KQ from around 10 to around 20 h), but it was clear that the effectiveness of the LP-s depends on the type and concentrations of the activators of the system. The LP-s activated using I^- as an electron donor was more effective than the LP-s activated using SCN^- as an electron donor, increasing the KQ by a further 6–8 h compared with SCN^-.

Keywords: Lactoperoxidase, thiocyanate, iodide, hydrogen peroxide, cow milk, keeping quality.

Lactoperoxidase (LP) is a natural enzyme with the ability to oxidize molecules at the expense of H_2O_2 and thus to generate antimicrobial compounds. Its antimicrobial effects also require the presence of thiocyanate (SCN^-) or a halogen as a second substrate (de Wit & van Hooydonk, 1996), to form the lactoperoxidase system (LP-s). Iodide (I^-) is the most readily oxidisable of all the halides whereas Cl^- requires a more powerful oxidant for its oxidation (de Wit & van Hooydonk, 1996). In the LP-s, the enzyme catalyses the oxidation of SCN^- to yield $OSCN^-$ and $HOSCN^-$ (Shin et al. 2001) and the oxidation of I^- to yield OI^- and HOI (Bosch et al. 2000). These compounds react with microbial sulphydryl groups to inhibit various cellular functions (Björck, 1985; Wolfson & Summer, 1993; Shin et al. 2001).

The concentration of enzyme necessary for the system to be active is only 1–2 mg/l, much less than normally present in bovine milk (approximately 30 mg/l; Björck et al. 1979; Siva et al. 1991). In the presence of non-limiting activity of LP, the antimicrobial effect is dependent on concentrations of SCN^- and H_2O_2 (Björck, 1981) which occur naturally but in sub-optimal levels (Reiter & Harnulv, 1984). FAO/WHO (1991) recommends the activation of the LP-s using 10:10 mg SCN^-:H_2O_2/l. However, literature data show inconsistency in concentrations of SCN^- and H_2O_2 used to activate the LP-s, but all reports demonstrated extension of shelf life (Ridley & Shalo, 1990). For example, the keeping quality (KQ) results obtained using different activation strategies in earlier studies show considerable variation. Siva et al. (1991) reported an extended KQ of 9·9, 12·2, and 13·6 h for raw milk treated with equal concentrations of 10:10, 20:20, and 30:30 mg/l SCN^-:H_2O_2 respectively at 30 °C. With excess concentrations of H_2O_2, Ewais et al. (1985) reported the KQ of buffalo and cow milk preserved at 20–22 °C treated with 10:25 and 10:30 mg SCN^-:H_2O_2/l to be 48 h, while the use of 10:70–80 mg SCN^-:H_2O_2/l kept the acidity unchanged for 24 h at 35 °C. Barraquio et al. (1994) observed a 7 h extended shelf life of raw milk treated with 14:30 mg SCN^-:H_2O_2/l at 27–32 °C. Using excess concentration of SCN^-, 15:10 mg SCN^-:H_2O_2/l (Chakraborty et al. 1986) recorded a 3 h extension in shelf life of buffalo milk at 37 °C

*For correspondence; e-mail: a.s.grandison@reading.ac.uk

and Björck et al. (1979) reported total lag phases of 7–8 h (30 °C), 11–12 h (25 °C), 15–16 h (20 °C), and 24–26 h (15 °C) using 10 : 8·5 mg SCN^- : H_2O_2/l. Increased fungicidal and bactericidal effects have also been demonstrated in LP-s enriched with iodide (Bosch et al. 2000), however little information is available about the LP/I^- : H_2O_2 system.

The aim of this study was to establish the most effective substrate (SCN^- : H_2O_2) combination for extension of shelf-life in the Tanzanian environment. Three strategies were adopted – i.e. equal concentrations, excess SCN^- and excess H_2O_2. A second aim was to compare the effect of different electron donors (SCN^- and I^-) on the performance of the LP-s.

Materials and Methods

Selection of cows

The study was carried out between September and December in Morogoro Municipal in Tanzania which is located between longitude 37–39 °E and latitude 6–5 °S. The area experiences a sub-humid climate with a bimodal rainfall pattern characterised by two rainfall seasons in a year with a dry season separating the short rains (October to December) and long rains (March to May/June). Three commercial farms and four traditional individual farmers from Kimbarai village in the outskirt of Morogoro Municipal were identified for the study. Lactating cows from different breeds, Ayrshire, Friesian and Tanzania Short Horn Zebu (TSHZ) were sampled based on the number of lactations and the last calving date. To eliminate the possibility of variation due to lactation number or stage, all cows selected for the study (50 in total) were in their second lactation and in mid-lactation stage (4–5 months). With the exception of one farm, all lactating cows from other farms and individual farmers, were hand milked. Cows owned by individual farmers were grazed on natural pastures while the farm managed cows were grazed on natural pastures and also supplemented with maize bran, hay, and/or cotton seed cakes.

Milk sampling

Milk from individual cows was used for determining SCN^- content and LP activity, while pooled milk was used for KQ assessment. Individual cows milk samples (250 ml) were collected and transported without cooling, within 1 h of milking, to the Laboratory of the Department of Food Science and Technology at Sokoine University of Agriculture in Tanzania for analysis. Bulk milk was obtained by pooling together 50 ml from each of 50 cows and thorough mixing. The cans used for pooling milk and other containers were sanitised using hot water and sodium hypochlorite solution (4%).

Thiocyanate determination

Thiocyanate content (mg/l) was determined spectrophotometrically in duplicate as $Fe(SCN)_3$ as described previously (FAO/WHO, 1991; Fonteh et al. 2002). Milk samples were first deproteinised by mixing 4 ml milk with 2 ml 20% (w/v) trichloroacetic acid. The filtrate (1·5 ml) was mixed with 1·5 ml ferric nitrate reagent (prepared by dissolving 16 g $Fe(NO_3)_3 \cdot 9H_2O$ in 50 ml 2 M-HNO_3) and the A_{460nm} was measured immediately. Thiocyanate content was determined from a standard curve prepared using 10, 20, 30, 40, 50, and 60 mg thiocyanate/l. The experiment was repeated with fresh milk samples one week later.

Lactoperoxidase activity

Lactoperoxidase activity was determined in four replicates per sample according to the method described by Fonteh et al. (2002). The milk sample (0·1 ml) was mixed with 2 ml 1 mM-2, 2′ azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS, Sigma-Aldrich Chemie GmbH, Germany) in phosphate buffer (0·1 M, pH 6·7) in a cuvette. Reaction was initiated by adding 1 ml 0·3 mm-H_2O_2 to the mixture. The reaction was monitored for 1 min and the absorbance recorded at 412 nm. Results are expressed in μmole product/min. The experiment was repeated on fresh milk samples one week later.

Activation of the LP-s in raw milk

Activation of the LP-s was achieved by addition of sodium thiocyanate (BDH chemicals, Ltd., Poole, UK) or potassium iodide (Fisher Scientific, Leicestershire, UK), followed by sodium carbonate peroxhydrate (Peroxide – Chemie, GmbH, Munich). The concentrations of activators quoted refer to the calculated concentrations of SCN^-, I^- or H_2O_2 added to the milk.

Three treatment strategies based on SCN^- were used with varying concentrations of the activators. The treatments involved were (i) equal concentrations of SCN^- : H_2O_2 i.e. 7 : 7, 10 : 10 and 15 : 15 mg/l, (ii) excess concentrations of SCN^- in the SCN^- : H_2O_2 combination i.e. 15 : 10, 20 : 10, and 25 : 10 mg/l and (iii) excess concentrations of H_2O_2 in the SCN^- : H_2O_2 combination i.e. 10 : 15, 10 : 20 and 10 : 25 mg/l.

To compare the performances of LP/SCN^- : H_2O_2 system and LP: I^- : H_2O_2 system, 15 : 15 mg/l of SCN^- : H_2O_2 and I^- : H_2O_2 were used to activate the two systems.

The treated and control milk samples were dispensed in 20 ml glass bottles (sterilised by oven drying at 100 °C) and stored overnight (for approximately 10 h) at room temperature which varied from 28–30 °C. The final design of the experiment was determined from preliminary trials (results not shown) which showed that fresh raw milk could keep for up to 10 h. Sample assay was carried out at 0 h and 10 h and then after every 2 h to an end point as determined by the quality prediction methods.

Table 1 Lactoperoxidase activity and thiocyanate concentration in milk from various cow breeds. Values are means ± standard deviations (SD) for *n* = Number of selected cows

		Enzyme activity (μM product/min)		Thiocyanate concentration (mg/l)	
Breed of cow	*n*	Range	Mean (SD)	Range	Mean (SD)
Friesian	17	3241–7977	4737 ± 1037[a]	4·35–20·6	11·7 ± 4·69[a]
Ayrshire	21	730–5817	2285 ± 1173[b]	4–24·2	13·1 ± 5·74[a]
TSHZ	12	959–3320	2240 ± 769[b]	6·64–20·5	13·2 ± 4·39[a]
Total/Range/Mean	50	730–7977	3108 ± 1562	4–24·2	12·7 ± 5·1

[a,b]Mean values in a column with different superscripts are significantly different ($P < 0·05$)
TSHZ – Tanzania Short Horn Zebu

Keeping quality determination

Keeping quality of raw milk at 28–30 °C was measured using pH, titratable acidity, clot on boiling test and alcohol stability test as described below. For all KQ measurements, duplicate readings were taken and reported values are means. Keeping quality is reported as the time (h) between milking and the first observation of spoilage criteria noted for each test.

Titratable acidity (*TA*)

To 10 ml milk was added 1 ml phenolphthalein solution (0·5% w/w). The mixture was titrated against 0·11 M-NaOH (VWR International Ltd, Poole, UK) to an end point marked by a pink colour persistent for at least 5 s. The milk was judged to be spoiled when TA had increased by 0·02% (Chakraborty et al. 1986).

Clot on boiling (*COB*)

The test was performed by boiling 2 ml milk sample in a test tube for 5 min, after which it was examined for curdling by tilting the tube gently. The sample was judged to be spoiled when clotting was observed.

Alcohol stability test (*AST*)

Equal volumes of milk and 70% ethanol prepared from 99% ethanol (VWR International Ltd) were mixed together in a test tube. The tube was inverted several times and then examined for coagulation. Coagulation of milk indicated spoilage.

pH

Spoilage was judged to have occurred by a fall in pH of 0·4 units.

Statistical analysis

Data were analysed by analysis of variance (ANOVA) and the means compared using a multiple comparison test of Least Significant Difference (LSD) at ($P \leqslant 0·05$).

Results

LP activity and thiocyanate concentration in different breeds

Large variations, both between and within breeds, were recorded in all breeds in both LP activity and SCN^- concentration (Table 1). The greatest variability in both parameters was observed in the Ayrshire breed. The variation was lowest in TSHZ in terms of LP activity, and in Friesian in terms of SCN^- concentration. The highest individual and mean LP activities were found for Friesians while the lowest individual activity was for the Ayrshires and the lowest mean activity was found for the TSHZ. In some cases, large differences in both LP activity and SCN^- concentration in individual cows were recorded over the weekly sampling interval. An extreme case of this variation for an individual cow was LP activities of 254 and 2951 μM product/min, and SCN^- concentrations of 6·1 and 35·2 mg/l.

Statistically significant variations ($P < 0·05$) in LP activity between both Friesian and Ayrshire, and Friesian and TSHZ were noted, but the differences between Ayrshire and TSHZ were not significant. The thiocyanate concentration varied quite widely between the different individuals, but there was no significant difference between the three breeds.

Keeping quality of LP-s activated raw milk

The KQ results for the different activation strategies of LP-s activated raw milk stored at 28–30 °C are summarised in Table 2. There was good agreement between the four methods of estimating KQ in terms of the ranking of the different treatments. Higher KQ estimates were generally observed with COB and pH measurements, while TA and AST gave similar results. These results are consistent with previous findings (Barrett et al. 1999).

All activation strategies resulted in improved KQ, however, quite different increases in KQ were apparent when levels of either one or both substrates were increased. The use of equal substrate concentrations (Table 2a) showed a consistent increase in KQ in all four tests, and KQ increased as the concentrations of

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Table 2 Keeping quality of LP-s activated raw milk with varying concentrations of $SCN^-:H_2O_2$, stored at 28–30 °C. TA = Titratable acidity, AST = Alcohol stability test and COB = Clot on boiling test

Values are means of two experiments

	Keeping quality (h)			
$SCN^-:H_2O_2$ (mg/l)	Decreased pH ⩽ 0·4	Increased TA > 0·02	Unstable to 70% ethanol (AST)	Unstable to boiling (COB)
a. Equal $SCN^-:H_2O_2$				
00 : 00	⩽10	⩽10	⩽10	⩽10
07 : 07	16	12	14	18
10 : 10	22	18	18	24
15 : 15	24	20	22	26
b. Excess SCN^-				
00 : 00	14	⩽10	⩽10	12
15 : 10	20	16	16	20
20 : 10	20	16	18	22
25 : 10	18	16	16	18
c. Excess H_2O_2				
00 : 00	⩽10	⩽10	⩽10	⩽10
10 : 15	24	18	20	22
10 : 20	24	18	20	24
10 : 25	24	20	22	24

Table 3 Keeping quality of LP-s activated raw milk treated with equal concentrations of $I^-:H_2O_2$ and $SCN^-:H_2O_2$ at 28–30 °C. TA = Titratable acidity, AST = Alcohol stability test and COB = Clot on boiling test

Values are means of two experiments

		Keeping quality (h)			
LP substrates	Concentration (mg/l)	Decreased pH < 0·4	Increased TA > 0·02	Unstable to 70% ethanol (AST)	Unstable to boiling (COB)
Control	00 : 00	⩽10	⩽10	⩽10	⩽10
$I^-:H_2O_2$	15 : 15	30	24	28	32
$SCN^-:H_2O_2$	15 : 15	22	18	20	24

the system initiators were increased, such that KQ was more than doubled at the highest concentrations of initiators.

Although, the treatment of raw milk with excess concentrations of SCN^- similarly extended the KQ at all activation levels and for all four methods of assessment, there was little difference in KQ between the different activation levels (Table 2b).

With excess concentration of H_2O_2, all three treatments again gave considerable increases in KQ above the control, but there was little difference between the three activation treatments (Table 2c).

Comparison of activation with LP/$SCN^-:H_2O_2$ and LP/$I^-:H_2O_2$

Activation using the LP/$I^-:H_2O_2$ system gave much greater increases in KQ compared with LP/$SCN^-:H_2O_2$, according to all four criteria (Table 3).

Discussion

The wide variations in LP activity in individual cows of the same or different breeds, and variations between samples from individual cows over a weekly sampling interval, are consistent with the variable pattern of the enzyme activity described in earlier studies (Fonteh et al. 2002). The significantly greater activity in the Friesian milk compared with the other breeds is an interesting observation, although care should be taken in ascribing too much importance to this in view of the wide variation and unpredictability of LP activity seen in this and earlier studies.

The mean SCN^- content reported in the present study is consistent with literature data, 1–15 mg/l (Reiter & Harnulv, 1984; Wolfson & Sumner, 1993; de Wit & van Hooydonk, 1996) and 4–59 mg/l (Korhonen, 1973). Although variations in SCN^- concentration were observed between and within individual cows during sampling, the overall mean did not show any significant variations between breeds. The results also demonstrated a lack of

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correlation between milk LP activity and SCN^- content ($R^2 = 0{\cdot}052$) as reported previously in cows' and goats' milk (Fonteh et al. 2002). In milk of healthy cows, the SCN^- level is highly dependent on the feeding regime of the cow, as many plants, particularly *Cruciferaceae*, contain SCN^- precursors (Korhonen, 2004). Lactoperoxidase activity, on the other hand, is not primarily affected by diet.

The use of equal concentrations of SCN^-:H_2O_2 resulted in a consistent increase in KQ with increased substrate concentration. The enhancement of the effects of the LP-s with increasing concentration of SCN^-:H_2O_2 has been demonstrated in previous studies (Björck, 1978; Siva et al. 1991). Chakraborty et al. (1986) described the increasing effectiveness of the system with increasing substrate concentration as resulting from generation of large amounts of intermediate compounds $OSCN^-$ and O_2SCN^- for a long duration.

There was clearly no advantage in providing excess concentrations of SCN^- relative to H_2O_2. This observation suggests that the effects of SCN^- on the antimicrobial properties of LP-s, depend on the relative amount of available H_2O_2. H_2O_2 is considered to be the most limiting factor to the LP-s as only traces of H_2O_2 have been detected in freshly secreted milk (Korhonen, 2004). Siva et al. (1991) recorded a residual level of 61·4 mg SCN^-/l in LP-s activated milk treated with 70 : 30 mg/l SCN^-:H_2O_2 at the end of their experiment. Similar observations were reported by Ridley & Shalo (1990) using 21·5 mg SCN^-/l and 7·3–14·4 mg H_2O_2/l; they recorded an average residue of 11·5 mg SCN^-/l in treated milk after 24 h. Thakar & Dave (1986) demonstrated that the residual SCN^- concentration in milk treated using equimolar concentration of SCN^-:H_2O_2 is equivalent to the concentration of SCN^- occurring naturally in milk. The residual SCN^- may also partly result from regeneration through interaction of oxidation products with SH-groups of proteins (Siva et al. 1991). It should also be noted that fresh milk contains a significant and variable concentration of SCN^-, as discussed above, so the optimum activation level could vary between milk samples. Overall these results suggest that there is no advantage in providing excess SCN^- above 10 mg/l, in agreement with Ewais et al. (1985), who demonstrated that SCN^- levels >10 mg/l have no advantage in KQ improvement, and Rossi & de Oliveira (1993) who showed an increased efficacy of the LP-s with increasing SCN^- concentration in the range of 3·5 to 12 mg/l, but no further increase at higher concentrations. Similarly, excess concentrations of H_2O_2 >10 mg/l did not have a significant impact on the overall KQ of milk. Rossi et al. (1995) reported a decrease in the effectiveness of LP-s at H_2O_2 >20 mg/l and linked the antimicrobial effect at this concentration to the direct action of H_2O_2 rather than the LP-s. Excess H_2O_2 is associated with irreversible inactivation of LP (Seif et al. 2005), however this occurs when the H_2O_2 concentration exceeds 300 mg/l (FAO/WHO, 1991). It can be concluded that equal concentrations of the two activators are effective in increasing the KQ, and there is no particular advantage in providing excess concentrations of either component.

The greater effectiveness of the LP/I^-:H_2O_2 system over the LP/SCN^-:H_2O_2 system may be due to the formation of more stable compounds between I^- and sulphydryl compounds from bacterial enzymes, thus offering a much longer inhibition effect or greater bacteriocidal effect compared with SCN^- (De Wit & van Hooydonk, 1996). Barraquio et al. (1994) reported the strong dependence of the effect of the antimicrobial products (OI^- and $OSCN^-$) on their special ability to bind to free SH-groups in various proteins. Enhanced antimicrobial effectiveness of the LP-s against *Candida albicans*, *Escherichia coli* and *Staphylococcus aureus* by addition of iodide has also been demonstrated previously (Bosch et al. 2000). Studies are currently in progress to compare the bacteriocidal and bacteriostatic effects of the two systems against a range of spoilage bacteria in milk.

The authors are unaware of any current practical applications of I^- addition to milk. However, the Codex Alimentarius standard permits addition of potassium iodide to food grade salt at levels of 30–200 mg ml^{-1} (FAO, 1996), which suggests that application of the LP/I^-:H_2O_2 system in milk should be acceptable. It is felt that the possibility of application of this system should be further explored.

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