ANTIOXIDATIVE–RELATED ACTIVITIES OF LACTOFERRIN AND LACTOPEROXIDASE IN MILK FROM COWS WITH DIFFERENT FORMS OF MASTITIS

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Abstract

Milk samples were taken from cows with acute, subacute, chronic, and subclinical mastitis and from healthy cows. The mean activity of lactoferrin (LF) in milk from mastitic cows ranged from 8.9 ±3.0 to 12.1 ±6.9 mU/g protein and was significantly lower than that in milk from healthy cows (29.5 ±15.0 mU/g protein). In group of mastitic cows the highest LF activity was found in cows with chronic mastitis, and the lowest in those with subclinical mastitis. The lactoperoxidase activity in cows with clinical and subclinical mastitis was significantly higher in comparison with healthy cows (1.3 ±1.1 mU/g protein) ranging from 5.5 ±2.6 mU/g protein in subclinical mastitic cows to 8.4 ±5.0 mU/g protein in chronic mastitic cows. Lower LF activities in cows with mastitis than in healthy animals may lead to a decreased antioxidative defence system in mastitic cows.

Key words: cows, mastitis, lactoferrin, lactoperoxidase, antioxidative defence.

Mastitis is the most common and most costly health problem amongst dairy cows (36). The disease may be clinical or subclinical and leads to reduced yield of milk and its lower quality (16).

It is known, that an inflammatory process is the main event related to reactive oxygen species (ROS) production (3, 4). At the inflammation site, the activating factors such as complement fragments, opsonised bacteria, viruses, immunoglobulins, chemotactic peptides induce rapid consumption of oxygen in the phagocytic cells, called the “respiratory burst” and production of ROS used by phagocytes to destroy the absorbed microorganisms (34). However, lack of proper control of ROS production by antioxidative mechanisms results in their overproduction and damage to the phagocytic cells, as well as adjacent tissue cells. The in vitro studies have demonstrated that ROS released by activated neutrophils and macrophages may be toxic for various somatic cells such as erythrocytes, epithelial cells, fibroblasts or platelets (15).

Living organisms have sophisticated and well-known antioxidant defence systems against ROS including antioxidative enzymes (glutathione peroxidase, superoxide dismutase, catalase) and low molecular weight antioxidants (12, 14). There is evidence that apart from them, proteins like lactoferrin (LF) and lactoperoxidase (LP) may exert antioxidative properties in milk (28, 31, 38).

Lactoferrin (LF) is an iron-binding glycoprotein belonging to the family of transferases (9). LF is synthesised in the udder by epithelial cells and neutrophils. The concentration of LF in milk from healthy udders is low but during mastitis, its milk concentration increases (10). The best-known biological function of LF is antibacterial activity. Most micro-organisms need iron for their growth and LF has the potential to inhibit the growth of bacteria and even kill them by depriving them of iron (9). Moreover, LF shows anti-inflammatory, immunomodulating, and anti-oxidative activities (22, 38).

Lactoperoxidase (LP), besides xanthine oxidase, is the most abundant enzyme in milk (21). LP is an enzyme catalysing hypothiocyanate synthesis from thiocyanate and hydrogen peroxide, which is characterised by potent bactericidal properties (21, 31). Moreover, lactoperoxidase exerts antiviral effects, degrades carcinogens, and shows anti-oxidative properties (21, 28, 31).

The aim of the study was to compare antioxidative-related activity of milk from cows with various forms of clinical mastitis and subclinical mastitis by the determination of activity of two proteins, which are supposed to have anti-oxidative properties - LF and LP.

Material and Methods

The material consisted of milk samples from single udder quarters from 74 cows with mastitis confirmed by clinical examinations of the udder, TOK test (equivalent of California mastitis test), somatic cell counts (SCC), and bacteriological examinations. Forty-
eight cows were diagnosed with clinical and 26 with subclinical mastitis. In the group with clinical mastitis, acute mastitis (elevated internal temperature, oedema, reddening, udder pain, changes in milk appearance, positive bacteriological culture) was observed in 14 cows, sub-acute mastitis (slight quarter oedema, changes in milk, positive TOK results, positive bacteriological culture) was recognised in 18 cows, and chronic clinical mastitis (long-term ineffective treatment, persistent hardening of the glandular tissue, changes in milk, positive TOK results, positive bacteriological culture) displayed 16 cows. Subclinical mastitis was diagnosed on the basis of the increase of SCC (TOK positive). The age of mastitic cows ranged from 2 to 10 years; they were between 15 and 200 d postpartum. The study material also included 14 milk samples from healthy cows aged 2-6 years and between the lactation day 20 and 180. All cows belonged to Holstein-Friesian (HF), Polish Black–White (PBW), and PBWx HF breeds.

About 10 ml of cyosternal milk was aseptically collected to sterile tubes. After their use for bacteriological tests, the milk samples were frozen at −76°C and stored until further analyses.

**Bacteriological testing.** The bacteriological examinations of milk samples were carried out according to commonly accepted methods (25).

**Determination of LF activity.** The determination of LF activity was carried out according to the procedure of Liu et al. (23), and Ye et al. (38). Milk (100 µl) was added to the mixture consisting of 2.27 ml of TRIS-HCL buffer (16 mmol/L, pH 8.0), 300 µl of dihydro-nicotinamide-adenine-dinucleotide-phosphate (78 µmol/L), 300 µl of nitroblue tetrazolium (NBT), 30 µl of phenazin methosulfate (PMS). The absorbance was measured at 0 and 10 min of reaction at 560 nm (Ultrspec 2000; Pharmacia, Sweden). L-Ascorbic acid was used as a control. The calculations were based on standard curve prepared with different concentrations of NBT. LF activity was expressed in mIU per gram of protein.

**Determination of LP activity.** The activity of LP was determined according to the method by Mansson-Rahemtull et al. (26). Stock of 5, 5′-dithiobis (2-nitrobenzoic acid) (DTNB) was prepared by dissolving 29.1 mg of DTNB in 7.5 ml of 0.1 mol/L K₂HPO₄ (pH 8.8) and filled up to 50 ml with distilled water. The NBS assay buffer was prepared by adding 0.8 ml of 10 mmol/L mercaptoethanol to 4 ml of stock DTNB to reduce DTNB to NBS. This solution was then filled up to a final volume of 100 ml by addition of 0.1 mol/L phosphate buffer (pH 5.66). This NBS assay buffer should give an absorbance of 0.8-1.0 at 412 nm. Next 50 µl of milk and 100 µl of the stock H₂O₂ were added to 2 ml of the NBS buffer. The absorbance of samples was read at 412 nm. The calculation was based on standard curve prepared with different concentrations of DTNB. The LP activity was expressed in mIU per gram of protein.

**Protein determination.** The protein content in milk was measured using the commercial kit (Total Protein Kit, Cormay, Poland) according to the producer’s protocol.

**Statistical analysis.** The results were statistically analysed defining the arithmetic mean and standard deviation (+SD). The inter-group differences were analysed using Statistica 5.0 software. P<0.05, P<0.01, and P<0.001 were considered as significant.

**Results**

The pathogens were found in all milk samples from mastitic cows. In milk samples from acute mastitis cows, the following microorganisms were isolated: *Stir. agalactiae* (1), *Staph. aureus* (10), and *E. coli* (3). The milk samples from cows with mild clinical mastitis were demonstrated to contain *Str. dysgalactiae* (8), *Str. uberis* (5), *Staph. aureus* (2), coagulase-negative staphylococci - CNS (1), and *E. coli* (2). The milk samples from cows with chronic clinical mastitis contained *Str. dysgalactiae* (6), *Str. uberis* (2), and *Candida* sp. (8). The bacteria isolated from cows with subclinical mastitis included: *Stir. agalactiae* (9), *Str. dysgalactiae* (4), *Str. uberis* (8), and CNS (5). No pathogens were isolated in milk from healthy cows.

The mean activity of LF in milk from healthy cows was 29.5 ±15.0 mU/g of protein and was significantly higher (P<0.001) than that in milk from cows with particular forms of mastitis. The comparison of mean LF values in milk from mastitic cows showed differences between individual forms of the disease. The highest LF activity was found in milk from cows with chronic mastitis (12.1 ±6.9 mU/g of protein), the lowest - in milk from cows with subclinical mastitis (8.9 ±3.0 mU/g of protein). The differences in LF activities between these groups were statistically significant (P<0.05).

The mean activity of LP in milk from healthy cows was 1.3 ±1.1 mU/g of protein and was significantly lower in comparison to cows with clinical and subclinical mastitis. The mean values of LP activities in milk from mastitic cows ranged from 5.5 ±2.6 to 8.4 ±5.0 mU/g of protein; the highest activity was observed in chronic mastitis whereas the lowest one in cows with subclinical mastitis. The differences in LP activities between these groups were statistically significant (P<0.05).

**Discussion**

It is evident that ROS are the key denominators in a number of pathological conditions, including inflammation (3, 13). Once the growth of these metabolites of molecular oxygen is uncontrollable, they are likely to cause oxidative injury to all main constituents of the cell (13).
The excess of ROS results in the damage and necrosis of cells through various mechanisms, including peroxidation of cellular membrane lipids, protein denaturation, and DNA damage (7). Moreover, ROS, such as superoxide anion radical or hydrogen peroxide, mediate the infiltration and accumulation of neutrophils at the inflammation site, and mobilise the changes of arachidonic acid (18). ROS are also involved in the activation of transcription factors, such as NF-kB and AP-1. The transcription factors are activated during inflammation of epithelial and inflammatory cells, in which they induce the expression of many encoding genes, e.g., T NFα, II-1, II-6, nitrogen oxide synthesis, MHC complex I antigens, and adhesive particles (4). Furthermore, it is believed that a large group of proteases, called metalloproteinases, is affected by ROS, which enhances the tissue damage at the inflammatory focus (34).

Deleterious effects of ROS are counteracted by the antioxidative defence system, which consists of non-enzymatic antioxidant molecules and the antioxidant enzymes (12, 14). The antioxidative defence system is responsible for maintaining appropriate levels of ROS. In the present study, less popular markers of antioxidative defence against ROS in milk of cows were analysed. Lactoferrin and lactoperoxidase are important non-specific protective elements of the mammary gland against mastitis pathogens (21, 30) but they also have antioxidative properties (28, 31, 38).

Lactoferrin may serve as an antioxidative defence mechanism by binding any catalytic iron generated during the course of cell destruction, minimising hydroxyl radical-mediated tissue injury associated with neutrophil-oxidant production during inflammation, and by binding lipopolysaccharide (LPS) with a consequent reduction in LPS bioactivity and ameliorating LPS-induced toxicity (38). The activation of leukocytes by LPS results in oxidative burst. Large quantities of LPS are supposed to cause ROS overproduction and in consequence tissue damage. LF can limit ROS production and tissue damage by its ability to inhibit superoxide radical generation.

The obtained results demonstrated that the LF activity in milk from mastitic cows was significantly lower in comparison to milk from healthy cows. The activity of LF in milk from healthy udders was similar to that demonstrated by Albera and Kanktofer (1). There was, however, no data concerning antioxidative activity of LF in milk mastitic cows. In contrast, in several studies, higher concentration of LF in milk from mastitic cows compared to milk from healthy udders has been reported (10, 11, 17, 32). This suggests that, the increase in LF content in milk of mastitic cows is accompanied by the decrease in antioxidative properties of this protein. The experiments of other authors revealed the decrease in the content of non-enzymatic antioxidants such as vitamin C and E in milk of cows suffering from mastitis (5, 35). Our findings demonstrate that the differences in LF activities depend on the form of mastitis. In milk from cows with clinical mastitis, activity of LF was higher than in cows with the subclinical form; the highest LF activity was found in milk from chronic mastitic cows. This corresponds to the results published by other authors, which showed that LF concentration during mastitis depended on the severity of mastitis (11, 17, 19). The highest concentrations were observed in E. coli acute mastitis, whereas the lowest values were found in subclinical mastitis (17, 19).

LP is a glycoprotein synthesised in the udder mainly by neutrophils (31). LP is probably a major consumer of H2O2 - one of the main RFT- that is constantly produced in mammary secretions (8). This enzyme uses H2O2 to oxidise the anion thiocyanate. In this reaction, hypothiocyanate is formed, which has potent bactericidal effects (21, 31). Moreover, LP induces the oxidation of SH-groups in cell membranes of bacteria, which leads to the loss of their capacity to transport glucose and to efflux potassium ions, amino acids, and peptides from the cell (21, 31). The LP may protect animal cells against toxic effects of H2O2 (28, 31). In our study, the LP activity was significantly higher in milk from cows with mastitis compared to healthy cows. The highest LP activity was observed in cows with chronic mastitis, whereas the lowest in those with subclinical mastitis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Acute mastitis</th>
<th>Subacute Mastitis</th>
<th>Chronic mastitis</th>
<th>Subclinical mastitis</th>
<th>Healthy cows</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactoferrin (mU/g of protein)</td>
<td>10.3 ±8.1***</td>
<td>9.7 ±2.6***</td>
<td>12.1 ±6.9***</td>
<td>8.9 ±3.0***</td>
<td>29.5 ±15.0</td>
</tr>
<tr>
<td>Lactoperoxidase (mU/g of protein)</td>
<td>6.7 ±5.2**</td>
<td>6.0 ±3.9***</td>
<td>8.4 ±5.0***</td>
<td>5.5 ±2.6***</td>
<td>1.3 ±1.1</td>
</tr>
</tbody>
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** - statistically significant differences compared to controls at P<0.01; *** - statistically significant differences compared to controls at P<0.001; a,b - values denoted with letters are statistically significantly different at P<0.05.
increase in LP activity in mastitic cows may be associated with excessive production of ROS in the site of inflammation. The increase in antioxidative enzyme activity is a natural response of organism to the new challenges, among others, to oxidative stress (14). There are very few literature data concerning LP activity in cow milk. Similar observations on LP activity in milk of healthy cows were made by Albera and Kankofer (1). According to Person et al. (27), there were no significant differences in LP activity in milk of healthy and mastitic cows. On the contrary, Asadpour et al. (2) demonstrated higher LP activity in milk from cows with subclinical mastitis compared to healthy cows. Furthermore, Schmedt et al. (32) showed a significantly higher level of LP in quarters affected by clinical mastitis in comparison to healthy quarters of the same udder. In their study, the LP activity did not correlate with the severity of clinical symptoms of the disease. In another study, LP activity was found to increase rapidly in the colostrum during postpartum mastitis and to return to normal values following appropriate treatment (37).

Higher antioxidative activity of both LF and LP in milk of cows with clinical form of mastitis as compared to subclinical inflammation may indicate that ROS overproduction that appears in clinical mastitis is significantly higher, and may lead to induction of a compensative increase in antioxidative systems. It is known that ROS production increases together with the progress of disease (33), which is confirmed in the obtained results, indicating higher intensity of oxidative stress in cows with chronic mastitis in comparison to other forms. These observations correspond with other studies on humans and animals, which demonstrated significant role of oxidative stress during chronic inflammation related to, among others: digestive tract (24), respiratory tract (29), joints (2), and eye (29). Opposite relationship of parameters between healthy cows and animals suffering from mastitis, observed in the present study might be related to different mechanisms used for scavenging ROS by appropriate antioxidants, which is not simply the sum of antioxidative activity of antioxidants present currently in biological sample.

In conclusion, the obtained results show significant differences in activities of LF and LP in milk from healthy and mastitic cows. The perturbation of activity of these proteins, which are supposed to have antioxidative properties, is more pronounced in milk of cows with clinical mastitis, particularly in chronic form of mastitis. Lower activities of LF in mastitic cows in comparison to the healthy animals may lead to a decreased antioxidative defence system in mastitic cows.

References


