Lysozyme Activity in Buffalo Milk: Effect of Lactation Period, Parity, Mastitis, Season in India, pH and Milk Processing Heat Treatment

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ABSTRACT : Lysozyme activity in buffalo milk in relation to the period of lactation, parity of animal, weather conditions and udder infections was studied. Effect of storage and heat processing of milk on lysozyme activity was determined. Lysozyme activity was higher in buffalo milk than in cow milk. Buffalo colostrum showed lysozyme activity 5 times of that in mature milk. Lysozyme activity in buffalo milk was not influenced by the parity of animal and the stage of lactation, however, it increased during extreme whether conditions (winter and summer). Lysozyme in both cow and buffalo milk exhibited maximum activity at pH 7.4. Buffalo milk lysozyme was fully stable while the cow milk lysozyme was partly inactivated by pasteurization (low temperature-long time as well as high temperature-short time treatments). Lysozyme in buffalo milk was more stable than in cow milk during storage and heat treatment. A 10 to 50-fold increase in milk lysozyme activity was observed in mastitic cows. An assay of lysozyme activity in milk can be used to diagnose mastitis in cattle but not in buffaloes. Some buffaloes exhibited 1000 fold greater lysozyme activity and moderately raised somatic cell count in milk, but there was no sign of mastitis in these animals. A possible role of milk lysozyme in prevention of mastitis in buffaloes is discussed. ***(Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 6 : 895-899)***

Key Words : Lysozyme, Buffalo Milk, Bovine Milk, Parity, Mastitis

INTRODUCTION

Milk supplies an array of defense factors in the form of antibacterial, anti-inflammatory and, immuno-modulatory agents that protect the recipient from enteric infections, and the donor from udder infections. Lysozyme, a low molecular weight basic protein, is an important component of antibacterial system in milk. It kills bacteria by cleaving the β 1, 4-glycosidic bond between *N*-acetyl glucosamine and *N*-acetyl muramic acid residues in the peptidoglycan of bacterial cell wall. Milk lysozyme concentration shows wide variation among species and is influenced by factors such as the period of lactation, health, age and the parity of animals. Bovine and human milk lysozymes are widely studied and, human milk is one of the richest sources of lysozyme (Chandan et al., 1964, 1965; Panif-Kuncewiez and Kisza, 1976).

Buffalo's udders are less susceptible to infections compared with that of cow (Chand et al., 1995), and buffalo's milk is more resistant to spoilage than the cow milk. Anti-microbial properties of buffalo milk are not fully understood; particularly no information exists about lysozyme in buffalo milk. In the present communication, the levels and the stability of lysozyme in buffalo milk as influenced by various factors are reported.

MATERIALS AND METHODS

Lyophilized cells of *Micrococcus lysodeikticus* and egg white lysozyme (EC 3.2.1.17) were procured from Sigma Chemical Company (St Louis, MO, USA). All other chemicals used were of analytical grade. Milk samples were collected from crossbred cows (Karan Swiss and Karan Fries) and Murrah buffaloes housed in the cattle yard of National Dairy Research Institute in Karnal, India. Milk samples were stored at 10°C, and used in the experiments within one hour.

Lysozyme activity was determined according to the method of Selested and Martinez (1980) with some modifications. A suspension of killed cells of *Micrococcus lysodeikticus* (35 mg%) in 0.05 M potassium phosphate buffer (pH 7.4) was used as substrate. The reaction mixture contained 2.1 ml of substrate, 0.3 ml each of bovine serum albumin (10 mg/ml) and sodium azide (0.1%), and 10-15 μl of diluted skim milk in a final volume of 3 ml made up with 0.05 M potassium phosphate buffer. The mixture was incubated at 37°C with mild agitation, and the absorbance measured at 450 nm using JASCO UVIDEC 610-Spectrophotometer (Japan Spectrophotometer, Tokyo, Japan). The reduction in absorbance relative to control (devoid of enzyme source) was taken as a measure of lysozyme activity. The reaction time was kept 6 h instead of 18 h used by Selested and Martinez (1980). The change in absorbance was linear with respect to the enzyme concentration, providing sensitivity of 10-50 ng egg white lysozyme, sufficient for the assay of lysozyme in diluted milk samples. The unit of lysozyme activity was defined as the change in unit absorbance per min per ml reaction mixture at 450 nm.

Somatic cells in milk were counted following fixing and staining with Newmann's stain. Statistical differences in

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experiments involving multiple treatments were determined by analysis of variance. Statistical analysis in single treatment experiments was performed using student *t* test (Snedecor and Cochran, 1989). The results are expressed as means±SE.

RESULT AND DISCUSSION

Lysozyme content in milk

Lysozyme activity was determined in milk from normal cows and buffaloes (somatic cell count $<5\times10^5$ cells/ml milk), and in buffalo colostrum. In cow milk, lysozyme activity ranged from 17.8×10^{-3} to 38.2×10^{-3} units/ml (table 1). The average lysozyme activity in cow milk (2.92 units/100 ml) is equivalent to 32 μg of standard egg white lysozyme. These values are in agreement with those reported in literature (Chandan et al., 1965; Panif-Kuncewiez and Kisza, 1976). Lysozyme activity in milk from normal buffaloes ranged 37.3×10^{-3} to 73.4×10^{-3} units/ml, which is higher than that reported for cow (Chandan et al., 1965; Panif-Kuncewiez and Kisza, 1976), goat (Chandan et al., 1965), sheep (Chandan et al., 1965) and camel (Elgamy, et al., 1996) milks. The higher lysozyme activity in buffalo milk possibly is one of the factors responsible for lesser incidences of udder infections in buffaloes.

The activity of lysozyme in buffalo colostrum (301.7×10^{-3} units/ml) was about 5 times the activity observed in mature buffalo milk (60×10^{-3} units/ml). In humans (Chandan et al., 1964) and bovines (Panif-Kuncewiez and Kisza, 1976), however, milk and colostrum contain lysozyme in similar concentrations. Hence, buffalo calf receives greater amounts of lysozyme during first few days after birth, which might play an important role in prevention of enteric infections.

Effect of parity, season and stage of lactation on lysozyme activity in buffalo milk

Buffalo milk lysozyme activity was determined at first, third and fifth calving. Results presented in table 2 show that there was no significant difference in milk lysozyme activity between different calving. The apparently higher activity at fifth calving was not significantly different from that at first and third calving. The effect of parity on milk lysozyme activity is not similar in different species. In humans milk, higher lysozyme activity has been reported in multiparous than in primiparous mothers (Younes et al., 1994) while in bovines, primiparous animals have greater milk lysozyme activity than in multiparous animals (Soldatov and Lyumbimov, 1986).

Lysozyme activity in buffalo milk was influenced by weather conditions (table 3). The activity of lysozyme in buffalo milk was higher during summer and winter seasons than during spring and autumn. Soldatov and Lyumbimov (1986) also reported higher lysozyme activity in cow milk during winter season. Therefore, milk lysozyme activity is increased in cattle and buffaloes during extreme climatic conditions.

The lactation period of buffaloes was divided into four stages and lysozyme activity determined in milk (table 4). There was no significant difference in milk lysozyme activity between different stages of lactation. The apparent decrease in lysozyme activity observed during 101-160 days of lactation was not statistically significant. There is a report (Meyer and Senft, 1979) showing an increase in lysozyme activity in cow milk with the advancement of lactation. On the contrary, Soldatov and Lyumbimov (1986) have reported a decline in milk lysozyme activity with the

Table 1. Lysozyme activity in milk

	No. of samples	Units$\times10^{-3}$/ml
Cow milk	15	29.1±1.5
Buffalo milk	15	60.0±2.9^a
Buffalo colostrum	10	301.0±15.2^b

Values are mean±SE.

Lysozyme activity was determined in animals having somatic cell count $<5\times10^5$ cells/ml.

[a] p<0.05 as compared to cow milk.

[b] p<0.05 as compared to buffalo milk.

Table 2. Lysozyme activity in buffalo milk at different calving

Calving	No. of samples	Units$\times10^{-3}$/ml
First	10	71.9±12.9
Third	9	72.9±8.3
Fifth	8	96.0±8.7

Values are mean±SE.

Lysozyme activity was determined in buffalo milk having somatic cell count $<5\times10^5$ cells/ml.

Differences between mean values are not significant (p<0.05).

Table 3. Effect of weather conditions on lysozyme activity in buffalo milk

Season[1]	No. of samples	Units$\times10^{-3}$/ml
Spring	10	59.4±3.1^a
Summer	12	96.9±4.9^b
Autumn	10	53.4±3.6^a
Winter	12	85.0±5.4^b

Values are mean±SE.

[1] The minimum to maximum out side temperature ranged around 12° to 25° in spring, 27° to 42° in summer, 17° to 29° in autumn and 6° to 15° in winter.

Lysozyme activity was determined in buffalo milk having somatic cell count $<5\times10^5$ cells/ml.

The values with different superscripts are significantly different (p<0.05).

Table 4. Effect of lactation period on lysozyme activity in buffalo milk

Lactation period (days)	Units×10^{-3}/ml
<100	59.7±8.6
101-106	36.9±7.5
161-220	61.4±8.9
>220	73.1±8.9

Values are mean±SE from 4 observations.

Lysozyme activity was determined in buffalo milk having somatic cell count <5×10^5 cells/ml.

Differences between the mean values are not significant ($p<0.05$).

advancement of lactation in cows. Barbour et al. (1984) observed a decrease in lysozyme activity in camel milk, while Younes et al. (1994) has reported an increase in lyzozyme activity in human milk with the enhancement of lactation stage. Therefore, milk lysozyme activity in different species is not uniformly influenced by the stage of lactation. There was some decrease in milk lysozyme activity in buffaloes during 101-160 days of lactation. Recently, Singh and Ludri (2001) have reported that somatic cell count in milk decreases to lowest value during 90 to 150 days of lactation in buffaloes, showing some kind of relationship between somatic cell count and lysozyme activity in milk.

Relationship between mastitis and lysozyme activity in milk

Some samples of buffalo milk exhibited lysozyme activity thousand fold greater then the others. In order to identify the reason for this unusual phenomenon, a comparative study was conducted between lysozyme activity and somatic cell count in buffalo milk. Based on somatic cell count, animals were divided into two groups, one with somatic cell count <5×10^5 cells/ml milk and, the second with somatic cell count >5×10^5 cells/ml milk. Results presented in table 5 show that in the first group, lysozyme activity ranged from 37.4×10^{-3} to 82×10^{-3} units/ml milk. In the second group, somatic cells in milk were between 5.3×10^5 to 102.9×10^5 cells/ml and the lysozyme activity ranged from 202×10^{-3} to 48,667×10^{-3} units/ml milk. However, there was no sign of mastitis in these animals. Buffaloes are much less susceptible to mastitis compared with bovines. A sharp rise in milk lysozyme activity could be the reason of some mild udder infection, and increased lysozyme activity might have protected buffaloes from development of mastitis.

Mastitis is very common in cows. In healthy cows, milk somatic cell count ranged 0.2×10^5 to 3.8×10^5 cells/ml and lysozyme activity from 11.7×10^{-3} to 59.3×10^{-3} units/ml milk. In mastitic cows, the range of milk somatic cell count was 72×10^5 to 1,014×10^5 cells/ml and that of lysozyme activity

Table 5. Relationship between lysozyme activity and somatic count in milk

Cows		Buffaloes	
Somatic cell counts×10^5/ml	Lysozyme activity Units×10^{-3}/ml	Somatic cell counts×10^5/ml	Lysozyme activity Units×10^{-3}/ml
Normal animals with somatic cell counts <5×10^5 cells/ml			
3.8	50.8	1.5	38.0
3.0	59.3	3.4	82.0
1.8	57.0	1.6	49.6
0.2	21.9	0.6	47.5
0.7	25.4	1.0	74.2
1.9	36.4	0.5	42.2
0.8	40.0	0.3	43.5
1.4	18.8	0.7	41.9
1.5	15.5	1.8	43.8
1.0	11.7	0.5	37.4
2.5	14.4	3.4	58.1
2.4	23.2		
Animals with somatic cell count >5×10^5cells/ml			
509	1,753	6.2	213
834	942	6.5	202
72	1,515	5.3	364
955	1,290	31.4	13,149
1,014	3,043	87.0	12,533
480	792	9.1	1,071
859	1,794	74.5	23,333
625	649	102.9	48,667
866	1,084	36.5	253

649×10^{-3} to 3,043×10^{-3} units/ml. These results show that milk lysozyme activity is increased 10 to 50 fold in mastitic cows, and are in agreement with the earlier reports (Sokolova et al., 1983). Further, an assay of milk lysozyme activity provides a simple method for diagnosis of mastitis in cattle.

Effect of heat treatment and pH on milk lysozyme activity

Buffalo milk when subjected to low temperature-long time (LTLT) and high temperature-short time (HTST) heat treatments followed by immediate cooling, retained 100% of its lysozyme activity, while cow milk lost 11.5 and 24% of lysozyme activity by these treatments, respectively (table 6). HTST treatment of buffalo milk followed by slow cooling caused a loss of only 7.5% of its lysozyme activity. Losses in lysozyme activity by heating to boil were 40 and 61% in buffalo and cow milk, respectively. The losses reported for lysozyme in human milk due to LTLT and HTST treatments were 47 and 30%, respectively (Chandan et al., 1964). Therefore lysozyme in buffalo milk is fully stable, while in cow and human milks it is partly inactivated during pasteurization.

Table 6. Effect of heat treatment on lysozyme activity in milk

Heat treatment	Buffalo milk	Cow milk
Fresh milk	51.7±0.2	22.9±0.1
LTLT1[1]		
Cooled immediately	52.7±0.1	20.3±0.1
Cooled slowly	-	19.0±0.2
HTST[2]		
Cooled immediately	52.2±0.1	17.4±0.0
Cooled slowly	47.9±0.6	14.2±0.1
Boiling[3]		
Cooled immediately	31.1±0.2	9.0±0.1

Values are mean±SE from 4 determinations and expressed in units$\times10^{-3}$ per ml milk.

[1] Low temperature, long time (63°C for 30 min).

[2] High temperature, short time (74°C for 16 sec).

[3] Milk taken in a beaker was heated on a hot plate with constant stirring till it started boiling.

Figure 1 depicts the effect of pH on lysozyme activity in cow and buffalo milk. The enzyme in both cow and buffalo milks was observed to be active over a wide range of pH with optimum pH 7.4.

Effect of storage on milk lysozyme activity

Raw milk samples were stored at different temperatures and, the lysozyme activity was determined. Raw cow milk samples when stored at 5° to 15°C did not curdle up to 24 h and there was no change in lysozyme activity. When stored for 48 h, the raw milk was curdled and the losses in lysozyme activity were 37% at 5°C and 47% at 10° to 15°C. When stored at 30° and 37°C for 24 h, cow milk was curdled and retained 77.3 and 72.3% of its lysozyme activity, respectively. Following storage for 48 h at these temperatures, the losses were 60 and 87%, respectively (table 7).

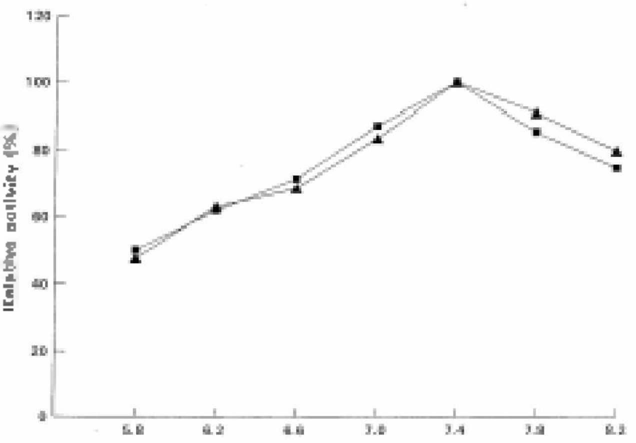

Figure 1. Effect of pH on the activity of lysozyme in bovine (▲) and buffalo (■) milk. Values are mean of 3 determinations and expressed as percentage of maximum activity.

Table 7. Lysozyme activity in milk stored at different temperatures

Temperature (°C)	Storage period (h)			
	6	12	24	48
Cow milk				
5	98.7	96.6	98.7	62.6
10	97.9	98.7	95.0	53.4
15	100	97.1	94.5	53.4
30	97.9	95.4	77.3	39.9
37	97.9	89.9	72.3	13.5
Buffalo milk				
5	96.2	97.8	99.0	94.2
10	97.8	97.8	98.7	94.5
15	96.7	98.4	97.5	97.6
30	98.1	98.5	88.5	79.9
37	97.4	97.0	84.1	71.1

Values are mean of 3 determinations and expressed as percentage of original activity in fresh in fresh milk.

Raw buffalo milk did not curdle up to 48 h when stored at 5° to 15°C. Even at higher temperature (30° to 37°C) no curdling was seen in buffalo milk when stored for 24 h. There was no significant loss in lysozyme activity in buffalo milk when stored at 5° to 15°C for 48 h. Also, at higher temperature (30° to 37°C), only 12 to 16% activity of lysozyme was lost following storage for 24 h (table 7). Therefore, during storage, lysozyme is more stable in buffalo milk than in cow milk. Better storage stability of buffalo milk then the cow milk may be due to higher activities of anti-microbial agents such as lysozyme, lactoferrin and lactoperoxidase. Buffalo milk contains lactoferrin 0.32 mg/ml (Bhatia and Valsa, 1994) opposed to 0.1 mg/ml in cow milk (Bhatia, 1997). The activity of lactoperoxidase is also higher in buffalo milk than in cow milk (Kumar, 1994).

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