



# Comparing the Activity and Thermal Inactivation Behavior of Lactoperoxidase in Iranian Cow and Buffalo Milk and Whey

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## HIGHLIGHTS

- Lactoperoxidase (LPO) activity was slightly, but not significantly, higher in buffalo milk than in cow milk.
- LPO activities in cow and buffalo whey were 16-19% lower than those of milk.
- LPO was heater labile in whey than milk, in both cattle and buffalo.

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### Acronyms and abbreviations

LPO=Lactoperoxidase  
HTST= High Temperature Short Time  
ABTS=2,2'-azino-bis-3-ethylbenzo thiazoline-6-sulphonic acid

## ABSTRACT

**Background:** Lactoperoxidase (LPO) is one of the most heat-stable enzymes in milk and its inactivation has been proposed for monitoring thermal processes. The aim of this study was to provide information on activity and thermal inactivation behavior of LPO in Iranian cow and buffalo milk and whey.

**Methods:** Sixty cow and buffalo milk samples were collected. The LPO activity was measured using spectrophotometer at 436 nm using a multimode microplate reader. Thermal inactivation behavior of LPO in milk and whey samples was investigated at temperatures 65, 70, 75, and 80 °C, using glass capillaries for quick temperature transfer. Data were analyzed using SPSS software (Chicago, IL, v. 16.0).

**Results:** LPO activity was slightly, but not significantly, higher in buffalo milk than in cow milk, where its activity was 4.15±0.13 U/ml and 4.02±0.1 U/ml in buffalo and cow milk, respectively. LPO activities in cow and buffalo whey were 16-19% lower than the respective values in milk, on average, 3.39±0.1 U/ml in buffalo whey and 3.36±0.08 U/ml in cow whey. No significant differences ( $p>0.05$ ) were observed between thermal stabilities of LPO in both milk types. In addition, at all the tested temperatures, LPO was heater labile in whey than milk, in both milk types.

**Conclusion:** There were some variations in LPO behavior against thermal processing in cow and buffalo milk and whey. So, these findings could be helpful in further studies about monitoring thermal processes in dairy industries.

## Introduction

Lactoperoxidase (LPO) is a natural enzyme found in plants and animals, belonging to the peroxidase family. It is thought to be an important component in the natural host-defense systems against bacterial infections (Kussendrager and van Hooijdonk, 2000). LPO system can also be used to increase the storage stability of milk at high ambient temperatures (FAO, 1999).

LPO is a natural antimicrobial system in milk, which is activated by increasing the concentrations of thiocyanate and hydrogen peroxide. This reaction is catalyzed by LPO enzyme which is intrinsically present in milk and causes the formation of antibacterial compounds (Seifu et al., 2005).

In bovine milk, LPO is the second most abundant en-

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zyme after xanthine oxidase (de Wit and van Hooydonk, 1996). Its concentration in bovine milk is about 30 mg/L constituting about 1% of the milk serum protein. LPO is one of the most heat-stable enzymes in bovine milk (Seifu et al., 2005). It retains its activity during holder pasteurization (63 °C for 30 min), as well as full or partial activity during High Temperature Short Time (HTST)-heating at 72 °C for 15 s, but it is rendered inactive at 80 °C for 2.5 s (Korhonen, 1980). So, LPO inactivation has been proposed for monitoring thermal processes for heat treatments above 78 °C for 15 s (Griffiths, 1986). Limited literature is available to provide data on the LPO activity in whey, despite the fact that, whey is produced in huge quantity during these years and LPO is commonly extracted from whey and used in the health and food industries. For example, LPO is used in preserving food and preparing toothpastes, cosmetics, and ophthalmic solutions (Horton, 1995).

Most studies on the activity and thermal inactivation behavior of LPO have been performed on cow milk and only few have focused on non-bovine milk. Growing markets for buffalo milk and its products may increase the research interests to this area. Although there are some studies performed with buffalo milk, but due to difference of the analytical methods used in the previous researches, it is important to perform a comparative study using the same experimental conditions. Therefore, the first aim of this work was to provide information on the activity and thermal inactivation behaviors of LPO in Iranian buffalo milk and compared with cow milk from viewpoint of monitoring heat treatments during typical pasteurization process. Also, it has taken into account that processing of buffalo milk may involve thermal processing parameters other than those for cow's milk. In addition, to the best of our knowledge, there are no comprehensive studies that have compared the thermal inactivation behavior of LPO in milk and whey. Hence, the second aim of this study was to provide information on the activity and thermal inactivation behavior of LPO in whey and compared with milk, in both species.

## Materials and methods

### Milk samples

A total of 60 bulk milk samples of cow (Holstein Friesian) as well as buffalo (Iranian river buffalo) were collected from the central tank of the dairy farms and put in cold portable insulated boxes and transferred to the laboratory. Table 1 shows the average content of fat, protein, and dry solids of the bulk milk samples which were analyzed using standard procedures (Wehr and Frank, 2004).

### Preparation of whey

Whey samples were prepared by incubating milk samples containing 0.1% rennet at 37 °C for 60 min followed by centrifugation at 4000 rpm for 5 min.

### Assay of enzyme activity

The LPO activity was measured using spectrophotometer at 436 nm, using a multimode microplate reader (Synergy HT, BioTek, USA). In brief, 10 µl of the milk or whey samples were transferred into each well of a 96-well microtiter plate which contained 210 µl of 100 mM potassium phosphate buffer (pH 5.5). Then, 70 µl of 10 mM 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) solution was added and mixed. To initiate the reaction, fresh prepared 0.025% H<sub>2</sub>O<sub>2</sub> solution was added and the absorbance was measured after 30 s and 90 s. The blank solution used in measurements was prepared by heating 1 ml of raw milk or whey for 1 min at 95 °C. One unit of activity (U) is defined as the amount of enzyme that catalyses the oxidation of 1 µmol of ABTS per min at 25 °C. All tests were performed in triplicate. Enzyme activity was calculated using the following equation (Keeseey, 1987).

$$\text{Units/ml enzyme} = (\Delta A_{436}/\text{min test} - \Delta A_{436}/\text{min blank}) / (\text{TV}) / (\text{DF}) / (29.3) / (\text{SV})$$

Where, TV is the total volume (ml) of the assay; DF is the dilution factor; 29.3 is millimolar extinction coefficient of oxidized ABTS at 436 nm and SV is the sample volume (ml) used in the assay.

### Determination of heat resistance

Determination of heat resistance was performed using glass capillaries for quick temperature transfer. Glass capillaries (length 10 cm, wall thickness 0.15 mm, inner diameter 1 mm) filled with whey or milk samples were sealed and immersed in a water bath set to temperatures of 65, 70, 75, and 80 °C for 30, 10, 3, and 1.5 min, respectively. At each sampling time, the glass capillaries were immediately immersed in the ice water to allow rapid cooling and stop the heat effect. After that, the LPO activity was measured using spectrophotometer as mentioned before.

### Statistical analysis

All experiments were performed in triplicate. Data were analyzed using the repeated measures ANOVA and T-test by SPSS software (Chicago, IL, v. 16.0). The significance levels were expressed at a 95% confidence level ( $p < 0.05$ ) throughout.

## Results

Results showed that, LPO activity was slightly higher in buffalo milk than in cow milk, where its activity was  $4.15 \pm 0.13$  U/ml and  $4.02 \pm 0.1$  U/ml in buffalo and cow milk, respectively ( $p > 0.05$ ). Some decreases in LPO activity was observed on the preparation of rennet whey, where, the activity of LPO in rennet whey was 81-84% of that found in milk; on average,  $3.39 \pm 0.1$  U/ml in buffalo whey and  $3.36 \pm 0.08$  U/ml in cow whey.

Heating at  $65^\circ\text{C}$  for up to 30 min did not affect the activities of LPO in cow and buffalo milk ( $p > 0.05$ ), however, during the same period of time, LPO activities in cow and buffalo whey were significantly ( $p < 0.05$ ) decreased from  $3.36 \pm 0.08$  U/ml to  $2.96 \pm 0.07$  U/ml and from  $3.39 \pm 0.1$  U/ml to  $3.05 \pm 0.1$  U/ml, respectively (Fig. 1A).

At  $70^\circ\text{C}$ , the activities of LPO in cow and buffalo milk were not significantly affected for the first 5 min of the heating time, however at the end of the heating time (10 min), significant decreases ( $p < 0.05$ ) were observed in

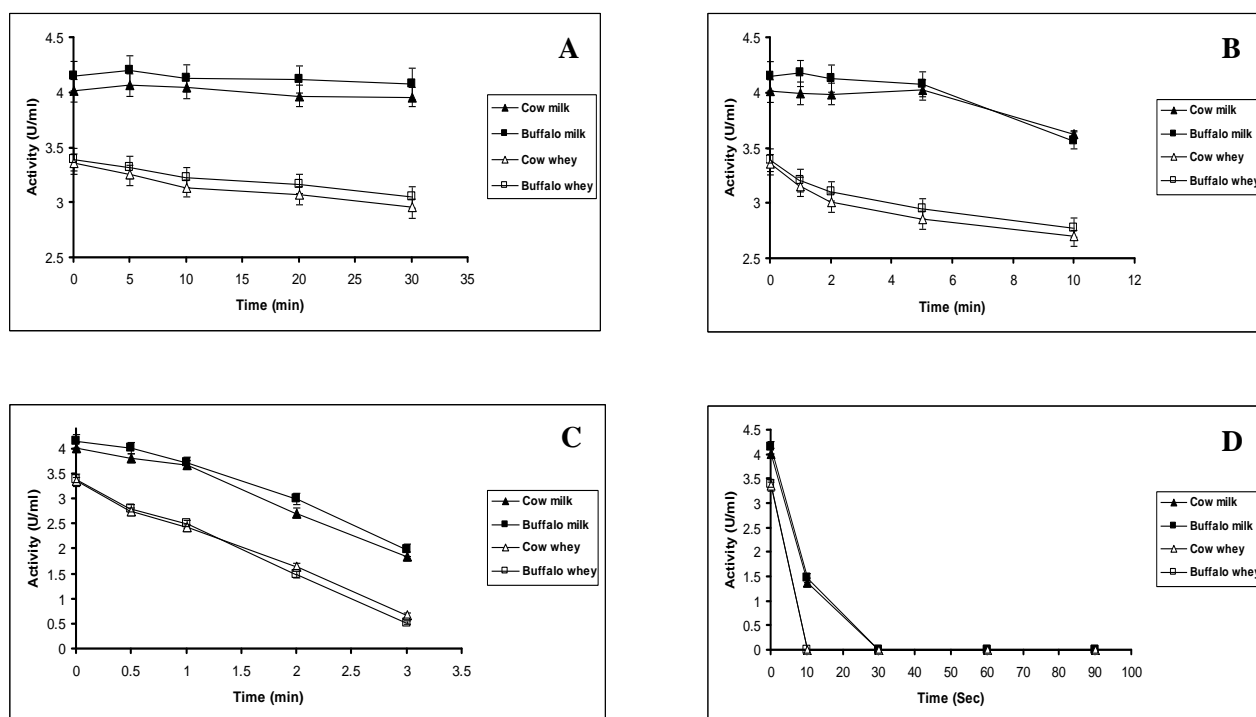
the LPO activity; from  $4.02 \pm 0.1$  U/ml to  $3.63 \pm 0.03$  U/ml in cow milk and from  $4.15 \pm 0.13$  U/ml to  $3.57 \pm 0.08$  U/ml in buffalo milk. In contrast, in cow and buffalo whey, obvious reduction trends ( $p < 0.05$ ) were found from the first min to the end of the heating time (Fig. 1B).

As shown in Fig. 1C, heating at  $75^\circ\text{C}$  resulted in a significant reduction ( $p < 0.05$ ) in the LPO activities of cow and buffalo milk. After 3 min of holding time, the residual LPO activities were 46% ( $1.85 \pm 0.09$  U/ml) and 47.4% ( $1.98 \pm 0.12$  U/ml) in cow and buffalo milk, respectively. In whey, the reduction trends were more severe.

LPO retained part of its activity, in both cow and buffalo milk, for at least 10 s at  $80^\circ\text{C}$  while, after 10 s of heating, the residual LPO activities were 34.5% and 35.6% in cow and buffalo milk, respectively and no activities were detected at the second sampling time (30 s of holding time) in both milk. In contrast, LPO was more ( $p < 0.05$ ) heat labile in whey than milk and became completely inactive after 10 s of heating, in both milk types (Fig. 1D).

**Table 1:** Average composition of the bulk milk samples

	No. of samples	Fat (%)	Protein (%)	Dry solids (%)
Cow milk	30	$3.41 \pm 0.19$	$3.97 \pm 0.28$	$11.09 \pm 0.74$
Buffalo milk	30	$7.06 \pm 0.48$	$4.72 \pm 0.23$	$16.57 \pm 1.46$



**Fig. 1:** Thermal inactivation of LPO in cow and buffalo milk and whey at  $65^\circ\text{C}$  (A),  $70^\circ\text{C}$  (B),  $75^\circ\text{C}$  (C), and  $80^\circ\text{C}$  (D)

## Discussion

In the present study, it was shown that LPO activity was slightly, but not significantly higher in buffalo milk

than in cow milk. According to Kumar and Bhatia (1999), LPO activity was higher in buffalo milk both at pH 6.0 ( $7.3 \pm 0.13$  U/ml in buffalo milk

against  $5.7 \pm 0.27$  U/ml in cow milk) and pH 4.4 ( $1.37 \pm 0.26$  U/ml in buffalo milk against  $1.26 \pm 0.23$  U/ml in cow milk); and also its activity was around five-fold higher at pH 6.0 than at pH 4.4 both in cow and buffalo milk samples. In contrast, Hamulv and Kandasamy (1982), reported higher LPO activity in cow milk (1.2 U/ml) than buffalo milk (0.9 U/ml) at pH 4.4. LPO activity in cow milk has been reported to be in the range of 1.2 to 19.4 U/ml (Seifu et al., 2005). Variations in the enzyme level were reported to depend on the season, breed, feeding regime, and the sexual cycle of the cow (Kussendrager and van Hooijdonk, 2000). In addition, because of the various chromogens used for LPO assay and the variability in the experimental conditions, data for LPO activity vary greatly in the literature. For example, according to Lorenzen et al. (2010), the LPO activity was 2.01, 2.80, and 5.19 U/ml for cow, sheep, and goat's milk, respectively. Mean LPO activity of 0.77 U/ml (Medina et al., 1989) and 3.46 U/ml (Althaus et al., 2001) have been reported for raw ewe milk. The LPO activity of goat milk reported in the literature varies widely. LPO activity in goat milk ranging from 0.05-3.55 U/ml (Zapico et al., 1990) to 0.04-0.16 U/ml (Fonteh et al., 2002) has been reported, previously.

Results of the present study showed that, LPO activities in cow and buffalo whey were 16-19% lower than the respective values in milk. Similar findings have been reported by Kumar and Bhatia (1999) who found a loss of 10-15% of LPO activity on the preparation of rennet whey, both in cow and buffalo whey samples. Furthermore, LPO activity was around five-fold higher at pH 6.0 ( $6.9 \pm 0.18$  U/ml in buffalo whey against  $5.25 \pm 0.19$  U/ml in cow whey) than at pH 4.4 ( $1.25 \pm 0.15$  U/ml in buffalo whey against  $1.11 \pm 0.24$  U/ml in cow whey).

According to Marks et al. (2001), the HTST pasteurization process does not inactivate the LPO in cow milk, while de Wit and van Hooijdonk (1996) reported that complete inactivation of LPO in cow milk needs  $78^\circ\text{C}$  for 15 s. Lorenzen et al. (2010) found that LPO activity of bovine milk was not affected by holder pasteurization ( $62\text{--}65^\circ\text{C}$  for 30-32 min), while the LPO activities in ovine and caprine milk were reduced by about 5%. In addition, they reported that, HTST heat treatment at  $75^\circ\text{C}$  for 28 s made 50 to 60% decrease in LPO activity without considerable differences between species. Furthermore, heating at  $85^\circ\text{C}$  for 90 s resulted in residual LPO activities of 0.03%, 0.65%, and 0.33% in cow, sheep, and goat, respectively. Dumitrascu et al. (2012) revealed that the enzyme was heater labile in goat and sheep milk in the lower temperature range ( $70\text{--}75^\circ\text{C}$ ), whereas at higher temperatures, the enzymes was more stable in goat milk when compared with cow and sheep milk. In another study, Chavarri et al. (1998) reported that pasteurization of sheep's milk reduced

activity by an average of 10–30%. Data regarding the thermal stability of LPO in buffalo milk are very limited. Van Nieuwenhove et al. (2004) found that average LPO activity in buffalo milk was  $2.49 \pm 0.86$  U/ml, and also, they reported 16% of thermal inactivation on the low pasteurization ( $65^\circ\text{C}$  for 30 min) and 80% on the high pasteurization ( $72^\circ\text{C}$  for 15 s). In the current research, the time-temperature combination needed for complete inactivation of the enzyme was higher than those reported in the literature. This could be due to the different heating methods and the variability in the experimental conditions. In addition, at all of the tested temperatures, LPO was more heat labile in whey than milk, in both milk types. This could be attributed to the different composition of the milk and whey (matrix effects).

## Conclusion

There were some variations in LPO behavior against thermal processing in milk and whey from cow and buffalo. So, these findings could be helpful in further studies about monitoring thermal processes in dairy industries.

## Conflicts of Interest

The authors declare that they have no conflict of interest in this research.

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