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Effect of Heat Treatment on Buffalo (*Bubalus bubalis*) Lactoperoxidase Activity in Raw Milk

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Abstract: Inactivation kinetics of buffalo milk lactoperoxidase toward thermal processes was studied at isothermal condition over a range of 67 to 71°C. The analysis of inactivation rate constant data for the process of thermal denaturation of lactoperoxidase showed good agreement with a first-order reaction. Based on the thermal death time model, which describes first-order heat inactivation kinetics in the area of food processing and preservation, D-values and z-value (2.45°C) were obtained. Thermodynamic parameters were also calculated. The high values obtained for activation energy (920.43 kJ mol⁻¹) and change in enthalpy of activation (~917 kJ mol⁻¹) indicate that lactoperoxidase is one of the most heat stable enzymes in buffalo milk which high amount of energy is needed to initiate its denaturation. The mean buffalo lactoperoxidase activity in raw milk also was found 16.84 U mL⁻¹.

Key words: Buffalo milk, lactoperoxidase, thermal denaturation

INTRODUCTION

Lactoperoxidase (EC 1.11.1.7) is an oxidoreductase enzyme secreted into milk, saliva and tears and plays important biological roles such as; airway Defense (Conner *et al.*, 2002), protecting the lactating mammary gland and the intestinal tract of the newborn infants against pathogenic microorganisms through a bacteriostatic or bactericidal effect (Naidu, 2000; Tenovuo, 2002; Touch *et al.*, 2004) and degradation of various carcinogens and protection of animal cells against peroxidative effects (Perraudin and Reiter, 1998). The biocidal activity of lactoperoxidase results from the products of the chemical reaction it catalyzes. For develop its function, lactoperoxidase needs the presence of hydrogen peroxide and thiocyanate, which have been called together lactoperoxidase system. The primary reaction product hypothiocyanite is known to react with the thiol groups of various proteins that are important for the viability of pathogens, thereby, inactivating crucial enzyme and protein systems (Sermon *et al.*, 2005). Lactoperoxidase in milk may be associated with NO metabolism and produced metabolites such as nitric dioxide (Silamkove *et al.*, 2005).

The antimicrobial property of lactoperoxidase gives it a practical application in different fields. The activation of the lactoperoxidase system has been used to preserve milk from microbial proliferation (souring) during its

collection, storage and transportation to processing place (Haddadin *et al.*, 1996). Furthermore, lactoperoxidase has been used for preservation of cosmetics, foodstuffs and protection of growing flowers, fruits, tubers, etc. (Touch *et al.*, 2004; Le Nguyen *et al.*, 2005).

The study of lactoperoxidase resistance to heat is of great interest mainly for two practical reasons. Firstly, lactoperoxidase activity has been proposed for monitoring thermal processes for treatments above 78°C for 15 sec (Fox and Kelly, 2006). Secondly, a lactoperoxidase system can be activated in some cases after treatment, thus contributing to extend the shelf-life of pasteurised milk in locations with inefficient cold storage conditions (Barrett *et al.*, 1999). While this enzyme has already been extensively examined in bovine milk (Martin-Hernandez *et al.*, 1990; Ludikhuyze *et al.*, 2001; Marin *et al.*, 2003), detailed quantitative kinetic thermal inactivation studies in domestic water buffalo (*Bubalus bubalis*) milk are lacking. The aim of this study is to determine the effect of heat treatments, in the temperature range from 67 to 71°C, on lactoperoxidase activity in water buffalo milk.

MATERIALS AND METHODS

This study was performed from June 2008 to October 2008 in Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran.

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Chemicals: 2, 2'-azinobis (3-ethylbenzthiazoline-sulfonic acid) (ABTS) was obtained from Sigma Chemical Co. (Deisenhofen, Germany). Hydrogen peroxide (30% solution) and all the other chemicals used in this research were obtained from Merck (Darmstadt, Germany) and were of reagent grade.

Milk sampling: Fresh raw buffalo (*Bubalus bubalis*) milk was obtained (June-July 2008) from a local dairy farm (Urmia, Iran) and divided into small portions (50 mL) and kept at -20°C until analysis (not later than 2 weeks).

Enzymatic activity assay: Milk lactoperoxidase activity was measured as described by Asadpour *et al.* (2008) where the H₂O₂-dependent oxidation of ABTS at 412 nm is followed using an extinction coefficient of 32.400 mM⁻¹ cm⁻¹ (Kumar and Bhatia, 1999). For the preparation of ABTS solutions (1 mM), 55 mg ABTS was dissolved in phosphate buffer (0.1 M, pH 6.6) and made up the volume too 100 mL. H₂O₂ stock solutions (3.2 mM) were prepared daily by appropriate dilution of 30% H₂O₂ in distilled water. Three milliliter of ABTS solution (1 mM in 0.1 M phosphate buffer, pH 6.6 which corresponds to the pH of raw milk) and milk sample (0.1 mL) were added together in cuvette. The reaction was initiated by the addition of 0.1 mL hydrogen peroxide solution (3.2 mM) and immediately the measurement of absorbance started at 412 nm as a function of time for 2 min at 15 sec intervals using an UNICO UV-2100 PC (USA) spectrophotometer. Measurements were carried out against the reagent blank containing ABTS and enzyme solution only. Reaction velocity was computed from linear slopes of absorbance-time curve. One unit of activity is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of ABTS per min at room temperature (~22-25°C). Results were average of at least three separate experiments.

Heat incubation: Thermal stability of milk lactoperoxidase were studied by incubating aliquots of milk at various temperatures (67, 69 and 71°C) up to 60 min in a thermostatic water bath and measuring their activity at room temperature after brief cooling in ice. As only the isothermal conditions were taken into account for data analysis, the initial phase of heating until reaching the desired temperature was excluded and the initial activity (A₀, t = 0) was defined as the lactoperoxidase activity in sample when temperature remained constant in time. The incubation was carried out in sealed vials to prevent change of volume of the sample and hence, the enzyme concentration due to evaporation. Assays at the different temperatures were done at least in 3 separate experiments and the mean values of data were used to obtain the different kinetic and thermodynamic parameters.

Kinetic data analysis: Inactivation kinetics of milk lactoperoxidase toward thermal processes was subjected to reaction kinetic analysis. This process behaves in an analogous way to a general rate reaction of order n according to this equation:

$$-dA/dt = k.A^n \quad (1)$$

where, -dA/dt represents the loss of lactoperoxidase activity rate, k the inactivation rate constant (min⁻¹), A the lactoperoxidase activity at each time of treatment and n the order of reaction. The experimental points are plotted according to the equation $\ln A/A_0 = k.t$ derived from Eq. 1, where A₀ is the initial response value (e.g., initial enzyme activity at isothermal condition at time t₀), A is the response value after heating treatment and t is the exposure time (min). When the assumption of first-order reaction is correct, straight lines must be obtained by linear regression with high coefficients of correlation (R²) and with a value of the ordinate intercept b (time t = 0) close to zero.

Based on the thermal death time model, which describes first-order heat inactivation kinetics in the area of food processing and preservation, we calculated D-values (decimal reduction time or time for one log reduction of the initial response value at a given temperature) and z-value (temperature necessary to reduce D-value by 1 logarithmic cycle) according to the expressions:

$$\text{Log}(A/A_0) = -1/D \times t \quad (2)$$

The z-value can be estimated by linear regression of log D-values versus corresponding temperatures. Linear regressions were performed using the Sigma Plot for windows version 10.0 (Systat software, Germany). The rate constant in a denaturation process and the temperature of treatment are related according to the Arrhenius equation:

$$\ln k = \ln A - E_a/RT \quad (3)$$

where, k is the rate constant, A is the Arrhenius constant, E_a the apparent activation energy, R the universal gas constant and T the absolute temperature. The slope of the line obtained permits to calculate the activation energy.

The values of the activation energy (E_a) allow the determination of different thermodynamic parameters such as variations in enthalpy (ΔH°), Gibbs free energy (ΔG°) and entropy (ΔS°) according to the following expressions:

$$\Delta H^\circ = E_a - RT \quad (4)$$

$$\Delta G^\circ = - RT \ln (kh/KT) \quad (5)$$

$$\Delta S^\circ = (\Delta H^\circ - \Delta G^\circ)/T \quad (6)$$

where, h and K are the Planck's and the Boltzmann's constants, respectively.

RESULTS AND DISCUSSION

The mean lactoperoxidase activity of buffalo milk used in the experiments was 16.84 U mL⁻¹. There are some significant differences between present results and with those given by Kumar and Bhatia (1999) and Ozdemir *et al.* (2002). They reported lower values of lactoperoxidase activity, in some cases, amounting 70% lower than ours. These discrepancies arise from the different method or various chromogens used for their assay and the variability in the assay conditions such as pH and buffer. Variations in enzyme level were also depend on the sexual cycle, season, feeding regime and breed (Kussendrager and van Hooijdonk, 2000).

The discrepancy for lactoperoxidase activity of cow milk also reported in the literature. Lactoperoxidase activity in cow milk ranging from 1.2 to 19.4 U mL⁻¹ (Gothefors and Marklund, 1975), 1.5 to 2.7 U mL⁻¹ (Fonteh *et al.*, 2002) and 4.63 to 6.49 U mL⁻¹ (Asadpour *et al.*, 2008) have been reported. The highest level of lactoperoxidase activity (22 U mL⁻¹) has been reported for guinea pig milk (Stephens *et al.*, 1979). Mean Lactoperoxidase activity of 0.77 U mL⁻¹ (Medina *et al.*, 1989) and 3.46 U mL⁻¹ (Althaus *et al.*, 2001) have been reported for ewe milk.

Effect of heat treatment on the enzymatic activity of lactoperoxidase on ABTS substrate at different temperatures is shown in Fig. 1. As shown in the Fig. 1, the degree of lactoperoxidase denaturation increases with temperature and time of treatment and linear relationship with high coefficients of correlation (R²) was observed between residual lactoperoxidase activity and time for each temperature (Table 1). This result indicates that thermal inactivation of buffalo milk lactoperoxidase follows a first-order kinetic model.

Lactoperoxidase is one of the most heat stable enzymes in milk (Shakeel-ur-Rehman and Farkye, 2002). Its destruction has been used as an index of pasteurization efficiency of milk. Lactoperoxidase is only partially inactivated by short time pasteurisation at 72°C for 15 sec, or long time heating during normal pasteurisation of cow milk (63°C for 30 min) but is destroyed at 80°C in 2.5 sec. On the other hand, de Wit and van Hooydonk (1996) reported that complete inactivation of Lactoperoxidase in cow milk requires 15 sec at 78°C. A recent report by Marks *et al.* (2001)

Table 1: Inactivation kinetic parameters of buffalo milk lactoperoxidase toward thermal processes, assuming a 1st-order reaction

Temperature (°C)	D-values (min)	R ²	k- values (min ⁻¹)
67	370.37	0.950	0.0061
69	57.80	0.968	0.0398
71	8.56	0.972	0.2689
z-value (°C)	2.45	0.999	

R²: Coefficients of correlation, D: Decimal reduction time, k: Rate constant, z: Temperature necessary to reduce D-value by 1 logarithmic cycle

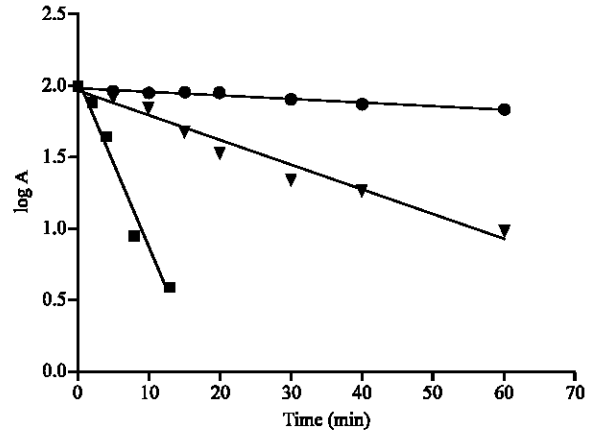


Fig. 1: Effect of heat treatment on buffalo milk lactoperoxidase activity as a function of treatment time at different temperatures: 67°C (●), 69°C (▼), 71°C (■). The activity expressed as the percentage of initial activity. Results were average of at least three separate experiments

confirms the fact that normal pasteurisation of milk does not inactivate lactoperoxidase in milk. Present research show that buffalo milk lactoperoxidase lost its enzymatic activity very slowly below 69°C and at 71°C there was a remarkable increase in the rate of deactivation. Thus, we found that at 67°C, 106 min were necessary to reduce lactoperoxidase activity to 50%, while at 71°C only 4 min of treatment caused the same reduction. Therefore, buffalo milk lactoperoxidase is more sensitive to increases of temperature above 71°C than to variations in the duration of treatment at lower temperatures. These results agree in general with those obtained by Martin-Hernandez *et al.* (1990) and Marin *et al.* (2003) for bovine milk lactoperoxidase.

Semi-logarithmic Plot of lactoperoxidase activity against time allows us to calculate decimal reduction time (D-values). As shown in Table 1, D-values decreased by increasing at temperature. D-values that obtained at 71 and 69°C were about 43 and 6.5 times lower than in comparison to D-value at 67°C, respectively. This remarkable decrease at D-value between 69 and 71°C indicate a potential thermal denaturation in buffalo milk lactoperoxidase.

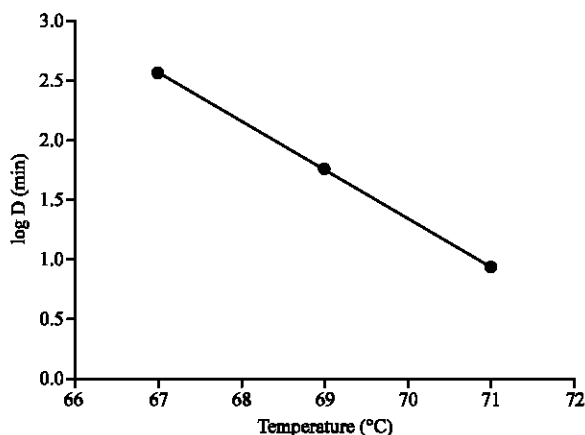


Fig. 2: Summary of the temperature sensitivity of inactivation rate constants for thermal inactivation of buffalo milk lactoperoxidase activity

The effect of temperature on D-values for inactivation of lactoperoxidase activity in buffalo milk is shown in Fig. 2. For the range of temperatures studied (67-71°C) z-value (2.45°C) was calculated from slope of the line. This value is very close to that reported for bovine milk (3.7°C) by Olszewski and Reuter (1992) and Ludikhuyze *et al.* (2001) and (3.1°C) by Marin *et al.* (2003). In general, high z-values mean more sensitivity to the duration of heat treatment and lower z-values mean more sensitivity to increases in temperature (Barrett *et al.*, 1999). Therefore, the z-value of 2.45°C for buffalo milk lactoperoxidase indicates that this enzyme is more sensitive to increases of temperature than to the extension of treatment time.

For determination of inactivation rate constants of buffalo milk lactoperoxidase toward thermal process, semi-logarithmic plot of the activity retention (A/A₀) as a function of treatment time were plotted (Fig. 3, Table 1). As shown in Fig. 3, the straight lines obtained have coefficients of correlation between 0.95-0.972 and intersects the ordinate close to the origin. These results indicate that the value of n = 1 (1st-order reaction) is the most appropriate to describe the process at the temperatures used, while other orders of reaction were also tried and did not give better fits.

Inactivation rate constants were used to draw the Arrhenius plot, from which slope activation energy was calculated and found to be 920.43 kJ mol⁻¹ (Fig. 4, Table 2). This value is higher than the values obtained for bovine milk lactoperoxidase by Martin-Hernandez *et al.* (1990) and Marin *et al.* (2003) which was 737.69 and 800 kJ mol⁻¹, respectively. The higher value found for the activation energy means that a higher amount of energy is needed to initiate denaturation (Bjorck, 1992), may be

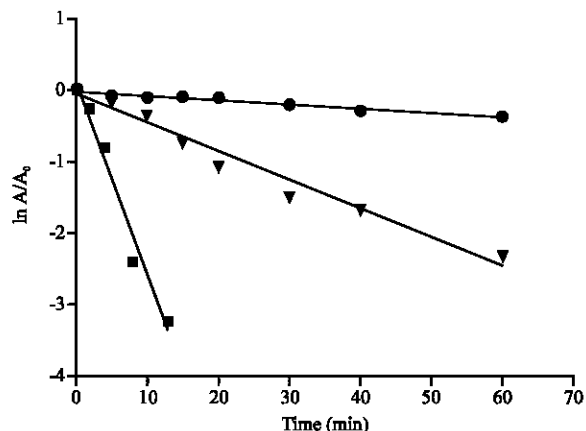


Fig. 3: Isothermal inactivation of buffalo milk lactoperoxidase activity as a function of treatment time, assuming a 1st-order reaction at 67°C (●), 69°C (▼) and 71°C (■)

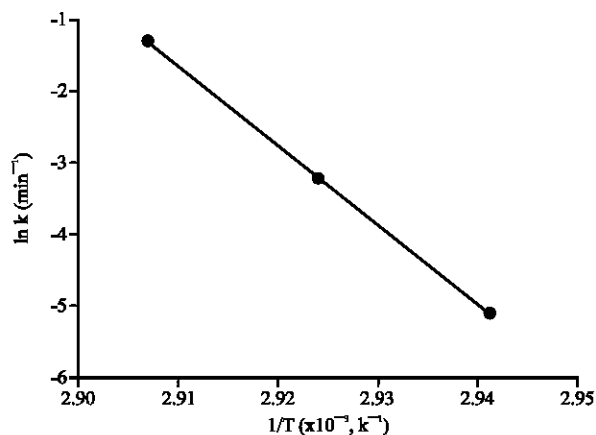


Fig. 4: Arrhenius plots for buffalo milk lactoperoxidase activity

Table 2: Thermodynamic parameters for inactivation of buffalo milk lactoperoxidase toward thermal processes, assuming a 1st-order reaction

Temperature (°C)	E _a = 920.43 kJ mol ⁻¹		R ² = 0.999
	ΔH° (kJ mol ⁻¹)	ΔS° (kJ mol ⁻¹)	
67	917.61	2.376	109.63
69	917.59	2.376	104.96
71	917.57	2.376	100.13

R²: Coefficients of correlation, E_a: Activation energy, ΔH°: Variations in enthalpy, ΔG°: Gibbs free energy, ΔS°: Entropy

due to the more compactness of buffalo milk lactoperoxidase in comparison to bovine milk lactoperoxidase.

Table 2 shows the thermodynamic values of variation in activation enthalpy (ΔH°), variation in activation entropy (ΔS°) and variation in Gibbs free energy (ΔG°) calculated for the different temperatures.

The values of the change in enthalpy of activation obtained at this study for buffalo milk lactoperoxidase ($\sim 917 \text{ kJ mol}^{-1}$) are higher than the values obtained for bovine milk lactoperoxidase ($\sim 734 \text{ kJ mol}^{-1}$) which reported by Marin *et al.* (2003). These results confirming the buffalo milk lactoperoxidase probably is more stable than bovine milk lactoperoxidase toward thermal processes, as suggested by higher value of activation energy. The positive values found for the variation in entropy of activation indicates that there are no significant processes of aggregation, since, if this would happen, the values of entropy would be negative (Anema and McKenna, 1996).

CONCLUSION

The investigation of thermal inactivation of buffalo milk lactoperoxidase indicated a monophasic inactivation pattern or first-order kinetic model. The high values of activation energy ($920.43 \text{ kJ mol}^{-1}$) and enthalpy ($\sim 917 \text{ kJ mol}^{-1}$) and low z-value (2.45°C) for buffalo milk lactoperoxidase suggest that lactoperoxidase is a thermostable enzyme in buffalo milk which has more sensitivity to increases of temperature than to the extension of treatment time.

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