journal homepage: www.fia.usv.ro/fiajournal
Journal of Faculty of Food Engineering,
Ştefan cel Mare University of Suceava, Romania
Volume XII, Issue 1 – 2013, pag. 53 - 58

FOOD AND ENVIRONMENT SAFETY

THERMAL INACTIVATION KINETICS OF LACTOPEROXIDASE IN MODEL SYSTEM, MILK AND WHEY

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Received 6 January 2013, accepted 10 February 2013

Abstract: *Inactivation of lactoperoxidase (LPO) in model system, milk and whey at atmospheric pressure was studied in a temperature range of 60-70 °C. The first order kinetics model allowed the estimation of the inactivation rate constants (k) and the thermal death times (D). D- and k-values decreased and increased, respectively with increasing temperature, indicating a more rapid LPO inactivation at higher temperatures. At 70°C the inactivation of LPO was achieved in milk after 6 minutes and in whey after 4 minutes of thermal treatment. At 67.5°C lactoperoxidase was completely inactivated after 14 minutes in phosphate buffer. In all systems studied the temperature dependence of lactoperoxidase inactivation in milk, whey and model system versus the reaction rates could be accurately described by the Arrhenius equation. The estimated activation energies were of 155.67 kJ/mol for phosphate buffer, 217.79 kJ/mol for milk and 235.57 kJ/mol for whey. The correspondent z_T values were estimated with the thermal death model and the values obtained were very close for all the three systems studied. For all the loglinear regression equations calculated SAS System for Windows 9 software was used.*
Lactoperoxidase is an important antimicrobial system and knowing its thermostability in milk, byproducts and model systems allows a better control of the enzyme activity.

Keywords: *lactoperoxidase, inactivation, enzymatic activity, kinetics*

1. Introduction

Lactoperoxidase (EC 1.11.1.7) is a member of the peroxidase family that has antimicrobial properties. The enzyme, naturally occurring in milk is part of the lactoperoxidase (LPO) system made of lactoperoxidase–thiocyanate–hydrogen peroxide. When activated, LPO catalyses, in the presence of H_2O_2, the oxidation of thiocyanate to compounds, such as hypothiocynate ($OSCN^-$) or higher oxyacids [1]. Not only the reaction products but also the intermediate ones are known to have antimicrobial effects against bacteria, fungi and viruses [2]. LPO activity in milk is variable due to the difference in thiocyanate concentration directly related with cyanglucozide presence. A high cyanglucozide concentration is associated mostly with feeding from green pastures. LPO activity is enhanced by the presence of xanthine-oxidase and sulhydryl oxidase.
The activation of the LPO has a bacteriostatic effect on the raw milk and

effectively extends the shelf life of raw milk for 7–8 hours under ambient temperatures of around 30°C. This ensures adequate time for the milk to be transported from the collection point to a processing centre without refrigeration. In developing countries LPO system is still used for milk preservation. LPO is effective in refrigerated raw milk. Researches have demonstrated that the activated LPO is successful also in prolonging the quality of raw milk for up to 5–6 days in refrigerated (+4°C) conditions [3].

In various experimental studies, the bacteriostatic or bactericidal effect of the LPO has been demonstrated against several human pathogenic microorganisms, such as *Streptococcus mutans, Aeromonas hydrophila, Candida albicans* and *Helicobacter pillory* [3].

Recently new products as dental creams and chewing gums were launched on the market, intended to improve the dental hygiene by increasing the enzyme activity in saliva [4]

Lactoperoxidase is considered one of the enzymes that could be used and an indicator for the high pasteurization treatment and it is inactivated at 80°C in 2.5 s.

The aim of this research study was to quantify the thermal inactivation kinetics of the LPO in milk, whey and buffer, in terms of rate constants and their associated temperature sensitivity.

2. Experimental

Assay of enzyme activity

The LPO activity was measured spectrophotometrically (UV-VIS Cintra) at 412 nm, using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid or (ABTS) as a substrate. A volume of (2,5 ml) ABTS 0,001 mol/L, pH=6, was mixed with 100 µL of sample (enzyme, milk or whey) and the reaction was initiated by the addition 100 µL of freshly prepared hydrogen peroxide (0.3%). The enzymatic activity was determined for 10 minutes as the slope of the absorbance versus reaction time. One unit of activity (U) is defined as the amount of enzyme that catalyses the oxidation of 1 mol of ABTS per min at 20°C. All tests were performed in duplicate.

Thermal treatment

The first experiment considered a thermostability screening study, whereby the samples are treated at different temperatures (25-70°C) for a fixed time interval (5min).

In order to determine the thermostability of LPO, glass Blaubrand capillary tubes were filled with 200 µL, to obtain "quasi" iso-thermal conditions. The tubes without air were sealed at both ends and incubated in a thermostated water bath at temperatures from 60°C to 70°C for fixed time intervals. The maximum period for the treatment was 15 minutes. Immediately after the treatment samples were could in an ice-bath to prevent the inactivation produced by the remanent thermal effect.

Kinetic analysis

Primary models

Thermal and pressure inactivation of enzymes frequently follows first-order kinetics. In this case, the decrease of enzyme activity as a function of the time, at constant processing conditions, can be described by Eq.(1):

$$A = A_0 \exp(-kt) \quad (1)$$

which can be linearized by a logarithmic transformation in Eq. (2)

$$\ln\left(\frac{A}{A_0}\right) = -kt \qquad (2)$$

where A is the enzymatic activity at time t; A_0 is the initial activity; t is the treatment time and k is the first-order inactivation rate constant. Next to the inactivation rate constant, the decimal reduction time (D) can be used to characterize the inactivation process. The relation between the decimal reduction time and the inactivation rate constant is given by Eq. (3):

$$D = 2.303/k \qquad (3)$$

Secondary models

The temperature dependency (at constant pressure) of the rate constant and the decimal reduction time can respectively be characterized by the activation energy (based on the Arrhenius equation, (4)) and the z_T-value (thermal death time equation, (5)).

$$\ln k_{obs} = \ln k_{ref} - \left[\frac{E_a}{R}\left(\frac{1}{T} - \frac{1}{T_{ref}}\right)\right] \qquad (4)$$

$$\log_{10}(D) = \log_{10}(D_{ref}) - ((T - T_{ref})/z_T) \qquad (5)$$

where k_{ref} is the rate constant at reference temperature T_{ref}, E_a is the activation energy, R is the universal gas constant (R= 8.314 J/mol K), D_{ref} is the decimal reduction time at reference temperature and z_T is the z-value. The activation energy and z-value were estimated using a linear regression analysis.

The thermal inactivation data were analyzed both according to the Arrhenius equation and the thermal death time model (in order to compare our data with existing literature data), while the high pressure-thermal inactivation data were analyzed according to the Arrhenius approach.

To model the results linear regression equations were applied for the data using SAS System for Windows 9 software.

3. Results and Discussion

The thermostablity screening studies indicated that LPO in phosphate buffer started to be inactivated at 50°C after 10 min of treatment; at 60°C a half of the initial activity was registered, while at 65°C the inactivation was almost ended after 10 min (Fig 1, Fig. 2).

A similar behavior could be noticed for the milk samples. However in this case the inactivation is very close to the one in phosphate buffer. At temperatures lower than 62.5°C LPO displays in milk a resistance to the inactivation similar to the one of the enzyme in buffer system. This behavior could be explained by the presence in milk of protective enzymes such as xanthine-oxidase and sulhydryl oxidase, up to the temperatures at which these enzymes starts to be inactivated.

Following the screening experiment, a detailed kinetic study (inactivation as function of temperature and time) was carried out on the LPO system in phospahate buffer, in milk, and in whey to obtain the inactivation rate constants (Figs. 3, 4 and 5).

Detailed thermal inactivation kinetics of the LPO system was studied at temperatures between 60°C and 70°C.

The applicability of the first-order kinetic model, which is frequently reported in the literature for enzyme inactivation [1] was confirmed for the LPO system under study in all media. Inactivation rate constants and decimal reduction time values were estimated by linear regression analysis and are presented in Table 1.

As expected, decimal reduction time decreases with temperature increase for each of the systems studied.

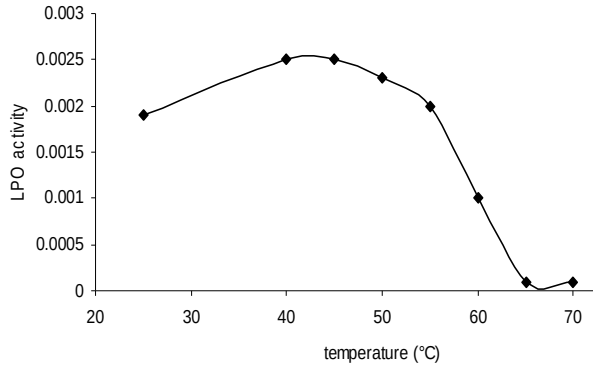

Figure 1. LPO activity at different temperatures after five minutes of isothermal treatment in phosphate buffer pH 6.

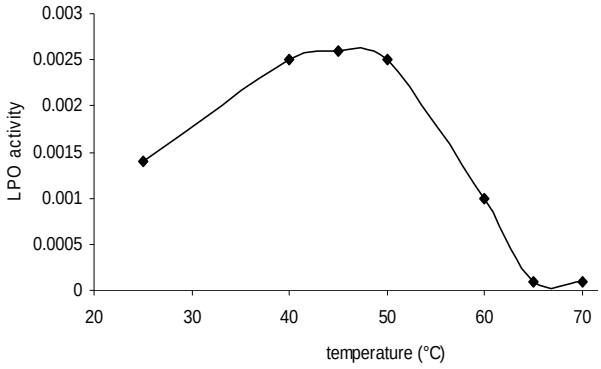

Figure 2. LPO activity at different temperatures after five minutes of isothermal treatment in milk

The inactivation of LPO goes very quickly in whey at 70°C, in 4 min., while it might take more than 6 min for the same enzyme to be inactivated in milk. The difference could be explained by the protective effect of milk fat on the LPO system in milk.

The enzyme activity was experimentally measured up to 67.5°C in phosphate buffered and up to 70°C in milk and whey.

At 62.5°C the inactivation rate constant is 0.0579 min^{-1} and is 1.03 fold higher in milk at the same temperature and 1.38 fold higher in whey.

The inactivation rate constant at 67.5°C is 0.1599 min^{-1} in phosphate buffer, 1.06 fold higher in milk and 1.76 fold higher in whey.

Tabel 1.
D-values for LPO inactivation in phosphate buffer, milk and whey

Tempe-rature (°C)	D (min)		
	Phosphate buffer	Milk	Whey
60.0	74.05±2.8[a]		
62.5	39.78±1.06	38.43±1.8	28.72±0.01
65.0	18.06±1.6	15.75±0.5	10.92±0.009
67.5	14.40±0.14	13.56±0.4	8.15±0.007
70.0		6.06±0.05	4.07±0.001

a) standard deviation

In general the purified enzyme in phosphate buffer displays a higher thermostablity in comparison with milk and whey.

The decimal reduction time is decreasing with the temperature increase. In phosphate buffer the deacrese from 60°C to 70°C was of 5.14, while in milk for the temperature range 62.5-70°C the deacrese in D-value was 6.38 fold. In whey the inactivation at 70°C goes 7.05 fold faster than at 62.5°C.

To express the temperature dependence of the inactivation rate constant, the Arrhenius equation (Eq. 4) was applied and the activation energies were estimated. Good correlation coefficients were obtained for the regression equations in all three systems studied (R^2 was 0.96 for phosphate buffer, 0.94 for milk 0.96 for whey). The highest activation energy estimated was registered for LPO in whey (235.5 kJ/mol) and the lowest for LPO in phosphate buffer (156.67 kJ/mol).

Tabel 2.
The activation energies and zT values for LPO inactivation

Variable	Phosphate buffer	Milk	Whey
Ea (kJ/mol)	155.67±10.3[a]	217.79±12.5	235.57±19.5
Z_T	10.10±0.5	10.12±0.7	9.354±0.6

a) standard deviation

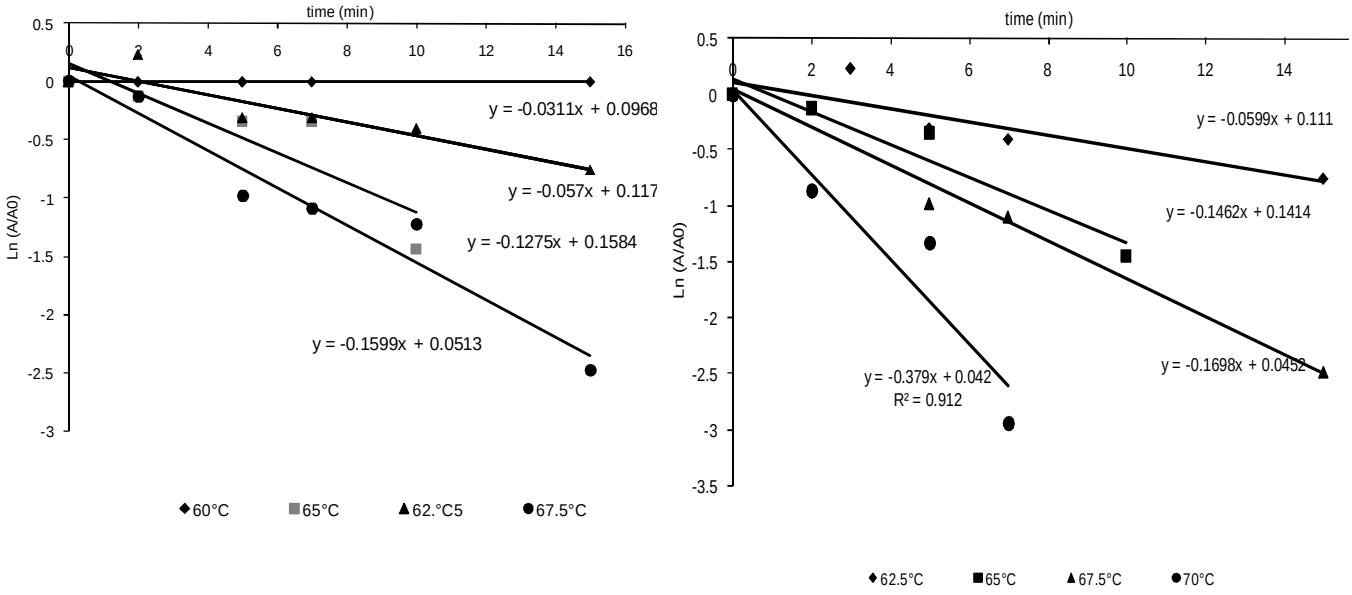

Figure 3. Thermal inactivation kinetics of the LPO in phosphate

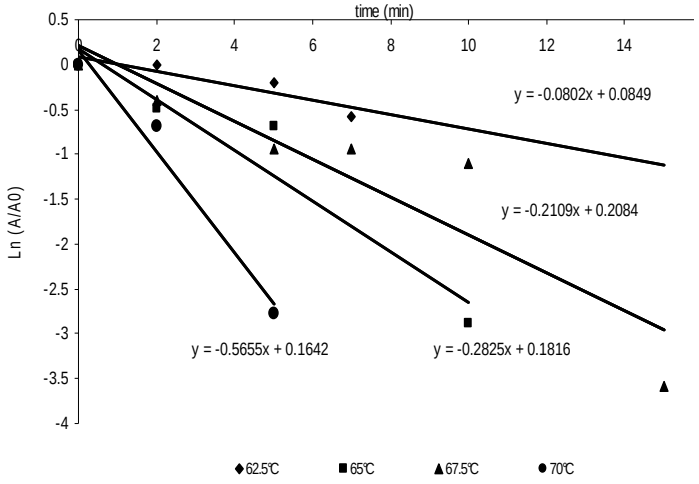

Figure 5. Thermal inactivation kinetics of the LPO in whey

The z_T values expressing the increase in temperature necessary for a 10 fold reduction in D-values obtained with Eq. 5 were very close for all three systems studied (Table 2).

The z_T values for LPO in milk and phosphate buffer are similar. For whey there is less than 1 unit difference between the z_T value in whey and the other two estimated values.

The standard deviation values for each of the estimated variables shows good estimation as indicate Table 1 and Table 2.

4. Conclusion

The present paper studied the thermal inactivation of LPO in model system, in milk and in whey. The kinetic study was done in the temperature range of 69°C to 70 °C. Based on primary kinetic equations the inactivation rate constant and the decimal time were estimated with linear regression. The fastest LPO inactivation was obtained for the whey system at 70°C of 4 minutes. The lowest inactivation time registered was for LPO in phosphate buffer at 60°C (74 minutes). Thermostability studies showed that the enzyme was more stabile in phosphate buffer, followed by milk and whey.

Secondary kinetic equations allowed to accurately estimate the activation energies and the D-values using Arrhenius equation and thermal death model.

The lowest activation energy was of 155.67 kJ/mol for the enzyme in phosphate buffer and the highest was in whey 235.57 kJ/mol.

The z_T values were very similar for all of three systems studies: milk-10.13, whey-9.35 and buffer10.10.

The results obtained are in line with other studies of LPO inactivation kinetics in goat milk [1].

5. Acknowledgments

The authors wish to thank Re-Spia project, SMIS code 11377, for the infrastructure provided in this study.

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