



Kinetic and thermodynamic parameters of alkaline phosphatase and γ -glutamyl transferase inactivation in bovine milk

Stănciuc, Ardelean, Diaconu, Râpeanu, Stanciu, Nicolau

► To cite this version:

Stănciuc, Ardelean, Diaconu, Râpeanu, Stanciu, et al.. Kinetic and thermodynamic parameters of alkaline phosphatase and γ -glutamyl transferase inactivation in bovine milk. Dairy Science & Technology, 2011, 91 (6), pp.701-717. 10.1007/s13594-011-0028-3 . hal-00930587

HAL Id: hal-00930587

<https://hal.science/hal-00930587>

Submitted on 11 May 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Kinetic and thermodynamic parameters of alkaline phosphatase and γ —glutamyl transferase inactivation in bovine milk

Nicoleta Stănciuc · Alina Ardelean ·
Veronica Diaconu · Gabriela Râpeanu ·
Silviu Stanciu · Anca Nicolau

Received: 9 January 2011 / Revised: 16 April 2011 / Accepted: 19 April 2011 /
Published online: 16 June 2011
© INRA and Springer Science+Business Media B.V. 2011

Abstract Kinetic and thermodynamic studies were carried out at different time–temperature combinations in order to evaluate the suitability of alkaline phosphatase (ALP) and γ -glutamyl transferase (GGT) as markers for the heat treatment of milk. The average activities of the enzymes in bovine milk were $0.46 \pm 0.02 \text{ U.mL}^{-1}$ for ALP and 4.05 ± 0.26 and $4.55 \pm 0.24 \text{ U.mL}^{-1}$ for GGT in milk with different fat levels. A detailed comparative kinetic study of ALP inactivation was performed in the temperature range of 50 to 75 °C in raw whole milk and buffer in order to assess the stabilizing effect of milk compounds on ALP. The degree and rate of inactivation of GGT in raw cow's milk with 1.5% and 2.5% fat were measured in the temperature range of 60 to 85 °C. Kinetic studies showed that the thermal inactivation of ALP and GGT followed first-order kinetics. The influence of temperature on the inactivation rate constant was quantified using the Arrhenius equation and was characterized by activation energy (E_a) values of 214.09 ± 12.9 and $222.02 \pm 5.6 \text{ kJ.mol}^{-1}$ for inactivation of ALP in buffer and raw milk. For GGT, E_a values ranged from 190.8 ± 6.06 to $183.7 \pm 8.4 \text{ kJ.mol}^{-1}$ for enzyme inactivation in low-fat milk and in milk containing 2.5% fat, respectively.

牛乳中碱性磷酸酶和 γ —谷氨酰转移酶失活的动力和热力学参数

摘要 通过研究不同温度和不同时间相结合条件下牛乳中碱性磷酸酶(ALP)和 γ —谷氨酰转移酶(GGT)的动力学和热力学参数来评价热处理乳的适宜性,并以此作为热处理乳的标志。牛乳

N. Stănciuc · A. Ardelean · V. Diaconu · G. Râpeanu · S. Stanciu (✉) · A. Nicolau
Faculty of Food Science and Engineering, “Dunărea de Jos” University of Galați, 111 Domneasca
Street, 800201 Galați, Romania
e-mail: Silviu.Stanciu@ugal.ro
URL: www.sia.ugal.ro
URL: www.trasilact.ugal.ro
URL: www.bioaliment.ugal.ro

中碱性磷酸酶的平均酶活是 $0.46 \pm 0.02 \text{ U} \cdot \text{mL}^{-1}$, 不同脂肪含量的牛乳中 γ -谷氨酰转移酶的平均酶活是 $4.55 \pm 0.24 \text{ U} \cdot \text{mL}^{-1}$ 。碱性磷酸酶失活的具体动力学研究在 $50\text{--}75^\circ\text{C}$ 温度范围内进行, 以全脂乳和缓冲剂进行对照, 缓冲剂用来评价乳中其他化合物对碱性磷酸酶稳定性的影响。在脂肪含量分别是 1.5% 和 2.5% 牛乳中, 分别检测了温度范围为 $60\text{--}85^\circ\text{C}$ 时 γ -谷氨酰转移酶 (GGT) 失活程度和失活速率。动力学研究结果显示碱性磷酸酶 (ALP) 和 γ -谷氨酰转移酶 (GGT) 失活遵循一级动力学方程。温度对失活率常数的影响用阿雷尼厄斯方程式定量, 碱性磷酸酶 (ALP) 的失活用活化能值来表示, 缓冲剂和原料乳中的活化能值分别是 214.09 ± 12.9 和 $222.02 \pm 5.6 \text{ kJ} \cdot \text{mol}^{-1}$ 。对于 γ -谷氨酰转移酶 (GGT), 在低脂肪奶和脂肪含量为 2.5% 的奶中, γ -谷氨酰转移酶失活的活化能值范围为 190.8 ± 6.06 到 $183.7 \pm 8.4 \text{ kJ} \cdot \text{mol}^{-1}$ 。

Keywords Milk enzymes · Heat treatment · Kinetic · Safety

关键词 乳中酶 · 热处理 · 动力学 · 安全

1 Introduction

The heat treatment of milk should involve the minimum required time/temperature combinations to obtain hygienic quality and extend the shelf life without causing significant heat-induced damage (Blé et al. 2002). An effective heat treatment of milk is essential to ensure the absence of pathogenic microorganisms and hence improve product safety for the shelf life of pasteurized milk. Several heat treatments induce chemical and physical changes in milk which could be used as indices of the severity of the heat treatment used. Furthermore, heat-induced changes of milk constituents are in general limited in the temperature range used for the thermization ($56\text{--}68^\circ\text{C}$) or pasteurization ($72\text{--}90^\circ\text{C}$). Since the activities of enzymes are (relatively) easily assayed in a large number of samples, their inactivation is easier to monitor than other heat-induced changes.

The sensitivity to heat of some indigenous enzymes makes them good indicators for the severity or effectiveness of the heat treatments used for milk and milk products, as they possess higher thermal resistance in comparison to most of the heat-resistant, nonspore-forming pathogens found in milk (Wilińska et al. 2007).

Alkaline phosphatase (ALP; EC 3.1.3.1) activity has been conventionally used as an index of adequate pasteurization, and the detection of ALP activity of thermally treated liquid milk products has become a common procedure for milk quality control (Murthy et al. 1992). The sensitivity of this test hinges on the initial concentration of ALP in raw milk. Hence, the higher the initial activity detected the more sensitive the test becomes. In addition, the sensitivity depends on the method of analysis of residual enzyme activity. Therefore, ALP is considered to be the most important enzymatic indicator both for legal reasons and ensuring the adequate pasteurization of milk resulting in the hygienic safety of pasteurized milk (Shakeel-Ur-Rehman et al. 2003).

Other indigenous milk enzymes are useful as time–temperature treatment indicators e.g. lactoperoxidase, which is used for distinguishing between high-temperature short-time (HTST) and high pasteurization ($>20 \text{ s}$ at 85°C) (Walstra et al. 1999). The xanthine oxidase (XO) and γ -glutamyl transpeptidase are other heat

labile enzymes (Zehetner et al. 1995) that may be valuable indicators of the thermal impact on milk. Andrews et al. (1987) considered XO as a suitable indicator for the thermal treatment of milk in the temperature range of 80 to 90 °C. On the other hand, Griffiths (1986) mentioned that the natural variability in the activity of XO in milk is too large to be used as a reliable index of heat treatment. XO is the most heat stable enzyme in cream but the least stable in skim milk. The enzyme was not completely inactivated after 120 s at 80 °C, and a *z* value of 6.8 was calculated (Griffiths 1986).

γ -Glutamyl transferase (GGT; EC 2.3.2.2) is a membrane-bound enzyme that has been shown to be distributed between skim milk (74%) and cream (23%) (Baumrucker 1979). Natural variations in the concentration of GGT in milk is low and heat inactivation follows first-order kinetics (Zehetner et al. 1995). The enzyme is relatively heat stable and has been proposed as a marker enzyme for milk pasteurized in the range of 72–80 °C for 15 s (Fox and McSweeney 1998).

Although the detection of ALP activity in milk is significant, due mainly to its accepted use as an index of HTST pasteurization, this enzyme may not be the most appropriate for this purpose because (a) reactivation of ALP under certain conditions causes difficulties in the interpretation of the test results; (b) the enzyme appears to be fully inactivated by subpasteurization conditions (70 °C for 16 s); and (c) the relationship between $\log_{10}\%$ initial activity and pasteurization equivalent is less linear than the relationship of lactoperoxidase or GGT (Fox 2003). Even though both enzymes are concentrated in the fat globule membrane and hence in cream (Fox 2003), Claeys et al. (2004) suggested that variations in the level of fat do not substantially affect the applicability of ALP as an intrinsic indicator for thermal treatment. On the other hand, McKellar (McKellar 1996) suggested that GGT was about nine times more stable in ice cream mix than in whole milk.

Therefore, the aim of this work was to investigate the potential roles of ALP and GGT activities as indicators of adequate pasteurization of milk. The inactivation kinetics of ALP was investigated in raw milk and in buffer with the pH value of milk to assess the stabilizing effect of milk components. Heat stability profiles of bovine milk GGT were examined to establish the suitability of this enzyme to be used for monitoring less stringent or even more severe heat treatments than typical thermization and pasteurization conditions. The inactivation kinetics of GGT was assessed in raw milk with different levels of fat (1.5% and 2.5%). In addition, the aim of the kinetic studies was to quantitatively describe the time–temperature inactivation of enzymes activities. Moreover, the reactivation of the ALP in milk was studied after heat treatment at different temperatures for 30 min and storage at room temperature for 48 h and 3 weeks.

2 Materials and methods

Bulk milk samples of an indigenous breed of cow (20 individuals, Romanian Simmental cows) were obtained from a local farm (Galati, Romania). The

samples were collected between May and July. Milk composition was determined using a Portable Milk Analyzer (Milk-Lab Ltd, Odham, Lancashire, UK), and pH measurements were performed on an InoLab pH meter 730 (WTW, Weilheim, Germany).

Alkaline phosphatase (EC 3.1.3.1) from calf intestine, grade I (120 U.mg⁻¹) was purchased from AppliChem GmbH (Darmstadt, Germany). *p*-Nitrophenylphosphate, L- γ -glutamyl *p*-nitroanilide (γ -GPNA) and glycylglycine were purchased from Alfa Aesar GmbH & Co. KG (Karlsruhe, Germany). All solvents and chemical reagents were of analytical grade.

The raw milk was stored at 4 °C for 1 day before being skimmed. Before centrifugation, the milk was heated to 35 °C. A portion of milk was skimmed by centrifugation (6,000 rpm/60 min) using an FT 15 Disc Bowl Centrifuge (Armfield Inc, Ringwood, England). Milk samples were standardized to a fat content of 1.5% and 2.5% fat content from the initial raw milk which contained 3.5% fat.

2.1 Isothermal inactivation of enzyme

Thermal inactivation kinetics of ALP and GGT in raw milk were conducted using the capillary tube method as an efficient way to allow fast heat transfer and accurate holding times. Glass capillaries (length, 100 mm; inner diameter, 1 mm; and wall thickness, 0.15 mm) were filled with 100 μ L of samples, sealed, and immersed in a water bath (Digibath-2 BAD 4, Raypa Trade, Spain) at the adjusted temperature for the different holding times (50–75 °C for ALP and 60–85 °C for GGT at 0–30 min). After thermal treatment, the capillaries were immediately immersed in ice water to allow rapid cooling.

2.2 Reactivation studies

A reactivation step was performed in order to check the reversibility of the reactions. For GGT activities, both the treated and untreated samples were stored at maximum temperature of 6 °C and to ensure that no reactivation had occurred after 1 week of storage.

The reactivation of ALP was measured for the heat-treated samples at different temperatures for 30 min and stored at room temperature for 48 h and 3 weeks.

2.3 Enzyme activity assays

ALP catalyzes the transformation of *p*-nitrophenylphosphate to phosphate and *p*-nitrophenol. The activity of ALP was determined spectrophotometrically at 405 nm and at 37 °C (Dinnella et al. 2004; Fadiloğlu et al. 2006). A volume of 2.9 mL of substrate solution (4 mmol.L⁻¹ *p*-nitrophenyl phosphate in sodium carbonate–bicarbonate buffer, pH 9.6) was introduced into a spectrophotometer cuvette containing 50 μ L of enzyme solution. The blank solution used in the measurements was prepared by heating 1 mL of raw milk for 1 min at 95 °C.

GGT activity was analyzed spectrophotometrically by quantifying *p*-nitroaniline according to a modification of the method described by Zehetner et al. (1995).

One mL of milk was diluted with 10 mL of double distilled water. Substrate buffer was prepared immediately before use and consisted of 0.1 mol.L⁻¹ Tris, 89 mmol.L⁻¹ NaCl, 48 mmol.L⁻¹ glycylglycine, and 4.8 mmol.L⁻¹ γ -GPNA, with the pH adjusted to 8.0 with 1 mol.L⁻¹ HCl.

The samples and substrate were preheated separately at 40 °C for about 3 min in a water bath. The (un)treated samples (50 μ L) were added to 2.5 mL substrate solution and the released *p*-nitroaniline was measured within 15 min in 3 mL cuvettes (10 mm path length) at a wavelength of 410 nm. The blank solution used in the measurements was prepared by using the same amount of substrate and distilled water instead of sample. The reaction rate was linear for at least 15 min.

The enzymes activities per milliliters were calculated using the following formula:

$$\text{Units.mL}^{-1} = (\Delta A \text{ min}^{-1}) \times (V_R) / (\varepsilon_\lambda) / (V_E) \times d \quad (1)$$

where: ΔA = the change in absorbance at 405 nm and 410 nm respectively, V_R = reaction volume, ε_λ = the millimolar absorption coefficient of *p*-nitrophenol at 405 nm (18.2 L⁻¹.cm⁻¹.mmol⁻¹) and of *p*-nitroaniline at 410 nm (8.8 L⁻¹.cm⁻¹.mmol⁻¹) respectively, V_E = enzyme volume (0.05 mL) and d is the dilution factor.

The residual activity of the enzymes was calculated using the following formula:

$$\text{Residual activity (\%)} = \frac{A}{A_0} \times 100 \quad (2)$$

where: A and A_0 are the activities of the treated and untreated enzyme solution respectively.

All spectrophotometric measurements were made using a UV–VIS GBC Cintra 202 spectrophotometer (Australia).

2.4 Kinetic data analysis

ALP and GGT inactivation was described by the first-order reaction (Eq. 3). The inactivation rate constant (k) was derived from the slope of the regression line obtained by plotting the natural logarithm of relative residual activity as a function of inactivation time (t).

$$\ln \frac{A}{A_0} = -kt \quad (3)$$

The kinetic parameters were estimated in a global fit using nonlinear regression analysis of relative residual response values (one step method) according to Eq. 4 (Claeys et al. 2001):

$$\log \left(\frac{A}{A_0} \right) = - \left(\frac{t}{D_{\text{ref}}} \right) \cdot 10^{\left[\frac{T - T_{\text{ref}}}{z} \right]} \quad (4)$$

where D is the decimal reduction time (i.e. time needed to reduce the initial activity

by one log unit at a constant temperature) and z is the temperature increase necessary to induce a 10-fold reduction of D .

The temperature dependence of the inactivation rate constants was estimated using the Arrhenius model (Eq. 5):

$$\ln(k) = \ln(k_0) + \left[\frac{E_a}{R} \cdot \left(\frac{1}{T_0} - \frac{1}{T} \right) \right] \quad (5)$$

where T and T_0 are the absolute and the reference temperature (K), k_0 is the rate constant at T_0 , E_a is the activation energy ($\text{kJ} \cdot \text{mol}^{-1}$) and R is the universal gas constant ($8.314 \text{ J}^{-1} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$). The activation energy was estimated using linear regression analysis on equation.

All statistical procedures were performed using the SAS software package, version 9.2 (SAS Institute, Cary, USA).

The thermodynamic parameters enthalpy (ΔH , $\text{kJ} \cdot \text{mol}^{-1}$), entropy (ΔS , $\text{kJ} \cdot \text{mol}^{-1}$), and free energy of activation (ΔG , $\text{kJ} \cdot \text{mol}^{-1}$) were calculated according to the following expressions:

$$\Delta H = E_a - RT \quad (6)$$

$$\Delta S = R(\ln A - \ln K_b/hp - \ln T) \quad (7)$$

$$\Delta G = \Delta H - T \Delta S \quad (8)$$

where $\ln A$ is the ordinate intersection of the straight line obtained by linear regression for E_a calculation (Eq. 5), K_b is the Boltzmann constant ($1.38066 \times 10^{-23} \text{ J} \cdot \text{K}^{-1}$), hp the Planck constant ($6.62618 \times 10^{-34} \text{ J} \cdot \text{s}^{-1}$), R the gas constant, and T the absolute temperature.

3 Results and discussions

3.1 Thermal inactivation

ALP is probably the most important indigenous milk enzyme from a dairy safety point of view, due to the almost universal usage of the enzyme test as an index of the efficiency of HTST pasteurization. Since milk is a vector for pathogenic bacteria (D'Aoust et al. 1988), the test is of great significance to public health as a mean of assessing the thoroughness of heat treatments or the addition of raw milk to heated products (Marchand et al. 2009). Evaluation of the effectiveness of pasteurization is based on the inactivation of ALP due to different time–temperature treatments required for effective pasteurization (Andrews 1992).

Detailed thermal inactivation of ALP was performed in the temperature range of 50 to 75 °C for 0 to 30 min in bovine milk in order to compare the kinetics of inactivation and to establish indices to control the efficacy of the heat treatment. Inactivation experiments were also performed in $10 \text{ mmol} \cdot \text{L}^{-1} \text{ MgCl}_2$ buffer solution pH 6.6, to assess the stabilizing or destabilizing influence of the milk components.

The average enzyme activities values in bovine milk ($n=20$) was 0.46 ± 0.02 U.mL⁻¹ for ALP and 4.05 ± 0.26 and 4.55 ± 0.24 U.mL⁻¹ for GGT in milk with different fat contents. Thus, the levels of GGT in raw milk were 8% to 10% higher than those of ALP, making it more sensitive and accurate as a testing marker.

A detection limit of 0.1% of raw milk diluted in commercial UHT milk was observed for ALP. The detection limit for GGT was 0.1% raw milk in the case of the milk with 1.5% fat and 0.2% in the case of the milk with 2.5% fat content.

Figure 1 shows the relative residual ALP (a) and GGT (b) activities in the different media plotted as a function of inactivation temperature after 5 min. At 50 °C, 7.4% of ALP activity in milk was lost after 5 min, whereas the loss in enzyme activity in buffer was about 2.3%. The enzyme inactivates both in milk and buffer after 5 min of thermal treatment at 70 °C. Regarding the GGT activity, it can be seen that at 60 °C, $15.9\pm1.2\%$ and $12.5\pm0.6\%$ of the initial activity were lost after 5 min of heating. In the higher temperature range, the enzyme was inactivated faster, with a complete inactivation at 76 °C.

The decrease in ALP activity in the two different media after heating, expressed as residual activity, is given in Fig. 2. Figure 2a represents the reduction in the activity of ALP in 10 mmol.L⁻¹ MgCl₂ buffer solution treated at different temperatures. There are significant differences in the activities of ALP depending on the temperature applied. In the temperature range of 50 to 62.5 °C, a decrease in activity from 22.8% to 99.9% was observed after 30 min of heating. ALP was completely inactivated after 20 min at 65 °C or 1 min at 75 °C.

Fig. 1 Thermal stability of **a** alkaline phosphatase (ALP) and **b** γ -glutamyl transferase (GGT) in raw milk (open squares) and buffer (closed squares) and in milk with 1.5% fat (open triangles) and 2.5% fat (closed triangles), respectively. Residual activity was measured after 5 min treatment at different temperatures. Experimental points were the average of three repetitions. For more details, see Section 2

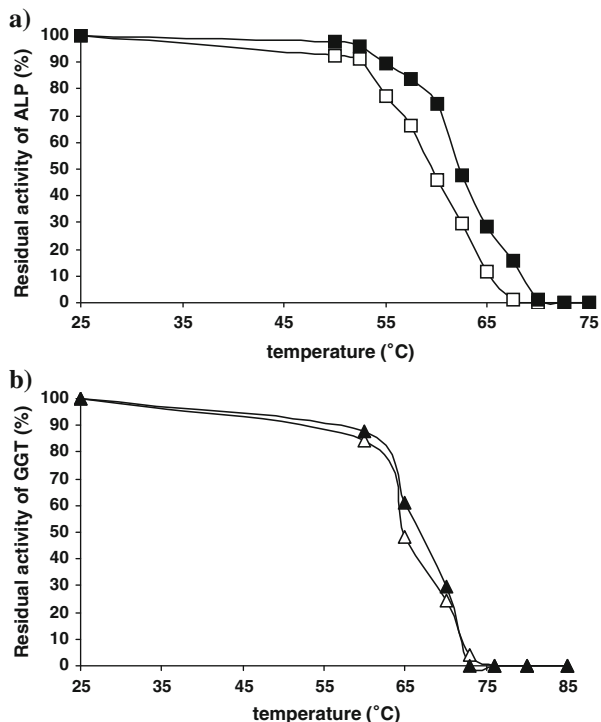
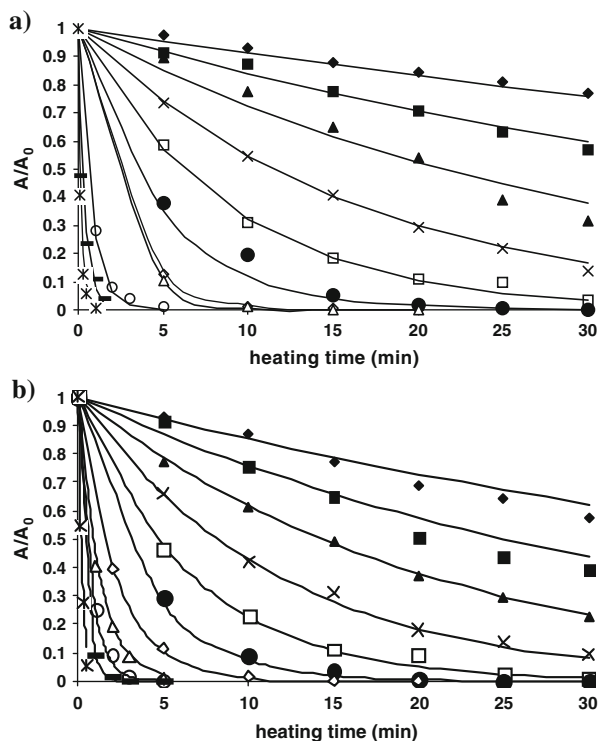


Fig. 2 Thermal inactivation of alkaline phosphatase (ALP) activity (A/A_0) at different temperatures: 50 (filled diamonds), 52.5 (filled squares), 55 (filled triangles), 57.5 (error marks), 60 (white squares), 62.5 (filled circles), 65 (white circles), 67.5 (white diamonds), 70 (white triangles), 72.5 (minus symbol), and 75 °C (asterisks). Treatments were performed in buffer (a) and raw milk (b). Plotted lines correspond to experimental data fit to a first-order kinetic model



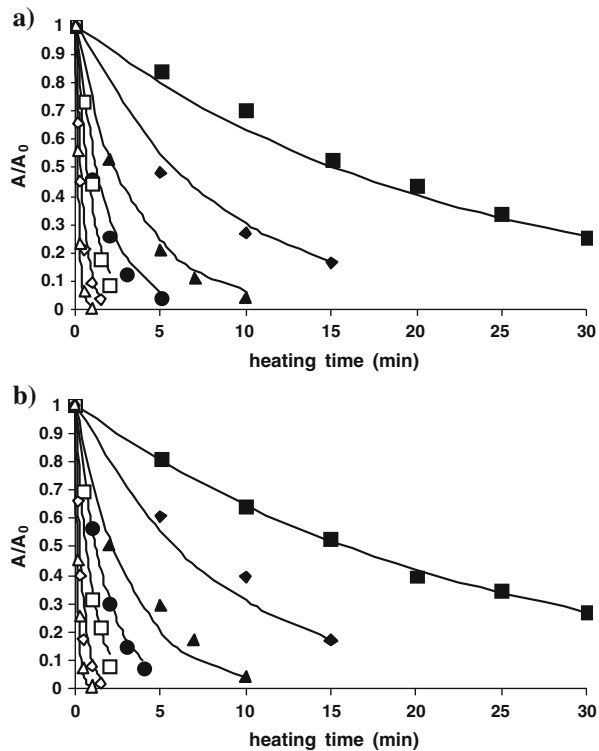
In raw milk, the inactivation of ALP was more rapid compared with buffer solution, especially in the higher temperature range (Fig. 2b). In the temperature range of 50 to 62.5 °C, the decrease in ALP activity varied from 42.7% to 99.5% after 30 min of heating. The time–temperature combination needed for complete inactivation of the enzyme was 70 °C/5 min or 75 °C/30 s. The ALP test is considered negative if the ALP activity measured in milk is not higher than 350 mU.L⁻¹ (Dinnella et al. 2004). This limiting value was achieved by the different time–temperature treatments applied in this study.

Figure 3 represents the reduction in the activity of GGT in milk with different fat contents. After 30 min of heating at 60 °C, the residual activity was 25.7±0.4% and 27.4±0.7% in milk with 1.5% and 2.5% fat, respectively. In the higher temperature range, the corresponding GGT activities after 30 s at 85 °C were 6.6±0.25% and about 7.3% in milk with 1.5% and 2.5% fat, respectively. A complete inactivation of GGT was observed at 85 °C after 1.5 min of heating for both media.

3.2 Kinetic and thermodynamic parameters

The relationship between the heat sensitivity of the target microorganism and of the thermal intrinsic indicator can be expressed in terms of D and z values which is the standard method of describing the heat inactivation kinetics of microorganisms according to the thermal death time model. Thermal inactivation of ALP and GGT could accurately be described by a first-order model, as indicated by the linear

Fig. 3 Thermal inactivation of γ -glutamyl transferase (GGT) activity (A/A_0) at different temperatures: 60 (filled squares); 65 (filled diamonds), 70 (filled triangles), 73 °C (filled circles), 76 (white squares), 80 (white diamonds), and 85 °C (white triangles). Treatments were performed in milk containing 1.5% fat (a) and 2.5% fat (b). Plotted lines correspond to experimental data fit to a first-order kinetic model



relationships obtained by plotting the retention values as a function of heating time on a logarithmic scale (Figs. 4 and 5) and confirmed by the r^2 values. The model was appropriate for the data, since residuals represented only the experimental errors and were randomly distributed when plotted. In all cases there was a good correlation between experimental and calculated values (r^2 0.99).

The D values were calculated according to the thermal death time model ($D=2.303/k$).

The corresponding D values for ALP together with standard errors and r^2 values are given in Table 1. The D values decreased with increasing temperature from 50 to 75 °C, indicating a faster inactivation of ALP at higher temperatures. The D values ranged from 121.8 ± 3.83 min to 0.40 ± 0.06 min for ALP inactivation in milk and from 258.7 ± 11.4 min to 0.41 ± 0.05 min in buffer solution. It is worth mentioning that at 50 °C, the D value for ALP inactivation in buffer solution was 2.1 times higher than the corresponding value for inactivation in raw milk. This is probably due to the relative higher thermal stability of the ALP in milk. It seems that the bovine milk environment had a protective effect on ALP inactivation as compared with buffer.

For the inactivation of ALP in raw milk, Claeys (2003) reported D values of 304.04 ± 24.85 min at 54 °C and 4.32 ± 0.67 min at 64 °C. Fadiloğlu et al. (2006) suggested D values of 217.0 ± 12.50 min at 50 °C, 9.3 ± 0.27 min at 60 °C, and 2.9 ± 0.09 min at 70 °C.

The corresponding D values for GGT inactivation together with standard errors and r^2 values are also given in Table 1. The D values decreased with increasing

Fig. 4 First-order thermal inactivation of alkaline phosphatase (ALP) in buffer (a) and raw milk (b) at different temperatures: 50 (filled diamonds), 52.5 (filled squares), 55 (filled triangles), 57.5 (error marks), 60 (white squares), 62.5 (filled circles), 65 (white circles), 67.5 (white diamonds), 70 (white triangles), 72.5 (minus symbol), and 75 °C (asterisks). A the enzyme activity at time t , A_0 the initial enzyme activity. For details, see Section 2.4

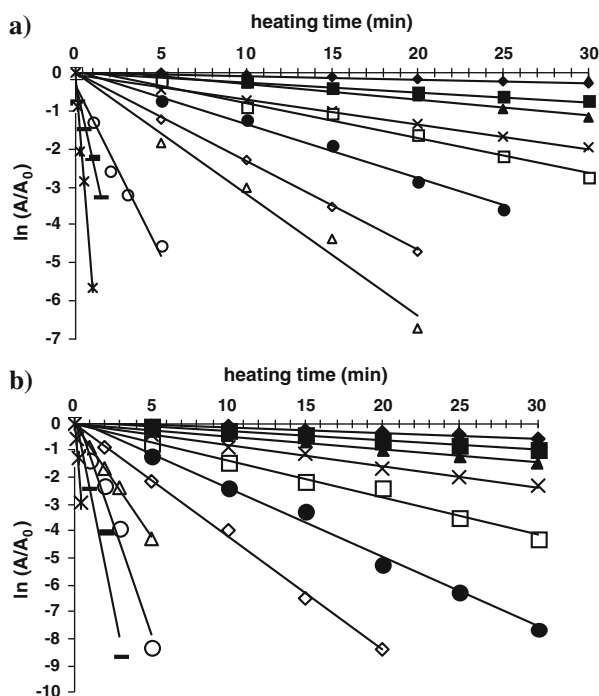


Fig. 5 First-order thermal inactivation of γ -glutamyl transferase (GGT) in milk containing 1.5% fat (a) and 2.5% fat (b) at different temperatures: 60 (filled squares), 65 (filled diamonds), 70 (filled triangles), 73 (filled circles), 76 (white squares), 80 (white diamonds), and 85 °C (white triangles). A the enzyme activity at time t , A_0 the initial enzyme activity. For details, see Section 2.4

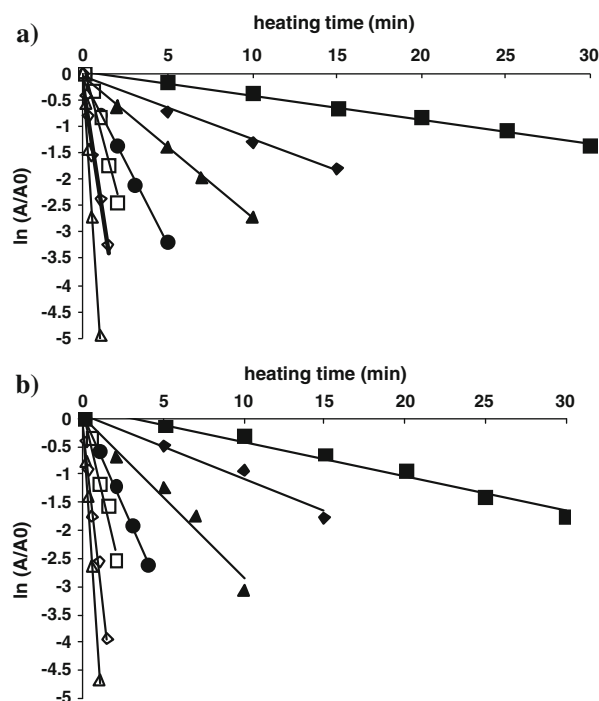


Table 1 Decimal reduction time (D) values and temperature resistance value (z) for alkaline phosphatase (ALP) and γ -glutamyl transferase (GGT)

Temperature (°C)	ALP				GGT			
	Buffer solution		Raw milk		Milk 1.5% fat		Milk 2.5% fat	
	D (min)	r^2	D (min)	r^2	D (min)	r^2	D (min)	r^2
50	258.76±11.41 ^a	0.99	121.85±3.83	0.99	n.d.			
52.5	89.96±6.03	0.99	68.74±12.35	0.99				
55	58.60±3.45	0.97	46.71±7.83	0.99				
57.5	35.76±1.47	0.99	29.26±5.77	0.97				
60	24.92±2.03	0.98	16.68±5.97	0.98	50.61±1.30	0.99	38.30±1.50	0.96
62.5	16.25±2.32	0.99	8.97±3.45	0.95	n.d.			
65	9.90±1.58	0.99	5.47±2.36	0.97	19.33±0.91	0.99	20.07±1.11	0.97
67.5	7.21±1.33	0.99	2.73±0.86	0.99	n.d.			
70	2.57±0.98	0.96	1.39±0.59	0.97	8.47±0.12	0.99	7.98±0.30	0.97
72.5	1.15±0.73	0.97	0.83±0.15	0.95	n.d.			
73	n.d.		n.d.		3.63±0.21	0.99	3.54±0.80	0.96
75	0.41±0.05	0.99	0.40±0.06	0.97	n.d.			
76	n.d.				1.84±0.50	0.96	1.85±0.70	0.97
80					1.06±0.60	0.97	0.90±0.03	0.98
85					0.45±0.05	0.99	0.49±0.04	0.99
D_{ref} (min)	1.66±0.12		1.54±0.13		2.70±0.10		2.71±0.11	
z (°C)	10.02±0.30		9.19±0.20		11.80±0.26		11.60±0.25	

D value: time required for 1 log reduction in activity at a specific temperature. z value: increase in temperature required for 1 log change in D value

n.d. not determined, D_{ref} decimal reduction time at a reference temperature

^a Standard error of regression

temperature from 60 to 85 °C, indicating a faster inactivation of GGT at higher temperatures. For GGT inactivation in milk with 1.5% fat, D values ranged from 50.6±1.3 min at 60 °C to 0.45±0.05 min at 85 °C. In milk with 2.5% fat content, D values decreased from 38.3±1.5 min at 60 °C to 0.49±0.04 min at 85 °C. Blel et al. (2002) reported D values of 7.2 min at 69 °C and 0.34 min at 75 °C. These results are roughly in agreement with D values reported elsewhere (Stănciuc et al. 2011) for the heat-induced inactivation of GGT in skimmed, whole milk, and cream.

The temperature resistance values (temperature increase needed for a 10-fold decrease of D) were estimated using a nonlinear regression analysis. The corresponding z values were 10.02±0.3 (°C) for the inactivation of ALP in buffer solution and 9.19±0.2 (°C) for the inactivation of ALP in milk, respectively.

For GGT inactivation, z values had similar values (11.8±0.26 °C for GGT inactivation in milk with 1.5% fat and 11.6±0.25 °C for inactivation in milk with 2.5% fat).

The z values obtained are higher than those previously reported in the literature. For example, Claeys (2003), Blel et al. (2002), Levieux et al. (2007), and Marchand et al.

(2009) reported z values ranging from 5 to 8 °C for thermal inactivation of ALP. The results obtained in this study are more consistent with values suggested by Fadiloğlu et al. (2006) who reported z values of 11.8 ± 0.69 °C for ALP inactivation in raw milk.

The z values for GGT inactivation are consistent with the values obtained previously for the heat sensitive enzyme in skimmed milk (10.8 ± 0.06 °C) and whole milk (12.27 ± 0.12 °C) (Stănciuc et al. 2011).

The inactivation rate values (k), calculated from the slope of Eq. 3 are given in Table 2. The inactivation of ALP was faster in milk in comparison to buffer solution, with the k value being 2.6 times higher for ALP inactivation in milk at 67.5 °C.

At 60 °C, the inactivation of GGT is faster in milk with a higher fat content, whereas at 85 °C the enzyme is slightly stable when compared with the inactivation rate in low-fat milk.

To calculate the activation energy (E_a), the natural logarithm of the inactivation rate constant k was plotted against the reciprocal of the absolute temperature in Kelvin (T) according to the Arrhenius Eq. 5. The linear regression and the coefficient of correlation of the Arrhenius plots were $y = -26,704x + 78.3$ ($r^2 = 0.994$) and $y = -25,748x + 75.06$ ($r^2 = 0.967$) for ALP inactivation in milk and buffer and $y = -22,952x + 65.8$ ($r^2 = 0.995$) and $y = -22,102x + 63.4$ ($r^2 = 0.989$) for GGT inactivation in milk with 1.5% and 2.5% fat, respectively, confirming that the inactivation of the enzymes follows first-order kinetics.

Table 2 Inactivation rate constant (k) and activation energy for alkaline phosphatase (ALP) and γ -glutamyl transferase (GGT) inactivation in buffer and raw milk

Temperature (°C)	$k \times 10^{-2}$ (min ⁻¹)			
	ALP		GGT	
	Buffer solution	Raw milk	1.5% fat	2.5% fat
50	0.89 ± 0.15^a	1.09 ± 0.18	n.d.	
52.5	2.56 ± 0.78	3.35 ± 0.69		
55	3.93 ± 0.84	4.93 ± 0.66		
57.5	6.44 ± 0.29	7.87 ± 0.54		
60	9.24 ± 0.26	13.80 ± 0.98	4.55 ± 0.23	6.01 ± 0.30
62.5	14.17 ± 0.13	25.65 ± 0.47	n.d.	
65	23.24 ± 0.49	42.03 ± 0.53	11.91 ± 0.27	11.47 ± 0.50
67.5	31.94 ± 0.55	84.23 ± 0.74	n.d.	
70	89.37 ± 0.54	165.02 ± 0.47	27.18 ± 1.25	28.85 ± 0.31
72.5	199.9 ± 0.88	275.19 ± 0.51	n.d.	
73	n.d.		63.34 ± 0.65	65.00 ± 0.63
75	557.15 ± 1.05	572.89 ± 0.62	125.11 ± 1.01	124.45 ± 1.20
80	n.d.		215.26 ± 1.23	255.5 ± 0.70
85			506.80 ± 2.36	468.99 ± 0.90
E_a (kJ.mol ⁻¹)	214.09 ± 12.90	222.02 ± 5.60	190.8 ± 6.06	183.7 ± 8.41

n.d. not determined

^a Standard error of regression

The temperature dependence of the rate constants for thermal inactivation of ALP and GGT in different media is depicted in Fig. 6. E_a values were $214.09 \pm 12.9 \text{ kJ} \cdot \text{mol}^{-1}$ ($r^2=0.96$) and $222.02 \pm 5.6 \text{ kJ} \cdot \text{mol}^{-1}$ ($r^2=0.99$) for the inactivation of ALP in buffer and raw milk, respectively. These values are in the same range as the activation energies reported by Fadiloğlu et al. (2006) ($207.8 \pm 17.1 \text{ kJ} \cdot \text{mol}^{-1}$), but significantly lower than those reported by Claeys (2003) ($432.1 \pm 6.3 \text{ kJ} \cdot \text{mol}^{-1}$) for ALP inactivation in bovine milk.

For GGT inactivation, E_a values ranged from $190.8 \pm 6.06 \text{ kJ} \cdot \text{mol}^{-1}$ ($r^2=0.99$) to $183.7 \pm 8.4 \text{ kJ} \cdot \text{mol}^{-1}$ ($r^2=0.98$) for enzyme inactivation in milk with 1.5% and 2.5% fat content, respectively. These values are significantly lower than those reported by Zehetner et al. (1995) who suggested an activation energy of $457 \text{ kJ} \cdot \text{mol}^{-1}$ but in close agreement with those suggested by Stănciuc et al. (2011) who reported values ranging from 214.8 to $174.5 \text{ kJ} \cdot \text{mol}^{-1}$ for GGT inactivation in skimmed milk and cream.

It is clear that the different environmental conditions that can affect system stability must be considered, including factors such as the presence of β -lactoglobulin, which can protect inactivation of ALP, or different thermal treatment conditions, when analyzing the differences in rate constants/decimal reduction times, and activation energies/ z values reported in the literature.

However, from Tables 1 and 2, it can be concluded that ALP inactivates faster in milk in comparison to buffer as indicated by both the z and E_a values. As regards GGT, the inactivation pattern as a function of fat content was similar.

Fig. 6 Arrhenius plot for thermal inactivation of **a** alkaline phosphatase (ALP) in (open squares) raw milk and (closed squares) buffer solution and **b** γ -glutamyl transferase (GGT) in milk with 1.5% fat (open triangles) content and milk with 2.5% fat content (closed triangles)

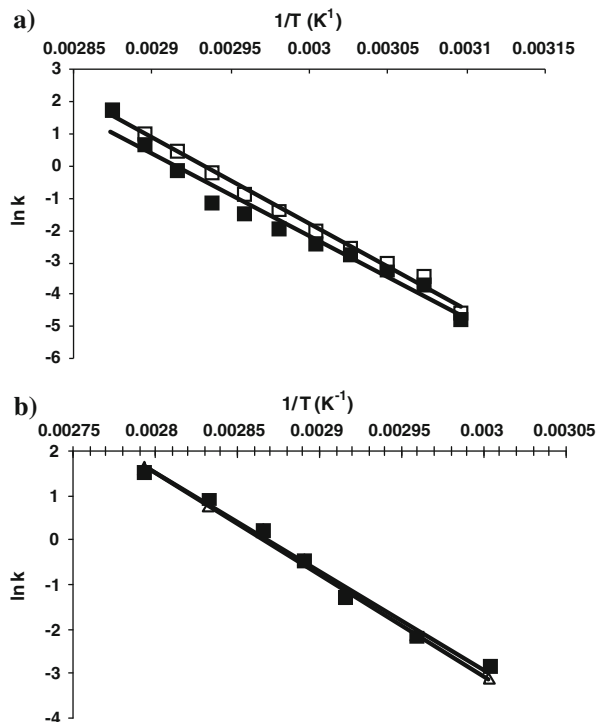


Table 3 Changes in enthalpy of activation (ΔH), free energy of activation (ΔG), and entropy of activation (ΔS) for alkaline phosphatase (ALP) and γ -glutamyl transferase (GGT) inactivation in milk with different complexity

Temperature		ALP				GGT			
		Buffer				Raw milk			
		ΔH (kJ.mol ⁻¹)	ΔG (kJ.mol ⁻¹)	ΔS (kJ.mol ⁻¹)	ΔH (kJ.mol ⁻¹)	ΔG (kJ.mol ⁻¹)	ΔS (kJ.mol ⁻¹)	ΔH (kJ.mol ⁻¹)	ΔG (kJ.mol ⁻¹)
°C	K								
50	323	211.40	92.01	0.260	219.35	91.47	0.260	n.d.	
52.5	325.5	211.38	89.88	0.259	219.33	89.15	0.260		
55	328	211.36	89.42	0.259	219.31	88.80	0.260		
57.5	330.5	211.34	88.77	0.259	219.29	88.22	0.260		
60	333	211.32	88.46	0.259	219.27	87.35	0.260	188.03	90.42
62.5	335.5	211.30	87.95	0.259	219.25	86.30	0.260	n.d.	
65	338	211.27	87.24	0.259	219.22	85.57	0.259	187.98	89.12
67.5	340.5	211.25	87.01	0.259	219.20	84.26	0.259	n.d.	
70	343	211.23	84.73	0.259	219.18	82.98	0.259	187.84	88.13
72.5	345.5	211.21	83.06	0.259	219.16	82.14	0.259	n.d.	
73	346	n.d.						187.92	86.49
75	348	211.19	80.71	0.259	219.14	80.63	0.259	n.d.	
76	349	n.d.						187.89	85.29
80	353							187.86	84.70
85	358							187.82	83.40

n.d. not determined

The activation energy value enabled the determination of enthalpy, entropy, and free energy of activation (Table 3).

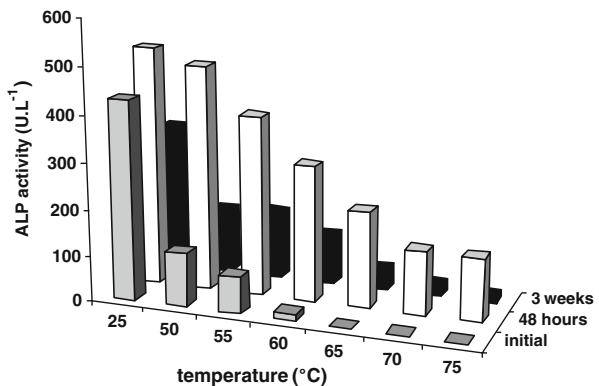
The high values of enthalpy and the positive values of the entropy and free energy indicate that, during thermal treatment, ALP and GGT underwent a large change in conformation. Concerning the influence of temperature on the conformation of ALP from bovine intestinal mucosa, Bortolato et al. (1999) suggested three denaturation steps. The first corresponds to the loss of ALP activity (at 45–60 °C), and was correlated to modifications of the enzyme conformation. The second temperature-induced step, appearing at about 60 °C, corresponds to the thermal unfolding transition of ALP probed by intrinsic fluorescence (at 65 °C) and the appearance of hydrophobic regions. This second step might correspond to the heat-induced monomerization. The third step, after 70 °C, might reflect the temperature-induced aggregation of ALP and corresponds to the formation of intermolecular β -sheets with a concomitant loss of some α -helices. Also, the positive value for ΔS indicates that the transition state is highly disordered compared with the native state. Degrees of freedom are liberated in going from the native state to the transition state, which, in turn, increase the rate of the reaction.

The results obtained in this study are in fair agreement with those reported by Dannenberg and Kessler (1988) for the heat denaturation of β -lactoglobulin in skim milk or whey (ΔH , 262–368 kJ.mol⁻¹; ΔG , 99–109 kJ.mol⁻¹; and ΔS , 0.44–0.63 kJ.mol⁻¹) and with those reported by Levieux et al. (2007) for the inactivation–denaturation kinetics of bovine milk alkaline phosphatase during mild heating using a monoclonal antibody-based immunoassay and colorimetric assay (ΔH , 303.48–308.14 kJ.mol⁻¹; ΔG , 77.71–84.85 kJ.mol⁻¹; and ΔS , 0.67–0.69 kJ.mol⁻¹). Data regarding the thermodynamic parameters of GGT inactivation were not found in the literature.

3.3 Reactivation studies

ALP totally inactivated by heat treatment can, under certain conditions, progressively recover part of its activity. Reactivation of ALP has considerable practical significance since the Romanian regulatory test for pasteurization assumes the negative reaction of ALP. From this point of view, it was decided to measure the enzyme activity after 48 h

Fig. 7 Reactivation of alkaline phosphatase (ALP) activity in raw whole milk during 3 weeks of storage at room temperature



and 3 weeks of storage at room temperature in raw milk samples previously heat treated at different temperatures for 30 min. The results are given in Fig. 7.

Significant reactivation of up to 43% of the original enzyme activity occurred after 48 h and then declined on prolonged storage. Lyster and Aschaffenburg (1962) also suggested a reactivation of ALP in milk of up to 30%.

There is evidence that the form of the enzyme which becomes reactivated is membrane bound, and several factors which influence reactivation have been established. Mg^{2+} and Zn^{2+} strongly promote reactivation (Fox 2003).

Reactivation of ALP in a complex medium such as milk, is strongly dependent on the presence of sulfhydryl ($-SH$) groups, provided by the presence of β -lactoglobulin. The one free thiol group per molecule becomes highly reactive during heat treatment especially at temperatures of up to 75 °C (Sava et al. 2005) and can be involved in reactivation. From this point of view, these results provide useful technical information for the relevant competent authorities in order to consider the reactivation of ALP during storage or a possible cross contamination during pasteurization.

4 Conclusions

Two possible intrinsic indicators for the heat treatment of bovine milk were studied in media of different complexities. For the different time–temperature combinations used in this study, both ALP and GGT showed first-order inactivation kinetics. The comparison of the rate constants of inactivation of ALP in media of different complexities revealed that the inactivation of the enzyme is faster in raw milk, especially at higher temperatures. These experiments confirmed that the stabilization of the enzymes by milk components had an entropic character since minimal differences among the activation energies of inactivation reactions were found. The obtained results suggested that the enzymes become reactivated to a certain degree after heat treatment and storage at room temperature.

The inactivation kinetics of GGT was compared in raw milk containing different levels of fat. No significant differences occurred for the kinetic parameters in the temperature range of 65 and 80 °C.

Additional experiments are needed in order to evaluate the possibilities to use these enzymes as indicators of industrial processing. Therefore, the kinetic data need to be compared with microbial inactivation kinetic data and with industrially applied and legally defined heat-treatment processes.

Acknowledgments The authors acknowledge financial support from the National University Research Council (NURC, PN-II-ID-PCE-2008-2, Idea, ID 517) Romania (www.trasilact.ugal.ro). The work of Alina Ardelean was supported by Project SOP HRD—EFICIENT 61445/2009. Bioaliment Research Platform (www.bioaliment.ugal.ro) is also acknowledged for providing technical support.

References

- Andrews AT (1992) In: Fox PF (ed) Food enzymology. Elsevier, London
- Andrews AT, Anderson M, Goodenough PW (1987) A study of the heat stabilities of a number of indigenous milk enzymes. *J Dairy Res* 54:237–246

- Baumrucker CR (1979) γ -Glutamyl transpeptidase of bovine milk membranes: distribution and characterization. *J Dairy Sci* 62:253–258
- Blel M, Guingamp MF, Gaillard JL, Humbert G (2002) Studies on the thermal sensitivity of γ -glutamyl transpeptidase measured with a modified test procedure and compared with that of alkaline phosphatase and lactoperoxidase in milk. *Lait* 82:555–566
- Bortolato M, Besson F, Roux B (1999) Role of metal ions on the secondary and quaternary structure of alkaline phosphatase from bovine intestinal mucosa. *Proteins Str Fun Gen* 37:310–318
- Claeys WL (2003) Intrinsic time temperature integrators for thermal and high pressure processing of milk. Ph.D. thesis, Katholieke Universiteit Leuven, Belgium
- Claeys WL, Ludikhuyze L, Van Loey A, Hendrickx ME (2001) Inactivation kinetics of alkaline phosphatase and lactoperoxidase, and denaturation kinetics of β -lactoglobulin in raw milk under isothermal and dynamic temperature conditions. *J Dairy Res* 68:95–107
- Claeys WL, Smout C, Van Loey A, Hendrickx M (2004) From time temperature integrator kinetics to time temperature integrator tolerance levels: heat-treated milk. *Biotechnol Prog* 20:1–12
- D'Aoust JY, Park CE, Szabo RA, Todd ECD, Emmons DB, McKellar R (1988) Thermal inactivation of *Campylobacter* species, *Yersinia enterocolitica*, and hemorrhagic *Escherichia coli* O157:H7 in fluid milk. *J Dairy Sci* 71:3230–3236
- Dannenberg F, Kessler HG (1988) Thermodynamic approach to kinetics of β -lactoglobulin denaturation in heated milk and sweet whey. *Milchwissenschaft* 43:139–142
- Dinnella C, Monteleone E, Farenga MF, Hourigan JM (2004) The use of enzymes for thermal process monitoring: modification of milk alkaline phosphatase heat resistance by means of an immobilization technique. *Food Control* 15:427–433
- Fadiloğlu S, Erkmén O, Şekeroğlu GS (2006) Thermal inactivation kinetics of alkaline phosphatase in buffer and milk. *J Food Pro Pres* 30:258–268
- Fox PF (2003) Significance of indigenous enzymes in milk and dairy products. In: Whitaker JR, Voraen AGJ, Wong DWS (eds) *Handbook of food enzymology*. Marcel Dekker, New York
- Fox PF, McSweeney PLH (1998) *Dairy chemistry and biochemistry*. Chapman & Hall, London
- Griffiths MW (1986) Use of milk enzymes as indices of heat treatment. *J Food Protect* 49:696–705
- Levieux D, Geneix N, Levieux A (2007) Inactivation-denaturation kinetics of bovine milk alkaline phosphatase during mild heating as determined by using a monoclonal antibody-based immunoassay. *J Dairy Res* 74:296–301
- Lyster RLJ, Aschaffenburg R (1962) The reactivation of milk alkaline phosphatase after heat treatment. *J Dairy Res* 29:21–35
- Marchand S, Merchiers M, Messens W, Coudijzer K, De Block J (2009) Thermal inactivation kinetics of alkaline phosphatase in equine milk. *Int Dairy J* 19:763–767
- McKellar RC (1996) Influence of ice-cream mix components on the thermal stability of bovine milk γ -glutamyl transpeptidase and *Listeria innocua*. *Int Dairy J* 6:1181–1189
- Murthy GK, Kleyn DH, Richardson T, Rocco RM (1992) In: Marshall RT (ed) *Standard methods for examination of dairy products*. American Public Health Association, Washington
- Sava N, van der Plancken I, Claeys W, Hendrickx ME (2005) The kinetics of heat-induced structural changes of β -lactoglobulin. *J Dairy Sci* 88:1646–1653
- Shakeel-Ur-Rehman FCM, Farkye NY, Fox PF (2003) Indigenous phosphatases in milk. In: Fox PF, McSweeney PLH (eds) *Advanced dairy chemistry*. Vol 1: proteins, 3rd edn. Plenum, New York
- Stănciuc N, Dumitrascu L, Stanciu S, Rapeanu G (2011) γ -glutamyl transferase inactivation in milk and cream: a comparative kinetic study. *Innov Food Sci Emerg Technol* 12:56–61
- Walstra P, Geurts TJ, Noomen A, Jellema A, Van Boekel MAJS (1999) *Dairy technology: principles of milk properties and processes*, 1st edn. Marcel Dekker, New York
- Wilnińska A, Bryjak J, Illeová V, Polaković M (2007) Kinetics of thermal inactivation of alkaline phosphatase in bovine and caprine milk and buffer. *Int Dairy J* 17:579–586
- Zehetner G, Bareuther C, Henle T, Klostermeyer H (1995) Inactivation kinetics of γ -glutamyl-transferase during the heating of milk. *Z Lebensmitt Unters Forsch* 201:336–338