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Kinetic and thermodynamic study of thermal inactivation of the antimicrobial peptide P34 in milk

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ABSTRACT

The knowledge on thermal inactivation of biopreservatives in a food matrix is essential to allow their proper utilisation in food industry, enabling the reduction of heating times and optimisation of heating temperatures. In this work, thermal inactivation of the antimicrobial peptide P34 in skimmed and fat milk was kinetically investigated within the temperature range of 90–120 °C. The inactivation kinetic follows a first-order reaction with *k*-values between 0.071 and 0.007 min^{-1} in skimmed milk, and 0.1346 and 0.0119 min^{-1} in fat milk. At high temperatures, peptide P34 was less resistant in fat milk, with a significant decrease in residual activity as compared with skimmed milk. At temperatures below 110 °C, the fat globules seem to have protective effect to the peptide P34. Results suggest that peptide P34 is heat stable in milk with activation energy of 90 kJ mol^{-1} in skimmed milk and 136 kJ mol^{-1} in fat milk.

1. Introduction

The preservation of foods is a current issue all over the world, despite of the advance of new technologies. The major challenges for the food industry are reduction of economic losses due to product spoilage through the food chain, lowering food processing costs and high retention of nutritional and sensory properties after food industrial processing (Gálvez, Abriouel, López, & Omar, 2007).

Thermal processing remains as the most widely method employed for food preservation and shelf-life extension. Recent developments in food processing aimed for technologies that may result in minimal damage to nutrients and sensory components by reducing heating times and optimising heating temperatures (Awuah, Ramaswamy, & Economides, 2007). Bacteriocins are antimicrobial peptides that bacteria use to compete against other microorganisms. Bacteriocins should be used in combination with other antimicrobial barriers, providing an additional hurdle to reduce the likelihood of food-borne diseases (Deegan, Cotter, Hill, & Ross, 2006). It is possible to reduce heat intensity combining bacteriocins with thermal processing, resulting in cost savings in heat treatment and reducing the impact of heat on foods properties (Gálvez et al., 2007).

Bacteriocins have been successfully used in dairy products to control pathogenic and spoilage bacteria (Bizani, Morrisy, Dominguez, & Brandelli, 2008; Malheiros, Daroit, Silveira, & Brandelli, 2010). The knowledge of kinetic parameters in thermal treatments would enable modulate processes to achieve desirable antimicrobial activity at the end of the heat operation, protecting the food by the maintenance of bioactivity during the shelf-life of the product (Sant'Anna, Utpott, Cladera-Olivera, & Brandelli, 2010). When nisin, a bacteriocin produced by *Lactococcus lactis*, was added to milk before thermal processing, the product was microbiologically acceptable and was superior in flavour, with no off-flavours within 32 days of storage (Wirjantoro, Lewis, Grandison, Williams, & Delves-Broughton, 2001).

Bacillus sp. P34, an isolate from Amazon basin, produces an antimicrobial peptide that inhibits important pathogenic and spoilage bacteria, such as *Listeria monocytogenes*, *Bacillus cereus* and *Erwinia carotovora*. This antimicrobial peptide maintains its activity within a broad range of pH and presents thermal stability (Motta, Cannavan, Tsai, & Brandelli, 2007). The peptide P34 also presents low cytotoxicity, equivalent to nisin, a bacteriocin used as biopreservative in more than 40 countries (Vaucher, Motta, & Brandelli, 2010). Statistical study on modelling the thermal inactivation of peptide P34 and the influence of pH and sodium chloride on kinetic parameters was previously described, showing that inactivation follows a first-order reaction (Sant'Anna et al., 2010; Sant'Anna, Utpott, Cladera-Olivera & Brandelli, 2011). However, the analyses were performed in sodium phosphate buffer, and the influence of food components on the inactivation behaviour was not evaluated.

The knowledge on kinetics of thermal inactivation of bacteriocins is important to allow their adequate use as biopreservatives in the food industry. However, this analysis in food matrixes is

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Nomenclature

A/A_0	residual antimicrobial activity	h	Boltzmann's constant (J/K)
D	decimal reduction time (min)	K_B	Planck's constant (J s)
k	inactivation rate constant (min^{-1})	Ea	activation energy (kJ/mol)
$t_{1/2}$	half-time (min)	$\Delta H^{\#}$	activation enthalpy (kJ/mol)
z	z-value (°C)	$\Delta S^{\#}$	activation entropy (J/mol K)
t	time (min)	$\Delta G^{\#}$	free energy inactivation (kJ/mol)
T	temperature (°C, K)	r^2	determination coefficient
R	universal gas constant (J/mol K)		

scarce. Therefore, the aims of this work were to evaluate the stability and to determinate kinetic and thermodynamic parameters of thermal inactivation of the antimicrobial peptide P34 in skimmed and fat milk during heat processing.

2. Materials and methods

2.1. Bacterial strains and media

Bacillus sp. strain P34, the producer strain, was previously isolated and characterised (Motta et al., 2007). The indicator strain for antimicrobial activity was *L. monocytogenes* ATCC 7644. The BHI medium (Oxoid, Basingstoke, UK) containing 20% (v/v) glycerol was used for maintenance of the strains at −20 °C. The cultivation of the strains was performed aerobically.

2.2. Bacteriocin production and activity assay

The protocol for production of antimicrobial peptide P34 was previously described by Motta et al. (2007). Shortly, the producer strain was cultivated aerobically in BHI broth at 30 °C for 24 h. The culture was centrifuged at 10,000 *g* for 15 min at 12 °C. Ammonium sulphate (Merck, Rio de Janeiro, Brazil) was added to the cell free supernatant to reach 20% (w/v) saturation. The resulting precipitate was suspended in 10 mM sodium phosphate buffer pH 7.0 and 1 ml were applied to a gel filtration column (Sephadex G-100, Pharmacia Biotech, Uppsala, Sweden) of 0.8 cm of diameter and 23 cm of length, and eluted with the same buffer. Fractions were collected in flow rate of 0.5 ml min^{-1} and those presenting antimicrobial activity were pooled and stored at 4 °C until used. The solution of partially purified bacteriocin has 12,800 AU ml^{-1} with a specific activity of 6050 AU mg^{-1} and purification factor of 80-fold.

Antimicrobial activity was determined diluting peptide P34 by the modified serial twofold dilution method (up to 1/128). An aliquot of 10 μl of each dilution was applied onto BHI agar plates previously inoculated with a 0.5 McFarland suspension of the indicator strain. Plates were incubated at 37 °C for 24 h. Activity was defined as the reciprocal of the dilution after the last serial dilution giving a zone of inhibition and was expressed as activity unit (AU) per milliliter (Motta & Brandelli, 2002).

2.3. Heat treatment

To evaluate the influence of powder milk in thermal degradation of peptide P34, 0.1 g of skimmed or fat powder milk (bought at local market, Porto Alegre, RS, Brazil) was added to 1 ml of partially purified peptide P34. The concentration utilised was the indicated by the manufacturer to reconstitute the powder into fluid milk. Kinetic parameters for the peptide P34 in 10 mM sodium phosphate buffer pH 7.0 had been determined before (Sant'Anna et al., 2010).

Thermal inactivation tests were performed as described elsewhere (Lappe, Cladera-Olivera, Dominguez, & Brandelli, 2009). Sealed tubes (1 mm of thickness, 7 mm of internal diameter and 3 cm of length) with 1.0 ml of bacteriocin solutions, added of powder milk, were placed in a thermostatically controlled dry bath (Accublock Digital Dry Baths, Labnet International In, NJ, USA). Tests were performed in temperature range from 90 to 120 °C as in previous work (Sant'Anna et al., 2010) for up to 240 min. At the end of each incubation time, tubes were immediately immersed in an ice bath, in order to stop heat inactivation.

The activity after 1 min of heating-up time ($t = 0$) was considered to be the initial activity, thereby eliminating the effects of heating-up (Schokker & van Boekel, 1999). Assays were done in duplicate.

2.4. Estimation of kinetic parameters

First-order reaction has been reported to describe heat inactivation of bacteriocins (Lappe et al., 2009), and it is algebraically described for the equation:

$$\frac{A}{A_0} = \exp(-kt) \tag{1}$$

where *A/Ao* is the residual bacteriocin activity at treatment time t (min), and k (min^{-1}) is the inactivation rate constant at a determined temperature. The inactivation rate constants (k-values) can be estimated by non-linear regression analysis.

Half-life ($t_{1/2}$) value of inactivation is given by the expression:

$$t_{1/2} = \frac{ln(2)}{k} \tag{2}$$

D-value is the time needed to reduce the initial activity 90%. It was related to k-values by Eq. (3) and mathematically expressed by (Espachs-Barroso, Loey, Hendrickx, & Martín-Belloso, 2006):

$$D = \frac{ln(10)}{k} \tag{3}$$

The z-value is the temperature needed to vary D-value one log-unit, and it was obtained by plotting log values of the D-values on a log scale versus the corresponding temperatures (Stumbo, 1973).

2.5. Thermodynamic analysis

Arrhenius' law is usually utilised to describe the temperature dependence of k-values, and it is algebraically given by:

$$ln(k) = ln(C) - \frac{E_a}{R.T} \tag{4}$$

where C is the Arrhenius constant, Ea (kJ/mol) the activation energy, R (8.31 J/mol K) the universal gas constant and T (K) is the absolute temperature. The Ea can be estimated by the slope of linear regression analysis of the natural logarithm of rate constant versus the reciprocal of the absolute temperature.

Obtained value of *Ea*, the activation enthalpy ($\Delta H^{\#}$) for each temperature was calculated was by:

$$\Delta H^{\#} = E_a - R.T \quad (5)$$

The free energy of inactivation ($\Delta G^{\#}$) can be determined according to the expression:

$$\Delta G^{\#} = -R.T.ln\left(\frac{k.h}{K_B T}\right) \quad (6)$$

where h (6.6262×10^{-34} J s) is the Planck's constant, K_B (1.3806×10^{-23} J/K) is the Boltzmann's constant, and k (s^{-1}) the inactivation rate constant of each temperature.

From Eq (5) and (6) it is possible to calculate the activation entropy ($\Delta S^{\#}$) by:

$$\Delta S^{\#} = \frac{\Delta H^{\#} - \Delta G^{\#}}{T} \quad (7)$$

2.6. Data analysis

Mean values were calculated from two independent experiments for each condition and duplicate assays of antimicrobial activity were performed for each experiment. Statistical analysis of the data was performed using the Statistica 7.0 software (Statsoft Inc., Tulsa, OK, USA) and plots using Microsoft Excel 2000 (MapInfo Corporation, Troy, NY, USA). Obtained *k*-values were compared using Tukey's test, and a $p < 0.05$ was considered statistically significant.

3. Results and discussion

The antimicrobial peptide P34 was heat treated in sodium phosphate buffer pH 7.0 and powder skimmed or fat milk was added to evaluate the influence of dairy compounds on thermal stability of the bacteriocin. The residual activity after heating-up time (1 min) was 100% for all temperatures tested.

During the tests, visual browning change in colour of the media was observed, indicating the formation of Maillard reaction products (MRPs). Some MRPs present antimicrobial activity (Einarsson, 1987; Rufián-Henares & Morales, 2008), thus control experiments without the presence of the peptide P34 were developed and tested for antimicrobial activity. No antimicrobial activity was observed in controls, indicating that the inhibition zones were due to the presence of the peptide P34 (data not shown).

The residual antimicrobial activities present exponential behaviour throughout time (Fig. 1). As expected, antimicrobial activity decreased as the heating time increased. For all treatments, bacteriocin inactivation follows first-order kinetics (Eq. (1)). In this figure, symbols refer to the average of experimental values, while lines correspond to the fitted values of Eq. (1) to experimental data.

Table 1 summarises the results of kinetic parameters for thermal processing of peptide P34 in skimmed and fat milk. The data in buffer solution has been previously described (Sant'Anna et al., 2010) and were used for comparison of heat stability. The rate of inactivation reaction was higher with increasing temperature. *k*-values for peptide P34 in skimmed milk for 115 and 120 °C were significantly higher ($p < 0.05$) than those found in buffer solution, indicating a decrease on thermal resistance at higher temperatures. *D*-Values ranged between 32 and 328 for temperatures between 90 and 120 °C. At 110 °C, the non-fat milk seem to protect the antimicrobial substance when compared with the behaviour in buffer solution, decreasing significantly ($p < 0.05$) *k*-values and consequently increasing the thermal resistance of peptide P34. It has already been suggested that protein content of foods may play a protective effect on bacteriocins during thermal processing

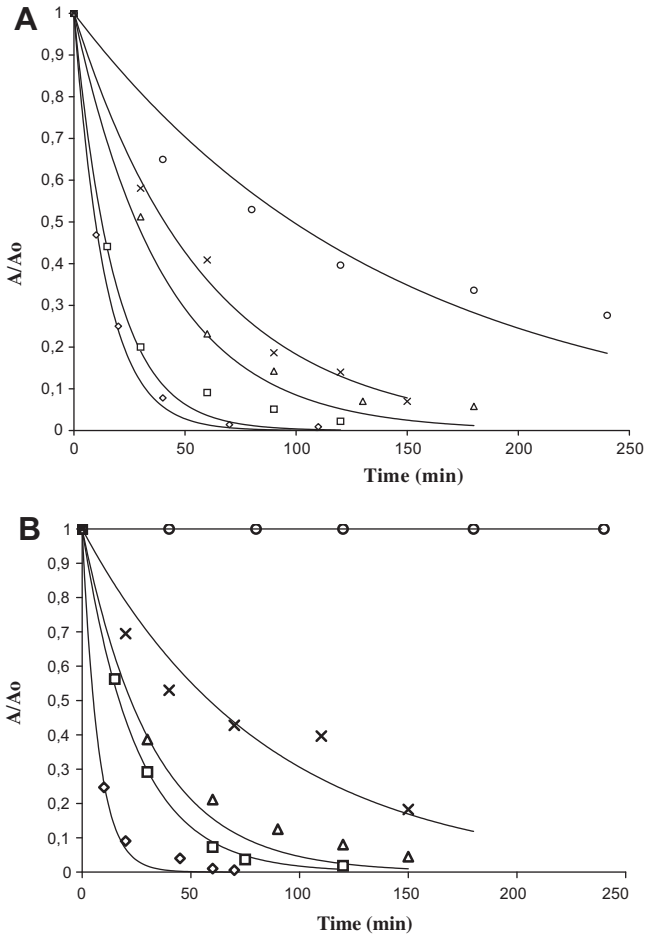

Fig. 1. Thermal inactivation of peptide P34 in skimmed milk (A) and fat milk (B). Residual activity was determined after incubation at 90 (o), 100 (x), 110 (Δ), 115 (□), 120 °C (◇). Values are means of duplicate assays of two independent experiments. The standard deviations were always lower than 7%.

(Lappe et al., 2009). However, the results suggest a possible interaction of peptide P34 with milk compounds. At high temperatures, beyond heat inactivation, the peptide could react with reducing sugars present in milk generating Maillard reaction products (MRPs), thus accelerating the loss of antimicrobial activity. Indeed, the antimicrobial activity of the bacteriocin nisin is significantly affected by glycation (Abdullah, Badaruddin, Ali, & Riaz, 2010). Peptide P34 resembles cyclic antimicrobial lipopeptides of *Bacillus* that lacks Lys residues (Stein, 2008), and thus reduced reactivity with lactose would be expected. However, partial loss of antimicrobial activity was observed after incubation of peptide P34 with MRPs resulting from lactose/glycine model systems (Sant'Anna, Malheiros, & Brandelli, 2011).

The fact that *Bacillus* sp. P34 produces only an antimicrobial peptide allows the use of a partially purified fraction (Motta et al., 2007). Although the degree of purity may have an influence on the thermal inactivation, the additional peptides present in the bacteriocin preparation would be negligible as compared with the amount of milk protein. The addition of sodium caseinate to extracellular protease of *Pseudomonas fluorescens* caused an increase in the inactivation rate, which was inferred to the possible association of the enzyme molecules with caseinate (Schokker & van Boekel, 1999). However, those authors observed that the kinetic parameters of purified protease did not differ significantly from the unpurified sample. Some enzymes are protected against thermal inactivation by companion proteins (Xiong, Liu, Song, & Ji,

Table 1
Kinetic parameters for inhibition of peptide P34 in skimmed and fat milk.

Medium	T (°C)	r^2	k (min^{-1})[b]	$t_{1/2}$ (min)	D (min)	z (°C)
Buffer[a]	120	0.996	0.059 ± 0.001	11.83	39.29	37.74
	115	0.972	0.037 ± 0.002	18.84	62.57	
	110	0.959	0.034 ± 0.001	20.39	67.72	
	100	0.898	0.012 ± 0.001	56.35	187.20	
	90	0.958	0.010 ± 0.001	67.96	225.74	
Skimmed milk	120	0.998	0.071 ± 0.003	9.71	32.26	30.21
	115	0.959	0.041 ± 0.001	12.54	41.67	
	110	0.963	0.028 ± 0.007	28.15	93.52	
	100	0.994	0.017 ± 0.001	40.80	135.53	
	90	0.930	0.007 ± 0.003	98.66	327.72	
Fat milk	120	0.997	0.135 ± 0.003	5.15	17.11	20.49
	115	0.999	0.055 ± 0.001	17.09	56.77	
	110	0.920	0.033 ± 0.002	21.04	69.88	
	100	0.917	0.012 ± 0.007	58.51	194.38	
	90	0.999	0.000 ± 0.00	∞	∞	

[a] Values from Sant'Anna et al. (2010) included for comparison.
[b] Values are means ± standard deviations of duplicate assays of two independent experiments. k-Values were statistically different ($p < 0.05$) in the range of 90–120 °C for each treatment.

2005), which could be associated to protection against proteolysis and partial detanuration during purification steps.

In fat milk, peptide P34 also had its resistance decreased at higher temperatures. At 115 and 120 °C, heat resistance was significantly lower ($p < 0.05$) as compared to skimmed milk and buffer solution. However, the inactivation of peptide P34 was not observed at 90 °C in fat milk, despite of continuous decreasing of residual activity in skimmed milk. The k-value in fat milk varied from 0.135 to 0.012 min^{-1}, and D-values from 17 to 194 min in the range 100–120 °C.

The z-values were obtained by analysing Fig. 2. Similarly to observed in experiments of pH reduction and addition of sodium chloride, where thermal stability of peptide P34 decreased at higher temperatures (Sant'Anna et al., 2011), the z-values were smaller compared to the z-value in buffer solution (Table 1). This indicates that a variation in temperature processing affects more intensely the stability of peptide P34 in fat milk than in skimmed milk. Calculated D-, z- and k-values indicate that the peptide P34 is heat stable in milk systems and also that it can be utilised in HTST (high temperature short time) and LTLT (low temperature long time) pasteurisation, where values of 75 °C for 1 min, or 85 °C for 15 s, and 65 °C for 30 min, respectively, are generally considered.

Comparison of thermal stability showed a lower resistance of peptide P34 in fat milk at higher temperatures. The inversion of the heat resistance can be observed in Figs. 2 and 3. At temperatures above 110 °C, the peptide P34 was more resistant in skimmed milk, and below 110 °C the resistance was higher in fat milk. The fat globules in milk are surrounded by a complex membrane, which has several distinct layers that are established during its synthesis in the mammary cell. This membrane is commonly referred as milk fat globule membrane (MFGM). The MFGM consists of complex mixtures of proteins, phospholipids, glycoproteins, triglycerides, cholesterol and other components of lesser importance, being highly affected by the processing of milk (Evers, 2004; Singh, 2006). It was already reported the influence of temperature on adsorption of milk proteins to MFGM (Ye, Singh, Taylor, & Anema, 2004). The exact mechanism of how proteins interact with MFGM is not entirely clear, however it may occur due to the breaking of MFGM during heating, leaving openings through which proteins can be absorbed by the fat exposed (Dalgleish & Banks, 1991). Ye et al. (2004) found that the adsorption of some dairy proteins with milk fat globules increases with temperature and processing time in the temperature range studied (65–95 °C). At high temperatures (above 110 °C), there was possibly wide dissociation of MFGM, and

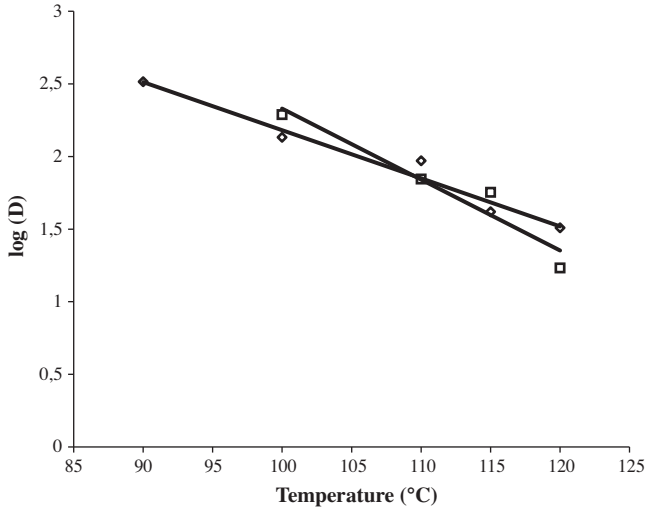

Fig. 2. Decimal reduction time (D) variation of peptide P34 in milk as a function of temperature. (◇) Skimmed milk, regression equation was determined as $\log(D) = -0.0331T + 5.4922 (r^2 = 0.968)$; (□) fat milk, regression equation was determined as $\log(D) = -0.0488T + 7.2134 (r^2 = 0.928)$.

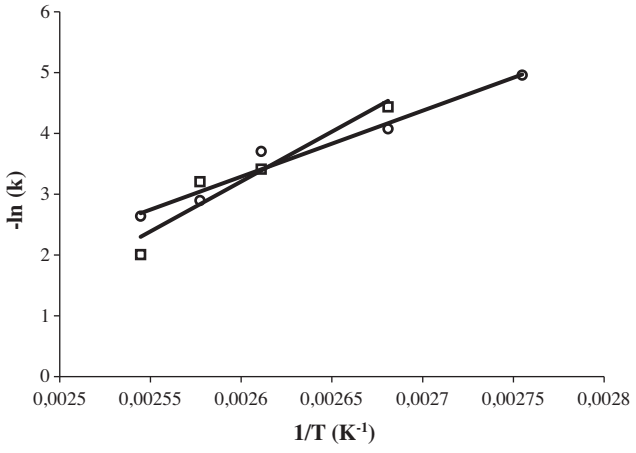

Fig. 3. Arrhenius' plot of thermal inactivation of peptide P34 in milk. (o) Skimmed milk, regression equation was determined as $-\ln(k) = 10,855/T - 24.93 (r^2 = 0.966)$; (□) fat milk, regression equation was determined as $-\ln(k) = 16,403/T - 39.44 (r^2 = 0.922)$.

the peptide P34 was quite exposed to the lipid portion of the fat globules, increasing interaction of the peptide with fat and therefore accelerating the loss of antimicrobial activity. At lower processing temperatures, the results suggest that there was not enough dissociation of MFGM and consequently low exposition of the peptide P34 to the fat content of the globules.

The onset of thermal degradation of many food compounds and many enzymes usually starts only when a certain temperature level is reached (Corradini & Peleg, 2004). The fat globules in milk seem to play a defensive action on peptide P34 against heat treatments at temperatures lower than 110 °C. The presence of fat globules in milk slows the formation of MRPs due to the thermal insulation given by the lipid content and consequently lowers heat transfer in the medium (Pellegrino, 1994; van Boekel, 1998).

Estimation of thermodynamic parameters is essential to understand the probable mechanism of denaturation, which is very important in thermal processes. Thermodynamic inactivation parameters in skimmed and fat milk are shown in Table 2. The *Ea* can be seen as the energy absorbed or released needed to the molecules be able to react (van Boekel, 2008). It can be estimated by analysis of Arrhenius' law expressed in Fig. 3. In milk, the absorption of energy needed to peptide P34 starts the inactivation reaction was higher than in buffer solution. In skimmed milk, *Ea* of peptide P34 was 90 kJ in the range 90–120 °C, and in fat milk it was 136 kJ in the range 100–120 °C. At 90 °C, in fat milk, the energy from the medium was not enough to start the reaction. High activation energy indicates strong temperature dependence, and that reaction will run very slowly at low temperature, but relatively fast at high temperatures (van Boekel, 2008).

From Table 2, it is observed the increasing of $\Delta H^{\#}$ and decreasing of $\Delta G^{\#}$ with increasing temperatures. The $\Delta S^{\#}$ values present a heterogeneous behaviour, which could result from the difficult evaluation of system disorder in such a small temperature variation. Values of $\Delta H^{\#}$ and $\Delta S^{\#}$ were higher, and $\Delta G^{\#}$ was quite similar comparing the peptide behaviour in buffer and milk. Higher values of E_a could indicate an increased stability at higher temperatures. However the peptide was inactivated faster at major temperatures in milk, probably due to the usual dominant role of $\Delta S^{\#}$ in the thermal inactivation of proteins in aqueous solutions (Bromberg, Marx, & Frishman, 2008). Protein unfolding results in a less organised molecule, due to the disruption of many relatively weak non-covalent bonds. As the $\Delta H^{\#}$ and $\Delta S^{\#}$ are parameters that provide a measure of the number of non-covalent bonds broken and the net enzyme/solvent disorder change associated with the formation of the transition state (Ortega, de Diego, Perez-Mateos, & Busto, 2004), it is suggested that the decrease in the *k*-values, or the increase in $\Delta S^{\#}$ and $\Delta H^{\#}$ values, are more reliable criteria to observe the heat degradation of bacteriocin. $\Delta H^{\#}$ values were higher in fat milk than in skimmed milk, what follows the higher *Ea* in fat milk. The increase of the $\Delta S^{\#}$ in fat and skimmed milk, compared to the buffer solution, compensates the high inactivation barrier, which causes the $\Delta G^{\#}$ to be low enough and the inactivation process to occur relatively fast (Ustok, Tari, & Harsa, 2009). In general, activation entropy has a dominant role in thermal inactivation of proteins in aqueous solutions (Bromberg et al., 2008).

Foods are unstable in the thermodynamic sense, which means that they have the tendency to change from a low-entropy, high enthalpy state to a high-entropy, low enthalpy state. Foods are so complex systems that there is a real concern in applying models directly to food when these models are based on fundamental reactions studied in model systems (van Boekel, 2008). Kinetics of thermal inactivation directly in real foods or in industrial scale are important to promote correctly and accurately use of bacteriocins in food industry, thus additional studies are essential to achieve this purpose.

Table 2
Thermodynamic parameters for heat inactivation of peptide P34 in skimmed and fat milk.

Medium	*Ea* (kJ/mol)	*T* (K)	$\Delta H^{\#}$ (kJ/mol)	$\Delta G^{\#}$ (kJ/mol)	$\Delta S^{\#}$ (J/mol K)
Buffer[a]	72	393	68.82	106.43	−95.69
		388	68.87	106.54	−97.10
		383	68.91	105.37	−95.21
		373	68.99	105.71	−98.44
		363	69.07	103.36	−94.45
Skimmed milk	90	393	86.94	105.73	−4.78
		388	86.98	105.16	−4.69
		383	87.02	106.34	−5.04
		373	87.11	104.63	−4.70
		363	87.19	104.41	−4.74
Fat milk	136	393	133.04	103.66	7.48
		388	133.08	106.16	6.94
		383	133.13	105.41	7.24
		373	133.21	105.75	7.36
		363	133.29	–	–

[a] Values from Sant'Anna et al. (2010) included for comparison.

4. Conclusion

Based on an isothermal experiment in the temperature range of 90–120 °C and using Arrhenius equation, the thermal inactivation of the peptide P34 in skimmed and fat milk can be explained by the first-order model. The presence of dairy solids, mainly fat content, decreased thermal stability of peptide P34 at temperatures above 110 °C. At temperatures below that, the bacteriocin was protected by the solid matrix of milk. *D*-, *z*- and *k*-values calculated indicates that the peptide P34 is heat stable in milk systems and also that it can be utilised in pasteurisation conditions, maintaining part of its biological activity. More studies about kinetics of thermal inactivation of antimicrobial peptides are necessary to allow their proper utilisation as natural biopreservatives in the food industry.

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