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Kinetic Modeling of the Thermal Inactivation of Bacteriocin-Like Inhibitory Substance P34

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Optimization of thermal processes relies on adequate degradation kinetic models to warrant food safety and quality. The knowledge on thermal inactivation kinetics of antimicrobial peptides is necessary to allow for their adequate use as natural biopreservatives in the food industry. In this work, thermal inactivation of the previously characterized bacteriocin-like inhibitory substance (BLIS) P34 was kinetically investigated within the temperature range of 90−120 °C. *Listeria monocytogenes* ATCC 7644 was used as the indicator microorganism for antimicrobial activity. Applicability of various inactivation models available in the literature was critically evaluated. The first-order model provided the best description of the kinetics of inactivation over the selected temperatures, with k values between 0.059 and 0.010 min^{-1}. D and k values decreased and increased, respectively, with increasing temperature, indicating a faster inactivation at higher temperatures. Results suggest that BLIS P34 is thermostable, with a z value of 37.74 °C and E_a of 72 kJ mol^{-1}.

KEYWORDS: Kinetic modeling; thermal inactivation; bacteriocin-like inhibitory substance P34; thermodynamic activation parameters

INTRODUCTION

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria that are active against other bacteria, in either the same species (narrow spectrum) or across genera (broad spectrum), and as defense peptides, cell-signaling mechanisms can also be involved (*1*). Their proteinaceous nature implies a putative degradation in the gastrointestinal tract of man and animals, suggesting that some bacteriocin-producing lactic acid bacteria (LAB) or purified bacteriocins could be used as natural preservatives in foods (*2*, *3*). Those produced by LAB are largely studied with the perspective to search for safe and food-grade preservatives of biological origin (*4*). Nisin is the most well-characterized bacteriocin, and its use in food is permitted in more than 40 countries (*3*).

The genus *Bacillus* includes a variety of industrially important species and has a history of safe use in both the food and pharmaceutical industry (*5*). Despite the intensive work on bacteriocins produced by LAB, several bacteriocins or bacteriocin-like inhibitory substances (BLISs) have been described for *Bacillus* species, including BLIS P40, cerein 8A, and thuricin 7 (*6*−*8*), presenting a broad spectrum of antibacterial activity. *Bacillus* sp. P34, an isolate from the Amazon basin, produces a BLIS that displays antibacterial activity against important food-borne pathogenic bacteria, such as *Listeria monocytogenes* and *Bacillus cereus*. This antimicrobial substance maintains its activity within a broad range of pH and presents thermal stability (*9*), which addresses the potential for use in heat-treated foods.

It is often suggested that bacteriocins should not be used as the primary processing step or barrier to prevent the growth or survival of pathogens but rather that they could provide an additional hurdle to reduce the likelihood of food-borne disease (*10*). The combination of heat treatment with bacteriocins is an interesting technique to obtain safe food with minimal destruction of food components, such as flavor, color, or nutrients, during processing. Nisin has been used in combination with heat to inactivate important food pathogens and spoilage microorganisms (*11*, *12*).

Thermal processing continues as the most widely used method employed for food preservation and shelf-life extension (*13*). However, excessive heating may produce considerable losses in the quality and, particularly, in the sensory properties of foods. Thermal processing methods emphasize the achievement of commercial sterility while minimizing changes in nutritional value and eating quality (*14*). In this context, recent developments in food processing have aimed at technologies that may result in minimal damage to nutrients and sensory components by reducing heating times and optimizing heating temperatures (*13*). Therefore, the combination of antimicrobial peptides with heat treatment must receive great emphasis to be adopted as an alternative to food conservation, reducing losses of bioactive components.

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Mathematical models consist of equations that provide an output based on set input data. It is a concise way to express the physical behavior in mathematical terms. The purpose of inactivation mathematical modeling is to assess the effect of different heat treatments on residual activity without performing numerous trial runs (*15*). Inactivation modeling has been described for microorganisms, food compounds, and enzymes (*12*, *16*, *17*). Considering that inactivation usually proceeds through a network of reactions including denaturation, aggregation, coagulation, and chemical decomposition, the kinetic models are based on different mechanisms: first-order, consecutive reactions, and parallel reactions (**Table 1**).

Modeling of thermal inactivation of antimicrobial peptides has not been found in the literature. In a recent experimental study, kinetic and thermodynamic parameters were determined for thermal inactivation of the bacteriocin cerein 8A, following a first-order model (*18*). This study was therefore undertaken to determine the best heat inactivation model for BLIS P34. On this basis, temperature dependence and thermodynamic parameters for thermal inactivation were determined.

MATERIALS AND METHODS

Bacterial Strains and Media. The producer strain was *Bacillus* sp. P34, isolated from fish (*Leporinus* sp.) of the Brazilian Amazon basin (*9*). The indicator strain was *L. monocytogenes* ATCC 7644. Strains were maintained in brain heart infusion (BHI) medium (Oxoid, Basingstoke, U.K.) containing 20% (v/v) glycerol at −20 °C. The cultivation of strains was performed aerobically.

Production of the Antimicrobial Substance. The BLIS produced by *Bacillus* sp. P34 was isolated as described elsewhere (*9*). *Bacillus* sp. P34 was cultivated aerobically in 500 mL Erlenmeyer flasks containing 200 mL of BHI broth at 30 °C and 180 rpm for 24 h. Cells were harvested by centrifugation at 10000*g* for 15 min at 12 °C, and the resulting supernatant was filtered through cellulose membranes (pore diameter of 2.2×10^{-4} mm; Millipore, Bedford, MA). The culture filtrate was submitted to precipitation with ammonium sulfate to 20% saturation. The resulting pellet was suspended in 10 mM sodium phosphate buffer at pH 7.0, applied to a gel filtration column (Sephadex G-100, Pharmacia Biotech, Uppsala), and eluted with 10 mM sodium phosphate buffer at pH 7.0. Fractions with antimicrobial activity were stored at 4 °C until used for the antimicrobial assay.

Antimicrobial Activity Assay. The antimicrobial activity was detected by the agar disk diffusion assay (*19*). Aliquots of 0.02 mL were applied to 6 mm cellulose discs onto agar plates previously inoculated with a swab submerged in a suspension of *L. monocytogenes* ATCC 7644, which corresponded to a 0.5 McFarland turbidity standard solution (10^8 CFU mL^{-1}). Plates were incubated at 37 °C. The bacteriocin titer was determined by the modified serial 2-fold dilution method (*19*). The reciprocal value of the highest dilution where an inhibition zone was observed was taken as activity units mL^{-1} (AU mL^{-1}).

Thermal Inactivation. Thermal inactivation of BLIS P34 was determined as described elsewhere (*18*). Bacteriocin solutions (1.0 mL) were heated in sealed tubes (thickness of 1 mm, internal diameter of 7 mm, and length of 3 cm) at 90, 100, 110, 115, and 120 °C, in a thermostatically controlled dry bath (Accublock, Labnet International, Inc., Woodbridge, NJ). Tubes were withdrawn at each time interval, immediately immersed in an ice bath, and antimicrobial activity was determined as described above. The activity after 1 min of heating time ($t = 0$) was considered to be the initial activity, thereby eliminating the effects of heating. Assays were performed in duplicate. Residual antimicrobial activity with respect to processing time at different processing temperatures was fit to several models (**Table 1**) using nonlinear regression (Statistica 7.0, StatSoft, Inc., Tulsa, OK).

Kinetic Analysis. Several models available in the literature to describe thermal inactivation are presented in **Table 1**. In the model equations, A represents antimicrobial activity (AU mL^{-1}) at time t (min), A_0 is the initial activity (AU mL^{-1}), and k (min^{-1}) is the reaction rate constant at a given temperature. First-order kinetics (eq 3) have been reported to describe thermal inactivation of cerein 8A (*18*) and several enzymes (*20*, *21*). The temperature dependence parameters for the first-order model are given by the Arrhenius equation, which describes the relationship of the thermal inactivation rate (k) and temperature

$$\ln(k) = \ln(A) - \frac{E_a}{RT} \quad (1)$$

where A is the Arrhenius constant, E_a is the activation energy, R is the universal gas constant, and T is the absolute temperature.

For enzymes, there are kinetic equations that suggest that their activity loss could be described by the summation of two exponential decays, one for the hypothesized heat-labile enzyme and the other for the hypothesized heat-stable enzyme (eqs 5−7). These models were tested to check if BLIS P34 has a labile and resistant heat fraction. A_L and A_R are the residual activities for the labile and resistant heat fractions, respectively. k_L and k_R are the first-order reaction rate constants for the labile and resistant heat fractions, respectively. The coefficient a represents the active fraction of the thermal-labile group in relation to the total activity (*22*, *23*). Fractional conversion refers to a first-order inactivation process and takes into account the non-zero enzyme activity upon prolonged heating because of the presence of an extremely heat-resistant fraction (*24*). n is the order of the reaction in nth-order model.

The Weibull distribution pattern (*25*) is based on the assumption that, under the conditions examined, the momentary rate of thermal sensitivity to heat is only a factor of the transient heating intensity and residual activity but not the rate at which the residual activity has been reached (*26*). Equation 4 represents the cumulative form of the Weibull distribution. The Weibull rate parameter b is characteristic of each reaction and emulates the thermal reaction rate. Its temperature dependence can be expressed by a log−logistic equation (*27*), according to eq **2**

$$b(T) = \log_e\{1 + \exp[k'(T - T_c)]\} \quad (2)$$

where T_c marks the temperature level where inactivation starts to accelerate and k' is the slope of $b(T)$.

Table 1. Kinetic Equations Used To Analyze Inactivation of BLIS P34

equation	model	equation	reference
3	first order	$\frac{A}{A_o} = \exp(-kt)$	*20*
4	Weibull distribution	$\frac{A}{A_o} = \exp(-bt^n)$	*25*
5	nth order	$\frac{A}{A_o} = \{A_o^{1-n} + (n-1)kt\}^{1/(1-n)}$	*21*
6	two fraction	$\frac{A}{A_o} = a\exp(-k_L t) + (1-a)\exp(-k_R t)$	*22*
7	fractional conversion	$\frac{A}{A_o} = A_r + (A_o - A_r)\exp(-kt)$	*24*

Comparison of Kinetic Models. For a comparison of fits obtained with nonlinear regression, statistical and physical criteria were considered. Statistical criteria include the coefficient of determination (r^2), χ^2, and Akaike information criterion (AIC) (*28*).

χ^2 was used to compare models of thermal inactivation of enzymes (*27*, *29*) and is given by

$$\chi^2 = \frac{\sum (a_{measured} - a_{predicted})^2}{(n-p)} \quad (8)$$

When models with different numbers of parameters are compared, the residual sum of squares does not give enough information to discriminate between these models. The minimum-corrected AIC produces ranking of parsimonious models when the number of experimental

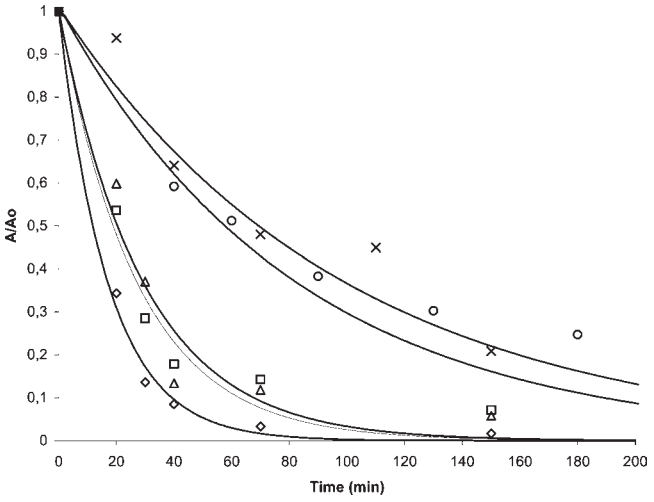

Figure 1. Thermal inactivation of BLIS P34 at 90 °C (○), 100 °C (×), 110 °C (△), 115 °C (□), and 120 °C (◇). Data presented are average values of two independent experiments. The standard deviations were always lower than 8%. Data were fitted to a first-order model.

data are small or when the number of fitted parameters are a moderate to large fraction of the number of data. This optimization criterion compares models by their sum of squares, corrected for the number of parameters (*28*).

AIC is the comparing statistical method of thermal inactivation for enzymes (*29*, *30*) and is defined as (*30*)

$$\mathrm{AIC} = n \ln\left(\frac{\sum (a_{\mathrm{measured}} - a_{\mathrm{predicted}})^2}{n}\right) + \frac{n(n+p)}{n-p-2} \qquad (9)$$

where n is the number of observations and p is the number of parameters.

The model with the lowest $\chi^2$and AIC and higher r^2 for the residual antimicrobial activity is the best choice from a statistical point of view. Estimation of negative parameters is a criterion to reject the inactivation model.

RESULTS AND DISCUSSION

BLIS P34 was previously reported as inhibitory to a broad range of indicator strains, including pathogenic and spoilage organisms, such as *L. monocytogenes*, *B. cereus*, and clinical isolates of *Aeromonas hydrophila*, and was stable for up to 100 °C and within a wide range of pH values (*9*). In this study, isothermal inactivation treatments of peptide P34 were evaluated, and residual activities were fitted for different thermal inactivation models. The results obtained for thermal inactivation at 90, 100, 110, 115, and 120 °C are shown in **Figure 1**, where an exponential behavior can be observed. As expected, antimicrobial activity decreased as the heating time increased, as well as at higher temperatures. Mass spectrometry analysis showed that BLIS P34 has a molecular mass of 1456 Da (*31*), and these results resemble the heat stability of some small antimicrobial peptides from *Bacillus* (*32*).

Several thermal inactivation kinetic equations were tested to fit the experimental data. Models that suggest that there is a labile and resistant fraction were also tested. The *n*th-order (eq 5) inactivation did not yield good r^2 values at high temperatures, and the AIC and χ^2 values obtained were quite higher compared to other models (**Table 2**). For the two-fraction model (eq 6), results showed that inactivation rate parameters for both fractions were the same, indicating single-step inactivation of the antimicrobial peptide. The fractional conversion (eq 7) model cannot explain the inactivation of BLIS P34, because parameter values obtained on the statistical analysis were negative. Thus, these models were rejected for modeling thermal inactivation of BLIS P34.

Discussion and validation of the applicability and usefulness of the Weibull distribution model have been recently published (*33*−*35*). It has been proposed to explain thermal inactivation of enzymes (*17*) and microorganisms (*36*); therefore, the Weibull distribution was tested for its applicability to the experimental data of BLIS P34 inactivation. First-order and Weibull distribution models gave a good fit for the data, with very similar r^2, χ^2, and AIC values. The r^2 values for the first-order model ranged from 0.899 to 0.995 (model fitted to the data is showed in **Figure 1**), while the r^2 values for the Weibull distribution model ranged from 0.898 to 0.997. The AIC values for first-order were lower than those for the Weibull pattern, ranging from −31 to −15 and from −24 and −11, respectively. The χ^2 ranged from 0.0006 to 0.0095 for the first-order model and from 0.0002 to 0.012 for the Weibull pattern, indicating a similar fit for both models. The values obtained for the criteria analyzed were very similar for both models, which does not allow for reliable choosing between them. Thus, dependence temperature parameters obtained for first-order (k values) and Weibull distribution (b values) models were fitted for Arrhenius (eq **1**) and log−logistic (eq **2**) equations, respectively. The r^2 for the Arrhenius equation gave a good fit to the data, with a value of 0.94 (**Figure 2**). For Weibull model parameters, the log−logistic equation had a very low r^2 value (0.10), as shown in **Figure 3**. This poor fit for the Weibull pattern to the experimental data indicates that the first-order model is the best model to explain the thermal inactivation for BLIS P34.

In heat processing, it is common to characterize first-order reactions in terms of D and z values (thermal death time concepts). The decimal reduction time (D value) is the time needed for a 10-fold reduction of the initial activity at a given temperature. It is obtained by plotting the antimicrobial activity values on a log scale against the corresponding inactivation times. The z value is the temperature needed to reduce the D value one log unit and is obtained by plotting the D values on a log scale against the corresponding temperatures (*37*).

Table 3 shows results obtained for k, D, and z values for BLIS P34 for heat treatments between 90 and 120 °C. The rate constant increased with the higher heating temperatures, and D values decreased with increasing temperature, indicating a faster inactivation at higher temperatures. It can be observed that D values are between 39 and 226 min at temperatures between 90 and 120 °C. The effect of the temperature on D values is shown in **Figure 4**, and the calculated z value for the range of temperatures studied (90−120 °C) was approximately 38 °C (**Table 3**).

The z values for cooking and nutrient degradation (25−45 °C) are generally greater than that for microbial inactivation (7−12 °C) (*13*). In fact, differences between the D and z values of microorganisms and nutrients are exploited to optimize thermal processes and can be exploited also to maintain antimicrobial activity after treatment. D and z values obtained for BLIS P34 indicate that it can be used in conditions of high-temperature short-time (HTST) pasteurization (often 72 °C for 15 s) or even in conditions of canned food sterilization, maintaining part of its antimicrobial activity.

To determine the industrial potential of bacteriocins, it is essential to understand their structure−stability relationships. Therefore, estimation of thermodynamic parameters helps to understand the probable mechanism of denaturation, which is very important in heat processes. The logarithm representation of the inactivation rate against the reciprocal temperature allows us to calculate the activation energy (E_a), comparing the experimental data equation to the Arrhenius equation (eq **1**). Activation

Table 2. Summary of Performance of Selected Models To Describe Inactivation of BLIS P34

model (equation)	r^2	χ^2	AIC	remarks
first order (3)	[0.899; 0.995]	[0.0006; 0.0095]	[−31; −15]	accepted
Weibull distribution (4)	[0.898; 0.997]	[0.0002; 0.012]	[−29; −11]	poor fit for dependence temperature parameters; rejected
*n*th order (5)	[0.47; 0.99]	[0.0002; 0.126]	[−32; 37]	low values for r^2 and high for AIC and SE; rejected
two fraction (6)	[0.959; 0.995]	[0.0006; 0.0147]	[−24; 24]	$k_L = k_R$; rejected
fractional convertion (7)	[0.908; 0.998]	[0.0002; 0.014]	[−31; 24]	negative parameters estimates; rejected

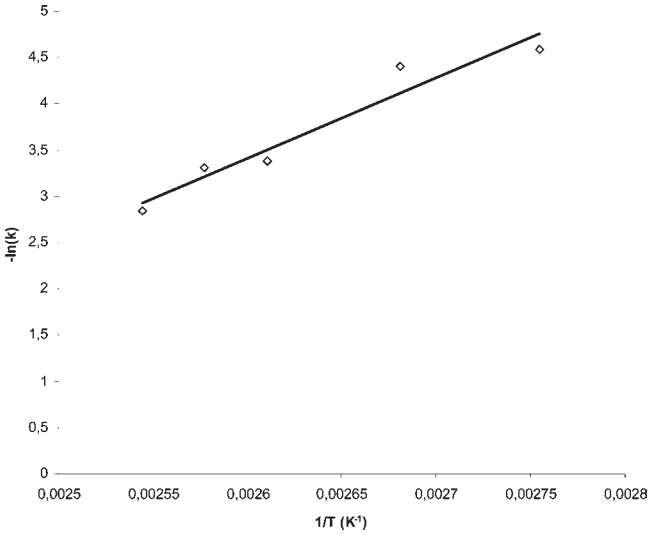

Figure 2. Arrhenius plot of inactivation rates of BLIS P34. The regression equation was determined as $y = -8671.1x + 191.135$ ($r^2 = 0.938$).

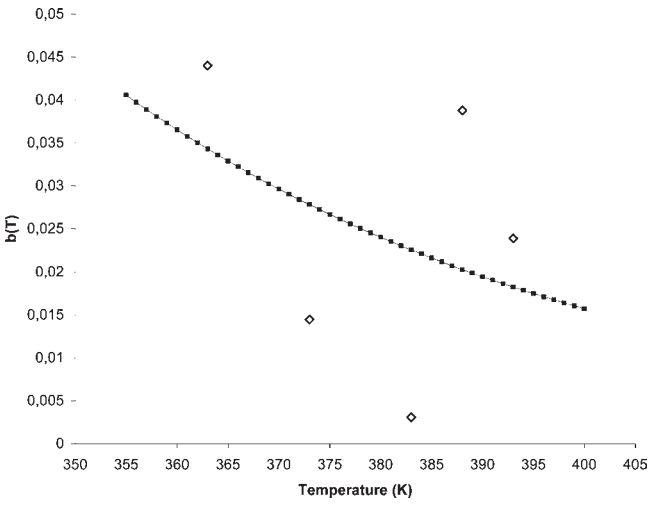

Figure 3. Dependence of the Weibull distribution coefficient *b* as a function of the log−logistic function. The regression equation was determined as $b(T) = \ln(1 + \exp(-0.02132(T - 205.643))$ ($r^2 = 0.10$).

enthalpy ($\Delta H^{\ddagger}$), free energy of inactivation ($\Delta G^{\ddagger}$), and activation entropy ($\Delta S^{\ddagger}$) can be calculated as described elsewhere (*18*). Inactivation is known to be a process where the secondary, tertiary, or quaternary structure of a protein changes without breaking covalent bonds (*38*). The changes in enthalpy and entropy, for the thermal inactivation of BLIS P34, which have been calculated using the transition-state theory, are presented in **Table 4**. Because the $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are heat and entropy change, respectively, these two parameters also provide a measure of the number of noncovalent bonds broken and the net enzyme/solvent disorder change associated with the formation of the transition state (*39*).

Table 3. Kinetic Parameters of Thermal Inactivation of BLIS P34

temperature (°C)	k (min^{-1})	r^2	$t_{1/2}$ (min)	D (min)	z (°C)
120	0.0586 ± 0.001[a]	0.9955	11.83	39.30	
115	0.0368 ± 0.002	0.9722	18.86	62.66	
110	0.0340 ± 0.001	0.9595	20.37	67.66	37.74
100	0.0123 ± 0.003	0.8979	56.58	187.97	
90	0.0102 ± 0.001	0.9583	68.29	226.86	

[a] Standard error of regression (95% confidence interval).

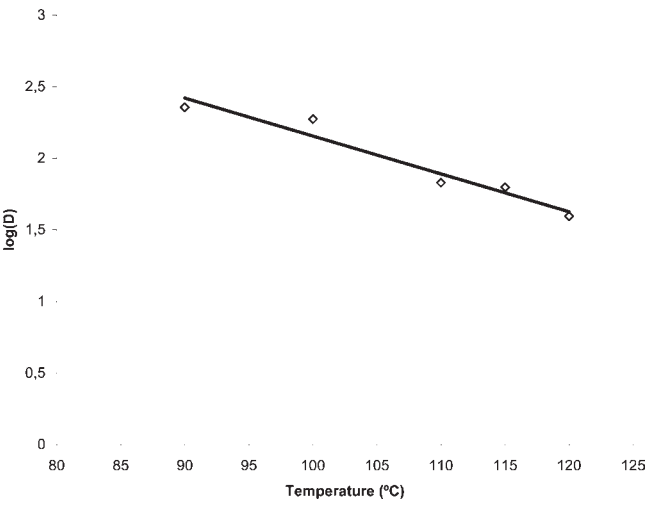

Figure 4. Variation of the decimal reduction time (*D*) with temperature for BLIS P34. The regression equation was determined as $y = -0.0265x + 4.81$ ($r^2 = 0.944$).

Table 4. Thermodynamic Parameter Values of Thermal Inactivation of BLIS P34 Activity

temperature (K)	$\Delta H^{\ddagger}$ (kJ mol^{-1})	$\Delta G^{\ddagger}$ (kJ mol^{-1})	$\Delta S^{\ddagger}$ (J mol^{-1} K^{-1})
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

The activation energy can be seen as the energy barrier that molecules need to cross to be able to react. The proportion of molecules able to do that increases with temperature, which qualitatively explains the effect of temperature on rates (*15*). For BLIS P34, it was necessary to absorb 72 kJ from the external medium to start its inactivation at temperatures between 90 and 120 °C. Cerein 8A, a relatively thermostable bacteriocin, needs 105.7 kJ to begin its inactivation (70−82 °C) (*18*). Foods are unstable in a 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 (*15*). The denaturation of many proteins, such as chicken egg albumin (*40*) and lactoglobulin, at low temperatures and in the presence of urea (*41*) shows

a negative $\Delta S^{\ddagger}$ because water ordering increases in the vicinity of nonpolar amino acids, which are exposed during unfolding (*42*). This ordering of water around hydrophobic residues is disrupted at higher temperatures (*43*). The negative entropy of activation ($\Delta S^{\ddagger}$) observed for invertase suggested that there was negligible disorderness at that of α-glucosidase from *Aspergillus wentii* (*44*). Protein unfolding is accompanied by the disruption of many relatively weak noncovalent bonds that form the folded structure and results in a less organized system. The increase of the activation entropy compensates for the high inactivation barrier, which causes the Gibbs free energy to be low enough and the inactivation process to occur relatively fast. The activation energies evaluated for immobilized enzymes in the solid matrix samples are much lower than those obtained in the aqueous solutions. In general, activation entropy has a dominant role in protein thermal inactivation in aqueous solutions (*45*). BLIS P34 may have intramolecular dissulfide bonds in the structure (*9*). This agrees with the fact that the inhibitory compound was thermostable.

Antimicrobial peptides may be used in combination with heat to obtain safe food with minimal destruction of food components, such as flavor, color, or nutrients, during processing. Adequate kinetic models of thermal destruction are essential to design new processes assuming a safe food product and giving a maximum retention of quality factors (*46*, *47*). However, it is necessary to point out that thermal inactivation was studied for BLIS P34 in buffer solution, and in a food system, this substance could be more stable because of the protective effect of food proteins or could react with other food compounds during heat processing. Some antimicrobial peptides may be inactivated by endogenous food enzymes or binding to components of the food matrix, which has been described for nisin, sakacin P, and lactoferricin B (*3*, *48*, *49*). In this concern, the knowledge about kinetics and thermodynamics of heat inactivation of antimicrobial peptides is an essential step to warrant their safe use.

In conclusion, the thermal residual activity curve indicates partial loss of the antimicrobial activity of BLIS P34 at high temperatures. A successful modeling will enable the processors to modulate their process to achieve desirable antimicrobial activity in the end of the thermal process. The first-order function can therefore be recommended to describe the heat inactivation kinetics of BLIS P34, provided that it satisfies the statistical and physical criteria of implementation.

On the basis of an isothermal experiment in the temperature range of 90−120 °C and using the Arrhenius equation, the thermal inactivation of BLIS P34 is satisfactorily explained by the first-order model. D, z, and k values calculated by nonlinear regression indicate that peptide P34 is a thermostable substance with a z value of 38 °C and E_a of 72 kJ mol^{-1}. More studies about kinetics of thermal inactivation of antimicrobial peptides are necessary to allow for their proper use as natural biopreservatives in the food industry.

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