

A Calorimetric Study of Thermal Denaturation of Recombinant Human Lactoferrin from Rice

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Thermal denaturation of recombinant human lactoferrin from transgenic rice with different degrees of iron saturation has been studied by differential scanning calorimetry. The maximum temperature, enthalpy change, and activation energy of denaturation were higher when recombinant lactoferrin was more saturated with iron, indicating an increase in the stability of the protein structure. Maximum temperature and activation energy values for apo- and holo-lactoferrins were practically identical to those reported for the same forms of lactoferrin from human milk, which indicates a similar thermal stability. However, the value of enthalpy change for denaturation of the recombinant lactoferrin was 2.5–3-fold lower than that found for the human milk protein. This finding may reflect the influence that the different glycosylation pattern may have in the relationship between lactoferrin domains. Denaturation of recombinant lactoferrin in milk was compared with denaturation in phosphate buffer, and results indicated that the protein was more heat-sensitive when treated in milk than in buffer.

KEYWORDS: Recombinant human lactoferrin; heat denaturation; calorimetry

INTRODUCTION

Lactoferrin is a glycoprotein that belongs to the family of iron-binding proteins that also includes transferrin, ovotransferrin, and melanotransferrin (1). It has a molecular mass of 80 kDa, and its three-dimensional structure has been determined by crystallographic analysis (2). It consists of a polypeptide chain, which forms two globular lobes with two domains each. There is one iron-binding site located in each lobe, and the iron atoms bound are coordinated by four protein ligands: two tyrosines, one histidine, and one aspartate. In the binding site, a CO_3^{2-} or HCO_3^- ion adjacent to an arginine side chain also participates (2).

The ability to bind iron gives lactoferrin several biological activities such as protection from pathogens by reducing the availability of iron to microorganisms, regulation of iron absorption in the intestine, or inhibition of oxidative reactions (3). On the other hand, there are other functions attributed to lactoferrin for which the level of iron bound is not essential, such as the bactericidal activity of lactoferrin by interacting with the bacterial surface (4), the modulation of some functions of the immune system (5), the promoting activity on cellular growth (6), or the antitumoral activity (7).

Human milk is particularly rich in lactoferrin; however, bovine milk contains very low levels of this protein (3). Because milk products, based on bovine milk, are practically devoid of lactoferrin, supplementation with this protein to make infant

formula more similar to human milk would be interesting. Up to now, bovine lactoferrin obtained from whey produced in the cheese-making process has been used as a supplement in special products. However, it is still not clear whether the activity of bovine lactoferrin is the same as that of human lactoferrin for all of the proposed functions. Therefore, the possibility of using human lactoferrin in products for human consumption would be interesting. Taking into account the difficulty in obtaining human lactoferrin in high amounts, some systems have been developed to produce recombinant human lactoferrin (rhLF), such as fungi cultures (8), transgenic cows (9), or plants such as tobacco (10), maize (11), and rice (12). The possibility of using the transgenic rice directly, without isolating lactoferrin, in some special products is very interesting because rice is a normal component of children's diet and it is a hypoallergenic food (12).

It is important to determine the thermal stability of rhLF to design treatments that ensure that the structure and biological activity of the protein are maintained. Although it has been reported that rhLF from rice possesses similar biological activities and a similar structure to human milk lactoferrin (12, 13), there are differences in the glycosylation pattern, which could be important in the behavior of the protein (14). Differences have also been reported in the glycans of the different transferrins (15) and also in the lactoferrin isolated from leucocytes as compared with that from milk (16). Furthermore, recombinant lactoferrin from *Aspergillus awamori* also has different glycan structures from those of lactoferrin from human milk (8). It has been reported that the unglycosy-

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lated lactoferrin is much more susceptible to degradation (17), and some studies have revealed that the susceptibility of lactoferrin to tryptic proteolysis depends on the type of bound glycans (18). Actually, human lactoferrin is more resistant to proteolysis than bovine lactoferrin due to differences in the glycan composition (19).

Thermal stability of lactoferrin has been studied in several works (20–24). The thermal parameters found for the thermal denaturation of bovine lactoferrin studied by calorimetry were lower (22) than those obtained for human lactoferrin (25), which indicates that the bovine protein is not as stable as the human one. We have reported in a previous work (25) that the behavior of rhLF from *A. awamori* is similar to lactoferrin from human milk when subjected to calorimetry, which reflects the high degree of structural similarity between the two proteins. Several works have demonstrated that the iron bound to lactoferrin confers more resistance to thermal denaturation (20, 22) and proteolytic digestion (26) to the protein structure.

The objective of this work was to determine the thermal stability of rhLF from rice by differential scanning calorimetry (DSC) in different conditions, to compare the results with those of lactoferrins from other origins and to facilitate the design of heat treatments that will maintain its integrity and biological activity.

MATERIALS AND METHODS

rhLF isolated from rice was kindly provided by Ventria Bioscience (Sacramento, CA). It was supplied in three forms, apo (0.05 mg Fe/g LF), holo (1.3 mg Fe/g LF), and as-isolated from rice (0.98 mg Fe/g LF). These proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and showed a main band that corresponded to a protein with a molecular mass of about 80 kDa; therefore, they were used in the calorimetric experiments without further purification.

Solutions of the different forms of rhLF described above were prepared in a buffer containing 15 mM potassium phosphate and 150 mM NaCl, pH 7.4 (phosphate-buffered saline, PBS), at a protein concentration of 100 mg/mL. Samples and references (10 μ L) were introduced into aluminum pans (TA Instruments, New Castle, United States) and sealed for analysis. The references consisted of pans containing the same volume of PBS or bovine skimmed milk.

DSC of proteins was performed in a DuPont thermal analyzer (model DSC 10, Nemours, Germany), using a thermal analyst 2000 system. DSC scans were programmed in the temperature range of 35–110 °C and at heating rates of 2, 3, 4, 5, 7, 10, and 20 °C/min. Samples were analyzed at least in triplicate. After the treatment of proteins, denatured samples were left to cool at room temperature and rescanned in the same conditions to evaluate if there was renaturation of the proteins analyzed.

From the transition peak obtained by DSC, it was possible to obtain several thermodynamic parameters. The enthalpy change of denaturation was calculated by integrating the peak area using a straight baseline drawn from the onset to the end of thermal transition. Values of temperature of maximum heat absorption (T_{\max}), onset temperature (T_s), and enthalpy change (ΔH_{cal}) of denaturation were plotted as a function of heating rate, and the same parameters were estimated by extrapolation to 0 °C. The measurement of the endothermic peak width at its half-height ($\Delta T_{1/2}$) was used to calculate the van't Hoff enthalpy (27):

$$\Delta H_{\text{VH}} = 4RT_{\max}^2/\Delta T_{1/2} \quad (1)$$

where R is the universal gas constant and T_{\max} is the maximum peak temperature. To check the irreversibility of the denaturation process, the value of the ratio $\Delta H_{\text{VH}}/\Delta H_{\text{cal}}$ was determined (28).

The kinetic parameters for denaturation were calculated by Kissinger's method (29). This method is based on the relationship between

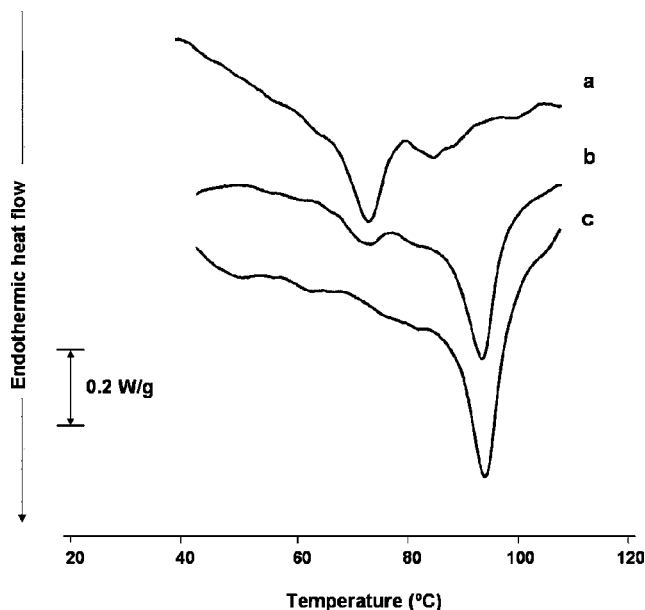


Figure 1. DSC thermograms of rhLF from rice as apo-form (a), as isolated form (b), and as holo-form (c). Scanning was performed at a heating rate of 10 °C/min.

the variation of the maximum heat temperature and the heating rate according to this expression:

$$(\beta/T_{\max}^2) = (AE_a/R)e^{-E_a/RT_{\max}} \quad (2)$$

where β is the scanning rate, T_{\max} is the maximum peak temperature, E_a is the activation energy, A is the preexponential factor, and R is the universal gas constant. E_a/R was determined from the slope of $\ln(\beta/T_{\max}^2)$ vs $1/T_{\max}$ without assuming any order of reaction. Data obtained were statistically evaluated by t test using the SPSS 13.0 package for Windows.

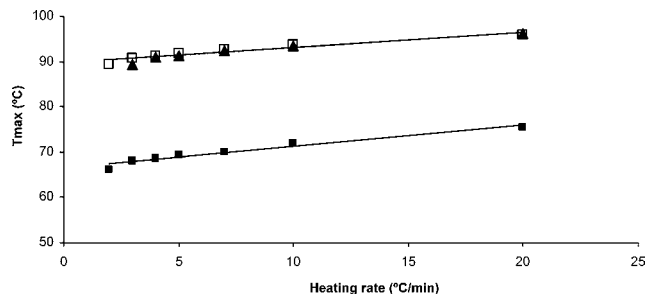
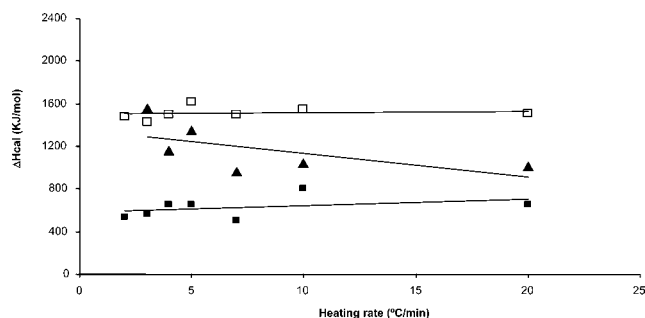
RESULTS

Thermal denaturation of rhLF was studied by DSC using different heating rates. The thermograms obtained for lactoferrin with different degrees of iron saturation at a heating rate of 10 °C/min are shown in **Figure 1**. The comparison between the three forms of lactoferrin is made considering the main peak of each form as the most representative. The apo- and holo-forms of rhLF show one main denaturation peak with differences in position and shape due to the different conformations that present the two forms of lactoferrin. The thermogram obtained for apo-lactoferrin presents a maximum temperature of denaturation at 72 °C, and only at the heating rate of 10 °C/min, a minor peak appears with a maximum temperature at 85 °C. The denaturation thermogram of as-isolated lactoferrin, with 60% iron saturation, shows a main peak coincident with that of holo-lactoferrin and a minor peak with the same maximum temperature as the main peak of apo-lactoferrin. Temperatures of maximum heat absorption and enthalpy change are higher when lactoferrin is more saturated with iron, as shown in **Table 1**. However, the half-peak height of the thermogram of apo-lactoferrin is wider than that of the iron-saturated forms. This fact could indicate that the apo-form presents lower cooperativity in the denaturation process. The van't Hoff enthalpy was calculated considering the maximum peak temperature and width at half-peak height, as has been described previously in the Materials and Methods. The ratio between the calorimetric enthalpy change and the van't Hoff enthalpy ($\Delta H_{\text{cal}}:\Delta H_{\text{VH}}$) was

Table 1. Thermal Parameters for Denaturation of rhLF from Rice in PBS at a Heating Rate of 10 °C/min^a

	T_{\max}^b (°C)	ΔH_{cal}^c (kJ/mol)	$\Delta T_{1/2}^d$ (°C)	ΔH_{VH}^e (kJ/mol)	$\Delta H_{\text{cal}}:\Delta H_{\text{VH}}$
Apo-rhLF	71.92 ± 0.23	801 ± 55	6.28 ± 0.53	634 ± 58	1.27 ± 0.14
As-isolated-rhLF	93.47 ± 0.09	1041 ± 150	5.35 ± 0.48	840 ± 80	1.26 ± 0.27
Holo-rhLF	93.84 ± 0.26	1549 ± 79	5.58 ± 0.26	804 ± 39	1.93 ± 0.19

^a Each value represents the mean ± SD of three or four replicates. ^b Peak maximum temperature. ^c Denaturation enthalpy change. ^d Half-width denaturation peak. ^e Van't Hoff enthalpy of denaturation.

**Figure 2.** Maximum peak temperature (T_{\max}) at different heating rates for denaturation of rhLF from rice in PBS as apo- (■), as isolated (▲), and as holo-forms (□).**Figure 3.** Enthalpy change (ΔH_{cal}) at different heating rates for denaturation of rhLF from rice in PBS as apo- (■), as isolated (▲), and as holo-forms (□).

also calculated. When the ratio $\Delta H_{\text{cal}}:\Delta H_{\text{VH}}$ is around 1, the calorimetric enthalpy change is equal to the van't Hoff enthalpy, which occurs in small single-domain globular proteins (28). However, in proteins in which the conformation is stabilized by interactions among several domains, the ratio $\Delta H_{\text{cal}}:\Delta H_{\text{VH}}$ is greater than 1. Thus, we have found a ratio $\Delta H_{\text{cal}}:\Delta H_{\text{VH}}$ above 1 for all forms of recombinant lactoferrin; for the iron-saturated protein, the ratio was very close to 2.

As shown in **Figures 2** and **3**, the maximum peak temperature and enthalpy change of denaturation are different depending on the heating rate, suggesting that the denaturation process is kinetically determined. The data were fitted to one line with correlation coefficients from 0.92 to 0.94 for the maximum peak temperature and below 0.37 for the calorimetric enthalpy change. For as-isolated rhLF, values of maximum peak temperature and enthalpy change of denaturation at a heating rate of 2 °C/min have not been considered because it was not possible to properly determine the baseline in any of the replicates. The maximum peak temperature parameters clearly increase with the heating rate; however, for the enthalpy change, the increase with the heating rate was slight for the apo-form, and this parameter even decreased for as-isolated lactoferrin. The values of maximum temperature of denaturation, denaturation enthalpy change, and onset temperature obtained by extrapolation to 0 °C/min are shown in **Table 2**. These parameters increased with the iron saturation degree of lactoferrin, mainly for the parameter of the

Table 2. Thermal Parameters for Denaturation of rhLF from Rice in PBS, Extrapolated to 0 °C/min

	T_{\max}^a (°C)	ΔH_{cal}^b (kJ/mol)	T_s^c (°C)
Apo-rhLF	66.47	583	63.10
As-isolated-rhLF	89.36	1361	85.07
Holo-rhLF	89.81	1500	85.45

^a Peak maximum temperature. ^b Denaturation enthalpy. ^c Onset temperature.

Table 3. Comparison of the Thermal Parameters for Denaturation of rhLF from Rice in PBS and in Bovine Skimmed Milk at a Heating Rate of 10 °C/min^a

	T_{\max} (°C)	ΔH_{cal} (kJ/mol)	T_s (°C)
PBS	71.92 ± 0.23	Apo-rhLF	
		801 ± 55	66.00 ± 0.65
milk	66.50 ^b ± 0.39	Apo-rhLF	
		602 ^b ± 130	59.62 ^b ± 1.68
PBS	93.47 ± 0.09	As-isolated-rhLF	
		1041 ± 150	88.32 ± 0.56
milk	91.51 ^b ± 0.07	As-isolated-rhLF	
		951 ± 98	87.51 ^b ± 0.21
PBS	93.84 ± 0.26	Holo-rhLF	
		1549 ± 79	89.10 ± 0.90
milk	91.56 ^b ± 0.10	Holo-rhLF	
		1281 ± 310	87.69 ^b ± 0.17

^a Each value represents the mean ± SD of three or four replicates. ^b Significant difference for $p < 0.05$.

denaturation enthalpy change, which is 2.5 times higher for holo-lactoferrin than for the apo-form.

The denaturation of rhLF in bovine skimmed milk has also been studied, and the results obtained are shown in **Table 3**, together with those obtained in PBS. The values of the maximum peak temperature, denaturation enthalpy change, and onset temperature of lactoferrin heated in bovine skimmed milk were significantly lower for the majority of parameters than those obtained when heated in PBS.

The Kissinger method was used to calculate the activation energy for thermal denaturation of rhLF. The Kissinger plots for the three forms of lactoferrin adjust very well to straight lines, with correlation coefficients from 0.98 to 0.99 (**Figure 4**). The activation energy values were calculated from the slopes of those straight lines. It has been observed that the activation energy increases with the iron saturation degree of lactoferrin; the values obtained were 240.0, 318.6, and 387.4 kJ/mol for the apo, as-isolated, and holo-forms, respectively.

DISCUSSION

In this work, we have studied the thermal behavior of rhLF from rice by DSC. The thermograms obtained showed higher values of maximum peak temperature and enthalpy change when lactoferrin was saturated with iron than when it was in its apo-form. These results indicate an increase in the protein stability with the increase in the degree of iron saturation that has also been found for human milk lactoferrin, rhLF from *A. awamori* (25), and bovine lactoferrin (20, 22). Denaturation of the holo-

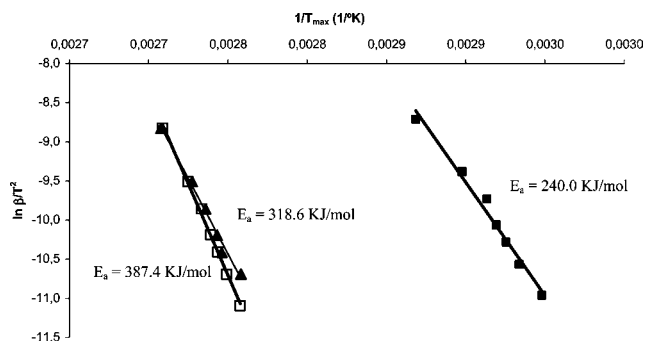


Figure 4. Kissinger plot for heat denaturation of rhLF from rice in PBS as apo- (■), as isolated (▲), and as holo-forms (□). T_{max} is the peak maximum temperature (degrees Kelvin), and β is the scanning rate. E_a = activation energy.

rhLF from rice gave a single peak, which indicates that the two lobes of the protein have the same thermosensitivity and denature simultaneously. The endotherm obtained for denaturation of the apo-form presented a single peak at all heating rates, except for the 10 °C/min heating rate, in which a second small peak appeared at a maximum temperature around 85 °C, a peak that might correspond to aggregated molecules. For as-isolated lactoferrin (60% iron saturation), an endotherm with two peaks has been found, a main peak coincident with that of holo-rhLF and a minor peak coincident with the main peak of apo-rhLF. The finding of a double peak in the endotherm had been previously reported for the 30% iron-saturated bovine lactoferrin and for the holo-bovine lactoferrin (22). The presence of these two peaks has been attributed to differences in the thermostability of the two lobes of lactoferrin (30), since the C-lobe appears more compact than the N-lobe in the iron-saturated protein (31), and it has also been explained by the presence of monoferric species (20).

The maximum temperatures of the endotherm obtained for rhLF from rice in the different forms are very similar to the values that we obtained in previous studies for human milk lactoferrin and for the recombinant protein from *A. awamori* (25), which means that all of the proteins present similar thermal stabilities. However, the value of enthalpy change for denaturation of rhLF from rice is about 2.5 or three times lower, depending on the degree of iron saturation, than that obtained for human milk lactoferrin or for the protein produced in *A. awamori*. This fact could be due to some differences in the structure of the protein. It has been reported that the molecular mass of the recombinant lactoferrin from rice is lower than that of lactoferrin from human milk, 78.5 and 80.6 kDa, respectively (12). This difference is likely the result of a lower degree of glycosylation for the recombinant protein, with 2.9% of glycans, as compared with the milk protein in which glycans represent 5.5% (12, 13). There are also differences in the type of glycans due to the different mechanisms of glycosylation that the vegetable cells have as compared to those of the mammary gland epithelial cells. Thus, lactoferrin from human milk has the typical glycans of mammals, such as α 2-6-linked neuraminic acid, β 1-4-linked galactose, and α 1-6-linked fucose (32), and lactoferrin from rice has the typical glycans of vegetables such as α 1-3-linked fucose and β 1-2-linked xilose (14). Differences in glycosylation have also been found in other rhLFs such as those produced in *A. awamori* (8), in the milk of transgenic cows (9), or in the milk of transgenic mice (33).

Glycans may be involved in intermolecular carbohydrate–carbohydrate or carbohydrate–protein interactions; thus, they can have an influence on the relationship between protein

domains (34). It is known that in the process of protein denaturation there are many endothermic reactions involved (break-up of electrostatic and van der Waals' interactions and hydrogen bonds) and also exothermic reactions (break-up of hydrophobic interactions), which can lower the overall observed enthalpy (35). Furthermore, in the case of large globular proteins, such as lactoferrin, the denaturation state is irreversible and aggregation processes, which are generally exothermic, can take place especially at high concentrations of protein, as those used in the DSC technique. For these reasons, the enthalpy change (ΔH) observed by DSC can vary depending on the denaturation temperature and on hydrophobicity and aggregation processes (35). Interactions among hydrophobic regions could be more strengthened in rhLF from rice than in human milk lactoferrin due to differences in glycosylation, and consequently, their break-up with thermal denaturation could decrease the enthalpy change value.

The analysis of the crystallographic structure of human lactoferrin revealed that the sites of glycan attachment are situated on the surface of the molecule (2). Furthermore, it has been reported that human lactoferrin bears three potential sites for N-glycosylation at Asn¹³⁸ in the N-lobe and Asn⁴⁷⁹ and Asn⁶²⁴ in the C-lobe (17); a study with glycosylation site mutants (18) has shown that Asn¹³⁸ and Asn⁴⁷⁹ are the preferential glycosylation sites. Although it is well-known that glycosylation affects the three-dimensional structure and the dynamics of a protein (34), any study has shown how the variations in position and type of glycans attached may influence the structure of lactoferrin.

A work carried out by van Berkel et al. (17) showed that unglycosylated rhLF from human kidney 293(S) cells was much more susceptible to degradation by trypsin than the glycosylated form. However, another study published by the same group revealed that the susceptibility of the protein to proteolysis was more affected by an altered conformation of glycans rather than an absence of glycosylation (18). It has been reported that the oligosaccharides bound to proteins confer resistance to proteolysis, either because they cause an increase of the protein rigidity or because glycans sterically protect the susceptible sites for proteolytic enzymes (34).

In the studies reported until now, no significant differences in the biological activities have been found between recombinant lactoferrin from rice and lactoferrin from human milk. These proteins bind the same amount of iron at various pH values, they have the same pH dependency in iron release, and they both inhibit the growth of human pathogens (12, 13). Moreover, hLF and rhLF from rice bind to the human colon carcinoma cell line Caco-2; the binding constant is similar for both proteins, although the number of binding sites reported was slightly higher for rhLF, a difference that has been attributed to differences in glycosylation (12).

The calculation of the van't Hoff enthalpy from the width at half-peak height of the transition peak gives information about the complexity of the denaturation process. When this value approaches the actual value of enthalpy obtained by integrating the endotherm, it means that the protein denaturation follows a two-state kinetic model (28). However, in the case of rhLF from rice, the ratio $\Delta H_{cal}:\Delta H_{VH}$ is above 1.0 for the three forms of lactoferrin; for the holo-rhLF, it is almost 2. Those results indicate that partially unfolded intermediates are in equilibrium with the native state during the thermal denaturation process. This occurs in proteins with a conformation that is stabilized by interactions among several domains. The three-dimensional structure of lactoferrin is in agreement with these results, as it

consists of two globular lobes, each of which is organized into two domains with the iron site at the domain interface (2). In the case of lactoferrin from human milk, the values obtained for the ratio $\Delta H_{\text{cal}}:\Delta H_{\text{VH}}$ were close to 4 (25), much higher than those obtained for the recombinant protein from rice, which also reflects differences between them in their thermal behavior.

The activation energy for denaturation of rhLF from rice has been calculated by the method of Kissinger (29). It has been observed that the activation energy increases with the iron saturation degree of lactoferrin, which is in agreement with previous results that show a higher thermal stability for iron-saturated lactoferrin. Although this method is applied to single denaturation reactions, which is not the case of lactoferrin, we have used it to obtain a kinetic approximation to the thermal denaturation of rhLF. The Kissinger plot obtained for rhLF gives a straight line, which indicates a good correspondence with single reactions (36). The values of activation energy obtained for lactoferrin from rice are very close to those obtained for lactoferrin from human milk and for the recombinant protein from *A. awamori* (25), which indicate a very close similarity in their structure.

To determine if the composition of the medium can influence the thermal behavior of rhLF from rice, its denaturation has also been studied by DSC in bovine skimmed milk. Milk subjected to DSC without added proteins did not show any endothermic peak. This is due to the low concentration of proteins in bovine whey and also to the absence of a signal given by casein when subjected to DSC (37). The results obtained for lactoferrin denaturation in milk were compared with those obtained for the protein denatured in PBS. We found that the peak maximum temperature and the onset temperature were significantly lower when lactoferrin was heated in bovine skimmed milk than in buffer. The denaturation enthalpy was also lower for the three forms of lactoferrin when heated in skimmed bovine milk, although the difference was found to be statistically significant only in the case of apo-rhLF. The lower values of thermal parameters obtained for lactoferrin denatured in bovine milk as compared to those in buffer could be due to the pH decrease that milk experiences when it is heated and also to changes in the balance of calcium (22). Similar results were also found for bovine lactoferrin (22), for human milk lactoferrin, and for recombinant lactoferrin produced in *A. awamori* (25). Therefore, the composition of the medium should be taken into account to determine the thermostability of lactoferrin in each product.

The results of this work did not show a different thermal stability of human recombinant lactoferrin from rice when compared to human lactoferrin from milk. However, the lower value of the enthalpy change observed for the recombinant protein may suggest the existence of differences in the relationship between the glycans and the domains of lactoferrin. Further experiments are needed to evaluate whether or not those differences may affect the various biological activities exerted by lactoferrin.

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