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Calorimetric and Raman investigation of cow's milk lactoferrin

Michele Iafisco · Ismaela Foltran · Michele Di Foggia · Sergio Bonora · Norberto Roveri

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Abstract Lactoferrin (LF), a non-heme iron-binding protein of blood plasma and milk with antioxidant, cariostatic, anticarcinogenic, and anti-inflammatory properties, has been studied by differential scanning calorimetry (DSC) and Raman spectroscopy over a wide pH range (4.0–9.0). Using these two techniques, the modifications in the quantity of iron bounded in the cow's milk LF and in the secondary structures, as a function of pH and heating, have been evaluated. DSC curves showed higher value of denaturation temperatures and enthalpy changes when LF was saturated with iron (holo-form) than when it was in its unsaturated form (apo-form). The denaturation curves of the protein solutions at pH $\geq$ 5.5 confirming that LF is a mix of apo- and holo-forms; on the contrary at pH 4.0, the holo-form is practically absent. Spectroscopic investigation showed that, as a function of pH, the content of α-helix increases up to pH 7.4, followed by a small decrease by further pH increase. The β-sheet percentage exhibits the opposite behavior, while the random-coil and turn structures do not change noticeably. In contrast, after heat-induced denaturation, strong variations were observed in the secondary structure, with an evident increase of β-sheet and decrease of the α-helix percentage. Finally, both thermal and spectroscopic analysis pointed out that the structure of cow's milk LF is strictly sensible to pH variation and it has the highest thermal stability at physiological pH.

Keywords Lactoferrin · Raman spectroscopy · Differential scanning calorimetry · Protein denaturation · Protein biophysics

M. Iafisco (✉) · I. Foltran · N. Roveri
Dipartimento di Chimica "G. Ciamician", Alma Mater Studiorum, Università di Bologna, Via Selmi 2, 40126 Bologna, Italy
e-mail: michele.iafisco@unibo.it

I. Foltran
e-mail: ismaela.foltran@unibo.it

M. Iafisco · I. Foltran
Dipartimento di Scienze Mediche, Università del Piemonte Orientale, Via Solaroli 4, 28100 Novara, Italy

M. Di Foggia · S. Bonora
Dipartimento di Biochimica 'G. Moruzzi' Alma Mater Studorium, Università di Bologna, Via Belmeloro 8/2, 40126 Bologna, Italy

Introduction

Lactoferrin (formerly known as lactotransferrin) (LF) is a non-heme iron-binding protein that is a part of the transferrin protein family, thus belonging to those protein capable of binding and transferring Fe^{3+} ions in blood serum [1]. LF is a glycoprotein with a molecular weight of about 80 kDa and has a basic isoelectric point (IP) ($\sim$9.0) [2]. It consists of a single polypeptide chain containing 703 amino acids folded into two symmetrical lobes (N and C lobes), which are highly homologous with each other (33–41% homology). These two lobes are connected by a hinge region containing parts of a α-helix between amino acids 333 and 343 in human LF. The polypeptide chain includes amino acids 1–332 for the N-lobe and 344–703 for the C-lobe, and the protein structure creates two domains for each lobe [3].

There are three forms of LF according to its iron saturation: apolactoferrin (iron free), monoferric form (one ferric ion), and hololactoferrin (binds two Fe^{3+} ions); the

tertiary structure in hololactoferrin and apolactoferrin is different [3–5]. In LF, each lobe can bind a metal atom in synergy with the carbonate ion (CO_3^{2-}) or bicarbonate ion (HCO_3^-). Four amino acid residues (histidine, two tyrosines, and aspartic acid) play a crucial role for the iron binding (Asp60, Tyr92, Tyr192, and His253 in the N-lobe and Asp395, Tyr433, Tyr526, and His595 in the C-lobe); while an arginine chain is responsible for the binding of the carbonate or bicarbonate ion [6]. LF has a great iron-binding affinity, and it is the only transferrin with the ability to retain this metal over a wide pH range. It also exhibits a great resistance to proteolysis by trypsin and trypsin-like enzymes, and the level of resistance is proportional to the degree of iron saturation. At physiological pH, LF molecule has a net positive charge, and its distribution in various tissues makes it a multifunctional protein. It is involved in several physiological functions: regulation of iron absorption in the bowel, immune response, antioxidant, cariostatic, antianemic, anticarcinogenic, and anti-inflammatory properties; moreover, it plays a role in the protection against microbial infection. The antimicrobial activity of LF is the most widely studied function to date, and it is mostly due to two mechanisms. The main antimicrobial mechanism of LF against the microorganisms that require iron is the ability of LF to chelate this metal, thereby, depriving them of the source of this nutrient [7]. Moreover, LF interacts with the cell membrane of some bacteria, leading to changes in the permeability and causing lysis with the release of lipopolysaccharide from the Gram-negative bacterial outer membrane [8].

The thermal stability of LF has been well reported in the literature [6–13], demonstrating that the iron bound to the LF confers more resistance to thermal denaturation [9, 10], as well as to the proteolytic digestion of the protein. Differences have been found in the thermal resistance among the species and, for example, bovine LF is less resistant than the human counterpart [10]. Moreover, it has been demonstrated that the biological properties of LF depend on its three-dimensional structure, which can be influenced by chemical and physical factors (pH, temperature, pressure, etc.) [3, 12].

Thermal analysis offers the best way to obtain precise and reproducible data on the overall denaturation process, while Raman spectroscopy has been proved to be a useful technique in revealing conformational changes of proteins, being very sensible both to changes in secondary structure as well as in the microenvironment of the side chains [11]. The aim of the present research was to study the thermal stability of cow's milk LF by differential scanning calorimetry (DSC) over a wide pH range (4.0–9.0) and combines these results with those obtained by using Raman spectroscopic technique.

Experimental

Materials

Lactoferrin, a 97% pure protein fraction from cow's milk was obtained from DMV International (Veghel, The Netherlands). Sigma-Aldrich supplied all common high-purity chemical reagents. Ultrapure water (0.22 μS at 25 °C) was used. The protein solutions (50 mg/mL) were prepared by dissolving LF in acetate buffer (pH 4.0), in ultrapure water (pH 5.5), in hepes buffer (pH 7.4), and in tris buffer (pH 9.0) in a 2-mL conical polyethylene Eppendorf tube. The buffer solutions were 0.01 M and, in order to mimic physiological conditions, 0.15 M NaCl was added. All pH values were tested using a microelectrode and adjusted to the request values by adding small amounts of NaOH/HCl solutions. To evaluate the long-term stability of the protein at the physiological temperature, the protein solutions were analyzed immediately after the preparation and after the thermal treatment in a bascule bath at 37.0 °C for 24 h.

Differential Scanning Calorimetry

Samples and references (20 μL) were introduced into aluminum pans and sealed for analysis. The references consisted of pans containing the same volume of the used buffers. DSC measurements were performed using a TA DSC-Q100 apparatus (TA Instruments, New Castle, DE, USA). Heating scans were run at 2 °C/min from 5 to 115 °C under nitrogen flow. Between heating scans, a constant rate cooling (2 °C/min) was applied to evaluate if the renaturation of the protein occurred. Thermal cycles were repeated on three different samples to ensure a good reproducibility of the data; the expected experimental errors were ±0.1 °C in the temperature and ±3% in the enthalpy change values (ΔH). Several thermodynamic parameters can be deduced from the DSC transition peak such as the temperature at the maximum of unfolding transition (T_d) and the enthalpy change (ΔH) for the denaturation of proteins. The last parameter (ΔH) was calculated by integrating the peak area using a straight baseline drawn from the onset to the end of the thermal transition.

To assess the complexity of the denaturation process, a parameter measured directly from the transition peak, the width at half-peak height ($\Delta T_{1/2}$) has been used. This parameter allows the evaluation of the van't Hoff enthalpy (ΔH_{VH}) for a two-state process by using the Eq. 1 [11]:

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

where T_d is the maximum peak temperature, and R is the gas constant. Therefore, the comparison of the values

obtained for ΔH_{VH} with the calorimetric enthalpy change (ΔH) indicates if the denaturation reaction could be approximate to a two-state process. Lack of correspondence between the two values indicates a decreased cooperatively due to the presence of intermediates or to intermolecular associations [14].

Raman spectroscopy

The FT-Raman spectra were recorded by a Bruker Multi-RAM spectrometer equipped with a Ge-diode detector. The excitation source was a Nd^{3+}-YAG laser (1064 nm) with a power on the sample of about 100 mW; the spectral resolution was 4 cm^{-1} and the number of scans for each spectrum was 5000.

Resonance Raman spectra were recorded using a Jasco NRS-2000C instrument in the back-scattering conditions with 4 cm^{-1} spectral resolution. The excitation source was an Ar^{+} laser (Innova Coherent 70), using the 514 nm line (power about 40 mW). The detector was a 160 K frozen CCD from Princeton Instruments Inc. and reported spectra result from the average of 10 measures. Resonance Raman is a spectroscopic technique widely used to characterize and assign vibrations directly connected with a chromophore, as well as for the assignment of the chromophore bands. The excitation frequency is applied close to the absorption maximum of the chromophore. The maximum of the visible LF absorption peak is near 460 nm, but we used the 514 nm line to reduce the interference due to the fluorescence of the protein, yet staying in pre-resonance conditions.

Both FT-Raman as well as resonance Raman spectra were recorded on the buffered LF 5% aqueous solutions (pH 4.0, 5.5, 7.4, and 9.0), both at low temperature (20 °C) on immediately prepared samples, both after heating for 10 min at a temperature 5 °C greater than the maximum of the corresponding first calorimetric peak in the heating thermal cycles (T_{d1}).

Results and discussion

Differential scanning calorimetry

Differential scanning calorimetry is a thermal technique that provides valuable information on the overall mechanism of protein denaturation, on its reversibility as well as on its cooperativity by studying temperature and enthalpy changes associated to the thermal transitions.

Figure 1a and b shows the heating DSC curves in the 40–75 and 65–115 °C range, respectively, obtained on freshly prepared cow's milk LF solutions at different pH. Each sample presents two endothermic transitions (T_d) due to the denaturation event: the first appears in the range

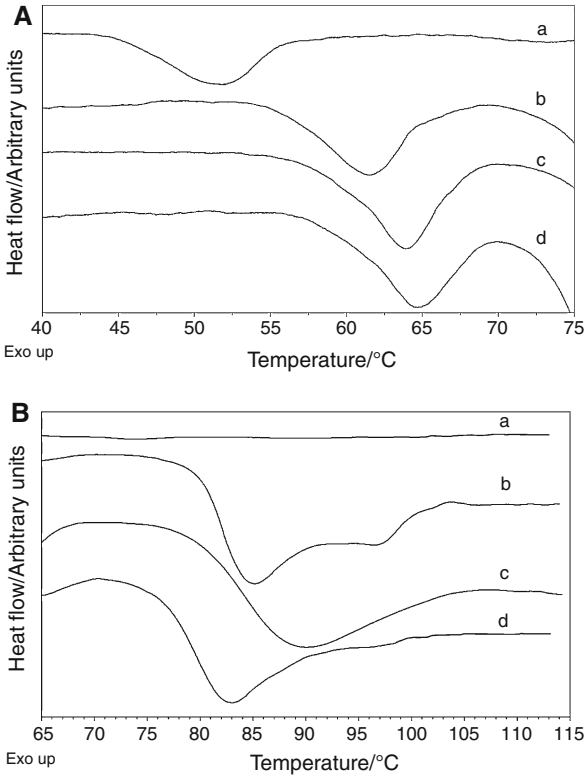

Fig. 1 DSC thermograms in the 40–75 °C (a) and 65–110 °C (**b**) range. LF solutions have been analyzed at pH 4.0 (*a*), pH 5.5 (*b*), pH 7.4 (*c*), and pH 9.0 (*d*)

52–65 °C and the second (very small at pH 4.0) in the range 74–89 °C.

It is well known that both apo- and holo-form show only one main denaturation peak, with differences in position and shape due to the different conformations of the two forms of LF. In fact, the curve for human apo-LF shows a maximum temperature of denaturation at 72 °C, whereas the holo-form presents the maximum temperature at 95 °C [15]. The presence of two denaturation peaks suggests that most part of LF is present in the apo- or holo-form or it could be also explained by the fact that the C-lobe is more compact than the N-lobe [16], thus, the C-lobe could be saturated with iron and the other one could be iron free. Therefore, the temperature of denaturation of the C-lobe may be higher than that of the N-lobe, as it was also reported for transferrin [17].

The denaturation curve of the native cow's milk LF at pH $\geq$ 5.5 (Fig. 1a, b) exhibits both peaks, with the second more intense than the first, confirming that the saturation iron percentage ranges from 50 to 100%. On the contrary, in the pH 4.0 curve (Fig. 1b) the peak of the holo-form is practically absent. Table 1 reports the observed temperatures of maximum heat absorptions (T_d), the half-width of the lower temperature peak ($\Delta T_{1/2}$), and the enthalpy changes (ΔH). Both T_{d1} and ΔH values increase by

Table 1 Thermal parameters of the denaturation of cow's milk LF solutions at different pH values

	T_{d1}/°C	T_{d2}/°C	ΔH_1/J g^{-1}	ΔH_2/J g^{-1}	$\Delta T^*_{1/2}$/°C	ΔH_{VH}/J g^{-1}	$\Delta H_1/\Delta H_{VH}$
pH 4.0	52	74	10.6	2	6.7	6.5	1.6
pH 5.5	62	85	14.4	154	5.7	8.2	1.8
pH 7.4	64	89	14.1	152	4.2	11.2	1.3
pH 9.0	65	83	12.5	140	4.7	9.9	1.3

*Half-width denaturation peak T_{d1}

increasing pH up to 7.4, at a condition in which LF is more iron saturated, and the half-peak height of the curve of apo-LF (pH 4.0) is wider than that of the same peak in other iron-bound forms.

These data suggest that the Fe^{3+} at acidic medium is released from native LF. This hypothesis is confirmed by the T_{d2} trend Which reaches its maximum value in hepes buffer solutions (pH 7.4) and decreases both in acidic as well as in basic solutions. Also the trend of the enthalpies agrees with that deduced from T_d behavior, exhibiting an increase up to pH 7.4, followed by a further decrease at pH 9.0. In particular, the ΔH_2 in acetate buffer has a negligible value, confirming the reduced LF capability to bind the Fe^{3+} ions in the acidic medium.

In Table 1 are reported the van't Hoff enthalpy of denaturation (ΔH_{VH}) and the corresponding ratio ($\Delta H_1/\Delta H_{VH}$). When this ratio is ≈ 1, the calorimetric enthalpy is equal to the van't Hoff enthalpy, observed in small single-domain globular proteins [18]. On the contrary, in proteins in which the conformation is stabilized by interactions among several domains, the ratio $\Delta H_1/\Delta H_{VH}$ is >1, in agreement with the found values for all the LF solutions at different pH. This finding suggests that partially unfolded intermediates exist in equilibrium with the native state during the thermal denaturation process, in agreement with the three-dimensional structure of LF which consists of two similar lobes, each of them further organized into two domains with a site for iron in the cleft between them [6].

The treatment of 24 h heating at 37 °C on LF solutions does not affect the previous described situation, and denaturation does not occur in such conditions, as it is demonstrated by the comparison of thermal curves whose differences are within the experimental errors, confirming the good stability of LF. In addition, it was observed that the denaturation of LF is irreversible in the whole pH range tested (4.0–9.0) after heating, as suggested by the absence of calorimetric peaks both in the cooling as well as in the re-heating cycle (data not shown).

FT-Raman spectroscopy

To detect changes in the overall protein structure, resulting from the exposure to heat, FT-Raman spectra of LF at four different pH, before and after the heating cycle were considered and are shown in Fig. 2a and b, respectively. In order to obtain the protein spectrum free of medium interferences in the Amide I band region, the spectrum of buffer was subtracted from the overall spectrum.

It is worth to mention that one of the potential advantages of Raman spectroscopy for the study of protein is the correlation between the vibrational wavenumbers of the peptide backbone and the various protein secondary

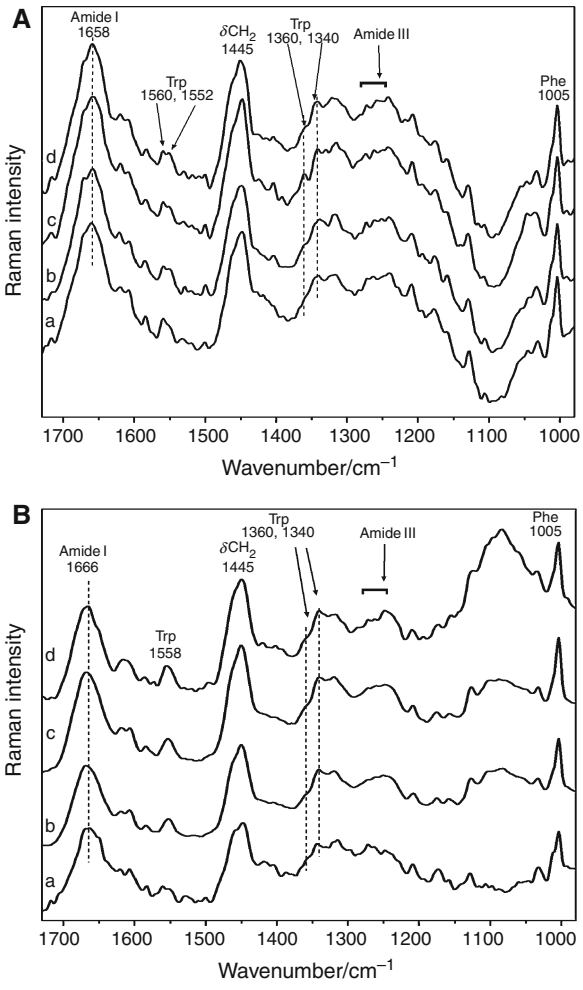

Fig. 2 FT-Raman spectra in the 1700–1000 cm^{-1} range of the native (**a**) and heated (**b**) LF samples. LF solutions have been analyzed at pH 4.0 (*a*), pH 5.5 (*b*), pH 7.4 (*c*), and pH 9.0 (*d*)

structures. In particular, the amide I Raman band, which appears in the 1620–1700 cm^{-1} spectral regions, may act as sensitive conformation marker [19]. A qualitative examination of the spectra evidences some slight differences in the spectral features of the amide I band. In particular, this band shifted from 1660 to 1658 cm^{-1} in the native LF and from 1664 to 1668 cm^{-1} in the heated LF, by increasing the pH from 4.0 to 7.4, suggesting a decrease in the β-sheet content in the first case and an increase in the second. On the contrary, at pH 9.0 a small opposite variation is observed (1660 cm^{-1} in the native and 1666 cm^{-1} in the heated solution).

To obtain an accurate evaluation of the heat-induced conformational changes, the percentages of the secondary structure were calculated by a method proposed by Alix et al. [20]. This method is based on Eq. 2 which allows to express the percentages of structural contents in a protein as a linear function of some parameters of the amide I Raman band, namely, the wavenumbers of the peak and the left and right widths at the half-height.

$$\% \text{ structure } = a_0 + a_{1\nu\max} + a_{2\nu\text{left}} + a_{3\nu\text{right}} \quad (2)$$

The a_n constants are coefficients calculated for each class of structure. This equation has been obtained by performing a statistical multi-parametric analysis of the correlations between structural data (obtained by X-ray crystallography) and spectroscopic Raman data using a large set of reference proteins. The results are reported in Fig. 3. Spectroscopic investigation showed that, for the native protein, a small increase in the α-helix is observed up to pH 7.4, followed by a small decrease by further pH increase. The β-sheet percent exhibits the opposite behavior, decreasing up to pH 7.4 and then rising again

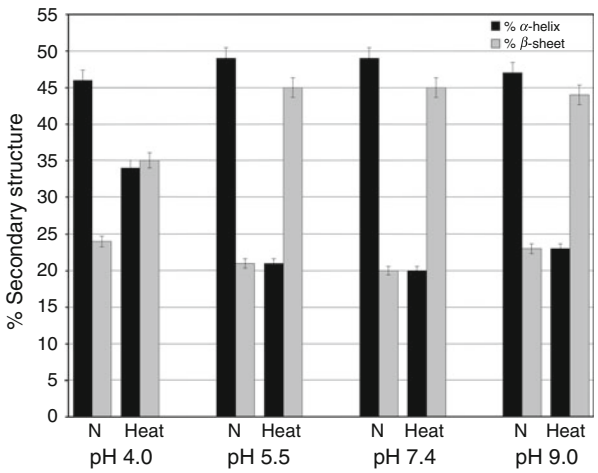

Fig. 3 Percentages of the secondary structure of LF at different pH values before (*N*) and after thermal treatment (*Heat*), measured by the analysis of the amide I Raman band

in basic solution, while the random-coil and turn structures do not change noticeably. On the contrary, after heat-induced denaturation, strong variations are observed in the secondary structure, with a noticeable increase of β-sheet and decrease of the α-helix percentage.

It should be noted that even the denaturation process is influenced by pH, as it can be deduced from the smaller structural variations observed in acid solutions. Furthermore, the pH influences the secondary structures of heated LF with a different trends compared with the modifications occurred at room temperature. In fact, Fig. 3 shows that the percentage variation reaches the maximum value in the case of LF in hepes buffer (pH 7.4) and this result can be related to the higher packing of protein molecules at this pH.

Moreover, in the native protein, the full width at half maximum of the amide I ($\Delta\nu_{1/2}$) increased by increasing the pH, reaching the maximum at pH 7.4, denoting a higher "dispersion" of structures, whereas the opposite trend was observed after heating. The found $\Delta\nu_{1/2}$ in the native protein was 50, 51, 54, and 50 cm^{-1}, whereas after heating they were 50, 48, 48, and 46 cm^{-1}, at pH 4.0, 5.5, 7.4, and 9.0, respectively.

On the contrary, the $\Delta\nu_{1/2}$ of the δCH_2 band at 1445 cm^{-1} does not change, suggesting that the hydrophobic domain of LF was not influenced by temperature and pH.

Another Raman band sensible to the secondary structure is the amide III located in the 1230–1300 cm^{-1} spectral region, but this band was poorly defined in our spectra because of the strong overlapping of aromatic amino acids side chains bands (in particular Tyr bands), and consequently this band could not be used as a diagnostic tool.

FT-Raman spectroscopy has been proved to be a useful technique in revealing conformational changes in the microenvironment of the side chains of proteins [11]. Spectral modifications were observed in the intensity of some bands attributed to Trp residues, particularly, in the doublet at 1340 and 1360 cm^{-1} (Fig. 2a), which is considered as a marker of the hydrophobicity of the molecular environment of Trp residue. It has been observed that the setting up of hydrophobic interactions between the Trp indole ring and the surrounding aliphatic groups cause the 1360 cm^{-1} peak intensity decrease and the 1340 cm^{-1} intensity increase [21]. By varying the pH, on the native sample, the intensity of the 1360 cm^{-1} band increases up to pH 7.4, decreasing again at pH 9.0 (Fig. 2a) suggesting that at physiological conditions, Trp residues are exposed and the protein presents the highest amount of hydrophilic interactions. After heating, a noticeable decrease in the intensity ratio (I_{1360}/I_{1340}) was observed, indicating an increase in the hydrophobic interactions between the Trp indole ring and other aliphatic groups (Fig. 2b).

Even the 1558 cm^{-1} Raman peak, marker of the orientation in the Trp indole ring with respect to the peptide backbone [22], changes as a function of the pH and thermal history of solutions. In most of the crude protein (pH $\geq$ 5.5) it appears as a doublet (1552 and 1560 cm^{-1}), suggesting the contemporary presence of two different orientations relative to the peptide backbone (Fig. 2a). On the contrary, after heating, it appears as single band, whose frequency ranges from 1552 to 1556 cm^{-1} (Fig. 3b) as a function of the pH, confirming that orientation of the indole ring changed to an intermediate angle. At pH 4.0, this band exhibits an opposite behavior, confirming that in the acidic medium the structure of the protein is slightly different.

Resonance Raman spectroscopy

Figure 4a reports the Resonance Raman spectra of native LF solutions at different pH. These spectra, excluding that at pH 4.0, are dominated by strong resonance-enhanced vibrational modes of Fe^{3+}-coordinated tyrosinate groups. Of the four resonance peaks reported in the literature at approximately 1600, 1500, 1260–1290, and 1170 cm^{-1} [23], we observed, in the excitation modality we used, only the 1520 and 1170 cm^{-1} peaks, that we assigned, according literature [23, 24], to the aromatic ring CC stretching of the tyrosine and to the CO bending of the phenolate ligand of the same molecule, respectively.

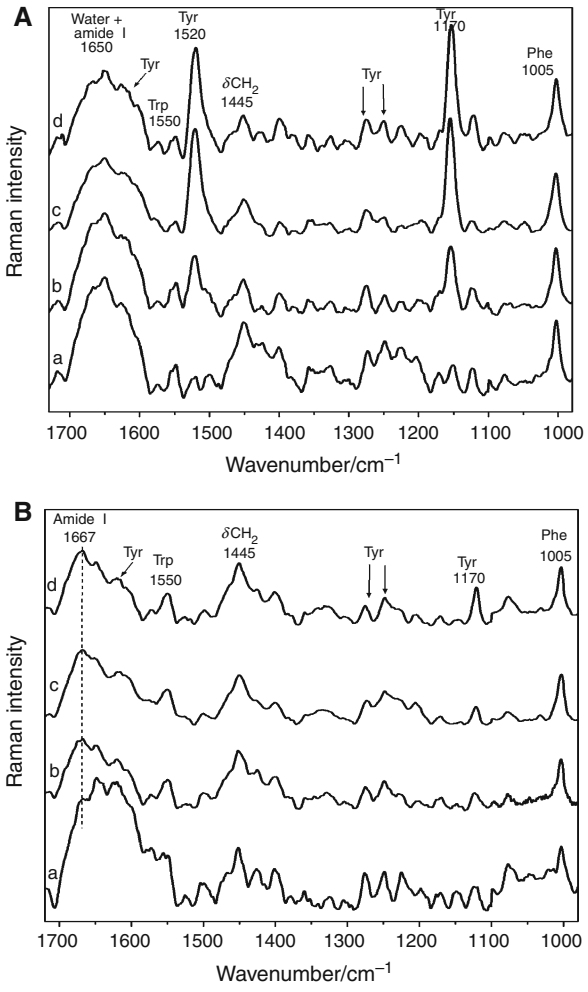

Fig. 4 Resonance Raman spectra in the 1700–1000 cm^{-1} range of the native (**a**) and heated (**b**) LF samples. LF solutions have been analyzed at pH 4.0 (*a*), pH 5.5 (*b*), pH 7.4 (*c*), and pH 9.0 (*d*)

Also some non-resonant features arising from protein motions were observed in the resonance spectra and located at $\sim$1450 cm^{-1} (CH_2 deformation) and $\sim$1005 cm^{-1} (phenylalanine ring breathing mode). The broad feature near 1650 cm^{-1} observed in all spectra (Fig. 4a) arises from the Amide I band overlapped with the solvent vibration (H_2O deformation).

Both 1520 and 1170 cm^{-1} band intensity increased by increasing the pH up to 7.4, confirming that the higher iron-binding capability of LF is reached at physiological pH.

After heating (Fig. 4b), both resonance bands can not be observed, suggesting that conformational structures change noticeably, interesting even the peptides surrounding Fe^{3+} ions, with a consequent iron release [presumably in the form of the insoluble $Fe(OH)_3$].

Conclusions

Calorimetric and spectroscopic investigations demonstrated that different pH values and heating induced conformational changes in LF. In particular, the modifications in the quantity of iron bounded in the protein and in the secondary structures are clearly visible using DSC and Raman. The DSC curves showed higher value of denaturation temperatures and enthalpy changes when LF was saturated with iron (holo-form) than when it was in its unsaturated form (apo-form). The denaturation curves of the native cow's milk LF solutions at pH $\geq$ 5.5 confirmed that the protein is a mix of apo- and holo-forms. On the contrary, at pH 4.0 the holo-form is practically absent, thus LF is present in the iron-free state.

Spectroscopic investigation allowed the evaluation of the secondary structure modifications of the protein as a function of pH, and showed that the content of α-helix increase up to pH 7.4, followed by a small decrease by further pH increase. The β-sheet percentage exhibits the opposite behavior, decreasing up to pH 7.4 and then rising again in basic solution, while the random-coil and turn structures do not change noticeably. On the contrary, after heat-induced denaturation, strong variations were observed in the secondary structure, with a clear increase of β-sheet and decrease of the α-helix percentage. It should be noted that even the denaturation process is influenced by pH, as

can be deduced by the very smaller structural variations observed in acid solutions. The pH influences the secondary structures of heated LF with a different trends compared with the modifications occurred at room temperature. As a matter of fact, the variation is more evident at pH 7.4, and this result can be related to the higher packing of protein molecules at this pH.

Finally, both thermal and spectroscopic analysis pointed out that the structure of cow's milk LF is strictly sensible to pH values, highlighting that, at physiological pH, LF has the highest thermal stability.

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