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Structural characteristic, pH and thermal stabilities of apo and holo forms of caprine and bovine lactoferrins

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Abstract Apo and holo forms of lactoferrin (LF) from caprine and bovine species have been characterized and compared with regard to the structural stability determined by thermal denaturation temperature values (T_m), at pH 2.0–8.0. The bovine lactoferrin (bLF) showed highest thermal stability with a T_m of 90 ± 1°C at pH 7.0 whereas caprine lactoferrin (cLF) showed a lower T_m value 68 ± 1°C. The holo form was much more stable than the apo form for the bLF as compared to cLF. When pH was gradually reduced to 3.0, the T_m values of both holo bLF and holo cLF were reduced showing T_m values of 49 ± 1 and 40 ± 1°C, respectively. Both apo and holo forms of cLF and bLF were found to be most stable at pH 7.0. A significant loss in the iron content of both holo and apo forms of the cLF and bLF was observed when pH was decreased from 7.0 to 2.0. At the same time a gradual unfolding of the apo and holo forms of both cLF and bLF was shown by maximum exposure of hydrophobic regions at pH 3.0. This was supported with a loss in α-helix structure together with an increase in the content of unordered (aperiodic) structure, while β structure seemed unchanged at all pH values. Since LF is used today as fortifier in many products, like infant formulas and exerts many biological functions in human, the structural changes, iron binding and release affected by pH and thermal denaturation temperature are important factors to be clarified for more than the bovine species.

Keywords Caprine lactoferrin · Bovine lactoferrin · Apo lactoferrin · Holo lactoferrin · Iron content · Structural stability · pH stability · Thermal denaturation temperature

Abbreviations

LF	Lactoferrin
apo LF	Apo lactoferrin
holo LF	Holo lactoferrin
cLF	Caprine lactoferrin
bLF	Bovine lactoferrin
Trp	Tryptophan
CD	Circular dichroism
far-UV CD	Far-ultraviolet circular dichroism
T_m	Thermal denaturation temperature

Introduction

Lactoferrin (LF), also known as lactotransferrin, is a globular multifunctional, iron (Fe) binding protein

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with many biological functions. Firstly, it is a part of the innate defense system of many species, second, in the transport and supply of iron to the body. LF is found in milk and mucosal secretions such as tears and saliva (Adlerova et al. 2008; Farnaud and Evans 2003; Jenness 1980). It is present in variable concentrations in milk (0.1–7.0 mg/ml) as reported, however, with a higher amount in human milk. (Masson and Heremans 1971; Sanchez et al. 1988; Steijns and van Hooijdonk 2000). LF is an iron transporting protein and supplies the human body with iron. The recommended daily intake (RCDI) in the diet is about 10 mg, but this may vary with respect to infants and adults (Neilands 1991). The bLF has been used in a wide variety of products such as infant formulas, probiotics, supplemental tablets, pet food, cosmetics and as a natural solubilizer of iron in food (Masco et al. 2005; Tomita et al. 2002, 2009; Uchida et al. 2006; Wakabayashi et al. 2006). The LF plays a major role in the first line of the human defense system against microbial infections. The antimicrobial and antifungal properties are the unique physiological functions of LF (Orsi 2004; Olakanmi et al. 2002). The LF exerts its antimicrobial activity by two different mechanisms. The bacteriostatic effect is most probably attributed by apo form, where the bacteria are deprived of iron that is necessary for cell growth. The bactericidal effect is a membrane mediated activity of negatively charged LF leading to cell death (van Hooijdonk et al. 2000). The biological function of LF is linked to the unique feature of transferrin chemistry. Lactoferrin is structurally similar to transferrin, a plasma iron transport protein, but has a much higher ($\sim$300 fold) affinity for iron (Brock 1997). LF, in general, has the ability to bind two Fe^{3+} ions, together with two CO_3^{2-} ions. The protein folds into two globular lobes, N and C. The lobes are further divided into two identical domains, N1, N2 and C1 and C2. The two iron (Fe) atoms are surrounded by each lobes; N1, N2 and C1 and C2. Recently Hu et al. (2008) proposed a new structural model of holo bLF compared to native bLF. Based on the multiferric Fe-binding they have concluded that while native LF exists as a monomer, the holo form (70 FeLF) exists in a multimeric form, similar to casein micelle. This supersaturated Fe^{3+}-LF structure may help the absorption of iron in vivo. Mainly, the antimicrobial mechanism of LF require iron and is due to the ability of LF to chelate this metal, thereby, depriving them of the source of this nutrient (Masson et al. 1966). Again, LF interacts with the cell membrane of some bacteria, leading to changes in the permeability and causing the release of lipopolysaccharide from the outer membrane of Gram-negative bacteria (Ellison et al. 1988). The antibacterial activities of cLF and bLF were reported earlier (Conesa et al. 2008). The cLF was found to be more active against *E. coli* as compared to bLF. In this point of view, the apo and holo LFs from caprine and bovine species may exhibit differences with respect to antibacterial spectrum of activity.

The three dimensional (3D) structures of LF from caprine (cLF) and bovine (bLF) are about 90% identical, however, the physicochemical and biophysical properties seem to vary. The iron binding properties seem to vary between LF from different species. LF has different Fe^{3+} binding status. The iron free (apo), the native, and the iron saturated (holo) form. The thermal denaturation of human LF in relation to Fe binding was studied by differential scanning calorimetry (Mata et al. 1998; Conesa et al. 2007). In a previous study (Sreedhara et al. 2010), a comparison between cLF and bLF was done with regard to the conformation and thermal stability. The native cLF showed a lower thermal stability than bLF. In a study on the thermal stability of LF from sheep, goat, human, camel, elephant and alpaca (Conesa et al. 2008), it was observed that the thermal transition temperature values were higher for iron saturated forms of LF as compared to respective native forms. Further human LF was reported to be most heat-resistant. It was shown that there were subtle differences among the structures of LFs from different species.

Till date, there were no reports available on the comparison of apo and holo LFs from caprine and bovine species within the pH range 2.0–7.0. The present paper aims to compare structural characteristics of holo and apo LFs from caprine and bovine species with respect to iron binding and release and the thermal stabilities at various pH values.

Materials and methods

Chemicals

Ferric chloride, 8-anilino-1-naphthalene-sulfonate (ANS), citric acid, glycine, ethylene-diamine-tetra-acetic-acid (EDTA), Tris (hydroxymethyl) amino-methane and acrylamide were of analytical grade and

purchased from Sigma-Aldrich Chemical Co., St. Louis, MO, USA. Dialysis membranes (with a molecular mass cutoff 6.0–8.0 kDa) were procured from Spectrum Laboratories, Inc, Rancho Dominguez, CA, USA. Sodium mono- and dihydrogen phosphate, sodium citrate and sodium hydrogen carbonate and all the other chemicals were analytical reagent grade and obtained from Merck, Darmstadt, Germany. All buffers and reagents were prepared in Milli-Q water.

The buffers used were 10 mM glycine–HCl, pH 2.0; 10 mM citrate–phosphate, pH 2.6, 3.0, 3.6, 4.0, 5.0 and 6.0; 10 mM sodium phosphate, pH 7.0 and 8.0.

Preparation of apo and holo forms of caprine and bovine lactoferrins

The bovine LF with 95% purity was supplied by DMV International (Veghel, Netherlands) and was stored at −20°C. Caprine LF was purified (>95%) using the cation exchange membrane chromatography method (Sreedhara et al. 2010).

Apo (iron deprived) LF from caprine (cLF) and bovine (bLF) LF was prepared according to the method of Khan et al. (2001) with a slight modification. The native LF (100 mg), bovine or caprine, was solubilized in 1 ml 10 mM Tris–HCl buffer, pH 8.0 and the solution was dialyzed against 20 volumes of 100 mM citric acid (pH 2.0) for 24 h followed by exhaustive dialysis against Milli-Q water at 4°C for 30 h. This colorless fraction was freeze dried and stored at −20°C.

Holo (iron saturated) LF from caprine and bovine was prepared according to Karthikeyan et al. (1999) with a slight modification: Apo LF (1 mM) and 2 mM ferric chloride were solubilized separately in 100 mM sodium bicarbonate–sodium citrate buffer, pH 8.0. They were brought to 26°C in a water bath for 15 min. The ferric chloride solution was then added to the protein solution at the same temperature and incubated for 24 h. The excess reagent was removed by exhaustive dialysis against Milli-Q water at 4°C for 30 h. This red colored fraction containing LF was freeze dried and stored at −20°C.

The total iron content bound to LF was measured by the standard procedure of Inductively Coupled Plasma (ICP) Optical Emission Spectrophotometer (Uchida et al. 2006). The freeze dried protein (1.0 mg/ml) was solubilized in 1% (v/v) HNO_3 before performing the analysis. The iron binding measurements were averaged for triplicate measurements.

Table 1 Iron saturation (% mol/mol) in apo and holo forms of caprine and bovine lactoferrins at pH 2.0–7.0

pH	Holo cLF	Holo bLF	Apo cLF	Apo bLF
2.0	20.00	20.70	0.23	0.58
3.0	31.10	36.20	0.81	0.85
5.0	41.80	47.30	3.80	4.00
7.0	73.50	88.10	4.60	5.30

Freeze dried protein samples (1.0 mg/ml) were solubilized in 1% (v/v) HNO_3 and analyzed by ICP. The values are given as a mean of three parallels

The LF concentration was determined by measuring the absorbance at 280 nm (Recio and Visser 1999, 2000). The extinction coefficient values for cLF and bLF were reported by Sreedhara et al. (2010).

Circular dichroism measurements

Circular dichroism (CD) measurements in the far-UV region were carried out with protein solutions in respective buffers with appropriate blanks. These measurements were done with a JASCO J-810 spectropolarimeter (JASCO, Tokyo, Japan) calibrated with ammonium d-10-camphor sulfonate. All protein solutions were dialyzed against the corresponding buffers at 4°C for 24 h, centrifuged at 11500×*g* for 5 min and the clear supernatants (protein) were used for the measurements. The protein concentration used was 0.25 mg/ml. The measurements were made at 25°C. A cell with a path length of 0.1 cm was used for the scans between 260 and 200 nm. Each spectrum was the average of three consecutive scans. The result was expressed as the mean residue ellipticity (MRE in deg cm^2/dmol), which is defined as:

$$\mathrm{MRE} = \theta_{\mathrm{obs}}(\mathrm{mdeg})/\left(10 \times n \times C_{\mathrm{p}} \times l\right) \quad (1)$$

θ_{obs} is the observed ellipticity in degrees, n is the number of peptide bonds, C_{p} is the molar concentration, and l is the length of the light path in cm. The estimation of the contents of α helix, β structure and unordered structure was performed according to Yang et al. (1986).

ANS induced fluorescence measurements

Fluorescence spectra were recorded at a slow speed using a Cary Eclipse Fluorescence Spectrophotometer

(Varian Instruments, Palo Alto, CA, USA) at 25°C. The protein concentration used was 0.02 mg/ml. The protein concentrations of the samples were measured with a NanoDrop UV–visible spectrophotometer (NanoDrop Technologies, Thermo Scientific Inc, Wilmington, DE, USA). The ANS concentration was 50 times the protein concentration. The ANS binding was measured by fluorescence emission with excitation at 380 nm and emission was recorded from 400 to 600 nm. The excitation and emission slit widths were adjusted to 10/10 nm, respectively. Measurements were done in triplicate.

Intrinsic tryptophan fluorescence

Intrinsic fluorescence emission spectra were recorded using a Cary Eclipse Spectrofluorimeter (Varian, Middelburg, The Netherlands) at 25°C. Protein samples were filtered using 50 kDa cut-off Ultracel membrane filters and concentrated to a final volume of about 10 µl. Protein samples having an absorbance value of 0.10 at 280 nm (equivalent to 0.08 mg/ml) were used for the measurements. The samples were equilibrated at room temperature for 30 min before tryptophan fluorescence measurements. Excitation and emission slit widths were kept at 5 nm. The emission spectra were recorded in the range 300–400 nm after exciting with a wavelength of 290 nm. All the fluorescence measurements were recorded 10 s after excitation. The spectra were scanned at slow speed. Appropriate blanks were used for the baseline correction of fluorescence intensity. Measurements were done in triplicate.

Fluorescence quenching studies

Fluorescence spectra of LF were recorded using a Cary Eclipse Spectrofluorimeter (Varian, Middelburg, The Netherlands) at 25°C. Quenching experiments were carried out by the addition of varying amounts (0.10–1.0 M) of acrylamide stock solution (5.0 M) to the protein solution (0.012 mg/ml) previously incubated at pH 2.0, 3.0, 5.0 and 7.0 at 25°C for 1 h and the fluorescence intensities were recorded. Protein sample was excited at 290 nm and the emission was recorded in the range 300–400 nm. The excitation and emission slit widths were adjusted to 5/5 nm, respectively. The quenching data were analyzed according to Stern–Volmer equation (Eftink and Ghiron 1981).

$$F_0/F = 1 + K_{SV}[Q] \quad (2)$$

where F_0 and F are the fluorescence intensities at an appropriate wavelength in the absence and presence of quencher, respectively, K_{SV} is the Stern–Volmer constant and $[Q]$ is the concentration of the quencher, acrylamide. Data points were averaged for triplicate measurements.

Thermal denaturation studies

UV–visible spectrophotometric method

The thermal denaturation studies of LF at various pH, were carried out using a Cary 100 Bio UV–vis spectrophotometer (Varian Instruments, Mulgrave, Victoria, Australia). Protein samples were prepared at each required pH by dialysis against the appropriate buffer for 24 h at 4°C. Concentration of LF used was 1.0 mg/ml. The spectra were recorded at 287 nm over a temperature range 35–90°C with 1°C increment per min using respective blanks. Measurements were made in triplicate. The apparent thermal denaturation temperature (T_m) was calculated either by first derivative plot of absorbance or by van't Hoff plot using a standard equation (Pace and Scholtz 1997).

Circular dichroism (far-UV) method

Thermal denaturation was monitored following the ellipticity at 222 nm using a band slit of 2 nm and a response time of 8 s. CD spectra and thermograms were recorded using a Jasco J-715 spectropolarimeter (JASCO, Easton, MD, USA). An Automatic Peltier Accessory (PFD 350S) allowed continuous monitoring of the thermal transition at a constant rate of 1.0°/min. Protein was dialyzed against the required buffer (pH 2.0–8.0) at 4°C for 24 h and then incubated at room temperature (25°C) for 1 h before the measurements. The protein concentration was 0.02 mg/ml. The temperature of the protein sample was monitored directly using a probe immersed in a cuvette and controlled with PFD-350S Peltier type FDCD attachment. Measurements were done in triplicate. The data were analyzed assuming a two-state reversible equilibrium transition (Koepf et al. 1999).

Results and discussion

Iron content in apo and holo lactoferrins

The iron content of the apo and holo forms of LF at pH 2.0–7.0 is shown in Table 1. The maximum iron binding was observed at pH 7.0 and showed 88.1% in holo bLF and 73.5% in holo cLF. The iron binding was reduced with acidification and minimum values were observed at pH 2.0. The apo bLF and apo cLF samples contained about 4–5% iron at pH 7.0. However, these values were also reduced following pH reduction to approximately 0.58 and 0.23%, respectively, at pH 2.0. As compared with native cLF and bLF, having iron contents of approximately 5 and 15%, respectively, the release of iron was in a similar manner when pH was reduced from 7.0 to 2.0 (Sreedhara et al. 2010). These results are in accordance with a report of Baker and Baker (2009). It seems that the electrostatic interactions between the LF molecule and the ferric ions for protein stability and iron release are important (Baker and Baker 2004; Hu et al. 2008; Sreedhara et al. 2010). In the two lobes (N- and C-) of LF, several hydrogen bonds are associated with Fe-binding sites. In addition, there are a few direct H-bonded interactions across the cleft between the two domains of each lobe. Such interactions are affected by pH which differs to various degrees in cLF and bLF. This could be an important factor for different Fe-saturation levels in LF at different pH levels. The domain movements will vary due to pH reduction, and this will give a reduced binding with reduction in pH and increased binding with increase in pH. In addition, the Fe^{3+}–HCO_3^{2-} coordination observed in bLF as in N- and C-lobes may differ in cLF and depend upon the balance between the closed and open conformations (Hu et al. 2008).

Another aspect is that the changes in the amino acid sequence in the iron binding area of the molecule may be attributed to the different iron binding capacity between cLF and bLF. Focusing on Ser393 in cLF sequence that is replaced by Asn393 in bLF means that these residues are close to the iron binding Asp395. The bond angles between iron binding residues in cLF (pdb code 1jw1) and bLF (pdb code 1blf) are different as observed in protein data bank or measured using Pymol software. This might influence the different iron binding levels of cLF and bLF at different pH values. Further, the surface properties of

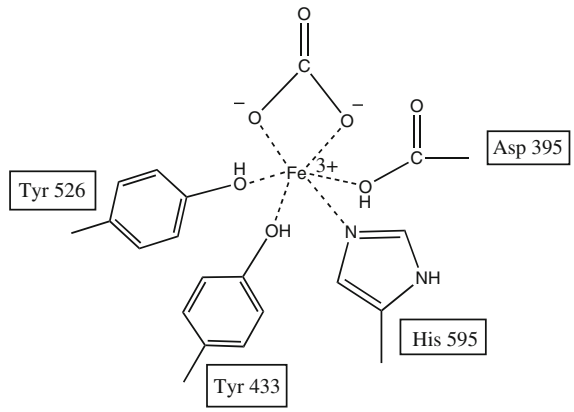

Fig. 1 The general type of ferric (Fe) ions bonded to bovine lactoferrin

apo and holo forms of cLF and bLF were different at different pH values (see ANS fluorescence data). Therefore, holo LFs from both species were not fully iron saturated at pH 7.0. Again, at pH 2.0, holo forms of cLF and bLF exhibited about 20% bound iron. However, the structure and mechanism of Fe binding and release of the holo forms of cLF and bLF with a reduction in pH from 7.0 to 2.0 is not clear. Figure 1 shows a structural model of iron binding to bLF. This model is based on a model proposed for bLF by Hu et al. (2008). But, the amino acid residue numbers that binds iron ion are different. The iron content in holo cLF and holo bLF at pH 2.0 is almost same, in contrast to the Fe contents at other pH values. The differences in Fe content at other pH values might be due to the different residue in the position 393 which in cLF is Ser and Asn in bLF and this difference is absent at pH 2 since Asp 395 most likely is then protonated and has no iron binding at that pH. The relatively higher differences in iron content of apo cLF and apo bLF at pH 2.0 could be due to a lower degree of ordered protein structure at that pH. More specifically, a higher exposure of tryptophans to the solvent.

pH dependent changes in the secondary structures of apo and holo forms of caprine and bovine lactoferrins

The CD spectra of a protein in the far-UV region (200–260 nm) are used to monitor the conformational changes in the polypeptide backbone. Figures 2 and 3 show the effect of pH on the far-UV CD spectra of

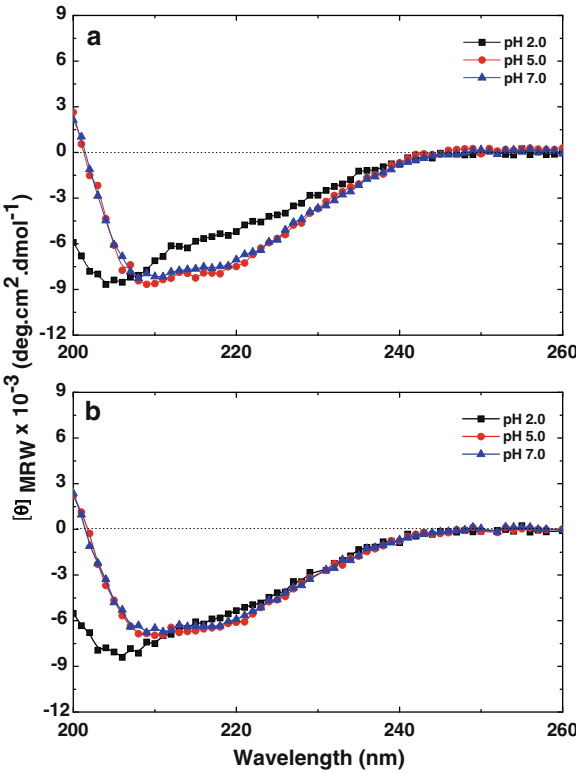

Fig. 2 Effect of pH on the CD secondary structures of **a** apo and **b** holo caprine lactoferrins (cLF). Buffers used are given under "Materials and methods"

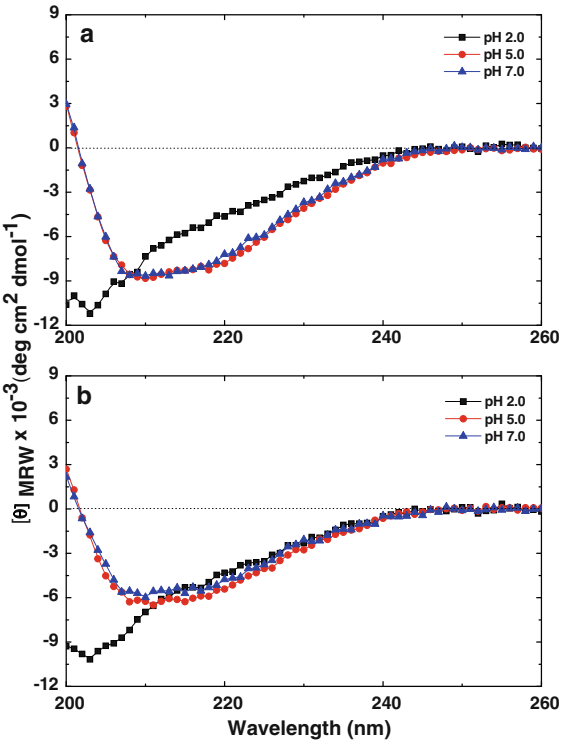

Fig. 3 Effect of pH on the CD secondary structures of **a** apo and **b** holo bovine lactoferrins (bLF). Buffers used are given under "Materials and methods"

apo and holo forms of caprine and bovine LFs, respectively. Bovine apo and holo LFs exhibited similar structures between pH 5.0 and 7.0. Two prominent peaks at 208 and 218 nm in the spectra of these LFs indicate the α/β structure of this protein (Nam et al. 1999). Between pH 4.0 and 8.0 (data shown at pH 5.0 and 7.0), the peaks observed at 208 and 218 nm in holo bLF seem to be slightly smaller as compared to that of apo bLF. All along, the far-UV CD spectrum (200–260 nm) of holo bLF showed no obvious difference from that of apo bLF, indicating that holo bLF maintained almost same secondary structure as apo bLF. From pH 7.0 to 5.0 α-helix content in apo and holo forms of cLF was increased (Table 2). But again from pH 5.0 to 2.0, there was an observed decrease in α-helix content. Further, a decrease in the β-structure between pH 7.0 and 2.0 was evident. However, from pH 7.0 to 2.0, there was also an observed increase in the aperiodic (random) structure content. A similar trend was observed for apo and holo cLF forms. But in this case, there was a complete loss of α-helix and a much higher amount of aperiodic (random) structure at pH 2.0. Hence, with acidification (pH 2.0), partly unfolded structures were

Table 2 Secondary structural contents (±0.25%) of lactoferrin as measured by circular dichroism (CD)

Lactoferrin		α helix	β structure	Unordered structure
Apo cLF	pH 2.0	7.0	55.0	38.0
	pH 5.0	21.0	56.0	23.0
	pH 7.0	19.0	58.0	23.0
Holo cLF	pH 2.0	8.0	55.0	37.0
	pH 5.0	19.5	57.5	23.0
	pH 7.0	14.0	65.0	21.0
Apo bLF	pH 2.0	0.0	58.5	41.5
	pH 5.0	23.0	54.0	23.0
	pH 7.0	20.0	59.0	21.0
Holo bLF	pH 2.0	0.5	59.0	40.5
	pH 5.0	17.0	62.0	21.0
	pH 7.0	11.5	70.0	18.5

The values are given as a mean of three parallel scans

observed for both apo and holo LF forms of both species (Figs. 2, 3). These results were found to be in a good agreement with the previous data on native cLF and bLF forms (Sreedhara et al. 2010). The conformations of native LFs from both species appeared similar to that of the corresponding apo and holo LF forms. Iron might not be involved in the conformational changes with respect to apo and holo forms of cLF and bLF in the pH range 2.0–7.0.

The intra-protein hydrophobic interactions are different for cLF and bLF (Tina et al. 2007). This might affect the thermal stabilities of apo and holo forms of caprine and bovine LFs to different extent.

Fluorescence measurements

ANS is a fluorescence hydrophobic probe used to detect hydrophobic regions on protein surfaces (Matulis et al. 1999). ANS binding to the apo and holo forms of cLF and bLF in the pH range 2.0–8.0 is shown in the Fig. 4a and b. The ANS binding to apo and holo LFs from both species was less in the pH range 5.0–8.0. Below pH 5.0, an increased binding of ANS was observed for both apo and holo forms of cLF and bLF. At pH 3.0, a maximum exposure of the hydrophobic regions in apo forms of both cLF and bLF was observed. This opening up of the LF molecule retains a minimum iron content. As seen in Fig. 4b, a marked increase (20 times) in the ANS fluorescence intensity of apo cLF from pH 3.5 to 3.0 along with an observed blue shift in the emission maximum (λ_{max}), indicating the exposure of hydrophobic regions of the protein. A similar trend was seen in holo cLF, where the fluorescence intensity was about seven times higher from pH 3.5 to 3.0 (Fig. 4b). Below pH 3.0, a decrease in the ANS fluorescence intensity values was observed suggesting the hydrophobic interactions leading to decreased surface hydrophobicity and an observed aggregation at pH <3.0. Whereas, from pH 3.5 to 3.0, for both apo and holo forms of bLF, a successive increase in the intensity by about five times was seen. These observations might suggest that this compact state (pH 3.0) with exposure of hydrophobic clusters can be an intermediate state observed with several proteins (Devaraja et al. 2009). At pH 2.0, the binding of ANS seems to be more in case of apo bLF as compared to apo cLF. Again, the binding of ANS to holo bLF at pH 2.0 was two times higher than that

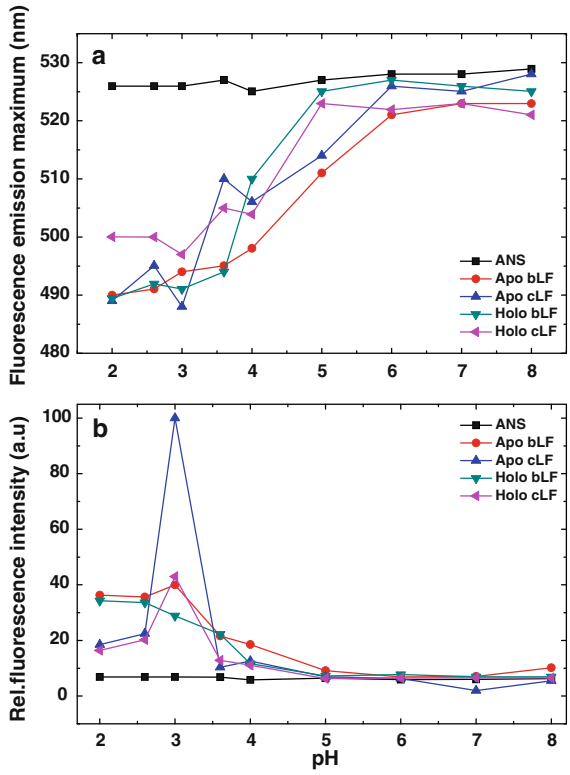

Fig. 4 **a** The 8-anilino-1-naphthalene-sulfonate (ANS) fluorescence emission maxima of apo and holo forms of caprine (cLF) and bovine (bLF) lactoferrins within the pH range of 2.0–8.0. **b** ANS fluorescence intensity of apo and holo forms of caprine (cLF) and bovine (bLF) lactoferrins within the pH range of 2.0–8.0. Buffers used are given under "Materials and methods"

of holo cLF. This depends on the overall 3D structural organization of apo and holo forms of cLF and bLF. A slow unfolding of apo bLF was evident at acidic pH (<4.0). But apo cLF exhibited a similar pattern of tryptophan exposure in that pH range. Apo bLF showed a higher ANS binding at pH 3.0 as compared to holo bLF. This was as observed from the intensity values in Fig. 4b. The extent of unfolding of apo and holo forms of cLF as well as bLF at lower pH is dependent on the 3D structures of individual LFs. These observations were made from the blue shifts in λ_{max} values. However, it was clear that apo and holo forms of cLF and bLF remain in the native state at pH 7.0 with little access to ANS binding. The structural changes start at pH between 6.0 and 5.0.

Intrinsic tryptophan fluorescence studies were done at different pH. The changes in the fluorescence

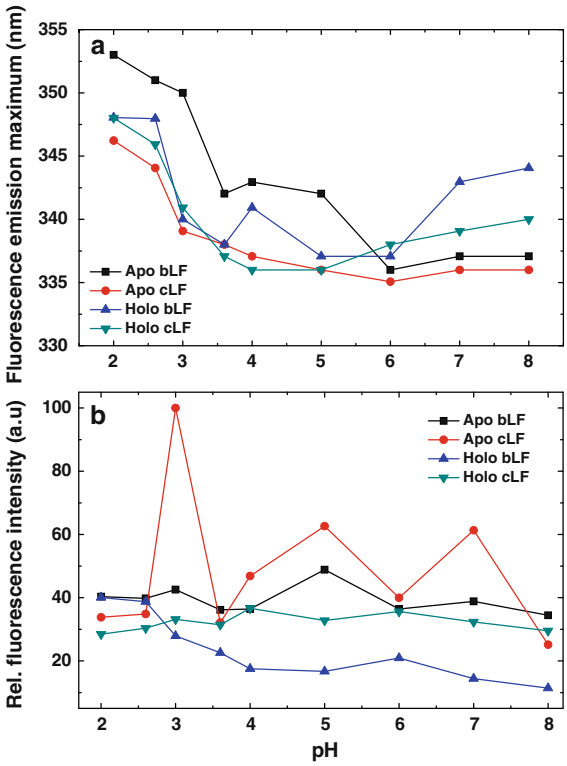

Fig. 5 **a** Fluorescence emission maxima (λ_{max}) of apo and holo forms of caprine (cLF) and bovine (bLF) lactoferrins at different pH. **b** Relative fluorescence intensity of apo and holo forms of caprine and bovine lactoferrins at different pH. Experimental conditions are as described under "Materials and methods"

emission maxima of apo and holo forms of cLF and bLF as a function of pH are as shown in the Fig. 5a. Figure 5b shows the changes in the relative fluorescence intensity values as a function of pH. Intrinsic fluorescence spectra provide a sensitive means of characterizing protein conformations. The spectra are determined mainly by the polarity of the environment of tryptophan and tyrosine residues, and their specific interactions. The emission maximum is a best parameter to monitor tryptophan polarity, and is sensitive to conformational changes (Gorinstein et al. 2000). Both apo and holo forms from cLF and bLF exhibited fluorescence when excited at 290 nm. At pH 7.0, for apo and holo bLF, the emission maximum (λ_{max}) values were found to be 337.07 and 342.96 nm, respectively, and at pH 2.0, these values were red shifted to 353 and 348.1 nm, respectively. So, as the pH was lowered from 7.0 to 2.0, a maximum red shift was observed between pH 2.0 and 3.5. This indicates the unfolding of the protein due to tryptophan exposure at acidic pH. This is also an indication of protein denaturation. A similar trend was observed with respect to the red shifts in the λ_{max} values of apo and holo forms of cLF (Fig. 5). At pH 3.0, apo cLF showed highest fluorescence intensity as compared to holo cLF. Further, an abrupt fall in the fluorescence intensity of apo cLF was evident at pH 2.0. Overall these data fits well with the results of ANS fluorescence. The results of fluorescence studies can be correlated with CD conformational studies in the pH range 2.0–7.0. In case of apo and holo LFs from both species, a marginal red shift of about 5–10 nm in the λ_{max} values from pH 4.0 to 2.0 was evident. Hence, the maximal exposure of tryptophans in all cases at low pH (2.0–3.0).

The data of ANS and tryptophan fluorescence showed a disordered pattern of the fluorescence changes of apo cLF, depending on either λ_{max} or fluorescence intensity values as a function of pH seems to emphasize changes in the range 2.0–4.0.

Fluorescence quenching of apo and holo forms of caprine and bovine lactoferrins at different pH

Quenching of tryptophan fluorescence by an external quencher is a common method to determine the solvent accessibility and microenvironment of tryptophan residues in proteins. The quenching of tryptophan fluorescence was determined based on the method of Eftink and Ghiron using uncharged molecules of acrylamide (Eftink and Ghiron 1981; Eftink and Selvidge 1982). Figure 6a and b represents the Stern–Volmer plots for the quenching of fluorescence by acrylamide in apo and holo forms of cLF and bLF at pH 2.0–7.0, respectively. Figure 7 shows the Stern–Volmer constants (K_{SV}) fitted to the linear part of the curves. K_{SV} value for apo cLF at pH 3.0 is 46.81 M^{-1} and is higher than that at pH 7.0. Further at pH 2.0 the value was reduced to 26.17 M^{-1}. These results were in good agreement with intrinsic fluorescence emission results. Tryptophans were more exposed to solvent at pH 2.0–3.0 than at pH 7.0. A similar trend was seen in case of apo bLF, where the values of K_{SV} from pH 7.0 to 2.0 were in an increasing order, the highest value was 45.94 M^{-1} at pH 2.0. Again, holo bLF was seen to be more hydrophobic as compared to holo cLF between pH 2.0 and 3.0. This is in accordance with the ANS binding data.

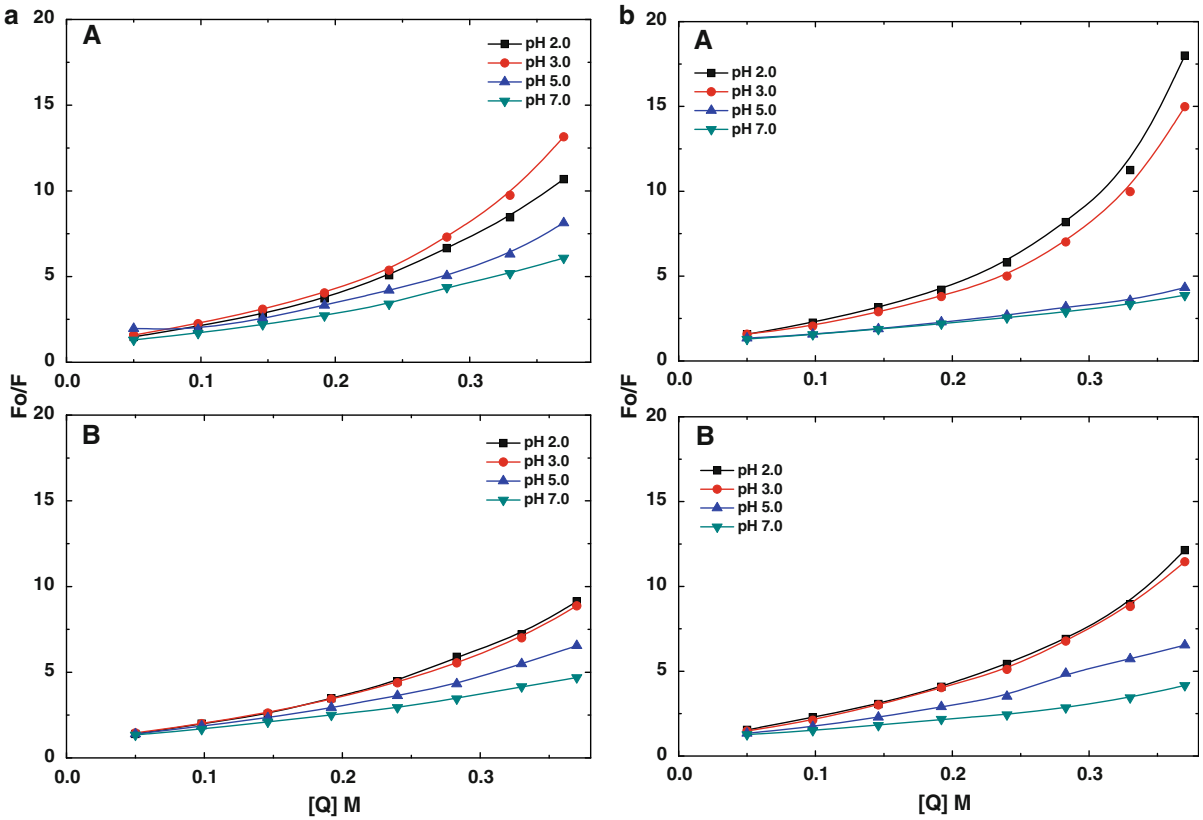

Fig. 6 **a** Fluorescence acrylamide quenching of *A* apo and *B* holo forms of caprine lactoferrin (cLF) at pH values 2.0–7.0. **b** Fluorescence acrylamide quenching of *A* apo and *B* holo forms of bovine lactoferrin (bLF) at pH values 2.0–7.0. Buffers used are given under "Materials and methods"

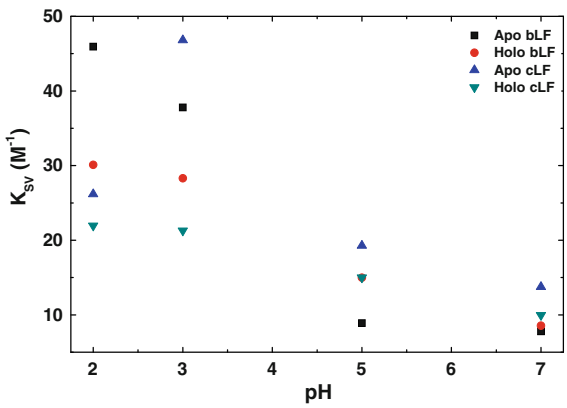

Fig. 7 Stern–Volmer constants (K_{SV}) for fluorescence quenching of apo and holo forms of caprine (cLF) and bovine (bLF) lactoferrin at pH values 2.0–7.0. Buffers used are given under "Materials and methods"

LF acquires open conformation at low pH (≤3.0) and this may play a significant role during the exposure of tryptophans to solvent. The closed structure of LF at pH 7.0 shows tryptophan to be fully embedded in the interior core as seen by ANS binding data.

Thermal denaturation profiles of apo and holo forms of caprine and bovine lactoferrins at pH 2.0–7.0

Apparent thermal denaturation temperature values (T_m) of the protein as determined by thermal response UV spectrophotometer (pH 6.0–8.0) and circular dichroism (pH 3.0–5.0) methods are depicted in Table 3. Figure 8a and b shows the thermal denaturation (T_m) profiles of the apo and holo forms of cLF and bLF in the pH range 6.0–8.0. The T_m measurements at pH 6.0, 7.0 and 8.0 by both methods were identical. The holo form of bLF exhibited the highest T_m (90 ± 1°C) at pH 7.0 as compared with the holo form of cLF (68 ± 1°C). There was a decrease in T_m values with reduction in pH. A large difference was

Table 3 Thermal denaturation temperature (±1.0°C) values of cLF and bLF at different pH

pH	3.0[a]	4.0[a]	5.0[a]	6.0	7.0	8.0
Apo cLF	23.00	32.50	49.50	63.0	64.0	64.00
Apo bLF	36.00	42.50	50.00	65.0	66.0	67.00
Holo cLF	40.00	56.50	58.00	65.0	68.0	67.00
Holo bLF	49.00	60.50	78.50	82.0	90.0	86.00

The values are given as a mean of three parallels

[a] T_m as measured by circular dichroism by monitoring the changes in secondary structure at 222 nm

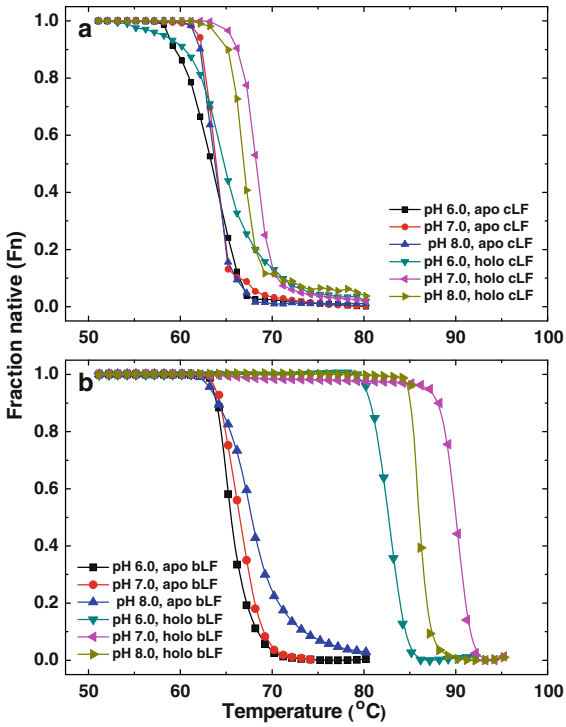

Fig. 8 Thermal denaturation profiles of apo and holo forms of caprine (cLF) and bovine (bLF) lactoferrins at different pH. **a** caprine, **b** bovine lactoferrin. The spectra were recorded at 287 nm over a temperature range 35–90°C with 1°C increment per min. Buffers used are given under "Materials and methods"

observed between the apo and holo forms of bLF. But this was not seen for apo and holo forms of cLF (Table 3). The reduction in pH from 7.0 to 3.0 showed reduced thermal stability for apo and holo LF forms from both species. Holo bLF retained a T_m of 49 ± 1°C even at pH 3.0. Whereas, holo cLF showed a T_m of 40 ± 1°C at that pH. A minimum T_m value of 23 ± 1°C was observed for apo cLF at pH 3.0. A low protein concentration was chosen to measure T_m below pH 6.0 to avoid precipitation. At pH <3.0 protein aggregation occured. This was identical for both apo and holo LF forms from both species. Previously, a comparison study was carried out with regard to the T_m values of native cLF and bLF forms in the pH range 2.0–8.0 (Sreedhara et al. 2010). It was shown that the thermal stability of LF from both species at different pH was dependent on iron binding. Again, the T_m values observed for native forms of cLF and bLF in the pH range 3.0–8.0 were in between apo and holo forms. The thermal stabilities of apo and holo forms of LFs from caprine and bovine were pH dependent. The amount of iron bound to cLF and bLF was reduced with a decrease in pH from 7.0 to 2.0. Hence the thermal stability of these proteins is dependent of the iron-binding capacity of cLF and bLF over the broad pH range studied. The total content of glycans may vary among cLF and bLF (Spik and Montreuil 1988). This may explain the variation in T_m values of cLF and bLF with respect to apo and holo forms.

Conclusions

Thermal denaturation (T_m) data of apo and holo forms of LF indicated that holo LF of both bovine and caprine species was much more stable than the respective apo form in the pH range 2.0–7.0. The bLF showed much higher thermal stability than the cLF. A significant loss in the iron content of both holo and apo forms of the cLF and bLF was observed when pH was decreased from 7.0 to 2.0. The conformation of apo and holo LFs from both caprine and bovine species showed an increased unfolded structure with reduced pH values. At pH 2.0, a higher content of aperiodic structure with an overall loss of α-helices was observed in case of apo and holo forms of bLF. Apo cLF and holo cLF showed 7–8% α-helix at low pH. This observation was supported by a maximum exposure of hydrophobic regions of the apo and holo LF forms of both species at pH 2.0–3.0. This data was also supported by acrylamide quenching studies. The results obtained to clarify structural conformation with respect to iron binding and release within the large pH range may be of importance to understand the behaviors of apo and holo LFs during the different gastrointestinal pH conditions in gut.

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