Structure, Stability and Digestion of Caprine and Bovine Lactoferrin. A Comparative Study

Struktur, Stabilitet og Fordøyelse av geit og storfe Laktoferrin. En komparativ studie

Philosophiae Doctor (PhD) Thesis

Ashoka Sreedhara

Dept. of Chemistry, Biotechnology and Food Science
Norwegian University of Life Sciences

Ås 2011

Thesis number 2011: 01
ISSN 1503-1667
ISBN 978-82-575-0966-8

CONTENTS

ACKNOWLEDGEMENTS

In this thesis the results are discussed of research carried out during 2006-2010 at the Department of Chemistry, Biotechnology and Food Science, at the Norwegian University of Life Sciences (UMB), Aas, Norway and the Department of Protein Chemistry and Technology, Central Food Technological Research Institute (CFTRI), Mysore (A Constituent Laboratory of CSIR Delhi)), India. Obviously, these results would never have reached without the assistance of other people. Due to the special set-up of my PhD project in an Indo-Norwegian Cooperation Research Program, the number of those, who have interacted with me and shared their experience, doubles. I would like to acknowledge the financial support from Norwegian Agency for Development Cooperation (NORAD), a directorate under Norwegian Ministry of Foreign Affairs (MFA) and Norwegian State Educational Loan Fund.

First and foremost, I would like to express my eternal gratitude to my Principal supervisor Prof. Gerd E. Vegarud (UMB) for her valuable guidance and advice with regular discussions during my PhD program. She inspired me greatly to work in this project. I have appreciated very much her genuine scientific spirit. I am grateful to her for her friendly interactions in my professional and personal life. It has made me to learn a lot!

I am highly grateful to Dr. V. Prakash (Director, CFTRI) for his supervision and valuable guidance during my PhD program. His incredible motivation and attention to details allowed me to go through all the challenges that came on the way and taught me to develop an eye for detail. He has been a very good teacher to me. His encouragement has made me to set appropriate and specific goals to accept and fulfill any challenges in professional and personal life.

Many thanks to my supervisor Prof. Ragnar Flengsrud (UMB) for his encouragements and for sharing his expertise with me both experimentally and in the writing process. I would like to thank my supervisor Prof. Thor Langsrud (UMB) for his inspiring discussions and helpful advises throughout my PhD program. I would like to extend my thanks to Dr. Purnima Kaul for her co-supervision and guidance during my research work in CFTRI. Special thanks to Dr. Tove G. Devold (UMB) for involving in interesting scientific discussions with enthusiasm. My sincere thanks to Dr. Sridevi A. Singh (CFTRI) for providing assistance during circular dichroism experiments.

I am grateful to Prof. Jacek Otlewski and Dr. Daniel Krowarsch, Faculty of Biotechnology, Wroclaw University, Wroclaw, Poland for providing circular dichroism facility. Interaction with Daniel regarding spectroscopy studies was interesting.

I would like to thank Toril A. Grønset, Proteomic Platform of University of Tromsø, Norway for her best technical assistance during LC-MS analyses. I am grateful to Morten Skaugen, Department of Chemistry, UMB for assistance and cooperation during proteomics analyses. My sincere thanks to Prof. Morten Sørlie, Department of Chemistry, UMB for his kind help with friendliness regarding thermodynamics (DSC and ICP) studies. Thanks to Solfrid Lohne, Department of Plant and Environmental Sciences, UMB for technical assistance during ICP analyses.

It is my pleasure to thank Dr. A. G. Appu Rao, Head, Department of Protein Chemistry and Technolgy, CFTRI, Mysore, India for his constant support during the project work in CFTRI.

I will cherish the moments that I spent in the laboratory at UMB and CFTRI.

My thanks go to the former and present colleagues in the laboratory of Food Science (UMB). Many thanks to Tor Bruun for his assistance during my experiments in the lab. Special thanks to Dr. Hilde Almaas, Dr. Camilla Sekse, Ragnhild A. Inglingstad, Irene Comi, Ellen K. Eriksen, Dr. Andrea Criscione, Dr. Flavio Tidona, Mestawet T. Asfaw, Camilla B. Furlund, Mohammad A. Islam and Diana Brumini for their nice interactions. Thanks to Rahul Agarwal (CIGENE, UMB) for sharing the bioinformatics knowledge with me.

Extending my special thanks to my former colleagues Dr. Srinivas Sistla, Dr. Guna Sekhar, Dr. Jay kant Yadav, Dr. Devaraj, Dr. Gajendra and present colleagues in the Department of Protein Chemistry and Technology (CFTRI). I would like to thank Roopesh, Arun, Shilpa, Vishwanath and Sahana for their friendly interactions during my work in CFTRI. Very special thanks to my friend Dr. Rajkumar for his friendly interactions during my PhD program.

I am grateful to the Masters students who I co-supervised during my PhD program. Teaching Masters/ PhD students has been an enjoyable experience for me! Adam Pomorski (Poland), Attiya Yasmin (Sweden) and Davide Capelli (Italy) have made me to invent the best ways of guiding and supervising students.

I would like to acknowledge all those who assisted me during my PhD studies at one stage or the other.

Finally, an honorable mention goes to my parents and wife Sowmya for their understanding and support to me in completing this project. Without help of the particular that mentioned above, we would face many difficulties while pursuing a PhD degree.

With everlasting gratitude,

29th November 2010 (thesis submission)

4th March 2011 (thesis final)

Ashoka Sreedhara, Ås, Norway

ABSTRACT

In this thesis, the structure, stability and digestion of caprine and bovine lactoferrin were compared. The work carried out in this project has been divided into four parts.

A comparative study was done on the thermal stabilities and conformational changes in the native forms of caprine lactoferrin (cLF) and bovine lactoferrin (bLF) in the pH range 2.0-8.0. In the pH range 2.0-8.0, bLF showed maximum thermal denaturation temperature (T_m) values than that of cLF. At pH 7.0, the T_m values of cLF and bLF were 67±1 and 70±1 ^oC, respectively. For both cLF and bLF, with reduced pH values a decrease in T_m values was observed. At pH 3.0, cLF and bLF showed 30±1 and 39±1 ^oC T_m values, respectively. At pH 2.0-3.0, the structural unfolding of cLF and bLF was observed. Both cLF and bLF were rich in β-structure (54 and 57%, respectively). Further at pH 2.0, tryptophans were exposed to the solvent to a greater extent in bLF than cLF. The thermal stability of bLF was higher than cLF and was pH dependent.

The structural characteristics and thermal stabilities of apo and holo forms of caprine and bovine LF were compared in the pH range 2.0-8.0. At pH 7.0, the holo forms of both cLF and bLF showed higher T_m values (68±1 and 90±1 ^oC, respectively) than the corresponding apo forms (64±1 and 66±1 ^oC, respectively). For both apo and holo forms of cLF and bLF, a continuous reduction in T_m values with a reduction in pH from 8.0 to 3.0 was evident. A reduction in pH from 7.0 to 2.0 showed significant loss in iron content of both apo and holo forms from both caprine and bovine LF. A higher exposure of hydrophobic surfaces at low pH for both apo and holo forms of cLF and bLF indicates the protein unfolding. These data were supported by the circular dichroism (CD) unfolding studies of both apo and holo forms of cLF and bLF at pH 2.0.

The interaction between zinc (Zn^{2+}) and LF from caprine and bovine was studied in the pH range 2.0-7.0. At pH 7.0, the zinc bound forms of cLF and bLF showed 67±1 and 83±1 ^oC T_m values, respectively. Thermal stability (T_m) values were decreased to 76±1 and 55±1 ^oC, respectively at pH 4.0. When the pH was reduced from 7.0 to 2.0, a significant loss in the zinc content of both cLF and bLF was observed. The CD results showed that at pH 2.0, the structure of zinc bound bLF (ZnbLF) was more unfolded than that of zinc bound cLF (ZncLF). The unfolding data was supported by the maximum exposure of tryptophan residues in ZnbLF than ZncLF at pH 2.0. Guanidine hydrochloride induced denaturation of ZncLF and ZnbLF indicated higher unfolding of the protein. In the pH range 2.0-7.0, a higher amount of iron binding to both cLF and bLF was observed when compared with the corresponding zinc bound forms. The thermal stabilities of ZncLF and ZnbLF were dependent of the pH and zinc binding.

A study was undertaken to identify peptides generated from bLF and cLF during *in vitro* digestion with human gastrointestinal enzymes, and to examine factors known to influence the outcome of protein degradation, 1) different concentrations of human gastric juice (HGJ) and human duodenal juice (HDJ), 2) different concentrations of bLF and 3) two different gastric pH values. Protein profiles of undigested and digested LF were obtained by SDS-PAGE. The degree of hydrolysis was assayed by the o-phthaldialdehyde (OPA) method. Peptides generated were identified by nano LC-MS. Protein degradation was highly dependent on gastric pH (2.5 and 4.0). At pH 2.5 lower content of intact LF and higher degrees of hydrolysis (~ 10.5) were observed. The peptide profiles from these samples revealed

higher number of peptides at pH 2.5 than at pH 4.0. Identical protein degradation patterns were seen in caprine and bovine LF samples. However, their peptide patterns showed differences with regard to number of different peptides and different sequence lengths. At pH 2.5 and 4.0, the apo and holo forms of bLF showed similar degradation patterns. More than 90% peptides were originated from the N-terminal part of bLF (native, apo and holo) or cLF (native) at pH 2.5 and 4.0. During the pH reduction to 2.5 or 4.0, the digested bLF with fast pH reduction generated more peptides when compared to that of slow pH reduction. After the action of HGJ and HDJ, more peptide fragments were detected in native bLF than that of native cLF at both pH values 2.5 and 4.0. The multiple sequence alignment of peptides from LF digests showed the presence of proline and leucine patterns at both pH values, 2.5 and 4.0. The use of *in vitro* digestion could contribute to a better knowledge about the generation of peptides during gastrointestinal digestion, however, this has to be confirmed by *in vivo* experiments.

LIST OF PAPERS

Paper I

A comparison of effects of pH on the thermal stability and conformation of caprine and bovine lactoferrin. Sreedhara A, Flengsrud R, Prakash V, Krowarsch D, Langsrud T, Kaul P, Devold TG & Vegarud GE. *Int. Dairy J. 20* (2010) 487-494.

Paper II

Structural characteristic, pH and thermal stabilities of apo and holo forms of caprine and bovine lactoferrins. Sreedhara A, Flengsrud R, Langsrud T, Kaul P, Prakash V, Vegarud GE. *Biometals 23* (2010)1159–1170.

Paper III

Zn (II) binding to lactoferrin at pH 2.0 - 7.0. A comparative study of caprine and bovine lactoferrin. Sreedhara A, Flengsrud R, Langsrud T, Prakash V, Vegarud GE. *''submitted manuscript''*

Paper IV

Peptides generated by the *in vitro* digestion of bovine and caprine lactoferrin

Furlund CB, Sreedhara A, Devold TG, Flengsrud R, Sekse C, Holm H, Jacobsen M, Vegarud GE (Manuscript)

1. INTRODUCTION

Milk - 'A perception of the traditional art to the modern science in India'

Every day, I get up in the morning and go to the market around the corner and buy a plastic bag of milk. It's fresh every morning, yummy! Buffalo milk is the best. The regular cow's milk is also available. This stuff isn't exactly low-fat, either, so it is really tasty… even in your coffee or tea! In rural India milk is delivered daily by a local milkman carrying bulk quantities in a metal container, usually on a bicycle; and in other parts of metropolitan India, milk is normally delivered in plastic bags via supermarkets.

Milk is as ancient as mankind itself, as it is the substance that feed the neonate of mammalian species from humans to whales. By 2000 B.C, the domesticated cow had appeared in North India, coinciding with the arrival of the Aryan nomads (Sanskrit word 'Arya' means noble). The Vedic civilization that ruled North India from about 1750 B.C to about 500 B.C depended much upon the dairy products of cows. Such a dependence on the cow milk was strengthened by the Vedas. Hence, the cow was considered as a sacred animal from ancient India to modern India!

Technological advances have come about in the history of milk consumption, and our generations will be the ones credited for having turned milk processing from art to science. The first modern dairy founded in India in 1946 under the brand name "AMUL" (Subrahmanyan et al., 1957; Misra, 1959). The cooperative was further developed with a technology breakthrough as it demonstrated that buffalo's milk, till then considered unsuitable for processing, could very well be formulated into a baby food powder. The first time on a commercial scale anywhere in the world. The availability of milk and milk products today in the modern world is a blend of centuries of old knowledge of traditional milk products with the applications of today's science and technology.

Milk has co-evolved with mammals and mankind to nourish their offspring and is a biological fluid of unique composition and richness. Milk is a complex fluid that contains 88% water and nutrients such as proteins, carbohydrates, fatty acids, minerals and vitamins secreted by the mammary gland (Shennan, 2008; Anderson et al., 2007; van Herwaarden et al., 2007; Lönnerdal, 2007; Bode, 2006). Milk contains all necessary nutrients for the growth and development of the newborn (German et al., 2008). The composition varies between different breeds and also between individuals within one breed (Ng-Kwai-Hang et al., 1982). The composition also varies between

different species like caprine, bovine and human (Anjaneyulu et al., 1985). Human milk contain macro- and micronutrients that influence the immune system and cognitive development, prevent pathogen colonization and positively modulate the intestinal microflora (Daniels & Adair, 2005; German et al., 2002; Harmsen et al., 2000; Kunz & Rudloff, 2006; Warner et al., 2001). Bovine milk is rich in bioactive compounds, which provide the newborn with protection from various infections (Korhonen & Pihlanto, 2006). The molecular understanding of biological milk function has emerged as a central theme in nutritional research (Ward & German, 2004).

1.1. Nutritional status of caprine, bovine and human milk

The composition of milk differs by the needs of the neonate of different species and can be affected by nutritional and non-nutritional (breed, parity, lactation, milk production and milk quality) factors (Arunvipas et al., 2003; Rook, 1961; DePeters & Cant, 1992). Human milk is the fit food for human infants for optimal growth and development (ESPGAN, 1982). While human milk is superior for the neonate, milk substituents (bovine or caprine milk) play a necessary role in nutrition and physiological effects as in infants when breast feeding is not possible, desirable or sufficient. The composition of caprine, bovine and human milk is shown in the **Table 1**. The nutritional composition varies among caprine, bovine and human milk. This may depend on the need of the neonate and the genetic set up of each species. The protein, fat and energy (cal) contents in caprine milk are higher than that of bovine and human milk. Caprine milk is significantly higher in the minerals calcium, phosphorous and sodium than bovine and human milk. The iron content in caprine, bovine and human milk is almost same, but the zinc content in caprine milk is almost two times lesser than that of human milk. It seems that there is a variable concentration of different vitamins in caprine, bovine and human milk (**Table 1**).

However, there were few negative effects of bovine milk on human health. Cow's milk protein (CMP) allergy is one of the most common food allergies and is potentially fatal (Brock et al., 2007; El-Agamy, 2007). The reported incidence of CMP allergy is in the range of 2 to 5%, of which only 60% are Immunoglobulin E (IgE) mediated (Host, 2002; Sampson, 2003). The rate of reported growing out of the allergy and the ability to tolerate milk also varies considerably and ranges between 29 and 76% for IgE-mediated cow's milk allergy (IgECMA). Cow milk allergy (CMA) is considered a common disease with a prevalence of 2.5% in children during the first 3 years of

life (Businco & Bellanti, 1993), occurring in 12–30% of infants less than 3 months old (Lothe et al., 1982), with an overall frequency in Scandinavia of 7–8% (Host et al., 1988), even as high as 20% in some areas (Nestle, 1987). Treatment with goat milk has resolved the CMA problems in humans to some extent (Sabbah et al., 1997; Reinert & Fabre, 1997). The nutritional and health benefits of goat milk have proved less allergenicity and better digestibility than cow milk (Fabre, 1997; Grzesiak, 1997).

Table 1. Composition of caprine milk in comparison to bovine and human milk

Component	Caprine	Bovine	Human
Moisture (%)	86.8	87.5	88.0
Protein (%)	3.3	3.2	1.1
Fat (%)	4.5	4.1	3.4
Carbohydrate (%)	4.6	4.4	7.4
Calories (cal)	72.0	67.0	65.0
Minerals (%)	0.8	0.8	0.1
Calcium (mg/100 g)	145.0	114.0	28.0
Phosphorous (mg/100 g)	130.0	90.0	11.0
Sodium (mg/100 g)	70.0	40.0	15.0
Potassium (mg/100 g)	136.5	160	55
Magnesium (mg/100 g)	15.0	12.0	4.0
Iron (mg/100 g)	0.3	0.2	0.2
Zinc (mg/100 g)	0.29	0.43	0.6
Selenium (mg/100 g)	1.33	0.96	1.52
Vitamin A (IU)	182.0	174.0	137.0
Vitamin C (mg)	1.0	2.0	3.0
Thiamine (µg)	50.0	50.0	20.0
Riboflavin (µg)	40.0	190.0	20.0
Niacin (µg)	300.0	100.0	-
Free folic acid (µg)	0.7	5.6	1.3
Total folic acid (µg)	1.3	8.5	-
Vitamin B_{12} (µg)	0.05	0.14	0.02

Anjaneyulu et al., 1985; Guo et al., 2001; Lindmark-Mansson et al., 2003; Leitner et al., 2004a; 2004b; Sahan et al., 2005; Garcia et al., 2006; Park & Chukwu, 1988; Park & Chukwu, 1989.

1.2. Milk proteins

Milk serves as one of the most excellent protein sources. Most of the milk proteins are synthesized in the mammary gland or, except for serum albumin that are retrieved from the blood. Mainly, milk proteins can be grouped into two classes: caseins (80%) and whey proteins (20%). In addition there are some minor proteins. Among these are the proteins that are related to milk fat globule membrane (MFGM). The caseins (αs1, αs2, β and κ) are organized in casein micelles. These micelles are large spherical particles of high molecular size (Dalgleish et al., 2004). The casein precipitates at pH 4.6, while the whey proteins remain soluble in the serum phase. A comparison of the quantities of caseins and whey proteins and minor proteins in caprine, bovine and human milk is shown in the **Table 2**. The total content of caseins in human milk is 4-5 times less as compared to bovine and caprine milk. Bovine milk has high content of whey proteins than caprine and human milk. Human milk lack beta-lactoglobulin (β-LG) and αS2-casein. But it has a higher content of α-lactalbumin (α-LA), lactoferrin (LF) and immunoglobulins (Igs) than bovine and caprine milk. Bovine milk has higher content of β-LG than caprine milk.

1.3. Whey proteins

Whey is a byproduct of cheese production and has until few years ago been regarded as a waste with low commercial value. Using new fractionation methods whey has been commercialized for the use in different products. Whey can be fractionated by membrane-based technology such as ultrafiltration (UF) or diafiltration (DF). UF mainly concentrates the proteins, whereas DF generates whey protein concentrates (WPC), in which lactose, minerals, and the low molecular weight fractions are removed. The protein content in WPC may differ between 35 and 80% (w/w) and even up to 90% (w/w), a so called whey protein isolate (WPI). The major whey proteins are β-lactoglobulin (β-LG), α-lactalbumin (α-LA), serum albumin (SA), immunoglobulins (Igs), lactoferrin (LF) and lactoperoxidase (LP). Since whey proteins have a high nutritional value, the individual whey proteins have been isolated and commercialized to be used as ingredients in many products (Kinsella & Whitehead, 1989).

Table 2. Total quantity (mg/ml) of caseins, whey proteins and minor proteins present in caprine, bovine and human milk

Protein	Caprine (mg/ml)	Bovine (mg/ml)	Human (mg/ml)
Total caseins	19.8-26.8	27.2	5.8
Whey proteins	3.3	4.5	2.1
Casein/ whey ratio	6.6-7.9*	6.0	2.8
αs1-Casein	0-7.0*	10.0	0.8
αs2-Casein	4.2	3.7	-
β-Casein	11.0	10.0	4.0
κ-Casein	4.6	3.5	1.0
α-Lactalbumin	1.2	1.2	1.6
β-Lactoglobulin	2.1	3.3	-
Lactoferrin	0.02-0.2	0.1-0.3	1.0-4.0
Serum albumin	0.26-0.30	0.1-0.4	0.1-0.4
Immunoglobulins	0.047-0.17	0.26	0.38
Lactoperoxidase	0.03	0.03	-

* The values correspond to the mean amounts recorded for goat homozygous 0/0 and A/A at the αS1-casein locus, respectively.

Brignon et al., 1985; Chtourou et al., 1985; Fox & McSweeney, 1998, 2003; Grøtte, 2001; Henart et al., 1991; Martin & Grosclaude, 1993; Martin et al., 1996; Masson et al., 1966; Masson & Heremans, 1971; Miranda et al., 2004; Sanchez et al., 1988; Drackova et al., 2009; Polis & Shmukler, 1953; de Wit & van Hooydonk, 1996; Park et al., 2007.

1.4. Lactoferrin

Lactoferrin (LF) was identified as a milk protein in 1960 (Groves, 1960). It is an 80 kDa iron-binding monomeric glycoprotein belonging to the transferrin (TF) family with an isoelectric point ~ 9.0 (Peter & Margaretha, 1995; Steijns and & Hooijdonk, 2000). Lactoferrin is widely distributed in mammalian milks, other secretory fluids and white blood cells, and it has a complex biology (Adlerova et al., 2008; Farnaud & Evans, 2003;

Jennes, 1980). The concentration of LF in milk of mammals is quite variable and dependent on the lactation stage. In human milk, LF is a major whey protein, with 6.0-8.0 mg/ml in colostrum and 1.0-4.0 mg/ml in milk (Henart et al., 1991; Masson & Heremans, 1971). Bovine colostrum and milk are much lower in LF content (~ 1.0 mg/ml and 0.1-0.3 mg/ml for colostrum and milk, respectively) (Masson et al., 1966; Sanchez et al., 1988). Caprine milk contains about 0.02 to 0.2 mg/ml of LF (Park et al., 2007). It can be purified on an industrial scale from whey by cation exchange chromatography (Law & Reiter, 1977; Yoshida et al., 2000; Ounis et al., 2008; Recio & Visser, 2000). The isolated LF can be used as a preserving agent in food, drugs, and cosmetics (Saito et al., 1994). This protein is homologous to the iron binding protein from serum, transferrin, but the proteins appear to differ from each other with respect to structure and function (Gordon et al., 1963). The LF has the ability to bind two Fe^{3+} ions together with two CO_3^{2-} ions (Baker, 1994). There are two metal-binding sites in two lobes, known as N- and C-. The lobes are further divided into two identical domains, N1, N2 and C1 and C2. The two iron atoms are surrounded by each lobes; N1, N2 and C1 and C2 (Baker & Baker, 2004). The LF shows visible absorption spectra at 465 nm (Recio & Visser, 1999, 2000).

1.4.1. Lactoferrin in different species

The high concentration of LF in human milk indicates that it plays a vital role in the growth of the newborn. Since 25 years, the bLF obtained from whey during cheese-making process has been used as a supplement in special products. The milk products based on bovine milk, have a low content of LF, therefore the supplementation with this protein to make infant formula more similar to human milk is of considerable interest. There has been an increasing demand for natural compounds acting as antibiotics to supplement specialty foods and pharmaceutical products, in which LF could exert such a role (Smithers et al., 1996). As the interest in LF production is growing, it is necessary to evaluate the thermal stability of this protein to design treatments which maintain the biological activity.

Current sequence databases contain LF sequences from nine species: human, mouse, sheep, bovine, horse, pig, caprine, buffalo and camel (Goodman & Schanbacher, 1991; Metz-Boutigue et al., 1984; Provost et al., 1994; Rado et al., 1987; Rey et al., 1990; Recio & Visser, 1999a). The mature LF from these species comprises 690 residues and share pair wise identities that range from a minimum of 65% to nearly 100%. They are 90% identical. Human

lactoferrin (hLF) shares about 68% sequence similarities with both cLF and bLF. The high sequence similarity in primary structures among LFs of the three species indicates that there might be small variations in their overall functional aspects. **Figure 1** shows the sequence alignments of cLF and bLF. The secondary structure of hLF was taken from Anderson et al. (1989). Lactoferrin from bovine is rich in α helices and β strands as compared with that of caprine (**Figure 1**). Along the bLF and cLF sequences, amino acid residues in the positions 10, 40, 42, 51, 61, 126, 129, 135, 151, 265, 269, 272, 327, 342, 356, 382, 393, 498 and 606 are different. These residues have resided mainly in α helices. This will either stabilize or destabilize the protein secondary structure. For example, Asp61 in cLF is replaced with Gly in bLF. This destabilizes the α helix in bLF. Similarly, Glu51 in bLF is replaced with Gly in cLF. Tyr135 in bLF is replaced with Pro in cLF. Pro gives structural rigidity to cLF. Such factors can be explained in more detail by 3D structural analysis of hLF (Anderson et al., 1989; Haridas et al., 1995).

1JW1 Signal sequence -19 MKLFVPALLSLGALGLCLA
1BLF MKLFVPALLSLGALGLCLA
1H43 MKLVFLVLLFLGALGLCLA

Clustered basic residues **Fe**
β β β β β αααααααααααα ββββββββ αααααααααα βββαα
1JW1 APRKNVRWCAISLPEWSKCYQWQRRMRKLGAPSITCVRRTSVLECIRAIAGKNADAVTLD 60
1BLF APRKNVRWCTISQPEWFKCRRWQWRMKKLGAPSITCVRRAFALECIRAIAEKKADAVTLD 60
1H43 GRRRSVQWCAVSQPEATKCFQWQRNMRKVRGPPVSCIKRDSPIQCIQAIAENRADAVTLD 60

Fe
αααααα ββββββββββ βββ ββββββββ
1JW1 DGMVFEAGRDPYKLRPVAAEIYGTEKSPQTHYYAVAVVKKGSNFKLDQLQGQKSCHMGLG 120
1BLF GGMVFEAGRDPYKLRPVAAEIYGTKESPQTHYYAVAVVKKGSNFQLDQLQGRKSCHTGLG 120
1H43 GGFIYEAGLAPYKLRPVAAEVYGTERQPRTHYYAVAVVKKGGSFQLNELQGLKSCHTGLR 120

CO_3^{2-}
ααααααααααα ααααααα βββ βββ
1JW1 RSAGWNIPVGILRPPLSWTESAEPLQGAVARFFSASCVPCVDGKAYPNLCQLCKGVGENK 180
1BLF RSAGWIIPMGILRPYLSWTESLEPLQGAVAKFFSASCVPCIDRQAYPNLCQLCKGEGENQ 180
1H43 RTAGWNVPIGTLRPFLNWTGPPEPIEAAVARFFSASCVPGADKGQFPNLCRLCAGTGENK 180

Fe
αααααααααα βββββααααααα αααααββββ
1JW1 CACSSQEPYFGYSGAFKCLQDGAGDVAFVKETTVFENLPEKADRDQYELLCLNNTRAPVD 240
1BLF CACSSREPYFGYSGAFKCLQDGAGDVAFVKETTVFENLPEKADRDQYELLCLNNSRAPVD 240
1H43 CAFSSQEPYFSYSGAFKCLRDGAGDVAFIRESTVFEDLSDEAERDEYELLCPDNTRKPVD 240

Fe
βββ βββββ ααααααααααααααα
1JW1 AFKECHLAQVPSHAVVARSVDGKENLIWELLRKAQEKFGKNKSQRFQLFGSPEGRRDLLF 300
1BLF AFKECHLAQVPSHAVVARSVDGKEDLIWKLLSKAQEKFGKNKSRSFQLFGSPPGQRDLLF 300
1H43 KFKDCHLARVPSHAVVARSVNGKEDAIWNLLRQAQEKFGKDKSPKFQLFGSPSGQKDLLF 300

Interlobe connecting helix
βββββ ααααααααααααααααααααααααααββββββααααααααααααα
1JW1 KDSALGFLRIPSKVDSALYLGSRYLTALKNLRETAEEVKARCTRVVWCAVGPEEQSKCQQ 360
1BLF KDSALGFLRIPSKVDSALYLGSRYLTTLKNLRETAEEVKARYTRVVWCAVGPEEQKKCQQ 360
1H43 KDSAIGFSRVPPRIDSGLYLGSGYFTAIQNLRKSEEEVAARRARVVWCAVGEQELRKCNQ 360

Fe
βββββββ ααααααααααα βββαααααααααα βββββββ
1JW1 WSEQSGQNVTCATASTTDDCIALVLKGEADALSLDGGYIYTAGKCGLVPVMAENRKSSKH 420
1BLF WSQQSGQNVTCATASTTDDCIVLVLKGEADALNLDGGYIYTAGKCGLVPVLAENRKSSKH 420
1H43 WSGLSEGSVTCSSASTTEDCIALVLKGEADAMSLDGGYVYTAGKCGLVPVLAENYKSQQS 420

Fe **CO_3^{2-}**
ββββββββ ββββ ααααααααααααα
1JW1 SSLD--CVLRPTEGYLAVAVVKKANEGLTWNSLKGKKSCHTAVDRTAGWNIPMGLIANQTGS 478

Fig. 1. A comparison of the sequences of caprine and bovine lactoferrin. The multiple sequence alignment (MSA) was done using CLUSTAL 2.0.10 software. The secondary structure of human lactoferrin was taken from Anderson et al. (1989). The different colors of residues indicate the amino acids with relevant properties.

1.5. Structure of lactoferrin

The three dimensional (3D) structure of this protein was determined in 1987, giving the first atomic view of any member of the transferrin family (Anderson et al., 1987). The structural organization of LF has been determined in detail for human lactoferrin (hLF) (Anderson et al., 1989; Haridas et al., 1995). The studies on the structures of LFs from bovine, caprine, mare, buffalo, and camel have shown that the same basic 3D structure is shared by all LFs (Karthikeyan et al., 1999; Khan et al., 2001; Moore et al., 1997; Sharma et al., 1998). The single polypeptide chain is folded into two globular lobes (**Figure 2**), representing its N- and C- terminal halves (residues 1-333 and 345-691 in human LF). The two lobes are connected by a peptide of 10-15 residues (residues 334-344 in human LF), which forms a 3-turn α-helix that are stabilized by hydrophobic interactions between them. Both lobes have the same fold.

The two lobes arose as product of gene duplication (Williams, 1982; Metz-Boutigue et al., 1984). The internal structure of LF is highly conserved, and is dedicated to bind iron (Fe^{3+}), which is sequestered in two almost identical sites, one in each lobe of the molecule. In each lobe, two α/β domains, referred to as N1 and N2, or C1 and C2, enclose a deep cleft within which is the iron binding site. This 2-lobe, 4-domain structure provides a key to understand the dynamic properties of LF. The iron binding and release are associated with a significant conformational change. The protein becomes highly compact when iron is bound (Baker et al., 1994).

The LFs from different species have identical metal and anion binding sites. The metal binding pocket contains four protein ligands, two Tyr, one Asp and one His. The three negative charges from the protein ligands are balanced with three positive charges of iron ion together with a helix N-terminus and Arg side chain whose positive charge balances the negative charge on the CO_3^{2-} anion. The LF structural characteristics relating to iron binding are strongly conserved.

Holo LF from human acquire a ‘‘closed’’ structure in which the two domains of each lobe enclose the bound Fe^{3+} ion, effectively sequestering it from the external environment. Four protein ligands plus the synergistically-bound CO_3^{2-} anion are covalently bound to the metal ion, which cross links the two domains (Anderson et al., 1987; Baker et al., 1987, 1994). This explains the high stability of this rigid structure and the difficulty of removing the bound metal.

Crystallographic studies on the apo form of hLF have shown that the release of iron involves rigid-body domain movements in each lobe, in which one domain swings away from the other to open up the binding cleft. This movement is made possible by a hinge in two polypeptide strands that run behind each iron binding site (Anderson et al., 1990; Gerstein et al., 1993; Jameson et al, 1998). In the absence of a bound metal ion (apo form) to lock the two domains of each lobe together, the apo form is flexible. Although it probably exists in the open form (the N lobe adopts open conformation while both the closed and open conformations have been observed for C lobe), the small energy difference between open and closed forms enables it to fluctuate between these states (Baker et al., 2002; Gerstein et al., 1993; Grossman et al., 1992, Jameson et al., 1998). This energy difference may be greater in the LFs of some species than others (Khan et al., 2001).

An analysis of hLF 3D structure shows a large domain movement in the N-terminal half of the molecule when compared with holo LF form (Norris et al., 1991). The superposition of N- and C- individual domains of human apo LF on to the corresponding domains of holo LF show a very little difference in the domain structure.

The glycans linked to the N-glycosylation sites in bLF possesses heterogeneous structures (Spik et al., 1982; Wei et al., 2001). Most of the glycosylation sites are highly exposed, on the protein surface, and the sugar residues have minimal interaction with the protein structure, at most a few hydrogen bonds.

Human and bovine LFs share 69% sequence homology and their tertiary level structures are very similar (Pierce et al., 1991). The superposition of cLF and bLF is shown in the **Figure 2 (C)**. The 3D structural superimposability of cLF and bLF shows that they are very similar. The 3D structures of cLF and bLF are 90% identical. The overall structure of cLF is essentially similar to that of bLF (Kumar et al., 2002). The LFs from caprine and bovine have the same basic architecture, with two similar lobes connected by a linker region and each lobe having two sub-domains that form a deep cleft in which iron is bound. The iron-binding environment in cLF is somewhat different, in which two CO_3^{2-} ions have low occupancies. The iron binding pattern of bLF is shown in the **Figure 2 (D)**.

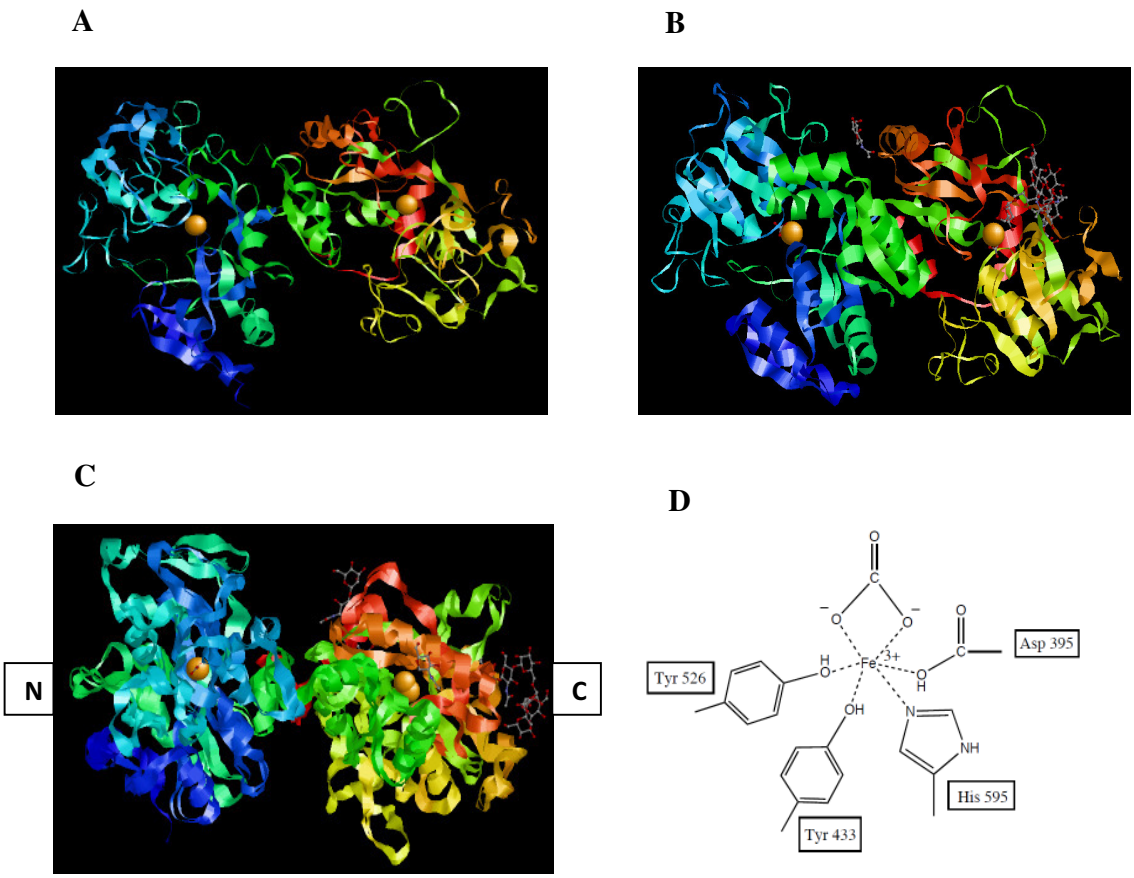

Fig. 2. 3D-structures of lactoferrin from (A) caprine (PDB file 1jw1) and (B) bovine (PDB file 1blf) species. (C) Super position of 3D-structures of caprine and bovine lactoferrin. (C) The general type of ferric (Fe) ions bonded to bovine lactoferrin. The iron (Fe) atoms are shown in yellow. The super positioned structure was a PYMOL Script (P1M format) from http://topmatch.services.came.sbg.ac.at/.

1.6. Lactoferricin

Lactoferricin (LFcin) is a highly basic peptide derived from the N-terminal region of LF which is not involved in iron binding (Bellamy et al., 1992a, b). It has a molecular weight ~ 3.15 kDa. Human lactoferricin (hLFcin), bovine lactoferricin (bLFcin) and caprine lactoferricin (cLFcin) corresponds to the amino acid residues 1-47, 17-41 and 18-42 (14-42), respectively, from the N-terminal region of the protein (Hunter et al., 2005; Hwang et al., 1998; Recio & Visser, 2000). All LFcins have an 18-residue loop stabilized by a disulfide bridge (Wakabayashi et al., 2003). The structure of hLFcin and bLFcin are shown in the **Figure 3**. The sequences of human and bovine LFcins

form a surface-exposed α-helix with a hydrophobic tail within the native proteins (Odell et al., 1996). The peptide adopts a different conformation when released from the parent protein (Schibli & Vogel, 2000). The solution structures of hLFcin and bLFcin were derived by NMR (Hunter et al., 2005; Hwang et al., 1998). Divergent from the β-sheet structure of the 25-residue bLFcin, hLFcin adopts a coiled structure under similar conditions. The hLFcin is larger in size as compared with that of bLFcin, hence the lack of amino acid sequence homology, leading to differences in charge distribution and hydrophobic character. The cLFcin structure is not available in PDB. The LFcin purified from caprine LF (cLFcin) shows a high similarity (72% sequence identity) to bLFcin (Recio & Visser, 2000). As part of the intact LF, the peptide forms a number of stabilizing, long range hydrophobic contacts with other parts of LF. Such long range hydrophobic interactions may encourage the formation of the α-helix in the intact protein and these would not be present in bLFcin peptide. The primary sequence of bLFcin contains many hydrophobic and positively charged residues, suggesting that it may interact with biological membranes (Hwang et al., 1998; Yamauchi et al., 1993).

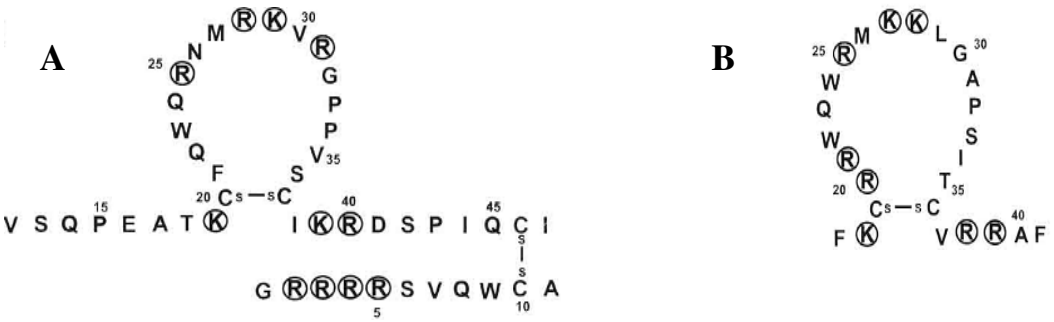

Fig. 3. Primary structures of (A) hLFcin and (B) bLFcin. Circles indicate the basic amino acid residues (Wakabayashi et al., 2003).

1.7. Stability and structure relationships of lactoferrin

The structural factors such as hydrogen bonding, hydrophobic interactions, disulfide bonds, amino acid composition and ligand binding play a major role in the stabilization of proteins (Kristjansson & Kinsella, 1991; Mozhaev & Mertinek, 1984). The stability of native proteins is a function of external variables such as pH,

temperature, ionic strength, and solvent, as they change the various forces that are responsible for the intrinsic stability of the protein (Privalov, 1979). The thermal stability is important when LF is used as a bioactive component in foods. The effect of heat treatment on bLF has been widely studied including the effect on some aspects related to its biological activity (Abe et al., 1991; Kawakami et al., 1992; Oria et al., 1993; Paulsson et al., 1993; Sanchez et al., 1992b). A study has shown that heating bovine milk at 85 ^{o}C for 30 min will denature LF and lead to a loss of the biological activity. (El-Agamy, 2000). Abe et al (1991) have studied the influence of pH 2.0-11.0 on the heat stability of bLF. At pH 4.0, when 1% bLF was preheated at 70 ^{o}C for 3 min followed by UHT at 130 ^{o}C for 2 s, only 3% loss of residual iron-binding capacity was shown compared with that of unheated sample. The bLF heated at pH 2.0 at 120 ^{o}C for 15 min had no iron-binding capacity (Saito et al., 1991). The thermal stability of bLF seemed to be affected by environmental conditions such as pH, salts, and whey proteins (Kussendrager, 1994). The heat-sensitivity of apo bLF and holo bLF was higher in milk than in phosphate buffer, where apo bLF was denatured faster than Fe-bLF (Sanchez et al., 1992). The study of the heat-induced denaturation of LF under the conditions of the application of interest is important. Mata et al (1998) studied the thermal denaturation of hLF and recombinant hLF by DSC. The thermal denaturation temperature (T_m) of hLF and iron saturated holo hLF were 67.0 and 90.6 ^{o}C, respectively. The thermal stability of hLF is higher than that of bLF (Mata et al., 1998). The binding of iron to LF may be an important factor in the thermal resistance of the protein structure. Resistance of LF to unfolding increases with iron saturation. Two thermal transitions have been observed for native bLF (Kussendrager, 1994; Paulson et al., 1993). The first transition (65 ^{o}C) corresponds to the apo LF form and the second (90-92 ^{o}C) to the holo LF form. The two transition peaks are due to N- and C- lobes in the structure of LF (Anderson et al., 1987). The effect of iron saturation on the thermal aggregation of bLF was characterized by Brisson et al (2007). It was shown that the iron saturation increased the thermal stability of LF and decreased aggregation. Spik et al (1988) have reported differences in the glycan composition of lactoferrin from different species such as human, mouse, bovine and caprine. The number and location of potential glycosylation sites, and the sites actually used, vary among LF in different species. The bLF and cLF have four glycans each. The relative proportions of glycan of oligomannosidic and of N-acetyllactosamine type vary with period of lactation. The primary structures of specific glycans bound to cLF and bLF seems to vary. The thermal stability of LF is influenced by the characteristics of glycans present. This differs in cLF and bLF (Spik et al., 1988; Van Berkel et al., 1996). The thermal denaturation temperature studies on the LF from milk of human, sheep, goat, camel, alpaca and elephant was done by Conesa et al (2008). The LF from

goat and sheep showed two thermal denaturation peaks. This pattern of denaturation was also found for iron saturated bLF (Sanchez et al., 1992c). This was explained by the different heat sensitivity between the two lobes of LF appearing in the C- lobe more compact than the N- lobe. The behavior could be also due to the formation of monoferric species, as was suggested that iron bound to lactoferrin might be sequestered by phosphate when temperature increases in the DSC analysis (Ruegg et al., 1977). The differences in the thermograms and the values of the thermodynamic parameters among LFs from milk of different species may indicate some diversity in their structure (Baker & Baker, 2005). The difference in the amino acid sequences of LFs from different mammal species is an important factor that influences the thermal stability of the protein. Nam et al (1999) have reported the circular dichroism secondary structure of goat LF. The secondary structural elements α helix and β structure could be related to the stabilities of LFs.

1.8. Role of metal ions

The metal ions enhance the structural-stability of a protein in the conformation required for biological function. The metal ions can alter the protein conformation upon binding (Rasmussen, 1990). The LF binds two Fe^{3+} *in vivo* in presence of two CO_3^{2-} (Anderson et al., 1987; Baker et al., 1994). The LF has a much higher (~ 300 fold) affinity for iron than TF (Brock, 1997). All LFs and TFs so far characterized have essentially identical metal and anion (CO_3^{2-}) binding sites, which appear to be optimized for binding Fe^{3+} and CO_3^{2-}. The iron bound holo form of LF is conformationally rigid and very stable. Other metal ions such as Ga^{3+}, Al^{3+}, VO^{2+}, Mn^{3+}, Co^{3+}, Cu^{2+} and Zn^{2+} can bind to LF with less affinity than Fe^{3+} (Ainscough et al., 1979; Baker, 1994; Baker et al., 1994; Swarts et al., 2000). The reaction of a metal ion, M^{2+} or M^{3+} with apo LF can be explained by the following equation (Harris & Stenback, 1988):

M^{2+} or $M^{3+}+HCO_3^-+$apo LF$\leftrightarrow$M-HCO_3-LF$+2H^+$

The protein folding process is in general driven by hydrogen bonding, disulfide cross-linking, simple steric interactions between specific amino acid side chains, and hydrophobic effects (Kraulis et al., 1992). In the case of LF, metal ions may also facilitate protein folding and stability by providing internal cross-links that directly lead to the final conformational state as an apo or a holo form. (Anderson et al., 1990; Baker et al., 2002; Wally &

Buchanan, 2007). Zn^{2+} ions play a wide range of structural and catalytic roles in natural proteins (Stillman & Presta, 2000; Underwood, 1977).

1.9. Biological functions of lactoferrin

Lactoferrin is a part of transferrin protein family which plays a key role in controlling the level of free iron in body fluids (Baker et al., 2002). It is an important component of the innate immune system. The LF is considered to be an important host defense molecule and has a diverse range of physiological functions such as antimicrobial/antiviral activities, immune modulatory activity, and antioxidant activity (Baveye et al., 1999; Chierici, 2001; Ward et al., 2005). The oral administration of LF exerts several beneficial effects on the health of humans and animals, including anti-infective, anticancer, and anti-inflammatory effects (Kawakami et al., 1988; Takeuchi et al., 2004; Sato et al., 1996; Togawa et al., 2002a, b; Dial et al., 2005; Tsuda et al., 2000). The bovine lactoferrin (bLF) has been reported to stimulate the growth of bifidobacteria and lactobacilli. The purified LF from caprine colostrum may constitute a novel anticancer agent for the food industry (Kim et al., 2009).

The LF could play a role in homeostasis of iron and other trace elements (Baker et al., 2000; Jabeen et al., 2005; Kozlowski et al., 2009; Kumar et al., 2000). The protein is involved in the transport of iron, zinc and cobalt and their regulation absorption (Marchetti et al., 1999). In the gastrointestinal tract of humans, preabsorptive processes substantially influence the zinc availability from LF. The gastric pH and/or intestinal pH could be important factors affecting both the solubilization of zinc in the stomach and its absorption by the intestine (McClain et al., 1980; Korhonen & Pihlanto, 2006). The bacteriostatic effect of LF is most probably attributed by apo form, where the bacteria are deprived of Fe 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). Lactoferrin possesses an intrinsic bactericidal activity that is unrelated to its capacity to bind iron (Arnold et al., 1977, 1980).

The high pI of LF (~ 9.0) and its strong cationic nature is a major factor in the ability of LF to bind to different anionic molecules and cells. The binding ability of LF to other macromolecules like DNA and proteins depend on the surface properties of LF. The LFcin domain in the N-terminal of LF is a major factor in the antibacterial activity of intact LF (Bellamy et al., 1992; Gifford et al., 1998). The N-lobe of hLF binds specifically

through LFcin region to the bacterial cell-surface protein, the pneumococcal surface protein A (PspA) from human pathogen Streptococcus pneumoniae (Senkovich et al., 2007). The LF inhibits the biofilm formation (Singh et al., 2002). Glycosylation plays an important role in the function of LF (Wei et al., 2001). Glycans of cell-surface are frequently targeted by viruses to gain entry to human cells, and some antiviral effects of LF may be provided by its carbohydrate (Spik et al., 1988; Valenti & Antonini, 2005).

1.10. Digestion of caprine and bovine lactoferrin and bioactive peptides

Bioactive peptides (BAP) may be generated *in vivo* through gastrointestinal processes. These peptides, encoded within the sequences of native protein precursors, may also be generated *in vitro* by enzymatic hydrolysis. The BAP are relatively short, with 3-20 amino acid residues and their activity is based on their amino acid sequence and composition (Korhonen et al., 1998). They are cationic, amphiphilic and active against bacteria, viruses, fungi and protozoa (Martin et al., 1995; Bellamy et al., 1992; Meisel & Schlimme, 1996; Pihlanto & Korhonen, 2003). The action of pepsin on LF generates peptides that have enhanced antimicrobial action as compared to LF. Many BAP serve in multifunctional capacities and often share common structural features based on a defined, biospecific role (Tomita et al., 1991). The bovine lactoferricin (LFcin B) is a potent bactericidal peptide specifically generated by pepsin degradation of LF (Bellamy et al., 1992; Jones et al., 1994; Tomita et al., 1991). This reaction is catalyzed at acidic pH by pepsin, and it has been shown that it can take place in the stomach releasing LFcin B into the intestine, where it is relatively stable (Kuwata et al., 1998a, 1998b). There is a direct evidence for the generation of LFcin in human stomach after the ingestion of LF (Kuwata et al., 1998a). The LFcin B is more potent than LFcin H with respect to the antibacterial activity (Chen et al., 2006; Vorland et al., 1998). There were studies on the BAP derived from the pepsin digested bLF (Dionysius & Milne, 1997; Recio & Visser, 2000). Those peptides were cationic and originated from the N-terminus of bLF in a region where LFcin B was identified. The peptide I corresponds to residues 17-42 (3195 Da). Peptide II consisted of two sequences, residues 1-16 and 43-48 (2673 Da), linked by a disulfide bond. Peptide III, a disulfide linked heterodimer, corresponds to residues 1-48 (5851 Da). Further, lactoferrampin (LFampin) peptides (residues corresponding to amino acids in the regions 268-284, 259-284, 265-296 and 265-284 in bLF) were generated by the proteolysis of bLF by pepsin (Bolscher et al., 2006). Two peptides with M_r values 2205.3 (residues 271-288) and 3494.1 (residues 14-42 corresponds to LFcin C) were

isolated from the peptic hydrolysate of cLF (Recio & Visser, 2000). There were reports on the synthesis of antimicrobial LFcin and LFampin peptides (Haney et al., 2007, 2009; van der Kraan et al., 2004, 2006).

There were *in vivo* studies on bLF digestion in stomach (Troost et al., 2001; Kuwata et al., 1998a, 1998b; Kuwata et al., 2001). More than 60% of administered bLF survives passage through the adult human stomach. Some parts of ingested LF were partly digested and remained in lower gastrointestinal tract. A peptide with residues 382-389 was generated from the C lobe of bLF in human duodenum 20 min after the milk ingestion (Chabance et al., 1998). A sixteen residue antimicrobial peptide corresponding to residues 25-35 in LF was observed in the pepsin hydrolysate of cLF (Kimura et al., 2000). An *in vitro* digestion model was developed by Almaas et al (2006) to study the milk protein degradation by human gastric juice (HGJ) and human duodenal juice (HGJ). The digestion profiles of caprine whey proteins were compared with respect to the digestion profiles of human and porcine enzymes. The proteins from caprine milk were shown to be degraded faster than that of bovine milk. The cLF present in whey was about 94% degraded (Eriksen et al., 2010). The holo form of LF was shown to be more resistant to degradation than apo LF (Baldi et al., 2005; Brines & Brock, 1983; Gonzalez-Chavez et al., 2009). Brines & Brock (1983) have showed that LF resists digestion by pepsin at pH 5.0. The physiological approaches (pH) with respect to the natural environment in human gastrointestinal system was reviewed (Ekmekcioglu, 2002). The absorption of iron during human digestion is important in the bioavailability studies (Kalantzti et al., 2006). A study on the effects of aging with an age range 18-98 years and gastritis on gastric acid and pepsin secretion in humans were done by Feldman et al (1996). 4000-5000 IU pepsin is necessary for optimal protein digestion (Ekmekcioglu, 2002). A study on human pancreatic exocrine response to nutrients in health and disease has explained the importance of activities of duodenal enzymes (Keller & Layer, 2005). Hence, considering the above literature, during the digestion studies of iron binding protein, LF, the different gastric and duodenal pH values, quantity and composition of digestive enzymes, the transit time in the various parts of the gastrointestinal tract were considered.

MAIN OBJECTIVES

The main objective for this project was to compare thermal stabilities, conformational characteristics and *in vitro* digestion patterns of caprine lactoferrin (cLF) and bovine lactoferrin (bLF) and to observe the differences between them. An observation of variations of these factors with regard to native, apo and holo forms of lactoferrin from the two species was also part of the objective. The influence of pH and iron content on the thermal stability and protein conformation were of importance.

I. A comparison of effects of pH on the thermal stability and conformation of caprine and bovine lactoferrin

The aim of the study was to compare the structural stability of bLF and cLF with respect to the influence of pH (2.0 - 8.0) and denaturation temperature values (T_m). This was investigated by tryptophan fluorescence, ANS-binding, acrylamide-quenching, circular dichroism and thermal denaturation measurements which shows changes in surface hydrophobicity values, α-helices and β-structures of the protein.

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

The aim of the paper was to compare the structural characteristics of holo and apo lactoferrin forms from caprine and bovine milk with respect to iron binding and release and the thermal denaturation values at different pH values (2.0 - 7.0) and to observe the differences from the native lactoferrin.

III. Zn (II) binding to lactoferrin at pH 2.0 - 7.0. A comparative study of caprine and bovine lactoferrin

The aim of this paper was to determine the conformational characteristics and thermal stabilities of the zinc bound forms of cLF and bLF with respect the influence of pH 2.0 - 7.0 and to observe differences from the iron bound forms of LF.

IV. Peptides generated by the *in vitro* digestion of bovine and caprine lactoferrin

The aim of the study was to identify and compare the peptides generated from cLF and bLF digested using human gastric juice (HGJ) and human duodenal juice (HDJ) varying the following factors. (1) Different activities of HGJ and HDJ, (2) Different concentration of bLF, and 3) different additions of acid to simulate two gastric pH values, 2.5 and 4.0. This constituted the *in vitro* digestion model.

MAIN RESULTS AND DISCUSSION

This section consists of a summary of the results and a general discussion of Papers I-IV. The **Figures** and **Tables** referred to in bold below are found in individual papers. The figures and complementary information in a broader perspective are found in the respective papers.

Paper I

A comparison of effects of pH on the thermal stability and conformation of caprine and bovine lactoferrin

Sreedhara A, Flengsrud R, Prakash V, Krowarsch D, Langsrud T, Kaul P, Devold TG & Vegarud GE. *Int. Dairy J. 20* (2010) 487-494.

In order to study the stability and conformation of a protein in detail, it must be pure and homogeneous. Protein purification is vital for the characterization of the structure, stability, function and interactions of the protein of interest. The lactoferrin (LF) purified from caprine whey was shown to be 95% pure and homogeneous (**Figure 1**). The molecular mass values of caprine lactoferrin (cLF) and bovine lactoferrin (bLF) were 78±1 and 79±1 kDa, respectively, and these values were consistent with previous results (Brock, 1985). The degree of iron (Fe) saturations in the native forms of cLF and bLF were 5.0 and 15.0%, respectively (**Figure 2**). At pH < 3.0, LF from both species released most iron. The amount of iron bound to bLF was higher than that of cLF in the pH range 2.0-8.0. The binding of Fe is sequential to the two lobes (N and C) of LF. But, both the Fe binding sites in LF are identical. The mechanism of Fe binding to LF is not clear (Baker & Baker, 2004). The N and C lobes in LF conformation contains two domains N1, N2 and C1, C2, respectively, with Fe binding sites situated in the interdomain clefts (Shimazaki et al., 1993). The diferric LF has the ability to bind two Fe-ions. The LF molecule is capable of solubilizing up to a 70-fold molar equivalent of Fe, which is much higher than the specific Fe-binding ability of LF (Kawakami et al., 1993). The electrostatic binding properties of cLF and bLF are different. (Baker & Baker, 2009; Hu et al., 2008; Lonnerdal, 1989; Nagasako et al., 1993). That affects the different iron-binding status of cLF and bLF in the pH range 2.0 – 8.0.

At pH 7.0 the thermal denaturation temperature values (T_m) of bLF and cLF were shown to be 70±1 and 67±1 ^oC, respectively (**Figure 3A and B**). The bLF had a greater thermal stability than cLF in the pH range 6.0-8.0. From pH 7.0 to 3.0, a gradual reduction in the T_m of both bLF and cLF was observed and reached 39±1 and 30±1

^{o}C, respectively (**Table 1**). The thermal stabilities of cLF and bLF decreased with a successive decrease in pH vales from 7.0 to 3.0. The T_m values obtained from both methods (CD and UV) were in close agreement with each other. The LF samples showed aggregation at pH 2.0 and it was difficult to measure the T_m at that pH. This is attributed to the maximum surface hydrophobicity of LF at pH 2.0. At pH 2.0-3.0, LF becomes flexible and more prone to thermal denaturation. But at pH 7.0, the molecule will be in a closed state with sequential iron binding to N- and C-lobes in LF (Baker & Baker, 2009). The T_m results obtained might depend on the isolation methods used for the cLF and bLF and the detection limits of the analytical equipment used. The total number of tryptophan residues in cLF and bLF were 12 and 13, respectively. This might affect the T_m of cLF and bLF.

The cLF showed a maximum emission value (λ_{max}) of 339 nm at pH 7.0. The λ_{max} was decreased to 336 nm at pH 5.0 and then increased to 347 nm at pH 2.0 (**Figure 4A**). A similar trend was observed for bLF. But at pH 2.0, the λ_{max} value was higher for bLF (349 nm) than cLF. A maxium exposure of tryptophan residues to the solvent at pH 2.0 was evident. ANS surface hydrophobicity studies indicated an unfolding of the cLF and bLF (pH<5) exposing the hydrophobic groups to which ANS will bind (**Figure 4B**). The λ_{max} values of cLF and bLF at pH 2.0 were observed to be 493 and 484 nm, respectively. This indicates a higher surface hydrophobicity of bLF than cLF at pH 2.0. However, a similar unfolding of LF from both species was evident below pH 5.0. For both cLF and bLF, the results of intrinsic tryptophan fluorescene and ANS binding studies were in agreement with each other. These data were supported by quenching studies of structural changes (**Figure 5A and B**).

The far UV-CD secondary structures of cLF and bLF were shown to be similar with minor differences in the pH range 5.0-8.0 (**Figure 6A and B**). Two prominent peaks at 208 and 218 nm in the spectra of cLF and bLF indicated the mixed type of α/β secondary structure of LF (Nam et al., 1999). But these peaks were smaller in case of cLF than bLF (pH 5.0-8.0). Both cLF and bLF were shown to be rich in β-structure in the pH range 5.0-8.0 (**Table 2**). At pH 2.0-3.0, partly unfolded structures of bLF and cLF were observed with relatively low content of α-helices (3 and 7%, respectively), but higher amount of β-structures (54 and 57%, respectively). The LF from both species acquired random conformation at pH 2.0. The results show that the unfolding starts at pH < 5.0 for both cLF and bLF (data shown at pH 2.0). Further, the loss of ellipticities of both LFs at pH 2.0 was evident. The shape of the spectra indicated that the conformations of cLF and bLF are almost identical. The present data can be correlated to the earlier reports (Hu et al., 2008; Shimazaki et al., 1991).

Shimazaki et al. (1991) have shown that CD spectra of bovine, caprine and ovine LF suggested comparable secondary structures at physiological pH. The present findings can be correlated with this report. The difference in the shape of the spectra might be due to the interaction between N and C-lobes in LF. The bLF and cLF have four glycans each. The relative proportions of glycan of oligomanosidic and of N-acetyllactosamine type vary with period of lactation. The primary structures of specific glycans bound to cLF and bLF seems to vary. The thermal stability is influenced by the characteristics of glycans present. This differs in cLF and bLF (Spik at al., 1988; Van Berkel et al., 1996). This might influence the thermal stability and CD conformational alterations in cLF and bLF. The lactoferricin (LFcin) peptide present in the N-terminal part of intact LF (residues 17-41) forms a number of stabilizing, long-range hydrophobic contacts with other parts of the protein (Hwang et al., 1998). This may contribute to the stabilities of cLF and bLF to different extent. The ionic and hydrophobic interactions influence the thermal stabilities of cLF anf bLF. Further the non-covalent interactions among N- and C- lobes of LF might affect the thermal stabilities and conformations of LF from both species. The thermal stability values of LF are also affected by the nature of the glycans present in cLF and bLF (Spik et al., 1988; Van Berkel et al., 1996). The total number of bonds in bLF and cLF are 5566 and 5404, respectively. As observed from 3D structural model (PDB) the number of α-helices in bLF and cLF are 26 and 23, respectively. This might impart stability to bLF and cLF. The bLF has more structure-stability as compared to cLF (Murzin et al., 1995). This factor supports the present research.

Paper II

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

Sreedhara A, Flengsrud R, Langsrud T, Kaul P, Prakash V, Vegarud GE. *Biometals 23* (2010) 1159 - 1170.

In this study, the apo and holo forms of lactoferrin (LF) from caprine and bovine milk were characterized and compared with respect to the conformation and thermal stabilities in the pH range 2.0-8.0. The holo forms of cLF and bLF showed an iron content of 73.5 and 88.1%, respectively at pH 7.0 (**Table 1**). The respective apo forms of cLF and bLF contained 4-5% iron at pH 7.0. A continuous reduction in the bound iron was observed with a decrease in pH from 7.0 to 2.0. This was evident for both apo and holo forms of cLF and bLF. In the pH range 2.0-7.0, the trend of iron release from apo and holo LFs from both species was similar to respective native forms (PAPER I).

This confirms the previous report on bLF (Baker & Baker, 2009). The domain movements will vary due to pH reduction, and this will give a reduced iron binding with reduction in pH and increased iron binding with increase in pH. In addition, the Fe^{3+}-HCO_3^{2-} coordination observed in bLF as in the N- and C- lobes may differ for cLF and depend upon the balance between the closed and open conformations (Hu et al., 2008). 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 between cLF and bLF. This could be an important factor for different Fe-saturation levels in cLF and bLF (pH 2.0-7.0). The electrostatic interactions also influence the iron binding to cLF and bLF to different extent (Baker & Baker 2004; Hu et al., 2008). The difference in bond angles between the iron binding residues in cLF and bLF might affect the iron saturation levels in these proteins. **Figure 1** shows a structural model of Fe binding to bLF. This model is based on the model proposed for bLF by Hu et al (2008). The Fe 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 2.0 since Asp 395 most likely is then protonated and have none Fe-binding at that pH. The relatively higher differences in the iron content between the apo forms of cLF and bLF at pH 2.0 could be due to the presence of higher amount of unordered structure at this pH.

The far UV-CD secondary structures of apo and holo forms of bLF were similar between pH 5.0 and 8.0 (**Figure 3a and b**). The far UV-CD spectrum of holo bLF showed no obvious difference from that of apo bLF in the pH range 4.0-8.0. A very similar trend was observed for the apo and holo cLF forms (**Figure 2a and b**). 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 is also an observed increase in the aperiodic (random) structure. A similar trend was observed for apo and holo cLF forms. But in this case, there is a complete loss of α-helix and much higher amount of aperiodic (random) structure at pH 2.0. Hence, with acidification (pH 2.0), partly unfolded structures are observed for both apo and holo LFs from both species (**Figure 2 and 3**). These results were in accordance with our previous data on native forms of cLF and bLF (PAPER I).

The 8-anilino-1-naphthalene-sulfonate (ANS) binding to apo and holo forms of both cLF and bLF was minimal in the pH range 5.0-8.0 (**Figure 4a and b**). In this pH range LF forms from both species seems to retain the native LF structure (PAPER I). For all LF forms, an increased ANS binding was observed at pH < 5.0. A considerable increase in the ANS fluorescence intensity was observed for apo cLF with a reduction in pH from 3.5 to 3.0. This indicates the greater exposure of tryptophan to the solvent. Below pH 3.0, the hydrophobic interactions lead to a decrease in hydrophobicity with an observed aggregation. The apo and holo forms of bLF showed a similar trend of increased hydrophobicity, but with a five time increase in intensity when pH was reduced from 3.5 to 3.0. The apo bLF binds more ANS than apo cLF at pH 2.0. Similarly more ANS was bound to holo bLF than holo cLF at that pH. The structural unfolding of apo and holo forms of cLF and bLF at low pH depends on the 3D structural organization of individual LFs. The unfolding of the protein at pH 2.0-3.5 was also observed by tryptophan fluorescence (**Figure 5a and b**). For apo and holo LFs from bovine, a maximum red shift was observed between pH 2.0 and 3.5 during lowering pH from 7.0 to 2.0. A similar trend was evident for corresponding forms of cLF (pH 2.0-7.0). A maximum exposure of tryptophan was observed at pH 2.0-3.0 in all cases. With respect to the apo and holo forms of cLF and bLF (pH 2.0-8.0), the results of tryptophan fluorescence studies were in accordance with the surface hydrophobicity data. These results were supported by fluorescence quenching studies (**Figure 6a and b**). **Figure 7** shows the Stern-Volmer constants (K_{SV}) for the fluorescence quenching of apo and holo forms of cLF and bLF at pH values 2.0-7.0.

The holo form of bLF showed a higher T_m value (90±1 ^oC) than holo cLF (68±1 ^oC) at pH 7.0 (**Figure 8a and b**). With a reduction in pH from 7.0 to 2.0, a continuous decrease in T_m values of apo and holo forms of cLF and bLF was observed. For either cLF or bLF, a prominent difference in T_m values was observed among the respective apo and holo forms (**Table 3**). A minimum T_m value of 23±1 ^oC was observed for apo cLF at pH 3.0. Below pH 3.0, protein aggregation occurred for apo and holo LFs of both species. The T_m values observed for the native forms of LF were showed to be between the corresponding T_m values of the apo and holo forms (PAPER I). Thermal denaturation of LF depends on the pH and iron binding. The total contents of glycans present in cLF and bLF may vary and this will affect the T_m of the protein (Spik & Montreuil, 1988). The intra-protein hydrophobic interactions among cLF and bLF are different (Tine et al., 2007). This might influences the thermal stabilities of apo and holo forms of cLF and bLF to different extent. The T_m also depends on the opening up of LF conformation at low pH (2.0-3.0) and the closing of the LF conformation at pH 7.0. This might take place in a different manner between cLF and bLF (Baker & Baker, 2004).

Paper III

Zn (II) binding to lactoferrin at pH 2.0 - 7.0. A comparative study of caprine and bovine lactoferrin

Sreedhara A, Flengsrud R, Langsrud T, Prakash V, Vegarud GE. *''Submitted manuscript''*

The binding of ligands such as metal ions to proteins is an important prerequisite for a variety of biological activities. The interaction between zinc (Zn^{2+}) and lactoferrin (LF) from caprine and bovine was studied in the pH range 2.0-7.0. The amount of zinc bound to cLF and bLF in the pH range 2.0 - 7.0 and in the presence of a denaturant (3.0 and 6.0 M GuHCl) is shown in **Table 1**. At pH 7.0, the zinc content in cLF and bLF was 74.8±0.5 and 90.1±0.5%, respectively. When the pH was reduced from 7.0 to 2.0, the zinc binding to LF from both species was decreased. At pH 2.0, about 20% zinc was still bound to LFs from both species. The zinc bound cLF (ZncLF) and zinc bound bLF (ZnbLF) were not completely saturated with zinc at pH 7.0. The results of zinc binding were compared with our previous report on iron binding to cLF and bLF (PAPER II). The trend of zinc binding to LFs from caprine and bovine was similar to that of iron binding. However, differences in the zinc and iron binding in the pH range 2.0-7.0 to the apo forms of either cLF or bLF depends on the electrostatic binding properties of these proteins (Baker & Baker, 2009; Hu et al., 2008). The 6.0 M GuHCl denatured forms of ZncLF and ZnbLF showed the lowest zinc content. Factors such as H-bonding interactions, differences in the amino acid sequence of LF in the zinc binding area, domain movements due to pH variation indicated that the bond angles between the zinc binding residues in LF and the overall 3D structure organization of the protein are somewhat different between cLF and bLF (Hu et al., 2008; Sreedhara et al., 2010b).

The far UV-CD conformations of ZncLF and ZnbLF showed the mixed α/β nature of the protein (Nam et al., 1999) (**Figure 1A and B, Figure 2A and B**). Two noticeable peaks at 208 and 218 nm were observed in the CD spectra of both ZncLF and ZnbLF. However, in the pH range 4.0-7.0, these peaks seem to be weaker in ZncLF than ZnbLF. The structures of ZncLF and ZnbLF were partly unfolded at pH 2.0. A higher unfolding of both ZncLF and ZnbLF was observed in presence of 6.0 M GuHCl. For both species, the gross conformations of zinc bound LF were almost similar to that of corresponding iron bound LFs (PAPER II). When the pH was reduced from 7.0 to 2.0, an increase in β-structure content with a concurrent decrease in α-helix content of ZncLF was observed (**Table 2**). A similar trend of secondary structures was evident for ZnbLF in the same pH range. Both ZncLF and ZnbLF retained about

50-60% β-structure in the whole pH range from 2.0-7.0. Both ZnLFs, showed a decrease in the α-helix content with a reduction in pH from 7.0 to 2.0. The secondary structural data of ZncLF and ZnbLF were compared with the respective FeLF forms in our previous study (PAPER II). The ZnLF forms from both species showed 8-10 % more α-helix and 10-15% less β-structure than that of corresponding FeLF forms. The changes in the amino acid sequences in the zinc binding area of cLF and bLF may have a consequence in relation to the changes in the α-helices and β-structures. So, zinc might be involved in the conformational changes of ZnLF from both species.

It was observed from the intrinsic fluorescence spectra that both ZncLF and ZnbLF showed a λ_{max} value of 334 nm at pH 7.0 (**Figure 3 and 4**). At pH 2.0, the λ_{max} values were shifted to 348 (ZncLF) and 351.1 nm (ZnbLF). Maximum exposure of tryptophans was observed at pH 2.0. For both ZncLF and ZnbLF, the GuHCl unfolded states exhibited larger red shift values indicating an even higher degree of unordered structures (**Figure 3 and 4**). This was also observed from the CD data of ZnLFs (**Table 2**). Further there was an observed minimal ANS binding at pH 7.0 for ZncLF and ZnbLF with the λ_{max} value between 520-525 nm (**Figure 5A and B**). With a reduction in pH from 7.0 to 2.0, these λ_{max} values were blue shifted to 493 (ZncLF) and 487 nm (ZnbLF). The tryptophan exposure was higher in case of ZnbLF than ZncLF at pH 2.0. These fluorescence results of ZnLF forms from both species were in a good agreement with that of respective iron saturated holo LF forms (PAPER II). Similar trends in ZncLF and ZnbLF were observed with respect to the ANS binding data. The structural perturbations start at pH ≤ 4.0. At pH 2.0, is the acid denatured state, usually LF molecule opens up, looses almost all the bound zinc. The trend of zinc binding to LF at pH 2.0 was almost similar to that of iron binding at that pH (PAPER II). The results of surface hydrophobicity studies were in a good agreement with the intrinsic tryptophan fluorescence data and the CD structural changes observed. The data were supported with the quenching studies of structural changes in the pH range 2.0-7.0 [**Figure 6 (i) and (ii)**]. The quenching results of ZncLF and ZnbLF can be correlated with apo and iron saturated LFs from caprine and bovine species at different pH (Sreedhara et al., 2010b).

At pH 7.0, ZnbLF showed a higher T_m (83±1 ^oC) than ZncLF (67±1 ^oC). By reducing pH to 4.0, T_m of ZncLF and ZnbLF decreased to 76±1 ^oC and 55±1 ^oC, respectively (**Figure 7A and B**). A difference of 17±1 ^oC in T_m was observed between ZnbLF and apo bLF at pH 7.0. However, only a small difference of 3±1 ^oC in T_m was observed between ZncLF and apo cLF at this pH. The ZnLFs from both species were unfolded at pH ≤ 3.0 (Surface hydrophobicity experiments) and hence the T_m measurements could not be done. The T_m data of ZncLF and ZnbLF

were compared with our previous studies on iron bound forms of cLF and bLF. The trend of decrease in T_m with a reduction in pH from 7.0 to 3.0 was similar for ZnLF and FeLF forms from both caprine and bovine. The T_m values observed for ZnLF were somewhat lower than that of FeLF in the pH range 3.0-7.0. The native forms of cLF and bLF have most stable forms at pH 7.0 and the thermal stabilities were highest at this pH (PAPER I). A comparative study on the thermal stabilities of apo and holo forms of caprine and bovine LFs showed that the thermal stability of LF was dependent of the Fe binding capacity of the protein and pH (PAPER II). Therefore, the thermal denaturation of ZncLF and ZnbLF depend on the pH and the amount of zinc bound to protein. The other factors like the contents of glycans present in cLF and bLF might have an influence on the T_m of iron bound LF. This factor might affect the thermal stabilities of ZnLF forms from caprine and bovine. The movements of the intra-protein hydrophobic interactions in cLF and bLF might affect the thermal stabilities of respective zinc bound forms (Tina et al., 2007).

Paper IV

Peptides generated by the *in vitro* digestion of bovine and caprine lactoferrin

Furlund CB, Sreedhara A, Devold TG, Flengsrud R, Sekse C, Holm H, Jacobsen M, Vegarud GE (Manuscript)

This study was undertaken in order to compare the digestion of bovine lactoferrin (bLF) and caprine lactoferrin (cLF) with human gastric juice (HGJ) and human duodenal juice (HDJ) and to observe the effect of two gastric pH values, 2.5 and 4.0. The highest activity of HGJ (20U/g) and HDJ (62.5U/g) used resulted in an increased degradation of bLF with many fragments in the molecular mass (M_r) range of 35-65 kDa and <20 kDa, however still a high amount of LF resisted digestion (**Figure 3**). With a decrease in bLF concentration from 10 to 1 mg/ml, extensive degradation was observed at both low and high activity of HGJ and HDJ (**Figure 3**, lanes 7-10). The bovine LF (0.1 mg/ml) was shown to be 100% digested by both low and high activities of HGJ and HDJ (data not shown).

Figure 4 illustrates differences in the degradation profiles of the digested bLF (10 mg/ml) at gastric pH values 2.5 and 4.0. The degradation of LF was highly dependent on gastric pH values 2.5 and 4.0. Highest degradation was shown at low gastric pH 2.5 compared to pH 4.0, independent of fast or slow pH reduction (**Figure 4**, lanes 3, 5, 7

and 9). More LF was degraded with HDJ giving rise to oligopeptides of lower molecular size. Fast reduction in pH down to 2.5, resulted in complete digestion of bLF by HGJ and further degradation of the peptides (M_r < 25 kDa) occurred when HDJ was added (**Figure 4**, lanes 7 and 8). A comparison of the digestion between apo and holo bLF forms resulted in a similar protein degradation pattern as the native bLF (**Figure S3**). Digestion of native caprine LF (10mg/ml) with HGJ and HDJ resulted in a protein degradation pattern very similar as shown for native bLF in **Figure 4**. The degree of hydrolysis (DH) of bLF (10 mg/ml) digested at pH values 2.5 (DH ~ 10.5) and 4.0 (DH ~ 9.0) is shown in the **Figure 5**. The highest DH was observed at pH 2.5 as compared to pH 4.0, whereas minor differences were seen between fast and slow pH reduction to pH 2.5 and 4.0.

The native LF of bovine and caprine generated peptides varying in lengths as a result of the action of HGJ and HDJ at pH values 2.5 and 4.0 (**Figure 6 and 7**). Always, more peptides were observed at pH 2.5 than at pH 4.0 and peptide fragments in the range of 5-32 residues were detected. The results also showed that less peptides were generated after the slow reduction of gastric pH as compared to the fast pH reduction and this was evident for both pH values 2.5 and 4.0. Thereafter addition of HDJ degraded longer peptides into shorter ones. However, two different peptide patterns between fast and slow gastric pH reduction were seen. Most of the peptides detected in bLF (native, apo or holo) and cLF (native) originated from the N-terminal part of LF. The common peptide fragments detected in native, apo and holo forms of bLF and native form of cLF are shown in **Table 2.** A comparison between native forms of bLF and cLF after the action of HGJ and HDJ at pH 2.5 and 4.0 has shown that less number of peptides were detected in cLF when compared with bLF (**Table 2**). The peptides also differed in sequence and length. Since there are differences in the amino acid sequences between bLF and cLF (**Figure 6 and 7**), the peptides originated within the same region of bLF and cLF may have different characteristics.

Holo bLF showed less number of peptides when compared with apo bLF and native bLF. The effect of iron could be important for the LF digestion. Peptide fragments corresponding to residues 267-288 and 271-288 were observed from HGJ digested of the holo bLF and native cLF forms, respectively. These peptides contain a part of the lactoferrampin (LFampin) sequence (Recio & Visser 1999, 2000). This peptide sequence was also detected as a part of the longer peptide fragment 267-299 for the holo bLF after HGJ digestion and in cLF after further digestion with HDJ at pH 2.5, respectively. The multiple sequence analysis (MSA) of peptides from LF digests showed the presence of proline and leucine patterns at pH values, 2.5. The peptides generated at pH 4.0 show a different pattern where lysine, alanine and multiple leucine residues show highest probability of a reliable pattern and indicating a

different proteolytic mechanism. At high pH 2.5 and 4.0 digestion the proline and leucine pattern is present, in addition to glycine residues.

In this study, an extensive degradation of bLF and cLF was observed showing the effective action of HGJ and HDJ, however, a high amount of LF resisted digestion. Studies done on bLF and cLF digestion using commercial porcine enzymes (Chatteron et al., 2004; Kimura et al., 2000; Recio & Visser, 2000; Dionysius & Milne, 1997; Elbarbary et al., 2010; Hoek et al., 1997), it was showed that many other peptides might have been generated during *in vitro* digestion in comparison with human enzymes. In an *in vivo* digestion study done by Troost etal (2001), up to 60-80% LF resisted gastric digestion and the present study is in accordance with that report. The low concentrations (1.0 and 0.1 mg/ml) of bLF used in the present study represent the LF content in human and bovine milk. The degradation was dependent of the LF concentration and activities of HGJ and HDJ used.

An antibacterial lactoferricin (LFcin) peptide region residing in the N- terminal part of LF was not detected in caprine or bovine LF digested by HGJ or HDJ. Even further digestion of LFcin was not evident with regard to the peptides generated thereafter. This may be due to the different separation techniques that have been used during fractionation or desalting steps or it might also depend on the sensitivity of the MS instrument used.

The current study has provided a detailed comparison between the digestion patterns of cLF and bLF under different conditions such as human gastrointestinal pH and different enzyme/ substrate ratios. The use of *in vitro* digestion could contribute to a better knowledge about the generation of peptides during gastrointestinal digestion *in vivo*.

4. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The specific objectives of this project were fulfilled through the work in paper I, II, III and IV.

The conclusions are provided in the respective papers. Additionally, the concluding remarks with respect to the overall study are provided in the following section.

The physicochemical/ biophysical properties of LF from caprine and bovine have been discussed and compared. Native bLF has showed higher thermal stability than cLF. Among apo, native and holo forms of cLF and bLF, differences were observed with respect to the influence of pH. The apo, native and holo forms of bLF showed higher amount of bound iron when compared with the respective apo, native and holo forms of cLF in the pH range 2.0 - 7.0. At low pH 2.0, iron was released from both the apo and holo forms of cLF and bLF with a highest release from cLF. Thermal denaturation studies showed that the apo, native and holo forms of bLF were more stable than the corresponding apo, native and holo forms of cLF at pH 2.0 - 7.0. The holo form of LF had the highest thermal stability compared to the apo form of both species. Further, with regard to the differences in metal binding, the iron bound LF form was showed to be more stable than the respective zinc bound form for both cLF and bLF (pH 2.0 - 7.0). The conformation of native cLF was more affected by pH when compared with native bLF with regard to structural unfolding studies. At pH 7.0, the structure of native bLF seemed to be more stable than native cLF. At pH 2.0, a total loss in α-helix content and a higher content of unordered structure for all LF forms was observed. However, only subtle differences were observed with respect to the conformations of apo, native and holo (iron and zinc bound) forms of LF from caprine and bovine species (pH 2.0 - 7.0). Bovine and caprine LF contain 13 and 12 tryptophan residues, respectively. The greater exposure of tryptophan residues in case of bLF (apo, native or holo) as compared to that of respective apo, native and holo forms of cLF indicated the higher degree of conformational unfolding of bLF at pH 2.0. The pH plays an important role in the changes in the thermal stability values of apo, native and holo LF forms of both species. For apo, native and holo forms of cLF and bLF an increase in the unfolded structure with a higher surface hydrophobicity and the reduction in thermal stability values at pH 2.0-3.0 are well correlated. This investigation provides a good approach for studying the pH induced changes on the thermal stability and conformational characteristics of LF using UV-visible, fluorescence and circular dichroic spectroscopy.

Previously, most reports on *in vitro* digestion studies of bLF have been carried out using commercial porcine enzymes. The current study has provided a detailed comparison between the digestion patterns of cLF and bLF under different conditions such as gastrointestinal pH in humans and different enzyme/ substrate ratios. This study has been done to simulate the digestion *in vivo*.

During the *in vitro* digestion of apo, native and holo forms of bLF and of native cLF, many peptides of varying lengths were generated at both pH 2.5 and 4.0. Different peptide patterns were evident for native forms of cLF and bLF. More number of peptides were observed at pH 2.5 compared to pH 4.0 after degradation with HGJ and HDJ of both cLF and bLF. The holo bLF formed showed less degradation than the native and the apo form. The effect of bound iron could be important for LF digestion.

The current study has provided a comparison between caprine and bovine lactoferrin (LF) resulting in information regarding iron binding, thermal denaturation and structural changes in a broad pH range 2.0 - 7.0 and the *in vitro* digestion by human gastro duodenal juices. Lactoferrin is reported to have bioactive functions in humans; the structural changes, iron binding and release under similar pH conditions as in gastrointestinal tract may thus be significant.

Future Perspectives

Based on the current study on the thermal stability, structure and *in vitro* digestion of caprine and bovine LF, following interesting recommendations are made for the continuation of present work.

The iron binding capacity of native cLF and bLF was decreased when the pH was reduced from 7.0 to 2.0. Also, the apo and holo forms of cLF and bLF exhibited difference in the iron content in the pH range 2.0 - 7.0. The secondary structural features may not provide detailed information regarding the ionic interactions which influence the iron binding to LF. The stabilizing aminoacids in the iron binding site may be important for the iron binding to LF at different pH. They are different in cLF and bLF. A structural analysis at tertiary level using NMR spectroscopy may be useful to get important information regarding the ionic interactions and domain movement at different pH values.

Zinc binds to the same sites as iron binds to LF. By calorimetric methods, it is possible to obtain information regarding the binding constant of iron and zinc. Further, a mixture of iron and zinc can be used to study the competition of zinc with iron binding to LF. Zinc may replace iron bound to LF. Interaction of LF with other metal ions like Ga^{3+}, Al^{3+}, VO^{2+}, Mn^{3+}, Co^{3+}, Cu^{2+} may be important as a comparative study between cLF and bLF.

Lactoferrin may interact with other proteins in milk. Such interaction studies may provide additional possibilities to modulate the conformational state of LF at secondary or tertiary level. This study may be important in many of the biological functions of LF from caprine or bovine. Protein-ligand interactions can be studied for LF using bioinformatics tools of Predicting protein interaction sites.

The peptides generated from LF after digestion exhibit some biological activity that might differ from the biological activity of native LF. The digestion of cLF and bLF showed difference in peptide patterns. Using chromatography methods, the digested LF can be partitioned into low and high mass fractions. It is important to study the antipathogenic/ antiviral spectra of such peptides.

An antibacterial LFcin peptide present in the N- terminal part of LF was not detected in caprine or bovine LF digested by HGJ or HDJ. Even further digestion of LFcin was not evident with regard to the peptides generated thereafter. This may be due to the different separation techniques that have been used during fractionation or desalting steps or it might also depend on the sensitivity of the MS instrument used. However, the findings on LFcin generation during LF digestion by pepsin hydrolysis were observed in literature. Hence, it may be important to understand why LFcin was not detected in the LF digests.

The present study on LF digestion *in vitro* simulates the gastro intestinal conditions in the human stomach. Further research is needed to throw light on the LF digestion *in vivo* that could be attributed to native, apo and holo forms of caprine and bovine LF. A study on the effect of iron on LF digestion could be interesting. It may be possible to measure the iron released from LF during different steps of digestion *in vitro* after the action of HGJ and HDJ.

REFERENCES

Abe H, Saito H, Miyakawa H, Tamura Y, Shimamura S, Nagao E., et al. Heat stability of bovine lactoferrin at acidic pH. J. Dairy Sci. 74 (1991) 65–71.

Adlerova L, Bartoskova A, Faldyna H. Lactoferrin: a review. Vet. Med. 53 (2008) 457-468.

Ainscough EW, Brodie AM, Plowman JE. The chromium, manganese, cobalt and copper complexes of human lactoferrin. Inorg Chim Acta 33 (1979) 149-153.

Almaas H, Anne-Laure C, Devold TG, Holm H, Langsrud T, Aabakken L., et al. *In vitro* digestion of bovine and caprine milk by human gastric and duodenal enzymes. Int. Dairy J. 16 (2006b) 961–968.

Anderson BF, Baker HM, Norris GE, Rice DW, Baker EN. Structure of human lactoferrin: crystallographic analysis and refinement at 2.8 Å resolution. J. Mol. Biol. 209 (1989) 711-734.

Anderson BF, Baker HM, Norris GE, Rumball SV, Baker EN. Apolactoferrin structure demonstrates ligand-induced conformational change in transferrins. Nature 344 (1990) 784-787.

Anderson BF, Baker HM, Norris GE, Rumball SV, Waters JM, Baker EN. The structure of human lactoferrin at 3.2 Å resolution. Proc. Natl. Acad. Sci. USA 84 (1987) 1769-1773.

Anderson SM, Rudolph MC, McManaman JL, Neville MC. Key stages in mammary gland development. Secretory activation in the mammary gland: it's not just about milk protein synthesis! Breast Cancer Res. 9 (2007) 204.

Anjaneyulu ASR, Lakshmanan V, Kesava Rao V. Status of meat and milk production from Indian goats. J. Fd. Sci. Technol. 22 (1985) 151-160.

Arnold RR, Brewer M, Gauthier JJ. Bactericidal activity for human lactoferrin: Sensitivity of a variety of microorganisms. Infect. Immun. 28 (1980) 893-898.

Arnold RR, Cole MF, McGhee A. Bactericidal effect for human lactoferrin. Science (Washington, D.C.) 197 (1977) 263-265.

Arunvipas P, Dohoo IR, VanLeeuwen JA, Keefe GP. The effect of non-nutritional factors on milk urea nitrogen levels in dairy cows in Prince Edward Island. Canada. Prev Vet Med. 59 (2003) 83-93.

Baker EN, Baker HM. A structural framework for understanding the multifunctional character of lactoferrin. Biochimie 91 (2009) 3-10.

Baker EN, Baker HM, Kidd RD. Lactoferrin and transferrin: functional variations on a common structural framework. Biochem. Cell. Biol. 80 (2002) 27-34.

Baker HM, Baker CJ, Smith CA, Baker EN. Metal substitution in transferrins: specific binding of cerium (IV) revealed by the crystal structure of cerium-substituted human lactoferrin. J. Biol. Inorg. Chem. 5 (2000) 692-698.

Baker EN, Baker HM. Molecular structure, binding properties and dynamics of lactoferrin. Cell. Mol. Life Sci. 62 (2005) 2531–2539.

Baker EN. Structure and reactivity of transferrins. Adv. Inorg. Chem. 41 (1994) 389-463.

Baker EN, Anderson BF, Baker HM, Day CL, Haridas M, Norris GE, Rumball SV, Smith CA, Thomas DH. Three-dimensional structure of lactoferrin in various functional states. Adv Exp Med Biol. 357 (1994) 1-12.

Baker HM, Baker EN. Lactoferrin and iron: structural and dynamic aspects of binding and release. Biometals 17 (2004) 209-216.

Baker EN, Rumball SV, Anderson BF. Transferrins: insights into structure and function from studies on lactoferrin. Trends. Biochem. Sci. 12 (1987) 350-353.

Baldi A, Ioannis P, Chiara P, Eleonora F, Roubini C, Vittorio DO. Biological effects of milk proteins and their peptides with emphasis on those related to the gastrointestinal ecosystem. J. Dairy Res. 72 (2005) 66-72.

Baldwin DA, Jenny RR, Aisen P. The effect of human serum transferrin and milk lactoferrin on hydroxyl radical formation from superoxide and hydrogen peroxide. J. Biol. Chem. 259 (1984) 13391-13394.

Baveye S, Elass E, Mazurier J, Spik G, Legrand D. Lactoferrin: A multifunctional glycoprotein involved in the modulation of the inflammatory process. Clin. Chem. Lab. Med. 37 (1999) 281-286.

Bellamy W, Takase M, Yamauchi K, Wakabayashi H, Kawase K, Tomita M. Identification of the bactericidal domain of lactoferrin. Biochim. Biophys. Acta 1121 (1992b) 130-136.

Bock SA, Munoz-Furlong A, Sampson HA. Further fatalities caused by anaphylactic reactions to food, 2001-2006. J. Allergy Clin. Immunol. 119 (2007) 1016-1018.

Bode L. Recent advances on structure, metabolism, and function of human milk oligosaccharides. J Nutr. 136 (2006) 2127–2130.

Bolscher JGM , van der Kraan MIA, Nazmi K, Kalay H, Grun CH, van't Hof W, Veerman ECI, Amerongen AVN. A one-enzyme strategy to release an antimicrobial peptide from the LFampin-domain of bovine lactoferrin. Peptides 27 (2006) 1-9.

Brignon G, Chtourou A, Ribadeauduman B. Preparation and amino acid sequence of human kappa-casein. Feb Letters 188 (1985) 48-54.

Brines RD, Brock JH. The effect of trypsin and chymotrypsin on the *in vitro* antimicrobial and iron-binding properties of lactoferrin in human milk and bovine colostrum. Unusual resistance of human apolactoferrin to proteolytic digestion. Biochim. Biophys. Acta 759 (1983) 229-235.

Brisson G, Britten M, Pouliot Y, Bullen JJ, Rogers HJ, Leigh L. Iron-binding proteins in milk and resistance to Escherichia coli infections in infants. Brit. Med. J. 1 (1972) 69-75.

Brock JH. Transferrins. In: Harrison, P.M. (Ed.), Metalloproteins. Macmillan, London pp. 183-262 (1985).

Brock JH. Lactoferrin structure function relationships: an overview. In: Hutchens TW, Lonnerdal B (eds) Lactoferrin interactions and biological functions. Human Press, Totowa, NJ, USA, pp 3-25 (1997).

Businco L, Bellanti J. Food allergy in childhood. Hypersensitivity to cow's milk allergens. Clin. Exp. Allergy. 23 (1993) 481- 483.

Chabance B, Marteau P, Rambaud JC, Samour DM, Boynard M, Perrotin P., et al. Casein peptide release and passage to the blood in humans during digestion of ilk or yogurt. Biochimie 80 (1998) 155-165.

Chatterton DEW, Rasmussen JT, Heegaard CW, Sorensen ES, Petersen TE. *In vitro* digestion of novel milk protein ingredients for use in infant formulas: Research on biological functions. Trends. Food Sci. Technol. 15 (2004) 373-83.

Chen HL, Yen CC, Lu CY, Yu CH, Chen CM. Synthetic porcine lactoferricin with a 20-residue peptide exhibits antimicrobial activity against Escherichia coli, Staphylococcus aureus, and Candida albicans. J. Agric. Food Chem. 54 (2006) 3277-82.

Chierici R. Antimicrobial actions of lactoferrin. Adv. Nutr. Res. 10 (2001) 247-269.

Chtourou A, Brignon G, Ribadeaudumas B. Quantification of beta-casein in human milk. J. Dairy Res. 52 (1985) 239-247.

Dalgleish DG, Spagnuolo PA, Goff HD. A possible structure of the casein micelle based on high-resolution field-emission scanningelectron microscopy. Int. Dairy J. 14 (2004) 1025–1031.

Daniels MC, Adair LS. Breast-feeding influences cognitive development in Filipino children. J Nutr. 135 (2005) 2589–2595.

DePeters EJ, Cant JP. Nutritional factors influencing the nitrogen composition of bovine milk. J. Dairy Sci. 75 (1992) 2043-2070.

de Wit JN, van Hooydonk ACM. Structure, functions and applications of lactoperoxidase in natural antimicrobial systems. Netherlands Milk & Dairy J. 50 (1996) 227-244.

Dial EJ, Dohrman AJ, Romero JJ, Lichtenberger LM. Recombinant human lactoferrin prevents NSAID-induced intestinal bleeding in rodents. J. Pharma. Pharmacol. 57 (2005) 93-99.

Dionysius DA, Milne JM. Antibacterial peptides of bovine lactoferrin: Purification and characterization. J. Dairy Sci. 80 (1997) 667-674.

Drackowa M, Borkovcova I, Janstova B, Naiserova M, Pridalova H, Navratilova P, Vorlova L. Determination of Lactoferrin in Goat Milk by HPLC Method. Czech J. Food Sci. 27 (2009) S102-S104.

Ekmekcioglu C. A physiological approach for preparing and conducting intestinal bioavailability studies using experimental systems. Food Chem. 76 (2002) 225-230.

El-Agamy EI. Effect of heat treatment on camel milk proteins with respect to antimicrobial factors: A comparison with cow and buffalo milk proteins. Food Chem. 68 (2000) 227-232.

El-Agamy EI. The challenge of cow milk protein allergy. Small Rum. Res. 68 (2007) 64-72.

Eriksen EK, Holm H, Jensen E, Aaboe R, Devold TG, Jacobsen M, Vegarud GE. Different digestion of caprine whey proteins by human and porcine gastrointestinal enzymes. Br J Nutr. 104 (2010) 374-81.

Elbarbary HA, Abdou AM, Park EY, Nakamura Y, Mohamed HA, Sato K. Novel antibacterial lactoferrin peptides generated by rennet digestion and auto focusing technique. Int. Dairy J. 20(2010) 646-51.

ESPGAN Committee on Nutrition. Guidelines on infant nutrition. III. Recommendations for infant feeding. Acta Paediatrica Scandinavica Suppl. 302 (1982) 1-27.

Fabre, A., 1997. Perspectives actuelles d'utilisation du lait de chevre dans l'alimentation infantile. In: Proceedings de Colloque Interets Nutritionnel et Dietetique du Lait de Chevre, vol. 81. Inst. Natl. Rech. Agron. Publ., Paris, France, pp. 123–126.

Farnaud S, Evans RW. Lactoferrin - a multifunctional protein with antimicrobial properties. Mol. Immunol. 40 (2003) 395–405.

Feldman M, Cryer B, McArthur KE, Huet BA, Lee E. Effects of aging and gastritis on gastric acid and pepsin secretion in humans: A prospective study. Gastroenterol. 110 (1996) 1043-1052.

Fox PF, McSweeney PLH. 1st Ed., Dairy Chemistry and Biochemistry. London, UK: Blackie Academic and Professional (1998).

Fox PF, McSweeney PLH. 3rd Ed., Advanced Dairy Chemistry-Volume 1, Proteins. NY, USA: Kluwer Academic/Plenum Publishers (2003).

Garcia MIB, Puerto PP, Baquero MF, Rodriguez ER, Martin JD, Romero CD. Mineral and trace element concentrations of dairy products from goats' milk produced in Tenerife (Canary Islands). Int. Dairy J. 16 (2006) 182-185.

German JB, Dillard CJ, Ward RE. Bioactive components in milk. Curr Opin Clin Nutr Metab Care. 5 (2002) 653–658.

German JB, Freeman SL, Lebrilla CB, Mills DA. Human milk oligosaccharides: evolution, structures and bioselectivity as substrates for intestinal bacteria. Nestle Nutr Workshop Ser Pediatr Progr. 62 (2008) 205-218.

Gerstein M, Anderson BF, Norris GE, Baker EN, Lesk AM, Chothia C. Domain closure in lactoferrin: two hinges produce a seesaw motion between alternative close-packed interfaces. J. Mol. Biol. 234 (1993) 357-372.

Gifford JL, Hunter HN, Vogel HJ. Lactoferricin: a lactoferrin-derived peptide with anti-microbial, antiviral, antitumor and immunological properties. Cell Mol. Life Sci. 62 (2005) 2588-2598.

Gonzalez-Chavez SA, Arevalo-Gallegos S, Rascon-Cruz Q. Lactoferrin: structure, function and applications. Int. J. Antimicrob. Agents 33 (2009) 301.e1-8.

Gordon WG, Groves ML, Basch JJ. Bovine milk ‘‘red protein’’: amino acid composition and comparison with blood transferrins. Biochem. 2 (1963) 817-820.

Grossmann JG, Neu M, Pantos E, Schwab FJ, Evans RW, Towns-Andrews E., et al. X-ray solution scattering reveals conformational changes upon iron uptake in lactoferrin, serum and ovotransferrins. J. Mol. Biol. 225 (1992) 811-819.

Grøtte BC. Lactoferrin in Norwegian goat milk. Master's Thesis, Norwegian University of Life Sciences, Department of Chemistry, Biotechnology and Food Science (2001).

Groves ML. the isolation of red protein from milk. J. Am. Chem. Soc. 82 (1960) 3345-3350.

Guo MR, Dixon PH, Park YW, Gilmore JA, Kindstedt PS. Seasonal changes in the chemical composition of commingled goat milk. J. Dairy Sci. 84 (2001) E79-E83.

Grzesiak T. Lait de chevre, lait d'avenir pour les nourrissons. In: Proceedings of Colloque Interets Nutritionnel et Dietetique du Lait de Chevre, vol. 81. Inst. Nat. Rech. Agron. Publ., Paris, France, (1997) pp. 127–148.

Haney EF, Lau F, Vogel HJ. Solution structures and model membrane interactions of lactoferrampin, an antimicrobial peptide derived from bovine lactoferrin. Biochimica et Biophysica Acta 1768 (2007) 2355-2364.

Haney EF, Nazmi K, Lau F, Bolscher JGM, Vogel HJ. Novel lactoferrampin antimicrobial peptides derived from human lactoferrin. Biochimie 91 (2009) 141-154.

Haridas M, Anderson BF, Baker EN. Structure of human diferric lactoferrin refined at 2.2 Å resolution. Acta. Cryst. D51 (1995) 629-646.

Harmsen HJ, Wildeboer-Veloo AC, Raangs GC, Wagendorp AA, Klijn N, Bindels JG, et al. Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. J Pediatr Gastroenterol Nutr. 30 (2000) 61–67.

Harris WR, Stenback JZ. The Bicarbonate-Dependence of Zinc (II)-Transferrin Binding. J Inorg Biochem 33 (1988) 211-223.

Hoek KS, Milne JM, Grieve PA, Dionysius DA, Smith R. Antibacterial activity of bovine lactoferrin-derived peptides. Antimicrob. Agents. Chemo. 41(1997) 54-59.

Host A, Husby S, Osterballe O. A prospective study of cow's milk allergy in exclusively breast-fed infants. Acta. Paediatr. Scand. 77 (1988) 663–670.

Host A. Frequency of cow's milk allergy in childhood. Ann. Allergy Asthma Immunol. 89 (2002) S33-37.

Hu F, Pan F, Sawano Y, Makino T, Kakehi Y., et al. Studies of the structure of multiferric ion-bound lactoferrin: A new antianemic edible material. Int. Dairy J. 18 (2008) 1051-1056.

Hunter HN, Demcoe AR, Jenssen , H, Gutteberg TJ, Vogel HJ. Human Lactoferricin Is Partially Folded in Aqueous Solution and Is Better Stabilized in a Membrane Mimetic Solvent. Antimicrob. Agents. Chemo. 49 (2005) 3387-3395.

Hwang PM, Zhou N, Shan X, Arrowsmith CH, Vogel HJ. Three dimensional solution structure of lactoferricin B, an antimicrobial peptide derived from bovine lactoferrin. Biochemistry. 37 (1998) 4288–4298.

Jabeen T, Sharma S, Singh N, Bhushan A, Singh TP. Structure of the zinc-saturated C-terminal half of bovine lactoferrin at 2.0Å resolution. Acta. Cryst. D61 (2005) 1107-1115.

Jameson GB, Anderson BF, Norris GE, Thomas DH, Baker EN. Structure of human apo lactoferrin at 2.0 Å resolution. Refinement and analysis of ligand-induced conformational change. Acta Cryst. D54 (1998) 1319-1335.

Jenness R. Composition and characteristics of goat milk: Review1968-1979. J. Dairy Sci. 63 (1980) 1605-1630.

Jones EM, Smart A, Bloomberg G, Burgess L, Millar MR. Lactoferricin, a new antimicrobial peptide. J. Appl. Bacteriol. 77 (1994) 208–214.

Kalantzi L, Goumas K, Kalioras V, Abrahamsson B, Dressman JB, Reppas C. Characterization of the human upper gastrointestinal contents under conditions simulating bioavailability/bioequivalence studies. Pharm. Res. 23 (2006) 165-76.

Karthikeyan S, Paramasivam M, Yadav S, Srinivasan A, Singh TP. Structure of buffalo lactoferrin at 2.5 Å resolution using crystals grown at 303 K shows different orientations of the N and C lobes. Acta Cryst. D55 (1999) 1805-1813.

Kawakami H, Hiratsuka M, Dosako S. Effects if iron-saturated lactoferrin on iron absorption. Agri. Biol. Chem. 52 (1988) 903-908.

Kawakami H, Tanaka M, Tatsumi K, Dosako S. Effect of ionic strength and pH on the thermostability of lactoferrin. Int. Dairy J. 2 (1992) 287-298.

Kawakami H, Dosako S, Nakajima I. Effect of lactoferrin on iron solubility under neutral conditions. Biosci. Biotechnol. Biochem. 57 (1993) 1376-1377.

Khan JA, Kumar P, Paramasivam M, Yadav RS, Sahani MS, Sharma S., et al. Camel lactoferrin e a transferrin cum-lactoferrin: crystal structure of camel apolactoferrin at 2.6 Å resolution and structural basis of its dual role, J. Mol. Biol. 309 (2001) 751-761.

Keller J, Layer P. Human pancreatic exocrine response to nutrients in health and disease. Gut 54. (2005) 1-28.

Kim Y, Kim MJ, Han KS, Imm JY, Oh S, Kim SH. Anticancer activity of lactoferrin isolated from caprine colostrum on human cancer cell lines. Int. J. Dairy Tech. 62 (2009) 277-281.

Kimura M, Nam MS, Ohkouchi Y, Kumura H, Shimazaki K, Yu DY. Antimicrobial peptide of Korean native goat lactoferrin and identification of the part essential for this activity. Biochem. Biophys. Res. Commun. 268 (2000) 333 - 336.

Kinsella JE, Whitehead DM. Proteins in whey: Chemical, physical and functional properties. Adv. Food Nutr. Res. 33 (1989) 343-438.

Korhonen H, Pihlanto-Leppala A, Rokka T. Angiotensin I converting enzyme inhibitory peptides derived from bovine milk proteins. Int. Dairy J. 8 (1998) 325-331.

Korhonen H, Pihlanto A. Bioactive peptides: production and functionality. Int. Dairy J. 16 (2006) 945-960.

Kozlowski H, Janicka-Klos A, Brasun J, Gaggelli E, Valensin D, Valensin G. Copper, iron, and zinc ions homeostasis and their role in neurodegenerative disorders (metal uptake, transport, distribution and regulation). Coord. Chem. Rev. 253 (2009) 2665 – 2685.

Kraulis PJ, Raine ARC, Ghadavi PL, Laue ED. Structure of the DNA-binding domain of zinc GAL4. Nature 356 (1992) 448-450.

Kristjansson MM, Kinsella JE. Protein and enzyme stability, structural, thermodynamic and experimental aspects. Adv. Food. Nutr. Res. 35 (1991), 237-316.

Kumar S, Sharma AK, Singh TP. Metal substitution in lactoferrins: the crystal structure of manganese lactoferrin at 3.4Å resolution. Ind. J. Phys. 74B (2000) 103-108.

Kumar P, Yadav S, Singh TP. Crystallization and structure determination of goat lactoferrin at 4.0 Å resolution: A new form of packing in lactoferrins with a high solvent content in crystals. Ind. J. Biochem. Biophys. 39 (2002) 16-21.

Kunz C, Rudloff S. Health promoting aspects of milk oligosaccahrides16 ed. 2006. p. 1341–1346.

Kussendrager K. Effects of heat treatment on structure and iron binding capacity of bovine lactoferrin. In IDF bulletin: Indigenous antimicrobial agents of milk: Recent developments. Uppsala, Sweden: IDF. (1994) pp. 133-146.

Kuwata HT, Yip T, Tomita M, Hutchens TW. Direct evidence of the generation in human stomach of an antimicrobial peptide domain (lactoferricin) from ingested lactoferrin. Biochim. Biophys. Acta 1429 (1998a) 129–141.

Kuwata HT, Yip T, Yamauchi K, Teraguchi S, Hayasawa H, Tomita M., et al. the survival of ingested lactoferrin in the gastrointestinal tract of adult mice. Biochem. J. 334 (1998b) 321-323.

Kuwata H, Yamauchi K, Teraguchi S, Ushida Y, Shimokawa Y, Toida T., et al. Functional fragments of ingested lactoferrin are resistant to proteolytic degradation in the gastrointestinal tract of adult rats. J. Nutr. 131 (2001). 2121–2127.

Law BA, Reiter B. The isolation and bacteriostatic properties of lactoferrin from bovine milk whey. J. Dairy Res. 44 (1977) 595–599.

Leitner G, Merin U, Silanikove N. Changes in milk composition as affected by subclinical mastitis in goats. J. Dairy Sci. 87 (2004a) 1719-1726.

Leitner G, Chaffer M, Shamay A, Shapiro F, Merin U, Ezra E, Saran A, Silanikove N. Changes in milk composition as affected by subclinical mastitis in sheep. J. Dairy Sci. 87 (2004b) 46-52.

Lindmark-Mansson H, Svenson U, Paulson M, Aleden G, Frank B, Johnsson G. Influence of milk components, somatic cells and supplemental zinc on milk process ability. Int. Dairy J. 10 (2000) 423-433.

Lönnerdal B. Trace element transport in the mammary gland. 27 ed. 2007. p. 165–77.

Lonnerdal B. The non-protein nitrogen components in human milk: biochemistry and potential functional role. In: Atkinson, S.A., Lonnerdal, B. (Eds.), Protein and Non-protein nitrogen in human milk. CRC Press, Florida, USA, pp. 29-42 (1989).

Lothe L, Lindberg T, Jacobson I. Cow's milk formula as a cause for infantile colic. Pediatrics 70 (1982) 7-10.

McClain C, Soutor C, Zieve L. Zinc deficiency: A complication of Crohn's disease. Gastroenterol 78 (1980) 272-279.

Marchetti M, Superti F, Ammendolia MG, Valenti PRP, Seganti L. Inhibition of poliovirus type 1 infection by iron-, manganese- and zinc-saturated lactoferrin. Med. Microbiol. Immunol. 187 (1999) 199-204.

Martin E, Gantz T, Lehrer R. Defensins and other endogenous peptide antibiotics of vertebrates. J. Leukoc. Biol. 58 (1995) 128–136.

Martin P, Brignon G, Furet JP, Leroux C. The gene encoding alpha (s1)-casein is expressed in human mammary epithelial cells during lactation. Lait 76 (1996) 523-535.

Martin P, Groclaude F. Improvement of milk protein-quality by gene technology. Livest Prod. Sci. 35 (1993) 95-115.

Masson PL, Heremans JF. Lactoferrin in milk from different species. Comp. Biochem. Physiol. B, 39 (1971) 119-129.

Masson PL, Heremens JF, Dive C. An iron-binding protein common to many external secretions. Clin. Chim. Acta 14 (1966) 735-739.

Mata L, Sanchez L, Headon DR, Calvo M. Thermal denaturation of human lactoferrin and its effect on the ability to bind iron. J. Agri. Food. Chem. 46 (1998) 3964-3970.

Metz-Boutigue MH, Jolles J, Mazurier J, Schoentgen F, Legrand D, Spik G., et al. Human lactotransferrin: amino acid sequence and structural comparisons with other transferrins. Eur. J. Biochem. 145 (1984), 659–676.

Meisel H, Schlimme E. Bioactive peptides derived from milk proteins: Ingredients for functional foods? Kieler Milchw. Forsch. 48 (1996) 343-357.

Miranda G, Mahe MF, Leroux C, Martin P. Proteomics tools to characterize the protein fraction of Equidae milk. Proteomics 4 (2004) 2496-2509.

Misra RP. Modified ''buffalo's milk'' (AMUL) as an infant food in health and disease. Indian J. Pediat. 26 (1959) 290.

Moore SA, Anderson BF, Groom CR, Haridas M, Baker EN. Three dimensional structure of diferric bovine lactoferrin at 2.8 Å resolution. J. Mol. Biol. 274 (1997) 222-236.

Mozhav VV, Martinek K. Structure-stability relationships in proteins, new approaches to to stabilizing enzymes. Enz. Microbiol. Technol. 6 (1984) 50-59.

Murzin AG, Brenner SE, Hubbard T, Chothia C. SCOP: a structural classification of proteins database for the investigation of sequences and structures. J. Mol. Biol. 247 (1995) 536-540.

Nagasako Y, Sainto H, Tamura Y, Shimamura S, Tomita M. Iron-binding properties of bovine lactoferrin in iron-rich solution. J. Dairy Sci. 76 (1993) 1876-1881.

Nam MS, Shimazaki K, Kumura H, Lee KK, Yu DY. Characterization of Korean native goat lactoferrin. Comp. Biochem. Physiol. B. 123 (1999) 201-208.

Nestle W. Allergy to cow milk proteins. Med. Enfance 9 (1987) 163–166.

Ng-Kwai-Hang KF, Hayes JF, Moxley JE, Monardes HG. Environmental influences on protein content and composition of bovine milk. J. Dairy Sci. 65 (1982) 1993-1998.

Norris GE, Anderson BF, Baker EN. Molecular replacement solution of the structure of apo LF, a protein displaying large-scale conformational change. Acta Cryst. B47 (1991) 998-1004.

Odell EW, Sarra R, Foxworthy M, Chapple DS, Evans RW. Antibacterial activity of peptides homologous to a loop region in human lactoferrin. FEBS Lett. 382 (1996) 175–178.

Oria R, Ismail M, Sa´nchez L, Calvo M, Brock H. Effect of heat treatment and other milk proteins on the interaction of lactoferrin with monocytes. J. Dairy Res. 60 (1993) 363-369.

Ounis WB, Gauthier SF, Turgeon SL, Roufik S, Pouliot Y. Separation of minor protein components from whey protein isolates by heparin affinity chromatography. Int. Dairy J. 18 (2008), 1043–1050.

Park YW, Chukwu HI. Macro-mineral concentrations in milk of two goat breeds at different stages of lactation. Small Rum. Res. 1 (1988) 157-166.

Park YW, Chukwu HI. Trace mineral concentrations in goat milk from French-Alpine and Anglo-Nubian breeds during the first five months of lactation. J. Food Composit. Analysis 2 (1989) 161-169.

Paulsson MA, Svensson U, Kishore AR, Naidu S. Thermal behavior of bovine lactoferrin in water and its relation to bacterial interaction and antibacterial activity. J. Dairy Sci. 76 (1993) 3711-3720.

Pierce A, Colavizza D, Benaissa M, Maes P, Tartra A, Montreuil J, Spik G. Molecular cloning and sequence analysis of bovine lactoferrin. Eur. J. Biochem. 196 (1991) 177-184.

Pihlanto A, Korhonen H. Advances in Food and Nutrition Research - Bioactive peptides and proteins. 1st Ed., Vol. 47. Amsterdam: Elsevier Academic Press.

Polis B, Shmukler H. Grystalline lactoperoxidase. I. Isolation by displacement chromatography. J. Biol. Chem. 201 (1953) 475-500.

Peter FL, Margaretha V. Lactoferrin: A general review. Haematologica 80 (1995) 252-267.

Privalov PL. Stability of proteins. Adv. Protein Chem. 33 (1979) 167-241.

Recio I, Slangen CJ, Visser S. Method for the production of antibacterial peptides from biological fluids at an ionic membrane. Application to the isolation of nisin and caprine lactoferricin. Lait 80 (2000) 187-195.

Recio I, Visser S. Two ion-exchange chromatographic methods for the isolation of antibacterial peptides from lactoferrin: In Situ enzymatic hydrolysis on an ion-exchange membrane. J. Chrom. A 831 (1999a) 191-201.

Recio I, Visser S. Antibacterial and binding characteristics of bovine, ovine and caprine lactoferrins: a comparative study. Int. Dairy J. 10 (2000) 597-605.

Reinert P, Fabre A. Utilisation du lait de chevre chez l'enfant. Experience de Creteil. In: Proceedings of the Colloque Interets Nutritionnel et Dietetique du Lait de Chevre, vol. 81. Inst. Nat. Rech. Agron. Publ., Paris, France, pp. 119-121 (1997).

Rook JAF. Variations in the chemical composition of the milk of the cow. Part I. Dairy Sci. Abstr. 23 (1961) 251-258.

Ruegg M, Moor U, Blanc B. A calorimetric study of the thermal denaturation of whey proteins in simulated milk ultrafiltrate. J. Dairy Res. 44 (1977) 509–520.

Sabbah A, Hassoun S, Drouet M. L'allergie au lait de vache et sa substitution par le lait de chevre. In: Proceedings of the Colloque Interets Nutritionnel et Dietetique du Lait de Chevre, vol. 81. Inst. Nat. Rech. Agron. Publ. (1997) Paris, France, pp. 111–118.

Sahan N, Say D, Kacar A. Changes in chemical and mineral contents of Awasi Ewe's milk during lactation. Turk. J. Vet. Anim. Sc. 29 (2005) 589 – 593.

Saito H, Miyakawa H, Tamura Y, Shimamura S, Tomita M. Potent bactericidal activity of bovine lactoferrin hydrolysate produced by heat treatment at acidic pH. J. Dairy Sci. 74 (1991) 3724-3730.

Sampson HA. Food Allergy. J. Allergy Clin. Immunol. 111 (2003) S540-547.

Sanchez L, Aranda P, Perez MD, Calvo M. Concentration of lactoferrin and transferrin throughout lactation in cow's colostrums and milk. Biological Chemistry Hoppe-Seyler, 369 (1988) 1005-1008.

Sanchez L, Peiro JM, Castillo H, Perez MD, Ena JM, Calvo, M. Kinetic parameters for denaturation of bovine milk lactoferrin. J. Food Sci. 57 (1992) 873-879.

Sato R, Inanami O, Tanaka Y, Takase M, Naito Y. Oral administration of bovine lactoferrin for treatment of intractable stomatitis in feline immunodeficiency virus (FIV)-positive and FIV-negative cats. Am. J. Vet. Res. 57 (1996) 1443-1446.

Senkovich O, Cook WJ, Mirza S, Hollingshead SK, Protasevich II, Briles DE, Chattopadhyay D. Structure of a complex of human lactoferrin N-lobe with pneumococcal surface protein A provides insight into microbial defense mechanism. J. Mol. Biol. 370 (2007) 701-713.

Sharma AK, Paramasivam M, Srinivasan A, Yadav MP, Singh TP. Three-dimensional structure of mare diferric lactoferrin at 2.6 Å resolution. J. Mol. Biol. 289 (1998) 303-317.

Shennan DB. Calcium transport by mammary secretory cells: mechanisms underlying transepithelial movement. Cell Mol Biol Lett. 13 (2008) 514–525.

Schibli DJ, Vogel HJ. Structural studies of lactoferricin B and its antimicrobial active peptide fragments. In: Lactoferrin, structure, function and application (2000). Eds. Shimazaki K, Elsevier Science, New York. Pp. 27-35.

Shimazaki K, Kawano N, Yoo YC. Comparison of bovine, sheep and goat milk lactoferrins in their electrophoretic behaviour, conformation, immunochemical properties and lectin reactivity. Comp. Biochem. Physiol. 98B (1991) 417-422.

Shimazaki K, Tanaka T, Kon H, Oota K, Kawaguchi A, Maki Y, Sato T. Separation and Characterization of the C-terminal Half Molecule of Bovine Lactoferrin. J. Dairy Sci. 76 (1993) 946-955.

Singh PK, Parsek MR, Greenberg EP, Welsh MJ. A component of innate immunity prevents bacterial biofilms development. Nature 417 (2002) 552-555.

Smithers GW, Ballard FJ, Copeland AD, De Silva KJ, Dionysius DA, Francis GL., et al. New opportunities from the isolation and utilization of whey proteins. J. Dairy Sci. 79 (1996) 1454-1459.

Spik BC, Montreuil J. Comparative study of the primary structures of sero-, lacto- and ovotransferrin glycans from different species. Biochimie 70 (1988) 1459-1469.

Spik G, Strecker G, Fournet B, Bouquelet S, Montreuil J, Dorland H., et al. Primary structure of the glycans from human lactotransferrin. Eur. J. Biochem. 121 (1982)413-419.

Steijns JM, van Hooijdonk ACM. Occurrence, structure, biochemical properties and technological characteristics of lactoferrin. British J. Nutr. 84 (2000) S11-S17.

Stillman MJ, Presta A. Characterizing metal ion interactions with biological molecules: The spectroscopy of metallothionein. In: Zalups RK, Koropatnick J (eds) Molecular Biology and Toxicology of Metals, Taylor & Francis., London, pp 1-33 (2000).

Subrahmanyan V, Chandrasekhara MR, Bhatia DS, Swaminathan M. Production of infant food and other products from buffalo milk in India. Food Sci. 6 (1957) 52.

Swarts JC, Swarts PJ, Baker EN, Baker HM. Spectroscopic studies on metal complexes of Aspergillus awamori-derived recombinant human lactoferrin. Inorg Chim Acta 298 (2000) 187-194.

Takeuchi T, Shimizu H, Ando K, Harada E. Bovine lactoferrin reduces plasma triacylglycerol and NEFA accompanied by decreased hepatic cholesterol and triacylglycerol contents in rodents. Brit. J. Nutr. 91 (2004), 533-538.

Tina KG, Bhadra R, Srinivasan N. PIC: Protein Interaction Calculator. Nucleic Acids Res. 35 (2007) W473-W476

Togawa J, Nagase H, Tanaka K, Inamori M, Nakajima A, Ueno N., et al. Oral administration of lactoferrin reduces colitis in rats via modulation of the immune system and correction of cytokine imbalance. J. Gastroenterol. Hepatol. 17 (2002a). 1291-1298.

Togawa J, Nagase H, Tanaka K, Inamori M, Umezawa T, Nakajima A., et al. Lactoferrin reduces colitis in rats via modulation of the immune system and correction of cytokine imbalance. A. J Physiol. Gastroint. Liver Physiol. 283 (2002b). G187-G195.

Tomita M, Bellamy W, Takase M, Yamauchi K, Wakabayashi H, Kawase K. Potent antimicrobial peptides generated by pepsin digestion of bovine lactoferrin. J Dairy Sci. 74 (1991) 4137-4142.

Troost FJ, Steijns J, Saris WHM, Brummer RJM. Gastric digestion of bovine lactoferrin *in vivo* in adults. J. Nutr. 131 (2001) 2101–2104.

Tsuda H, Sekine K, Ushida Y, Kuhara T, Takasuka N, Iigo M., et al. Milk and dairy products in cancer prevention: focus on bovine lactoferrin. Mut. Res. 462 (2000) 227-233.

Underwood EJ. Trace elements in human and animal nutrition. 4th edn. Academic Press, New York, pp 196-242 (1977).

Valenti P, Antonini G, Lactoferrin: an important host defence against microbial and viral attack, Cell. Mol. Life Sci. 62 (2005) 2576-2587.

Van Berkel PHC, van Veen HA, Geerts MEJ, de Boer HA, Nuijens JH. Heterogeneity in utilization of N-glycosylation sites Asn624 and Asn138 in human lactoferrin: a study with glycosylation-site mutants. Biochem. J. 319 (1996) 117-122.

Van Herwaarden AE, Wagenaar E, Merino G, Jonker JW, Rosing H, Beijnen JH, et al. Multidrug transporter ABCG2/ breast cancer resistance protein secretes riboflavin (vitamin B2) into milk. Mol Cell Biol. 27 (2007) 1247–1253.

Van Hooijdonk ACM, Kussendrager KD, Steijns JM. *In vivo* antimicrobial and antiviral activity of components in bovine milk and colostrum involved in non-specific defence. Brit. J. Nutri. 84 (2000) 127-134.

Van der Kraan MIA, Groenink J, Nazmi K, Veerman ECI, Bolscher JGM, Amerongen AVN. Lactoferrampin: a novel antimicrobial peptide in the N1-domain of bovine lactoferrin. Peptides 25 (2004) 177-183.

Van der Kraan MIA, Nazmi K, van't Hof W, Amerongen AVN, Veerman ECI, Bolscher JGM. Distinct bactericidal activities of bovine lactoferrin peptides LFampin 268-284 and LFampin 265-284: Asp-Leu-Ile makes a difference. Biochem. Cell Biol. 84 (2006) 358-362.

Vorland LH, Ulvatne H, Anderson J, Haukland HH, Rekdal Ø, Svendsen JS., et al. Lactoferricin of bovine origin is more active than lactoferricins of human, murine and caprine origin. Scand. J. Infect. Dis. 30 (1998) 513-517.

Wakabayashi H, Takase M, Tomita M. Lactoferricin Derived From Milk Protein Lactoferrin. Curr. Pharma. Design 9 (2003) 1277-1287.

Wally J, Buchanan SK. A structural comparison of human serum transferrin and human lactoferrin. Biometals 20 (2007) 249–262.

Ward RE, German JB. Understanding milk's bioactive components: a goal for the genomics toolbox. J. Nutr. 134 (2004) 962S–967S.

Ward PP, Paz E, Conneely OM. Multifunctional roles of lactoferrin: a critical overview. Cell. Mol. Life Sci. 62 (2005) 2540-2548.

Warner EA, Kanekanian AD, Andrews AT. Bioactivity of milk proteins: 1. Anticariogenicity of whey proteins. Int. J. Dairy Technol. 54 (2001) 151–153.

Wei Z, Nishimura T, Yoshida S. Characterization of glycans in a lactoferrin isoform, lactoferrin-a. J. Dairy Sci. 84 (2001) 2584-2590.

Williams J. The evolution of transferrins. Trend. Biochem. Sci. 7 (1982) 394–397.

Wood RA. The natural history of food allergy. Pediatrics. 111 (2003) 1631-1637.

Yamauchi K, Tomita M, Giehl TJ, Ellison RT. Antibacterial Activity of Lactoferrin and a Pepsin-Derived Lactoferrin Peptide Fragment. Infect. Immun. 61 (1993) 719-728.

Yoshida S, Wei Z, Shinmura Y, Fukunaga N. Separation of lactoferrin-a and -b from bovine colostrums. J. Dairy Sci. 83 (2000) 2211–2215.

Paper I

International Dairy Journal 20 (2010) 487–494

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

A comparison of effects of pH on the thermal stability and conformation of caprine and bovine lactoferrin

Ashoka Sreedhara [a,b,*], Ragnar Flengsrud [a], Vishweshwaraiah Prakash [b], Daniel Krowarsch [c], Thor Langsrud [a], Purnima Kaul [b], Tove G. Devold [a], Gerd E. Vegarud [a]

[a] Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Aas, N-1432 Norway
[b] Department of Protein Chemistry and Technology, Central Food Technological Research Institute (A Constituent Laboratory of CSIR, Delhi), Mysore, 570 020, India
[c] Faculty of Biotechnology, University of Wroclaw, Tamka 2, 50-137 Wroclaw, Poland

ARTICLE INFO

Article history:
Received 26 August 2009
Received in revised form
21 December 2009
Accepted 17 February 2010

ABSTRACT

Thermal stability and structural changes in caprine lactoferrin (cLF) and bovine lactoferrin (bLF) at pH 2.0–8.0 were measured using thermal denaturation temperature (T_m) analysis, fluorescence spectroscopy and circular dichroism (CD). Thermal stability analysis indicated a T_m of 70 °C for bLF and 67 °C for cLF at pH 7.0. From pH 7.0 to 3.0, a gradual reduction in the T_m of both bLF and cLF was observed and reached a value of 39 °C and 30 °C, respectively. At pH 2.0–3.0, a partly unfolded structure of bLF and cLF was observed with a relatively low content of α-helix structure (3% and 7%, respectively), but still rich in β-structure (54% and 57%, respectively). A higher exposure of hydrophobic surfaces at low pH for bLF compared with cLF was proved by fluorescence studies. In conclusion, the structure of cLF was more affected by pH and showed lower temperature stability than bLF.

1. Introduction

The red iron-binding protein in milk was named lactoferrin (LF) in 1961 (Blanc & Isliker, 1961). This protein is homologous to the iron (Fe)-binding protein from serum, transferrin, but the proteins appear to differ from each other with regard to structure and function (Gordon, Groves, & Basch, 1963). The structure of LF has two homologous globular lobes (N-lobe and C-lobe), each further divided into two domains (N1 and N2, C1 and C2, respectively) and they have identical Fe-binding sites, one per lobe, giving them the capacity to bind two Fe^{3+} ions per molecule (Baker, 1994). LF is a whey protein present in variable concentrations in milk (0.1–7.0 mg mL^{-1}) from a number of species (Masson & Heremans, 1971; Sanchez, Aranda, Perez, & Calvo, 1988). LF has been proposed to be involved in various biological functions (Farnaud & Evans, 2005) and the protein, in addition, is active against a wide range of pathogenic bacteria (Kolb, 2001).

LF has high basic pI ~ 9.0 (Steijns & van Hooijdonk, 2000), and can therefore easily be purified on cation-exchange resins (Ounis, Gauthier, Turgeon, Roufik, & Pouliot, 2008). However, the protein might lose its stability and activity (Qiu et al., 1998; Ye, Wang, Liu, & Ng, 2000) during various steps of purification. This protein may also interact with other proteins in milk and whey. It is essential to overcome such difficulties during purification. Most of the methods used to date result in lower purity than desirable for high precision analytical work (Recio & Visser, 2000), and are not easily scaled up.

The biological properties of a protein depend on its three-dimensional structure, which can be influenced by chemical and physical factors (ligands, pH, temperature, pressure, etc.) (Patel, Singh, Anema, & Creamer, 2006; Privalov, Griko, Venyaminov, & Kutyshenko, 1986). In view of the variability and the concentration of LF in milk from different species, it seemed worthwhile to undertake a detailed investigation of the physicochemical/biophysical properties of LF from caprine (cLF) and bovine milk (bLF). Previously, experiments were carried out mainly at physiological pH (Ainscough et al., 1980; Castellino, Fish, & Manna, 1970; Rossi et al., 2002). This report is a comparative study on the detailed biophysical characterization of purified cLF and bLF over a wide pH range, 2.0–8.0. Since LF is reported to have bioactive functions in humans (Korhonen & Pihlanto, 2006), the structural differences under similar pH conditions as in the gastrointestinal tract are important.

Current sequence databases contain LF sequences from nine species: human, mouse, sheep, cow, horse, pig, goat, buffalo and camel. The mature LF from these species comprises 690 residues and share pair-wise identities that range from a minimum of 65% to nearly 100%. The complete sequences of cLF and bLF were reported earlier (Goodman & Schanbacher, 1991; Provost, Nocart, Guerin, &

* Corresponding author. Tel.: +47 64 96 61 53; fax: +47 64 96 59 01.
E-mail address: ashoka.sreedhara@umb.no (A. Sreedhara).

0958-6946/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.idairyj.2010.02.003

Martin, 1994; Recio & Visser, 1999). They are 90% identical and this may influence their structure and function.

A few reports are available on the thermodynamic characterization of the corresponding molecule from cows, goats, sheep and elephants (Conesa et al., 2008; Paulsson, Svensson, Kishore, & Naidu, 1993). For cLF, two transitions were observed at pH 7.4. At neutral pH, LF was observed to maintain a stable conformation, which was evident from circular dichroism studies, and bLF was found to be stable against heat treatment under acidic conditions (Abe et al., 1991). Heat treatment (at >70 °C) caused denaturation and aggregation through disulfide cross-linking and hydrophobic interactions. Although these mechanisms are generally accepted, the contributions of alterations in conformation at secondary and tertiary structural levels are not fully understood.

The aim of the study was to compare the structural stability of bLF and cLF with respect to the influence of pH (2.0–8.0) and temperature. This was investigated by tryptophan fluorescence, ANS-binding, acrylamide-quenching, circular dichroism and thermal denaturation measurements.

2. Materials and methods

2.1. Purification of lactoferrin

Whey protein concentrate (WPCG) was prepared from caprine milk according to the method of Almaas, Holm, Langsrud, Flengsrud, & Vegarud (2006). LF was purified from WPCG using membrane cation-exchange chromatography (Etzel & Chiu, 1997). Sartobind strong cation-exchange micro-porous membranes S75x and S100x were procured from Sartorius Stedim Biotech GmbH (Gottingen, Germany). The WPCG, dissolved in 10 mM phosphate buffer, pH 6.5 at a concentration of 6 g L^{-1}, was centrifuged at 20,462 × *g* for 50 min at 10 °C using a Sorvall RC-5B centrifuge (Block Scientific, Inc., Nutley, New Jersey, USA). WPCG solution and all buffers were passed through 0.22 μm filters (Stericup, Millipore). The column was pre-equilibrated using 10 mM phosphate buffer pH 7.0. WPCG was passed through the columns at a flow-rate of 50 mL min^{-1} for S75x and 80 mL min^{-1} for S100x using a Watson Marlow-Sci-Q 300 series peristaltic pump (323E/D). To remove impurities and bound proteins other than LF, the column was washed with 10 mM phosphate buffer, pH 7.0, followed by washing with the same buffer containing 0.2 M NaCl. The bound LF was then eluted with the same buffer containing 1.0 M NaCl (2.0 mL min^{-1}). This fraction was pale red. In this manner, three cycles of loading/washing/elution were performed (20 °C) with the purification. The protein fractions were desalted and concentrated using a filter with a cut-off of 30 kDa (Amicon Ultra 15, Millipore). The LF fractions were lyophilized and stored at −20 °C. Native bLF with 95% purity was supplied by DMV International (Veghel, Netherlands) and was stored at −20 °C.

The protein concentration and iron saturation of LF were determined by measuring the absorbance values at 280 and 465 nm, respectively (Recio & Visser, 1999, 2000). Homogeneity of the proteins was tested by SDS-PAGE (10% gel, 10% T, 2.7% C) using a discontinuous buffer system (Laemmli, 1970). The total iron (Fe) content bound to LF at different pH was measured by the standard method of Inductively Coupled Plasma (ICP) Optical Emission Spectrophotometer (Uchida, Oda, Sato, & Kawakami, 2006). The freeze-dried protein (1.0 mg mL^{-1}) was solubilized in 1% (v/v) HNO_3 before performing the analysis. The Fe-binding measurements were done by ICP in triplicates and averaged for two consecutive protein purification batches.

2.2. Thermal denaturation analysis

2.2.1. 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 Pty Ltd, Mulgrave, VIC, Australia). Protein samples were prepared at each required pH by dialysis against the appropriate buffer for 24 h at 4 °C. The concentration of LF used was 12.5 μM and 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 plots using a standard equation (Pace, Shirely, & Thomson, 1989).

2.2.2. Circular dichroism (Far-UV) method

Thermal denaturation was monitored following the ellipticity at 222 nm using a band slit of 2 nm and response time of 8 s. CD spectra and thermograms were recorded on a Jasco J-715 spectropolarimeter (JASCO Inc., Easton, MD, USA). An automatic peltier accessory (PFD-350S) allowed continuous monitoring of the thermal transition at a constant rate of 1.0 deg min^{-1}. 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 performing the measurements. The concentration of protein used was 0.25 μM. 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, Petrassi, Sudol, & Kelly, 1999).

2.3. Fluorescence spectroscopy and circular dichroism

Tryptophan fluorescence emission spectra and acrylamide-quenching experiments were done using a Cary Eclipse Spectrofluorimeter (Varian BV, Middelburg, The Netherlands). For tryptophan fluorescence measurements, the LF concentration used was 1.0 μM. Protein samples were filtered through 50 kDa cut-off Ultracel membrane filters and concentrated to a final volume of about 10 μL with the required buffer. The sample was excited at 290 nm and the emission was recorded in the range 300–400 nm using slit widths of 5 nm for both excitation and emission (Bagshaw & Harris, 1987). Spectra were recorded 10 s after excitation.

Quenching experiments were carried out by the addition of varying amounts (0.1–1.0 M) of acrylamide stock solution (5.0 M) to the protein solution (1.90 μM) previously incubated at pH 2.0, 5.0 and 8.0 at 25 °C for 1 h, and the fluorescence intensities were recorded. The quenching data were analyzed according to Stern–Volmer equation (Eftink & Ghiron, 1982).

For ANS-binding studies, fluorescence spectra were recorded using a Cary Eclipse Fluorescence Spectrophotometer (Varian Instruments, Walnut Creek, CA, USA). LF concentration used was 0.2 μM. ANS concentration was 50 times the protein concentration. The sample was excited at 380 nm and the emission was recorded in the range 400–600 nm using slit width of 10 nm for both excitation and emission (Kato & Nakai, 1980). All fluorescence spectra were scanned with slow speed, and data points are means of data from triplicate measurements.

Circular Dichroism (CD) measurements in the far-UV region were carried out with a JASCO J-810 spectropolarimeter (JASCO, Tokyo, Japan) calibrated with ammonium d-10-camphor sulfonate. All protein solutions were dialyzed against buffers with the required pH for 24 h at 4 °C. These samples were centrifuged at 11,500 × *g* for 5 min and clear supernatants (protein) were used for the experiments. LF concentration used was 3.1 μM. A cell of path

length 0.1 cm was used for scans between 265 and 195 nm. Each spectrum was the average of three consecutive scans. The CD result was expressed as the mean residue ellipticity (MRE in deg cm^2 $dmol^{-1}$), which is defined as:

$$\mathrm{MRE} = \theta_{\mathrm{obs}}(\mathrm{mdeg})/(10 \times n \times C_{\mathrm{p}} \times l).$$

where 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 light path in cm. The estimation of the contents of α-helix, β-structure and unordered structure was performed according to Yang, Wu, and Martinez (1986).

The fluorescence and CD measurements were done at 25 °C. Buffers used were: 10 mM glycine–HCl, pH 2.0; 10 mM citrate-phosphate buffer, pH 2.6, 3.0, 3.6, 4.0, 5.0 and 6.0; 10 mM sodium-phosphate buffer, pH 7.0 and 8.0.

2.4. Structural data

The X-ray diffraction data of cLF and bLF were obtained from protein database (http://www.rscb.org). The parameters were compared between cLF and bLF.

3. Results and discussion

3.1. Purification of caprine lactoferrin

The protein was more than 95% pure and homogeneous, as measured by SDS-PAGE (Fig. 1). The SDS-PAGE pattern of purified cLF was compared with native bLF, and the molecular masses were determined as 78 ± 1 and 79 ± 1 kDa for bLF and cLF, respectively. The molecular mass values reported previously for purified LFs from different species falls in the range 76–82 kDa (Brock, 1985), consistent with the present results. The LF molecule has a maximum stability in the pH range 7.0–7.4 (Brock, 1997). The cLF preparation was pale red, whereas bLF was red. This colour is a good indication of the total Fe bound to native (purified) LF. Maximum recovery of cLF was 40–50 mg from 1 L WPCG. The extinction coefficients ($E_{1\ \mathrm{cm,\ 280\ nm}}^{1\%}$) for cLF and bLF were 12 ± 0.5 and 12.5 ± 0.2, respectively. The extinction coefficients ($E_{1\ \mathrm{cm,\ 465\ nm}}^{1\%}$) for cLF and bLF were 0.21 ± 0.05 and 0.57 ± 0.02, respectively.

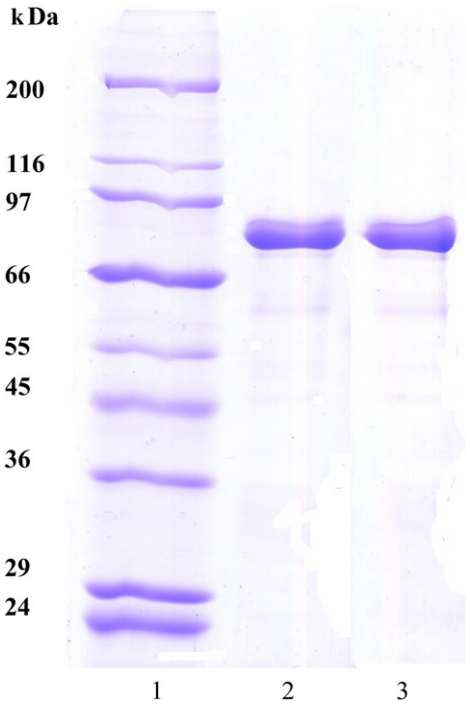

Fig. 1. SDS-PAGE electrophoretograms of: 1, Wide range molecular mass marker; 2, bovine lactoferrin; 3, caprine lactoferrin. The amount of protein loaded was 15.0 μg. The gel (10%) was stained with Coomassie Brilliant Blue R-250.

3.2. Binding of iron to lactoferrin at pH 2.0–8.0

The amount of Fe bound to LF over a broad pH range (2.0–8.0) is shown in Fig. 2. At pH 7.0, which is the most stable form of LF (Brock, 1997), the degree of Fe saturations in bLF and cLF were 15.0 and 5.0% (mol mol^{-1}), respectively. Below this pH, there was an observed decrease in the bound Fe content. Furthermore, at pH < 3.0, LF released most Fe, giving rise to the Fe-deprived form of the molecule. This confirms previous reports on bLF (Baker & Baker, 2009). As compared with the Fe bound to cLF (pH 2.0–8.0), bLF binds higher amount of Fe over the whole pH range. The binding of Fe occurs sequentially to the two lobes (N and C) of LF. However, both the Fe-binding sites in LF are identical. The mechanism of Fe binding to LF is not clear (Baker & Baker, 2004). The N- and C-lobes of LF contain two domains, N1, N2 and C1, C2, respectively, with Fe-binding sites situated in the interdomain clefts (Shimazaki et al., 1993). The diferric LF has the ability to bind two Fe-ions. The LF molecule is capable of solubilizing up to a 70-fold molar equivalent of Fe, which is much higher than the specific Fe-binding ability of LF (Kawakami, Dosako, & Nakajima, 1993). The molecular surface of LF leads to its Fe-binding properties (Baker & Baker, 2009; Hu et al., 2008; Lonnerdal, 1989; Nagasako, Sainto, Tamura, Shimamura, & Tomita, 1993). The cLF and bLF may have different electrostatic binding properties. This may influence the binding of Fe to cLF and bLF at various pH values (Fig. 2).

3.3. Effect of pH on thermal denaturation temperature of lactoferrin

Apparent thermal denaturation temperatures (T_{m}) of bLF and cLF were measured either by a thermal response UV spectrophotometry or by circular dichroism (CD). All the T_{m} values obtained with CD are depicted in Table 1. Fig. 3A and B shows the T_{m} profile of cLF and bLF, respectively, at pH 6.0, 7.0 and 8.0. The T_{m} values obtained from both methods (CD and UV) were in close agreement with each other. At pH 7.0, LF from both species exhibited maximum T_{m} (bLF, 70 ± 1 °C, and cLF, 66 ± 1 °C). The values obtained with bLF were consistent with a previous report on the thermal stability of bLF (Hu et al., 2008). It is

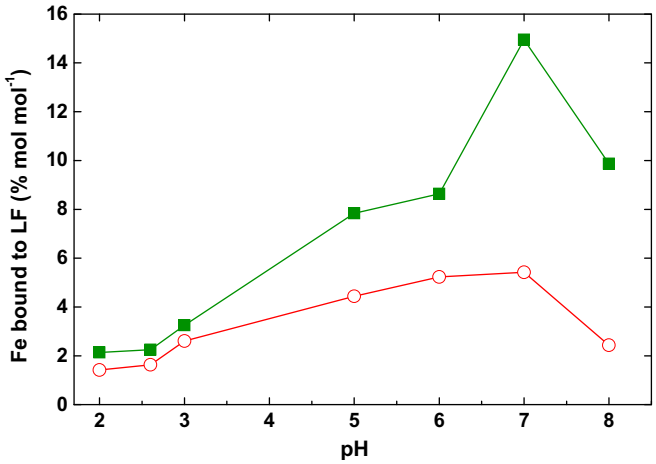

Fig. 2. Binding of Fe to caprine (○) and bovine (■) lactoferrin at pH 2.0–8.0. The protein (1.0 mg mL^{-1}) was solubilized in 1% (v/v) HNO_3 before ICP measurement (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Table 1
Thermal denaturation temperature ($T_m \pm 1$ °C) values of caprine and bovine lactoferrin in the pH range 3.0–8.0.

pH	3.0[a]	4.0[a]	5.0[a]	6.0	7.0	8.0
Bovine lactoferrin	39.0	45.0	50.0	67.0	70.0	67.0
Caprine lactoferrin	30.0	44.0	56.0	64.0	66.0	65.0

[a] T_m as monitored by the change in the secondary structure (CD) at 222 nm.

apparent from Table 1 that the binding of Fe to LF affects the thermal stability of LF, which is decreased with reduced Fe content at reduced pH. In addition, it was observed that the denaturation of cLF and bLF is irreversible (data not shown) in the pH range tested (2.0–8.0). It was difficult to measure the accurate T_m at pH 2.0, because LF samples were denatured at 25.0 ± 1 °C. This is attributed to the maximum hydrophobicity of LF at pH 2.0 (Section 3.5 of this article). A comparison of T_m profiles of cLF and bLF (Fig. 3A and B) indicated that, at physiological pH, bLF had a higher thermal stability than cLF (pH 6.0–8.0). The T_m decreased for cLF with reducing pH and reached a minimum of 30 ± 1 °C at pH 3.0 (Table 1); a similar trend was observed for bLF. At pH 2.0–3.0, LF becomes flexible and more prone to thermal denaturation. Initial Fe-binding is to one domain of LF, but as the protein acquires the closed state (neutral pH), through thermal fluctuations, it will be locked and closed as it completes its binding to ligands on the other domain (Baker & Baker, 2004).

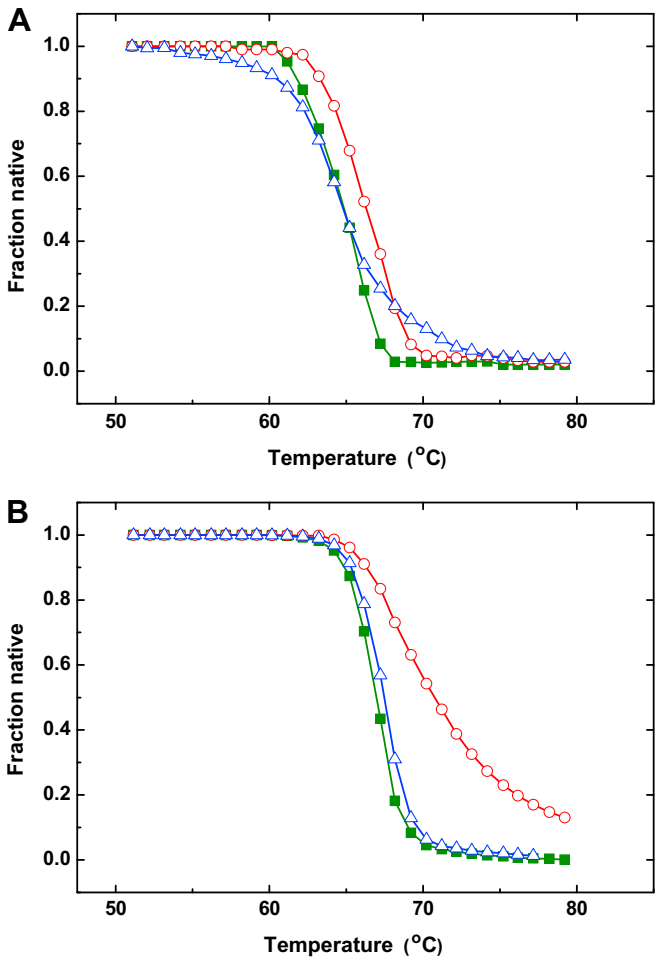

Fig. 3. Effect of pH on the thermal denaturation of lactoferrin as monitored by changes in the absorbance at 287 nm as a function of temperature for (A) caprine, (B) bovine lactoferrin in 10 mM sodium-phosphate buffer, pH 6.0 (■), pH 7.0 (○), or pH 8.0 (△) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Thermal stability data of LF from different species have been reported (Bezwoda & Mansoor, 1989; Conesa et al., 2008; Mata, Sanchez, Headon, & Calvo, 1998; Nam, Shimazaki, Kumura, Lee, & Yu, 1999; Paulsson et al., 1993). In the present study, one thermal transition peak was evident for both cLF and bLF in the temperature range between 35 and 90 °C. This depends on the detection limits of the analytical equipment used. The isolation methods used for cLF and bLF may affect the thermal stability parameters of the protein. Furthermore, the total number of tryptophan residues in bLF and cLF are 13 and 12, respectively. This is one of the factors that might affect the T_m of bLF and cLF. A slight modification of a single tryptophan residue produces a large decrease in the heat stability of proteins (Okajima, Kawata, & Hamaguchi, 1990).

3.4. Effect of pH on tryptophan fluorescence

The fluorescence properties of intrinsic tryptophan residues in proteins invariably change on denaturation of the protein. The average energy of emission of the tryptophan residues usually shifts to the red on unfolding due to solvent exposure, augmenting solvent relaxation in the unfolded state. In the unfolded state, most tryptophan residues in proteins have a maximum emission at ~355 nm.

LF exhibited fluorescence when excited at 290 nm (excitation maxima). The native cLF (pH 7.0) have a maximum emission of 339 nm. The emission maxima decreased to 336 nm at pH 5.0 and then increased to 347 nm at pH 2.0, indicating greater exposure of tryptophan residues to the solvent. A similar trend was observed for bLF, compared with cLF down to pH 2.6 and 2.0, where the emission maxima were higher for bLF. Maximum exposure of tryptophans at reduced pH was observed (Fig. 4A).

3.5. Effect of pH on surface hydrophobicity

The extensive binding of the aromatic chromophore 1-anilino-8-naphthalene sulfonate (ANS) to a protein surface is associated with the exposure of hydrophobic regions of the macromolecule (blue shift in the wavelength of maximum emission) that are generally buried and inaccessible to the probe. ANS-binding experiments with cLF at different pH values (2.0–8.0) decreased the maximum emission wavelength of the ANS-bound protein, when excited at 380 nm (Fig. 4B). This indicates the unfolding of the protein (pH < 5) exposing the hydrophobic groups to which ANS will bind. The emission maximum of ANS-bound cLF shifted from 524 to 493 nm at pH 2.0, and this blue shift of ANS fluorescence is due to the increased surface hydrophobicity. A similar unfolding between pH 8.0 and 2.0 was obvious for bLF. However, at pH 2.0, the emission maximum shifted to 484 nm, which indicates a higher surface hydrophobicity for bLF than cLF at this pH. A corresponding increase in the intensity was also evident (not shown). With decreasing pH values below pH 5.0, the unfolding of both LFs exposed the hydrophobic amino acids to the environment. This acid-denatured state has been shown to bind more ANS compared with the normal native state of LF from both species (pH 7.0).

3.6. Quenching studies of structural changes

Fluorescence quenching of tryptophan residues by different types of quenchers has been shown to provide information about the accessibility to solvent of these residues in proteins and the polarity of their microenvironment (Pawar & Deshpande, 2000). The Stern–Volmer plot for quenching of intrinsic fluorescence by acrylamide at pH 2.0, 5.0 and 8.0 is depicted in Fig. 5. The quenching constants (K_{SV}) calculated at pH 2.0, 5.0 and 8.0 are 16.86, 7.84 and 7.16 (M^{-1}), respectively, for bLF and 13.4, 11.7 and 8.0 (M^{-1}), respectively, for cLF. The Stern–Volmer plot indicates that the

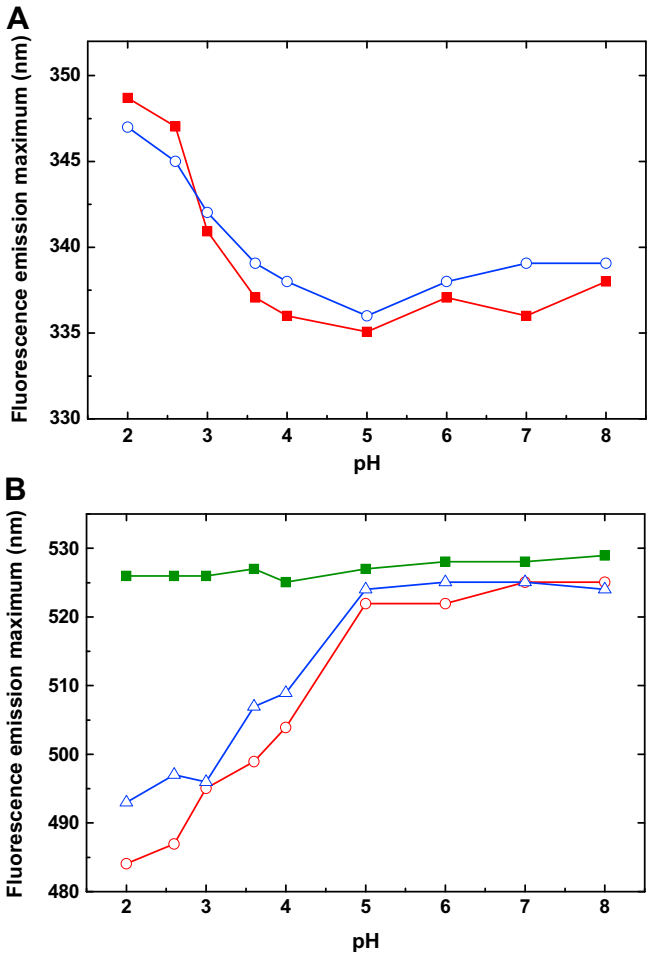

Fig. 4. (A) Effect of pH (2.0–8.0) on intrinsic tryptophan fluorescence of bovine (■) and caprine (○) lactoferrin. Excitation wavelength, 290 nm; emission range, 300–400 nm. Protein samples were equilibrated at 25 °C for 30 min before recording fluorescence spectra. (B) ANS fluorescence spectra of lactoferrin at pH values 2.0–8.0 in buffer (■), for bovine (○) and caprine (△) lactoferrin. Excitation wavelength was 380 nm; emission scan range was 400–600 nm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

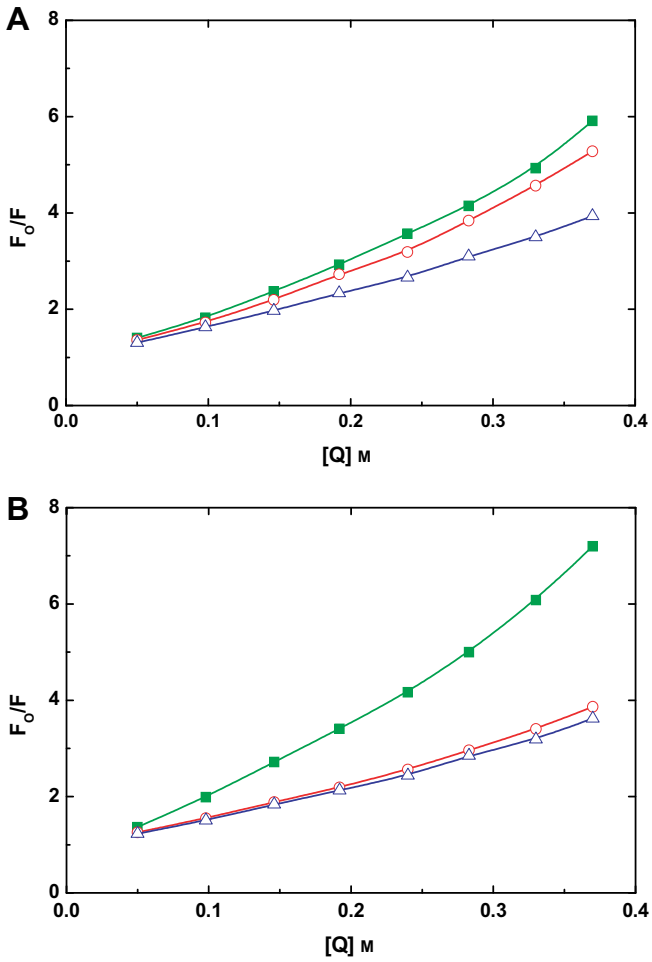

Fig. 5. Stern–Volmer plots of fluorescence quenching by acrylamide at pH 2.0 (■), pH 5.0 (○) and pH 8.0 (△) for (A) caprine, and (B) bovine lactoferrin. Values shown are the ratios of fluorescence in the absence of acrylamide (F_0) to the fluorescence at the concentration of quencher (F) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

aromatic amino acids were more exposed at pH 2.0 compared with the native folded conformation at pH 8.0 in cases of both cLF and bLF. Tryptophan fluorescence was more quenched in bLF.

3.7. Conformational changes in circular dichroism patterns induced by pH

The pH-dependence of the structural changes of cLF and bLF were characterized using circular dichroism (CD). The far-UV-CD spectra (190–260 nm) of cLF and bLF are presented in Fig. 6A and B, respectively. Hu et al. (2008) have reported the far-UV-CD structural changes of bLF as a function of guanidine hydrochloride concentration. In the present investigation, measurements over a wide pH range (pH 2.0–8.0) were performed. Two prominent peaks at 208 and 218 nm in the spectra of bLF and cLF indicated that these LFs are mixed α/β type (Nam et al., 1999). The secondary structures of bLF in the pH range 5.0–8.0 are constant with minor changes; a similar pattern was observed in case of cLF (Fig. 6A). Furthermore, a small shoulder around 218 nm and a negative band around 208 nm were evident in the case of cLF (pH 5.0–8.0). The contents of α-helix, β-structure and unordered structure were estimated from the CD spectra (190–260 nm) (Table 2). The α-helical content of bLF is higher than that of cLF as observed here. LF from both species was rich in β-structure in the pH range 5.0–8.0. At pH 2.0–3.0, partly unfolded structures of bLF and cLF were observed, with relatively low content of α-helices (3 and 7%), but higher amounts of β-structures (54 and 57%). Thus, this observation has proved that the state of the molecule under acidic conditions is different from the native state. Again, the molecule has acquired random conformation in case of LF from both species at pH 2.0. It seems that β-structure imparts stability to the conformation of LF from both species (Table 2).

The refolding of LF when pH was changed from 2.0 to 8.0 was observed by CD spectra and the protein regained all original spectral characteristics (data not shown). Apo LF was also used as a reference in this study and the secondary structure was similar (unpublished results). Fe might not be involved in the conformational changes in this case, compared with studies of reversibility of unfolding. Furthermore, there was no visible aggregation upon refolding to neutral pH. The binding of Fe to LF is reversible in accordance with the earlier reports (Baker, 1994; Baker & Baker, 2004, 2009). The shape of the spectra observed at pH 5.0–8.0 were identical (data shown at pH 8.0) and there was observed loss of ellipticities below pH 5.0 (data shown at pH 2.0). This is an indication of loss of α-helix or β-structure in the LF from both species (Table 2). It was apparent that the protein structure is stable

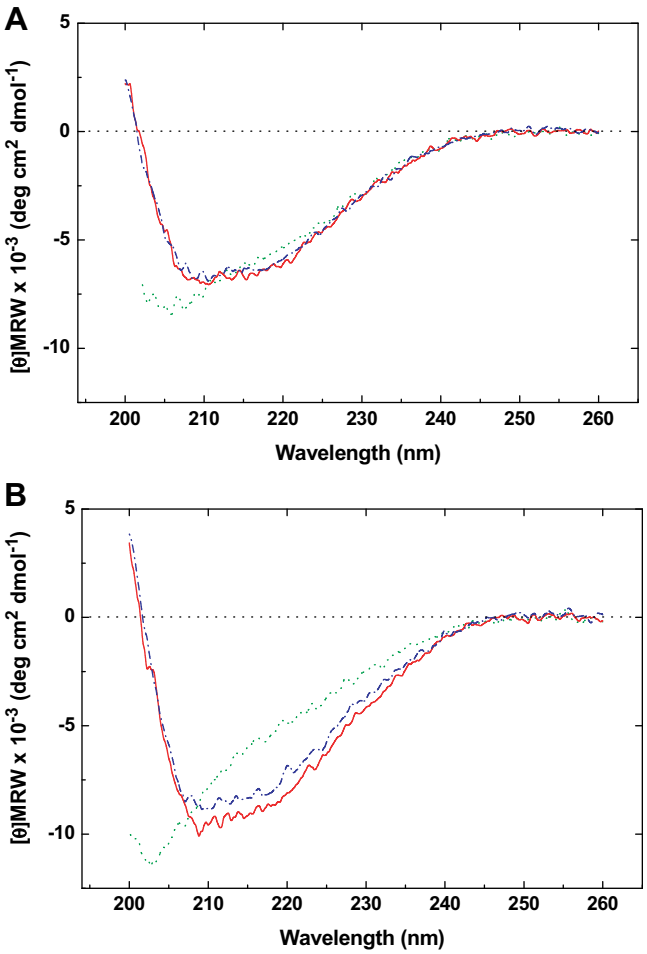

Fig. 6. Effect of pH on far-UV circular dichroic spectra of (A) caprine lactoferrin and (B) bovine lactoferrin at pH 2.0 (···), pH 5.0 (—) and pH 8.0 (-- --). Mean residue weight of 115 was used to calculate the molar ellipticity (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

in the pH range 5.0–8.0 and that unfolding starts at pH < 5.0 for both LFs (data shown at pH 2.0). Furthermore, the loss of ellipticity at acidic pH was evident in both LF-species, although bLF seems to be more stable than cLF. The shape of the spectra indicated that the conformation of cLF may be nearly identical with bLF.

Shimazaki, Kawano, and Yoo (1991) have shown that CD spectra of bovine, caprine and ovine LF suggested comparable secondary structures at physiological pH. The present findings can be correlated with this report. The difference in the shape of the spectra might be due to the interaction between N- and C-lobes in LF. In the N-terminal part of LF, the twenty five amino acid residues (17–41) form the lactoferricin peptide which has antiparallel β-sheet conformation. As part of intact LF, the peptide forms a number of stabilizing, long-range hydrophobic contacts with other parts of the protein (Hwang, Zhou, Shan, Arrowsmith, & Vogel, 1998). This may play an important role in providing stability to cLF and bLF to different extent.

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

Lactoferrin	pH	α-Helix	β-Structure	Aperiodic
Caprine	2.0	7.0	57.0	36.0
	5.0	16.0	63.0	21.0
	8.0	15.0	63.0	22.0
Bovine	2.0	3.0	54.0	43.0
	5.0	25.0	50.5	24.5
	8.0	20.0	60.0	20.0

To support the present research, 3D structural elements (bonds and helices) in bLF (PDB code 1blf) and cLF (PDB code 1jw1) were compared. The total number of bonds in bLF and cLF are 5566 and 5404, respectively. As observed from 3D structural modelling (PDB), the number of α-helices in bLF and cLF are 26 and 23, respectively. This might impart stability to bLF and cLF. The bLF has more structure–stability as compared with cLF (Murzin, Brenner, Hubbard, & Chothia, 1995). From CD data (Table 2) it can be seen that the total α-helix content at pH 5.0–8.0 was higher in bLF compared with cLF. All these factors might influence the shape of the thermograms of bLF and cLF (Conesa et al., 2008). The hydrophobic interactions and ionic interactions contribute to the stability of proteins (Grimsley et al., 1999). These interactions could be different among cLF and bLF. There are non-covalent interactions between N- and C-lobes of LF, which depend upon the positions of different amino acids within the sequence of LF. These factors might influence the structure and thermal stability of cLF and bLF.

LF is a glycosylated protein, but the number and location of potential glycosylation sites, and the sites actually used, vary among species. The bLF and cLF have four glycans each. The relative proportions of glycan of oligomannosidic and of N-acetyllactosamine type vary with period of lactation. The primary structures of specific glycans bound to cLF and bLF seems to vary. The thermal stability is influenced by the characteristics of glycans present. This differs in cLF and bLF (Spik, Coddeville, & Montreuil, 1988; Van Berkel, van Veen, Geerts, de Boer, & Nuijens, 1996). This might influence the thermal stability and CD conformational alterations in cLF and bLF.

4. Conclusions

The homogeneity of purified cLF was confirmed by SDS-PAGE and was compared with that of bLF. Based on the present research, the purification method can be efficiently applied to the industrial scale production of LF. The physicochemical/biophysical properties of LF from both species have been evaluated. Thermal transition temperature analysis revealed the higher stability of bLF compared with cLF in the pH range 2.0–8.0. Fluorescence investigations (intrinsic fluorescence, ANS-binding and acrylamide quenching) indicated the maximum tryptophan exposure at pH 2.0–3.0. The variation in pH was strong enough to induce protein changes measurable by fluorescence spectroscopy. The CD structures of both bLF and cLF were partially unfolded at pH 2.0–3.0. The CD data supported the observations done by fluorescence methods. Overall, this investigation provides a reasonable approach for studying the pH induced changes on the structure–stability of bLF and cLF using UV–visible, fluorescence and CD measurements. LF is reported to have bioactive functions in humans; the structural differences under similar pH conditions as in gastrointestinal tract may thus be significant.

Acknowledgements

This work is a part of a joint cooperation project between the Department of Protein Chemistry and Technology, Central Food Technological Research Institute (CFTRI), Mysore (A Constituent Laboratory of CSIR), India and the Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Norway. The author gratefully acknowledges the financial support from Norwegian Agency for Development Cooperation (NORAD), directorate under Norwegian Ministry of Foreign Affairs (MFA) and Norwegian State Educational Loan Fund.

References

Abe, H., Saito, H., Miyakawa, H., Tamura, Y., Shimamura, S., Nagao, E., et al. (1991). Heat stability of bovine lactoferrin at acidic pH. *Journal of Dairy Science, 74*, 65–71.

Ainscough, E. W., Brodie, A. M., Plowman, J. E., Bloor, S. J., Loehr, J. S., & Loehr, T. M. (1980). Studies on human lactoferrin by electron paramagnetic resonance, fluorescence, and resonance Raman spectroscopy. *Biochemistry, 19*, 4072–4079.

Almaas, H., Holm, H., Langsrud, T., Flengsrud, R., & Vegarud, G. E. (2006). In vitro studies of the digestion of caprine whey proteins by human gastric and the effects on selected microorganisms. *British Journal of Nutrition, 96*, 562–569.

Baker, E. N. (1994). Structure and reactivity of transferrins. *Advances in Inorganic Chemistry, 41*, 389–463.

Baker, E. N., & Baker, H. M. (2004). Lactoferrin and iron: structural and dynamic aspects of binding and release. *Biometals, 17*, 209–216.

Baker, E. N., & Baker, H. M. (2009). A structural framework for understanding the multifunctional character of lactoferrin. *Biochimie, 91*, 3–10.

Bezwoda, W. R., & Mansoor, N. (1989). Lactoferrin from human breast milk and from neutrophil granulocytes. Comparative studies of isolation, quantitation, characterization and iron binding properties. *Biomedical Chromatography, 3*, 121–126.

Bagshaw, C. R., & Harris, D. A. (1987). Measurement of ligand binding to proteins. In D. A. Harris, & C. L. Bashford (Eds.), *Spectrophotometry and spectrofluorimetry: A practical approach* (pp. 91–113). Oxford, UK: IRL Press.

Blanc, B., & Isliker, H. (1961). Isolation and characterization of the red siderophilic protein of human milk, lactotransferrin. *Bulletin of Society of Chemical Biology, 43*, 929–943.

Brock, J. H. (1985). Transferrins. In P. M. Harrison (Ed.), *Metalloproteins* (pp. 183–262). Porters South, London, UK: Macmillan.

Brock, J. H. (1997). Lactoferrin structure function relationships: an overview. In T. W. Hutchens, & B. Lonnerdal (Eds.), *Lactoferrin interactions and biological functions* (pp. 3–25). Totowa, NJ, USA: Humana Press.

Castellino, F. J., Fish, W. T. V., & Manna, K. G. (1970). Structural studies on bovine lactoferrin. *Journal of Biological Chemistry, 245*, 4269–4275.

Conesa, C., Sanchez, L., Rota, C., Perez, M., Calvo, M., Farnaud, S., et al. (2008). Isolation of lactoferrin from milk of different species: calorimetric and antimicrobial studies. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 150*, 131–139.

Eftink, M. R., & Ghiron, C. A. (1982). Fluorescence quenching studies with proteins. *Analytical Biochemistry, 114*, 199–227.

Etzel, M. R., & Chiu, C. K. (1997). Fractionation of lactoperoxidase and lactoferrin from bovine whey using a cation exchange membrane. *Journal of Food Science, 62*, 996–1000.

Farnaud, S., & Evans, R. W. (2005). Lactoferrin: the conductor of the immunological system? In B. A. Veskler (Ed.), *New research on immunology* (pp. 47–76) New York, USA: Nova Science Publishers.

Goodman, R. E., & Schanbacher, F. L. (1991). Bovine lactoferrin mRNA: sequence, analysis, and expression in the mammary gland. *Biochemical and Biophysical Research Communications, 180*, 75–84.

Gordon, W. G., Groves, M. L., & Basch, J. J. (1963). Bovine milk "red protein": amino acid composition and comparison with blood transferrins. *Biochemistry, 2*, 817–820.

Grimsley, G. R., Shaw, K. L., Fee, L. R., Alston, R. W., Huyghues-Despointes, B. M. P., Thurlkill, R. L., et al. (1999). Increasing protein stability by altering long-range coulombic interactions. *Protein Science, 8*, 1843–1849.

Hu, F., Pan, F., Sawano, Y., Makino, T., Kakehi, Y., Komiyama, M., et al. (2008). Studies of the structure of multiferric ion-bound lactoferrin: a new antianemic edible material. *International Dairy Journal, 18*, 1051–1056.

Hwang, P. M., Zhou, N., Shan, X., Arrowsmith, C. H., & Vogel, H. J. (1998). Three dimensional solution structure of lactoferricin B, an antimicrobial peptide derived from bovine lactoferrin. *Biochemistry, 37*, 4288–4298.

Kato, A., & Nakai, S. (1980). Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. *Biochimica et Biophysica Acta, 624*, 13–20.

Kawakami, H., Dosako, S., & Nakajima, I. (1993). Effect of lactoferrin on iron solubility under neutral conditions. *Bioscience, Biotechnology, and Biochemistry, 57*, 1376–1377.

Kolb, F. A. (2001). The prospects of modifying the antimicrobial properties of milk. *Biotechnology Advances, 19*, 299–316.

Koepf, E. K., Petrassi, H. M., Sudol, M., & Kelly, J. W. (1999). WW: an isolated three-stranded antiparallel β-sheet domain that unfolds and refolds reversibly; evidence for a structured hydrophobic cluster in urea and GdnHCl and a disordered thermal unfolded state. *Protein Science, 8*, 841–853.

Korhonen, H., & Pihlanto, A. (2006). Bioactive peptides: production and functionality. *International Dairy Journal, 16*, 945–960.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*, 680–685.

Lonnerdal, B. (1989). Non-protein nitrogen components in human milk: biochemistry and potential functional role. In S. Atkinson, & B. Lonnerdal (Eds.), *Protein and non-protein nitrogen in human milk* (pp. 29–42). Florida, USA: CRC Press.

Masson, P. L., & Heremans, J. F. (1971). Lactoferrin in milk from different species. *Comparative Biochemistry and Physiology B, 39*, 119–129.

Mata, L., Sanchez, L., Headon, D. R., & Calvo, M. (1998). Thermal denaturation of human lactoferrin and its effect on the ability to bind iron. *Journal of Agricultural and Food Chemistry, 46*, 3964–3970.

Murzin, A. G., Brenner, S. E., Hubbard, T., & Chothia, C. (1995). SCOP: a structural classification of proteins database for the investigation of sequences and structures. *Journal of Molecular Biology, 247*, 536–540.

Nagasako, Y., Sainto, H., Tamura, Y., Shimamura, S., & Tomita, M. (1993). Iron-binding properties of bovine lactoferrin in iron-rich solution. *Journal of Dairy Science, 76*, 1876–1881.

Nam, M. S., Shimazaki, K., Kumura, H., Lee, K. K., & Yu, D. Y. (1999). Characterization of Korean native goat lactoferrin. *Comparative Biochemistry and Physiology B, 123*, 201–208.

Okajima, T., Kawata, Y., & Hamaguchi, K. (1990). Chemical modification of tryptophan residues and stability changes in proteins. *Biochemistry, 29*, 9168–9175.

Ounis, W. B., Gauthier, S. F., Turgeon, S. L., Roufik, S., & Pouliot, Y. (2008). Separation of minor protein components from whey protein isolates by heparin affinity chromatography. *International Dairy Journal, 18*, 1043–1050.

Pace, C. N., Shirely, B. A., & Thomson, J. A. (1989). In T. E. Creighton (Ed.), *Protein structure: A practical approach* (pp. 311–329). Oxford, UK: IRL Press.

Patel, H. A., Singh, H., Anema, S. G., & Creamer, L. K. (2006). Effects of heat and high hydrostatic pressure treatments on disulfide bonding interchanges among the proteins in skim milk. *Journal of Agricultural and Food Chemistry, 54*, 3409–3420.

Paulsson, M. A., Svensson, U., Kishore, A. R., & Naidu, A. S. (1993). Thermal behaviour of bovine lactoferrin in water and its relation to bacterial interaction and antibacterial activity. *Journal of Dairy Science, 76*, 3711–3720.

Pawar, S. A., & Deshpande, V. V. (2000). Characterization of acid-induced unfolding intermediates of glucose/xylose isomerase. *European Journal of Biochemistry, 267*, 6331–6338.

Privalov, P. L., Griko, Y. U. V., Venyaminov, S. Y. U., & Kutyshenko, V. P. (1986). Cold denaturation of myoglobin. *Journal of Molecular Biology, 190*, 487–498.

Provost, F. L., Nocart, M., Guerin, G., & Martin, P. (1994). Characterization of the goat lactoferrin cDNA: assignment to the relevant locus to bovine U12 synteny group. *Biochemical and Biophysical Research Communications, 203*, 1324–1332.

Qiu, J., Hendrixson, D. R., Baker, E. N., Murphy, T. F., St Geme, J. W., & Plaut, A. G. (1998). Human milk lactoferrin inactivates two putative colonization factors expressed by *Haemophilus influenzae*. *Proceedings of the National Academy of Sciences of the United States of America, 95*, 12641–12646.

Recio, I., & Visser, S. (1999). Two ion-exchange chromatographic methods for the isolation of antibacterial peptides from lactoferrin: in situ enzymatic hydrolysis on an ion-exchange membrane. *Journal of Chromatography A, 831*, 191–201.

Recio, I., & Visser, S. (2000). Antibacterial and binding characteristics of bovine, ovine and caprine lactoferrins: a comparative study. *International Dairy Journal, 10*, 597–605.

Rossi, P., Giansanti, F., Boffi, A., Ajello, M., Valenti, P., Chiancone, E., et al. (2002). Ca^{2+} binding to bovine lactoferrin enhances protein stability and influences the release of bacterial lipopolysaccharide. *Biochemistry and Cell Biology, 80*, 41–48.

Sanchez, L., Aranda, P., Perez, M. D., & Calvo, M. (1988). Concentration of lactoferrin and transferrin throughout lacatation in cow's colostrums and milk. *Biological Chemistry Hoppe-Seyler, 369*, 1005–1008.

Shimazaki, K., Kawano, N., & Yoo, Y. C. (1991). Comparison of bovine, sheep and goat milk lactoferrins in their electrophoretic behaviour, conformation, immunochemical properties and lectin reactivity. *Comparative Biochemistry and Physiology B, 98*, 417–422.

Shimazaki, K., Tanaka, T., Kon, H., Oota, K., Kawaguchi, A., Maki, Y., et al. (1993). Separation and characterization of the C-terminal half molecule of bovine lactoferrin. *Journal of Dairy Science, 76*, 946–955.

Spik, G., Coddeville, B., & Montreuil, J. (1988). Comparative study of the primary structures of sero-, lacto- and ovotransferrin glycans from different species. *Biochimie, 70*, 1459–1469.

Steijns, J. M., & van Hooijdonk, A. C. M. (2000). Occurrence, structure, biochemical properties and technological characteristics of lactoferrin. *British Journal of Nutrition, 84*, S11–S17.

Uchida, T., Oda, T., Sato, K., & Kawakami, H. (2006). Availability of lactoferrin as a natural solubilizer of iron for food products. *International Dairy Journal, 16*, 95–101.

Van Berkel, P. H. C., van Veen, H. A., Geerts, M. E. J., de Boer, H. A., & Nuijens, J. H. (1996). Heterogeneity in utilization of N-glycosylation sites Asn624 and Asn138 in human lactoferrin: a study with glycosylation-site mutants. *Biochemical Journal, 319*, 117–122.

Yang, J. T., Wu, C. S., & Martinez, H. M. (1986). Calculation of protein conformation from circular dichroism. In C. H. Hirs, & S. N. Timasheff (Eds.), *Methods in Enzymology, Vol. 130* (pp. 208–269). New York, NY, USA: Academic Press.

Ye, X. Y., Wang, H. X., Liu, F., & Ng, T. B. (2000). Ribonuclease, cell-free translation-inhibitory and superoxide radical scavenging activities of the iron-binding protein lactoferrin from bovine milk. *International Journal of Biochemistry and Cell Biology, 32*, 235–241.

Paper II

Biometals (2010) 23:1159–1170
DOI 10.1007/s10534-010-9366-5

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

Ashoka Sreedhara · Ragnar Flengsrud · Thor Langsrud · Purnima Kaul · Vishweshwaraiah Prakash · Gerd Elisabeth Vegarud

Received: 31 May 2010 / Accepted: 15 July 2010 / Published online: 3 August 2010

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

A. Sreedhara (✉) · R. Flengsrud · T. Langsrud · G. E. Vegarud
Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Aas 1432, Norway
e-mail: ashoka.sreedhara@umb.no; ashoka.sreedhara@gmail.com

A. Sreedhara · P. Kaul · V. Prakash
Department of Protein Chemistry and Technology, Central Food Technological Research Institute (A Constituent Laboratory of CSIR, Delhi), Mysore 570 020, India

Introduction

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

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

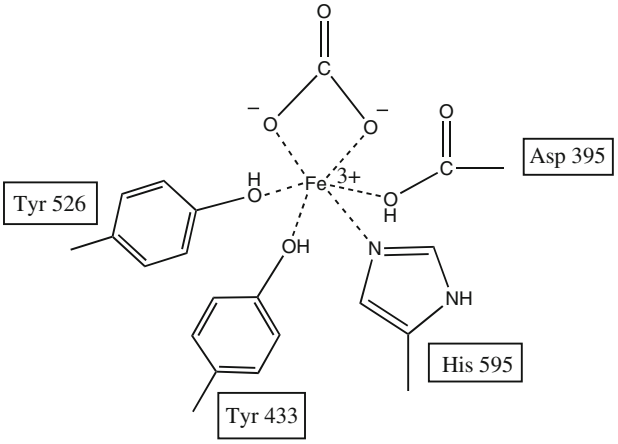

Fig. 1 The general type of ferric (Fe) ions bonded to bovine lactoferrin

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

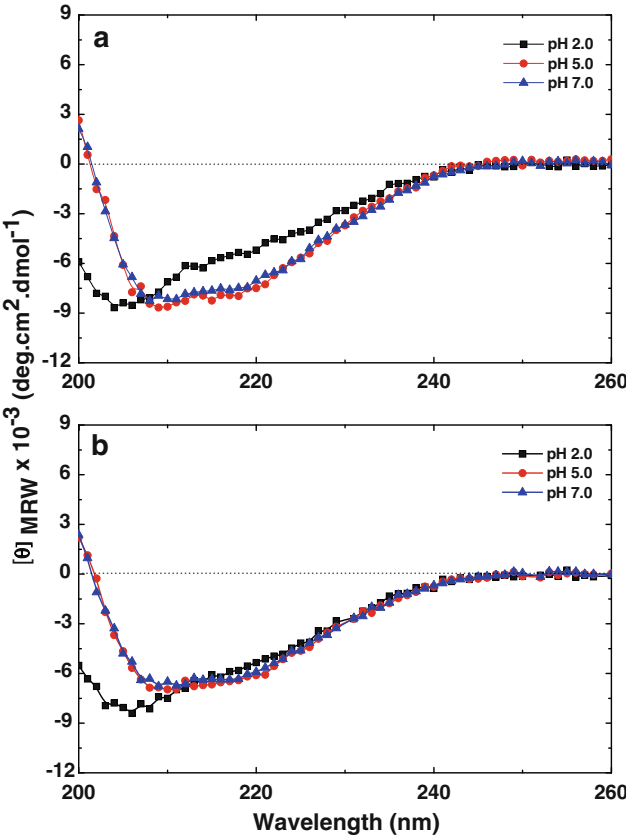

Fig. 2 Effect of pH on the CD secondary structures of **a** apo and **b** holo caprine lactoferrins (cLF). Buffers used are given under "Materials and methods"

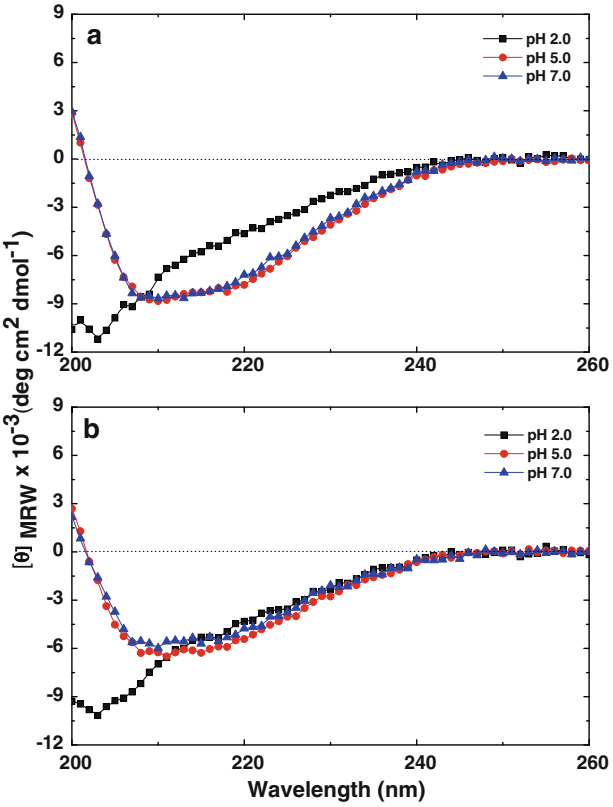

Fig. 3 Effect of pH on the CD secondary structures of **a** apo and **b** holo bovine lactoferrins (bLF). Buffers used are given under "Materials and methods"

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

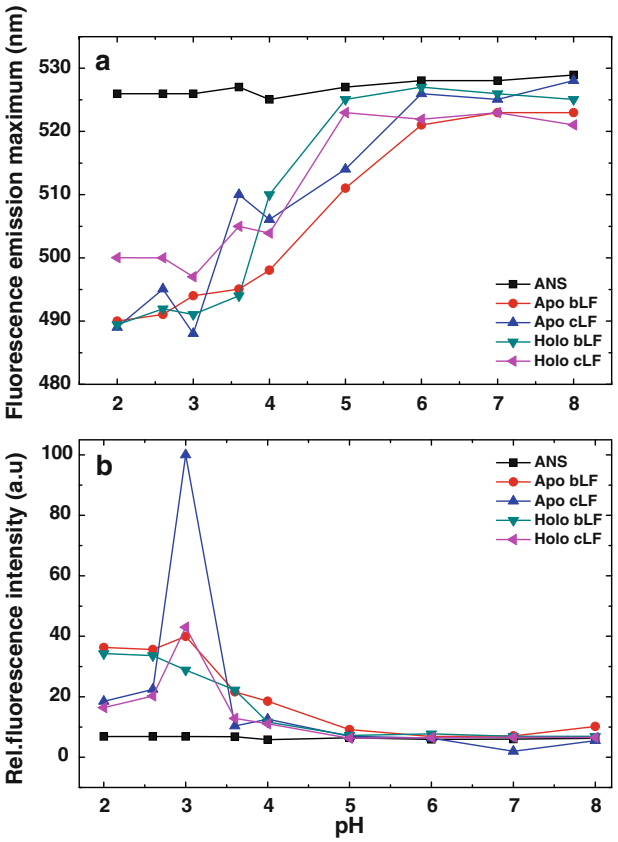

Fig. 4 **a** The 8-anilino-1-naphthalene-sulfonate (ANS) fluorescence emission maxima of apo and holo forms of caprine (cLF) and bovine (bLF) lactoferrins within the pH range of 2.0–8.0. **b** ANS fluorescence intensity of apo and holo forms of caprine (cLF) and bovine (bLF) lactoferrins within the pH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

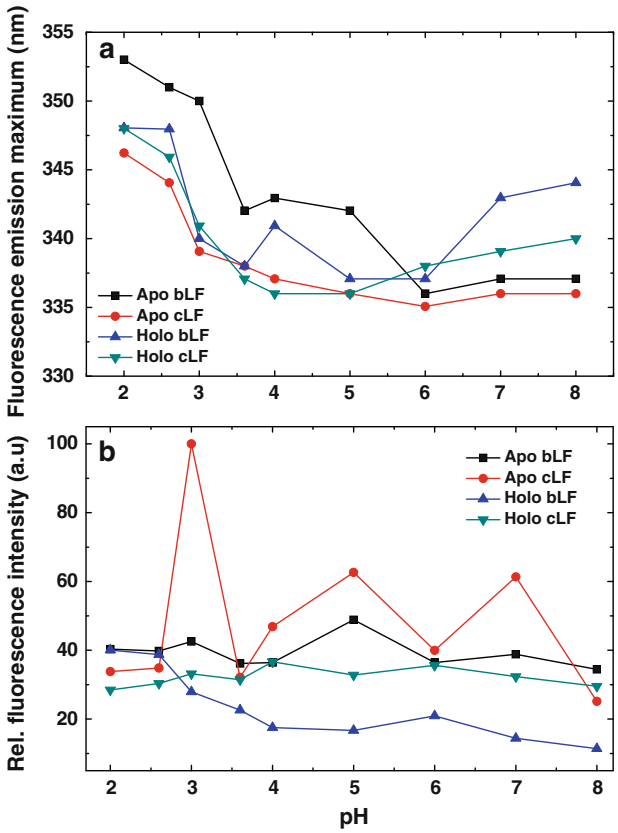

Fig. 5 **a** Fluorescence emission maxima (λ_{max}) of apo and holo forms of caprine (cLF) and bovine (bLF) lactoferrins at different pH. **b** Relative fluorescence intensity of apo and holo forms of caprine and bovine lactoferrins at different pH. Experimental conditions are as described under "Materials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

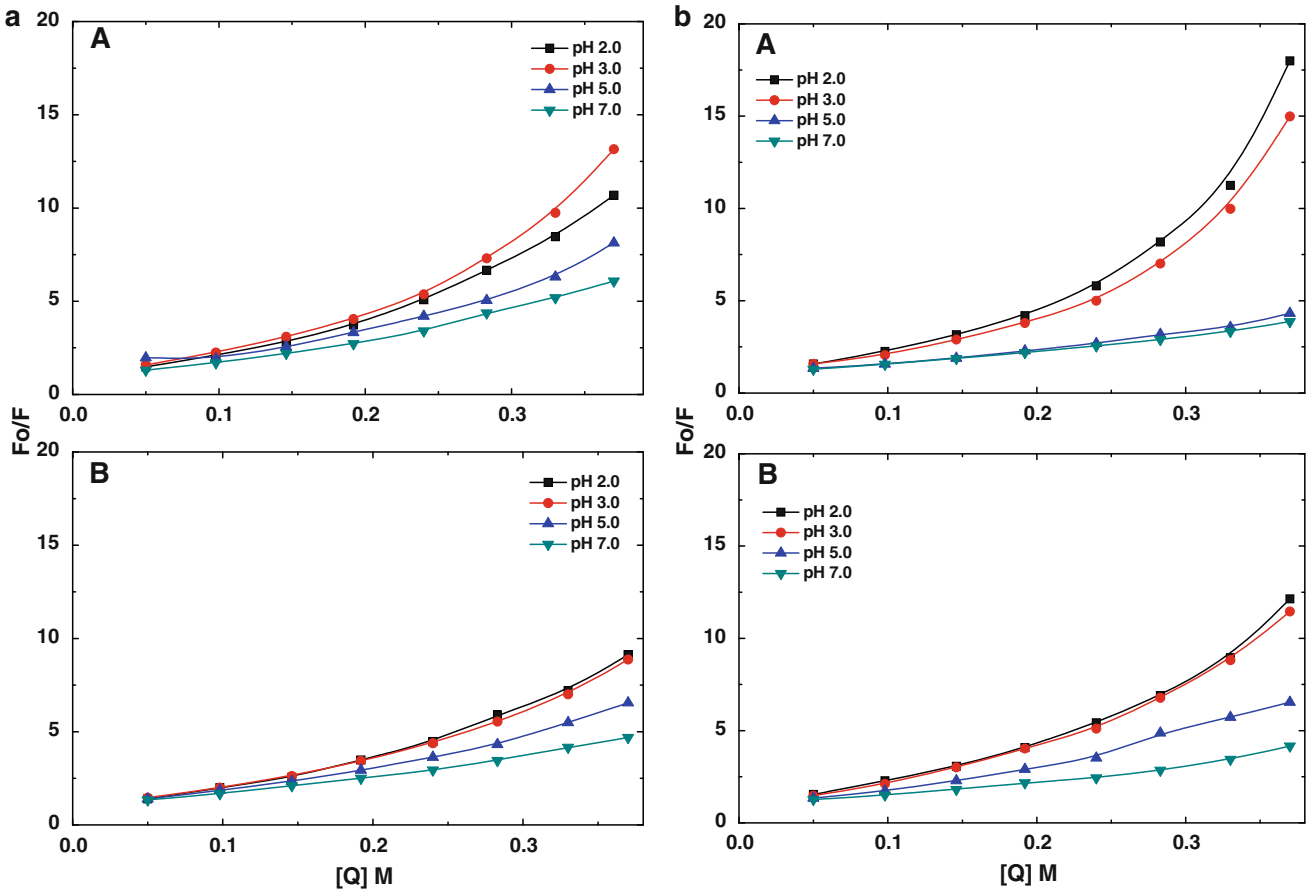

Fig. 6 **a** Fluorescence acrylamide quenching of *A* apo and *B* holo forms of caprine lactoferrin (cLF) at pH values 2.0–7.0. **b** Fluorescence acrylamide quenching of *A* apo and *B* holo forms of bovine lactoferrin (bLF) at pH values 2.0–7.0. Buffers used are given under "Materials and methods"

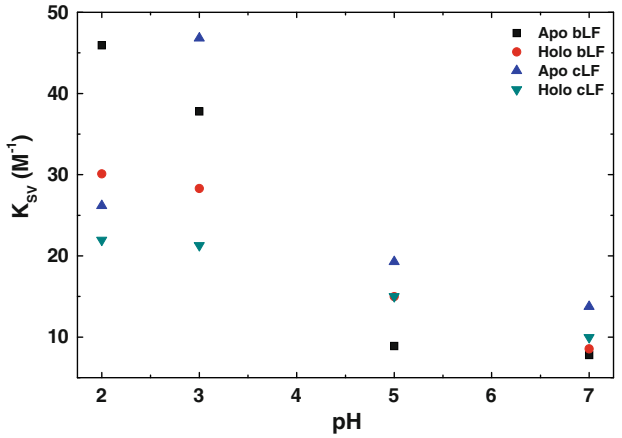

Fig. 7 Stern–Volmer constants (K_{SV}) for fluorescence quenching of apo and holo forms of caprine (cLF) and bovine (bLF) lactoferrin at pH values 2.0–7.0. Buffers used are given under "Materials and methods"

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

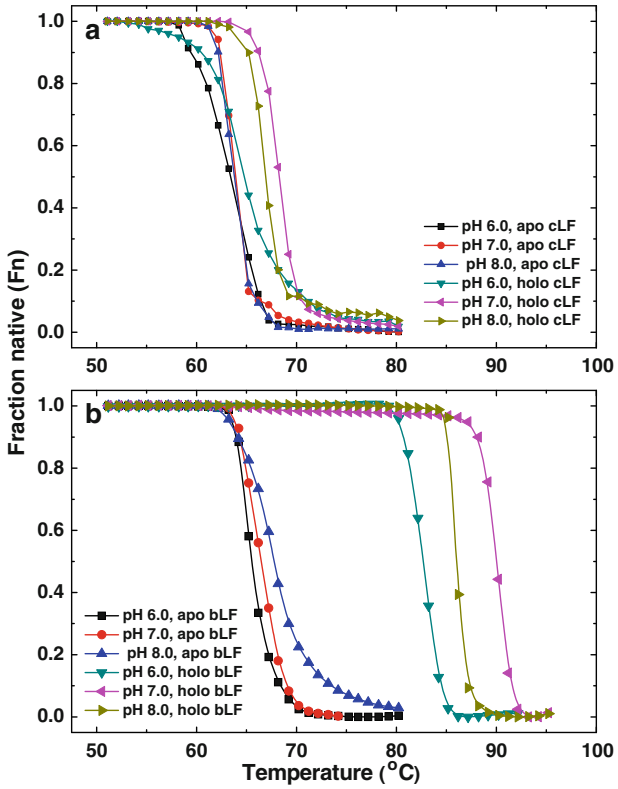

Fig. 8 Thermal denaturation profiles of apo and holo forms of caprine (cLF) and bovine (bLF) lactoferrins at different pH. **a** caprine, **b** bovine lactoferrin. The spectra were recorded at 287 nm over a temperature range 35–90°C with 1°C increment per min. Buffers used are given under "Materials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

Acknowledgments The author gratefully acknowledges Daniel Krowarsch, Faculty of Biotechnology, University of Wroclaw, Poland for providing circular dichroism facility and technical assistance. Further, the author is thankful to Tor Bruun, Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences for assistance during protein purification.

References

Adlerova L, Bartoskova A, Faldyna H (2008) Lactoferrin: a review. Vet Med 53:457–468

Baker HM, Baker EN (2004) Lactoferrin and Iron: structural and dynamic aspects of binding and release. Biometals 17:209–216

Baker EN, Baker HM (2009) A structural framework for understanding the multifunctional character of lactoferrin. Biochimie 91(1):3–10

Brock JH (1997) Lactoferrin structure function relationships: an overview. In: Hutchens TW, Lonnerdal B (eds) Lactoferrin interactions and biological functions. Human Press, Totowa, pp 3–25

Conesa C, Sanchez L, Rota C, Perez M, Calvo M (2007) A calorimetric study of thermal denaturation of recombinant human lactoferrin from rice. J Agric Food Chem 55:4848–4853

Conesa C, Sanchez L, Rota C, Perez M, Calvo M, Farnaud S, Evans RW (2008) Isolation of lactoferrin from milk of different species: calorimetric and antimicrobial studies. Comp Biochem Physiol B 150:131–139

Devaraja KB, Kumara PR, Prakash V (2009) Characterization of acid-induced molten globule like state of ficin. Int J Biol Macromol 45(3):248–254

Eftink MR, Ghiron CA (1981) Fluorescence quenching studies with proteins. Anal Biochem 114:199–227

Eftink MR, Selvidge LA (1982) Fluorescence quenching of liver alcohol dehydrogenase by acrylamide. Biochemistry 21(1):117–125

Ellison RT III, Giehl TJ, LaForce M (1988) Damage of the outer membrane of enteric gram-negative bacteria by lactoferrin and transferrin. Infect Immun 56: 2774–2781

Farnaud S, Evans RW (2003) Lactoferrin—a multifunctional protein with antimicrobial properties. Mol Immunol 40:395–405

Gorinstein S, Goshev I, Moncheva S, Zemser M, Weisz M, Caspi A, Libman I, Lerner HT, Trakhtenberg S, Martín-Belloso O (2000) Intrinsic tryptophan fluorescence of human serum proteins and related conformational changes. J Protein Chem 19(8):637–642

Hu F, Pan F, Sawano Y, Makino T, Kakehi Y, Komiyama M, Kawakami H, Tanokura M (2008) Studies of the structure of multiferric ion-bound lactoferrin: a new antianemic edible material. Int Dairy J 18:1051–1056

Jenness R (1980) Composition and characteristics of goat milk: review1968–1979. J Dairy Sci 63:1605–1630

Karthikeyan S, Sharma S, Sharma AK, Paramasivam M, Yadav S, Srinivasan A, Singh TP (1999) Structural variability and functional convergence in lactoferrins. Curr Sci 77(2):241–255

Khan JA, Kumar P, Srinivasan A, Singh TP (2001) Protein intermediate trapped by the simultaneous crystallization process. Crystal structure of an iron-saturated intermediate in the Fe^{3+} binding pathway of camel lactoferrin at 2.7 Å resolution. J Biol Chem 276:36817–36823

Koepf EK, Petrassi HM, Sudol M, Kelly JW (1999) WW: an isolated three-stranded antiparallel β-sheet domain that unfolds and refolds reversibly; evidence for a structured hydrophobic cluster in urea and GdnHCl and a disordered thermal unfolded state. Protein Sci 8:841–853

Masco L, Huys TG, De Brandt E, Temmerman R, Swings J (2005) Culture-dependent and culture-independent qualitative analysis of probiotic products claimed to contain bifidobacteria. Int J Food Microbiol 102:221–230

Masson PL, Heremans JF (1971) Lactoferrin in milk from different species. Comp Biochem Physiol B 39:119–129

Masson PL, Heremans JF, Prignot JJ, Wauters G (1966) Immunohistochemical localization and bacteriostatic properties of an iron-binding protein from bronchial mucus. Thorax 21:538–544

Mata L, Sanchez L, Headon DR, Calvo M (1998) Thermal denaturation of human lactoferrin and its effect on the ability to bind iron. J Agric Food Chem 46:3964–3970

Matulis D, Baumann CG, Bloomfield VA, Lovrien RE (1999) 1-Anilino-8-naphthalene sulfonate as a protein conformational tightening agent. Biopolymers 49(6):451–458

Nam MS, Shimazaki K, Kumura H, Lee KK, Yu DY (1999) Characterization of Korean native goat lactoferrin. Comp Biochem Physiol B 123:201–208

Neilands JB (1991) A brief history of iron metabolism. Biometals 4:1–6

Olakanmi O, Rasmussen GT, Lewis TS, Stokes JB, Kemp JD, Britigan BE (2002) Multivalent metal-induced iron acquisition from transferrin and lactoferrin by myeloid cells. J Immunol 169:2076–2084

Orsi N (2004) The antimicrobial activity of lactoferrin: current status and perspectives. Biometals 17:189–196

Pace CN, Scholtz JM (1997) Measuring the conformational stability of a protein. In: Creighton TE (ed) Protein structure: a practical approach, 2nd edn. IRL Press, Oxford, pp 299–321

Recio I, Visser S (1999) Two ion-exchange chromatographic methods for the isolation of antibacterial peptides from lactoferrin: in situ enzymatic hydrolysis on an ion-exchange membrane. J Chromatogr A 831:191–201

Recio I, Visser S (2000) Antibacterial and binding characteristics of bovine, ovine and caprine lactoferrins: a comparative study. Int Dairy J 10:597–605

Sanchez L, Aranda P, Perez MD, Calvo M (1988) Concentration of lactoferrin and transferrin throughout lactation in cow's colostrums and milk. Biol Chem Hoppe-Seyler 369:1005–1008

Spik BC, Montreuil J (1988) Comparative study of the primary structures of sero-, lacto- and ovotransferrin glycans from different species. Biochimie 70:1459–1469

Sreedhara A, Flengsrud R, Prakash V, Krowarsch D, Langsrud T, Kaul P, Devold TG, Vegarud GE (2010) A comparison of effects of pH on the thermal stability and

conformation of caprine and bovine lactoferrin. Int Dairy J 20:487–494

Steijns JM, van Hooijdonk ACM (2000) Occurrence, structure, biochemical properties and technological characteristics of lactoferrin. Br J Nutr 84:S11–S17

Tina KG, Bhadra R, Srinivasan N (2007) PIC: protein interaction calculator. Nucleic Acids Res 35:W473–W476

Tomita M, Wakabayashi H, Yamauchi K, Teraguchi S, Hayasawa H (2002) Bovine lactoferrin and lactoferricin derived from milk: production and applications. Biochem Cell Biol 80(1):109–112

Tomita M, Wakabayashi H, Shin K, Yamauchi K, Yaeshima T, Iwatsuki K (2009) Twenty-five years of research on bovine lactoferrin applications. Biochimie 91:52–57

Uchida T, Oda T, Sato K, Kawakami H (2006) Availability of lactoferrin as a natural solubilizer of iron for food products. Int Dairy J 16:95–101

Van Hooijdonk ACM, Kussendrager KD, Steijns JM (2000) In vivo antimicrobial and antiviral activity of components in bovine milk and colostrum involved in non-specific defence. Br J Nutr 84:127–134

Wakabayashi H, Yamauchi K, Takase M (2006) Lactoferrin research, technology and applications. Int Dairy J 16:1241–1251

Yang JT, Wu CS, Martinez HM (1986) Calculation of protein conformation from circular dichroism. In: Hirs CH, Timasheff SN (eds) Methods in enzymology, vol 130. Academic Press, New York, pp 208–269

Paper III

Zn (II) binding to lactoferrin at pH 2.0 - 7.0
A comparative study of caprine and bovine lactoferrin

Ashoka Sreedhara,*[,†,‡] Ragnar Flengsrud,[†] Thor Langsrud,[†] Vishweshwaraiah Prakash,[‡] Gerd Elisabeth Vegarud[†]

[†]Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Aas, N-1432, Norway

[‡]Department of Protein Chemistry and Technology, Central Food Technological Research Institute (A Constituent Laboratory of CSIR, Delhi), Mysore, 570 020, India

*Corresponding author (Telephone +47 64 96 61 53; Fax + 47 64 96 59 01; E-mail ashoka.sreedhara@umb.no, ashoka.sreedhara@gmail.com)

Abstract

The interaction between zinc (Zn^{2+}) and lactoferrin (LF) from caprine and bovine species was studied in the pH range of 2.0-7.0. The conformational changes and thermal stabilities of the apo and zinc bound holo LF (ZnLF) forms were compared among caprine and bovine species. At pH 7.0, zinc bound bovine LF (ZnbLF) showed a higher T_m (83±1 ^oC) as compared to zinc bound caprine LF (ZncLF) (67±1 ^oC). By reducing pH to 4.0, T_m of ZncLF and ZnbLF decreased to 76±1 ^oC and 55±1 ^oC, respectively. The apo and zinc bound LF forms from both species showed highest stability at pH 7.0. A significant loss in the zinc content of both cLF and bLF was observed when the pH was reduced from 7.0 to 2.0. At pH 2.0, the structure of ZnbLF was more unfolded than ZncLF having 54% β and 45% unordered structures and lowest α-helix structure (1%). Whereas, ZncLF retained unfolded structure with 57.5% β structure and 6% α-helix at that pH. A higher exposure of hydrophobic surfaces at pH 2.0 for ZnbLF as compared to ZncLF was shown. The results were supported by guanidine hydrochloride induced denaturation studies. Further, zinc bound LFs from both species were more stable than the respective apo LF forms. Since zinc is a trace element and plays a vital role in many biological systems in humans, the zinc binding and structural properties of LF obtained at different pH are significant with regard to simulated gastrointestinal conditions. Hence, comparison of the structural properties of cLF and bLF with regard to zinc binding/ release to LF could contribute as a better understanding of the absorption of trace elements ions in humans.

Keywords: caprine lactoferrin, bovine lactoferrin, pH effect, thermal denaturation, zinc binding, structural stability

Introduction

Lactoferrin (LF) is a mammalian iron-transport metalloprotein of molecular weight 80 kDa which is present in bodily fluids such as milk, tears, saliva, mucosal and genital excretions (Masson et al. 1966). This protein belongs to the transferrin (TF) family. Transferrins are a family of eukaryotic iron (Fe) binding glycoproteins that share the common function of controlling the level of free iron in biological fluids (Crichton et al. 1987). Lactoferrin has two metal-binding sites in two lobes, known as N- and C- lobe. Most studies on LF have been focused on its iron binding capacity. All LFs and TFs so far characterized have essentially identical metal and anion (CO_3^{2-}) binding sites, which appear to be optimized for the binding of Fe^{3+} and CO_3^{2-}. The iron bound holo form of LF is conformationally rigid and very stable. Other metal ions such as Ga^{3+}, Al^{3+}, VO^{2+}, Mn^{3+}, Co^{3+}, Cu^{2+} and Zn^{2+} can also bind to LF with less affinity than Fe^{3+} (Ainscough et al. 1979; Baker 1994; Baker et al. 1994; Swarts et al. 2000). The reaction of Zn (II) with apo LF can be explained by the following equation (Harris and Stenback 1988):

$$Zn^{2+}+HCO_3^-+\text{apo LF}\leftrightarrow\text{Zn-}HCO_3\text{-LF}+2H^+$$

Among the first-row transition metals, zinc (Zn) is second only to iron (Fe) in terms of abundance and importance in biological systems. Lactoferrin is involved in the transport of iron, zinc and cobalt and their regulation absorption (Baker and Ghio 2009; Marchetti et al. 1999). Lactoferrin could play a role in homeostasis of iron and other trace elements (Baker et al. 2000; Jabeen et al. 2005; Kozlowski et al. 2009; Kumar et al. 2000). In the gastrointestinal tract of humans, preabsorptive processes substantially influence the zinc availability from LF. The gastric pH and/or intestinal pH could be important factors affecting both the solubilization of zinc in the stomach and its absorption by the intestine (McClain et al. 1980; Korhonen and Pihlanto 2006).

A study on zinc bound LF (ZnLF) is important because Zn^{2+} ions play a wide range of structural and catalytic roles in natural proteins (Stillman and Presta 2000; Underwood 1977). The role of zinc as a cofactor in LF depends on the three dimensional (3D) conformation of LF. The protein folding process is in general driven by hydrogen bonding, disulfide cross-linking, simple steric interactions between specific amino acid side chains, and hydrophobic effects (Kraulis et al. 1992). In the case of LF, metal ions like zinc may facilitate protein folding and stability by providing internal cross-links that directly lead to the final conformational state as an open (apo) and a closed (holo or metal saturated) form. (Anderson et al. 1990; Baker et al. 2002; Wally and Buchanan 2007).

Although there were many reports on the iron binding and structural properties of LFs, and bLF in particular, no precise data were known about cLF. In a previous study, a comparison between iron bound forms of cLF and bLF was done with regard to the conformation and thermal stability at different pH values (Sreedhara et al. 2010b). The thermal stabilities of iron saturated LF forms from both species were found to be much higher than the corresponding apo forms. Partly unfolded secondary structures of apo and iron saturated forms of cLF and bLF were observed at pH 2.0-3.0 (Sreedhara et al. 2010a). The mechanisms for iron and zinc binding may be different in cLF and bLF.

The aim of this paper was to determine the conformational characteristics and thermal stabilities of the zinc bound forms of cLF and bLF with respect the influence of pH 2.0 - 7.0 and to observe differences from the iron bound forms of LF.

Materials and methods

Materials

All the 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 4.0 and 10 mM sodium phosphate, pH 7.0.

Preparation of apo and zinc bound form of caprine and bovine lactoferrin

Bovine LF with 95 % purity was supplied by DMV International (Veghel, Netherlands) and was stored at -20 ^{o}C. Caprine LF was purified (> 95 %) using the cation exchange membrane chromatography method (Sreedhara et al. 2010a).

Apo (iron deprived) LF from caprine (cLF) and bovine (bLF) species was prepared according to the method of Khan et al (2001) with a slight modification (Sreedhara et al. 2010b).

Zinc bound LF from caprine (cLF) and bovine (bLF) species was prepared according to the holo LF preparation method of Karthikeyan et al (1999) with a slight modification (Sreedhara et al. 2010b).

The concentrations of cLF and bLF were determined by measuring the absorbance values of protein solutions at 280 nm (Recio and Visser 1999, 2000; Sreedhara et al. 2010a). The extinction coefficients for cLF and bLF were reported by Sreedhara et al (2010a). The zinc content in LF was determined using a Shimadzu atomic absorption spectrophotometer (AA-6701F, Shimadzu, Kyoto, Japan) calibrated with appropriate reference standards (Arnaud et al. 1992). The zinc saturation levels of cLF and bLF were 90±0.5% and 75±0.5% (mol/ mol), respectively. No iron was detected in zinc bound LF forms as measured by the atomic absorption method.

Circular dichroic spectral measurements

Circular dichroism (CD) measurements in the range 195-260 nm 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 ^{o}C for 24 h, centrifuged at 11500 x g for 5 min and clear supernatants (protein) were used to record the spectra. The protein concentration used was 3.1μM. The measurements were made at 25 ^{o}C. The path length of the cell used was 0.1cm. Each spectrum was the average of three subsequent scans. The results were expressed as the mean residue ellipticity (MRE in deg.cm^2/dmol), which is defined as:

$$MRE = \theta_{obs} (mdeg)/ (10 \times n \times C_p \times l) \ldots\ldots. (3)$$

Where 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 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).

118 Fluorescence measurements

119 For 8-anilino-1-naphthalene-sulfonate (ANS) induced fluorescence measurements, the spectra were
120 recorded using a Cary Eclipse Fluorescence Spectrophotometer (Varian Instruments, CA, USA) at 25 ^{o}C. Protein
121 concentration was 0.02 mg/ml. ANS concentration was 50 times the protein concentration. ANS binding was
122 measured by fluorescence emission with an excitation at 380 nm and the emission was recorded from 400 to 600 nm
123 (Kato and Nakai 1980). The excitation and emission slit widths were adjusted to 10 nm.

124 Intrinsic tryptophan fluorescence and acrylamide quenching studies were carried out using a Cary Eclipse
125 Spectrofluorimeter (M/S Varian, B. V, 4330 EA Middelburg, The Netherlands) at 25 ^{o}C.

126 For tryptophan fluorescence measurements, the LF concentration used was 0.08 mg/ml. The protein
127 samples were filtered using 50 kDa cut-off Ultracel membrane filters and concentrated to a final volume of about 10
128 µL while washing with the required buffer. The protein samples were equilibrated at room temperature for 30 min
129 before recording for tryptophan fluorescence measurements. Excitation and emission slit widths were kept at 5 nm.
130 The emission spectra were recorded in the range 300 - 400 nm after exciting with a wavelength of 290 nm (Bagshaw
131 and Harris 1987). The fluorescence measurements were recorded 10 sec after excitation.

132 Quenching experiments were carried out at the concentrations of 0.10-1.0 M of acrylamide stock solution
133 (5.0 M) to a final protein concentration of 0.012 mg/ml. The protein samples were incubated at required pH for 1 h
134 at 25 ^{o}C and the fluorescence intensities were recorded. Protein sample was excited at 290 nm and the emission was
135 recorded in the range 300 - 400 nm. The excitation and emission slit widths were adjusted to 5 nm. The quenching
136 data were analyzed according to Stern-Volmer equation (Eftink et al. 1981):

137 $F_0/F = 1+K_{SV}[Q]$ ……. (2)

138 where F_0 and F are the fluorescence intensities at an appropriate wavelength in the absence and presence of
139 quencher, K_{SV} is the Stern-Volmer constant and [Q] is the concentration of the quencher, acrylamide. All
140 fluorescence spectra were scanned at a slow speed, and the measurements were done in triplicate.

141

142 Thermal denaturation studies

143 The apparent thermal denaturation temperature (T_m) measurements of apo and zinc bound LF forms at pH
144 2.0 - 7.0, were carried out using a Cary 100 Bio UV-vis spectrophotometer (Varian Instruments Pty Ltd, Mulgrave
145 VIC, Australia). Protein samples were prepared at each required pH by dialysis against the appropriate buffer at 4 ^{o}C
146 for 24 h. The concentration of LF used was 1.0 mg/ml. The spectra were recorded at 287 nm over a temperature
147 range 35 - 90 ^{o}C with 1 ^{o}C increment per min using respective blanks. Measurements were made in triplicate. The
148 T_m value was calculated either by first derivative plot of absorbance or by van't Hoff plot using a standard equation
149 (Pace et al. 1997).

150

151 **Results and discussion**

152

153 Zinc binding to caprine and bovine lactoferrin at pH 2.0 - 7.0

154 The zinc bound to cLF and bLF in the pH range pH 2.0 - 7.0 and in the presence of a denaturant (3.0 and
155 6.0 M GuHCl) is shown in Table 1. The degrees of zinc binding at pH 7.0 in cLF and bLF were 90.1±0.5% and
156 74.8±0.5%, respectively. When pH was reduced to 2.0, both cLF and bLF showed a large decrease in the bound zinc
157 content (~ 20%). At low pH, LF loses the bound metal and the molecule opens up and unfolds. Furthermore, the 6.0
158 M GuHCl denatured forms of ZncLF and ZnbLF showed very less content of bound zinc. The LF forms from both
159 species were not fully zinc saturated at pH 7.0. The different binding of zinc to cLF and bLF could be due to the
160 difference in electrostatic binding properties of cLF and bLF. Previously, iron binding studies on the native forms of
161 cLF and bLF was done at pH 2.0-8.0 (Sreedhara et al. 2010a). At pH 7.0, native cLF with 5% iron content showed a
162 lower thermal stability (66±1 ^{o}C) than bLF with 15% iron content (70±1 ^{o}C). The release of bound zinc to LF was in
163 a similar manner when pH was reduced from 7.0 to 2.0. The data of zinc binding was in agreement with iron binding
164 data to apo LF (Sreedhara et al. 2010b). A significant loss in the iron content of both holo and apo forms of cLF and
165 bLF was observed when pH was reduced from 7.0 to 2.0. The iron binding properties of cLF and bLF showed that
166 the holo form was much more stable than the apo form of bLF as compared with cLF (Sreedhara et al. 2010b). The

molecular surface of LF leads to its zinc binding properties (Baker and Baker 2009; Hu et al. 2008; Sreedhara et al. 2010a). The H-bonded interactions across the cleft between the two domains of N- and C- lobes in LF are affected by pH to different degrees in cLF and bLF. This might influence the different zinc binding in cLF and bLF in the pH range 2.0-7.0. Other important factors like the movement of domains during pH variation, changes in the amino acid sequence in the zinc binding area, the bond angles between the zinc binding residues in cLF and bLF could affect the different binding of zinc to LFs from caprine and bovine species in the pH range studied (Hu et al. 2008; Sreedhara et al. 2010b).

Conformational changes in circular dichroism (CD) patterns of zinc bound forms of caprine and bovine lactoferrin at pH 2.0 - 7.0

The CD spectrum of a protein in the far-UV (195-260 nm) region is particularly sensitive to its secondary structure. The CD unfolding measurements of ZncLF (Fig. 1A and 1B) and ZnbLF (Fig. 2A and 2B) over pH range 2.0 - 7.0 and in presence of 3.0 and 6.0 M GuHCl, were performed. Apo cLF and apo bLF were used as controls for ZncLF and ZnbLF, respectively. Two significant peaks at 208 and 218 nm in the spectra of ZnbLF indicated the α/β mixed nature of LF (Nam et al. 1999). In the pH range 4.0 - 7.0, two prominent peaks observed at 208 and 218 nm were weaker in apo bLF structure than ZnbLF (Fig. 2A and 2B). As seen in the Fig. 1B, in the pH range 4.0 - 7.0, the negative extreme around 218 nm for ZncLF was weaker when compared with ZnbLF (Fig. 2B). In addition, a peak at 208 nm seems to weaker than the peak in ZnbLF. It was observed that the far-UV CD spectra of ZnLF from both species exhibited no reasonable differences when compared with the respective controls, apo LFs. The gross conformations of ZncLF and ZnbLF were almost identical with the corresponding iron saturated forms of cLF and bLF (Sreedhara et al. 2010b). At pH 2.0, partly unfolded structures of native forms of cLF and bLF were observed and were different from the corresponding native states at pH 7.0 (Sreedhara et al. 2010a). The secondary structures of ZncLF and ZnbLF were also partly unfolded at pH 2.0.

With a decrease in pH from 7.0 to 2.0, there was an observed increase in β-structure content with a concomitant decrease in α-helix content in ZncLF (Table 2). At pH 2.0, the partially unfolded structures of apo and zinc bound forms of cLF and bLF retained about 50 - 60% β-structure. For both ZnLFs, the trend of change in α-

193 helix content by the reduction in pH from 7.0 to 2.0 is similar to the respective native LF forms (Sreedhara et al.
194 2010a). At pH 7.0, as compared to iron bound LF forms of caprine and bovine species, the zinc bound LF forms
195 from both species showed about 8-10% more α-helix and 10-15% less β-structure (Sreedhara et al. 2010b). There is
196 about 2-3% observed difference in the total zinc and iron bound to LF among each species at pH 7.0. The changes in
197 the amino acid sequences in the zinc binding area of cLF and bLF may have a consequence in relation to the
198 changes in the α-helices and β-structures. So, zinc might be involved in the conformational changes of ZnLF from
199 both species. Further, among both species, at pH 2.0, the contents of α-helices and β-structures of ZnLF resemble
200 the respective iron bound holo forms (Sreedhara et al. 2010b). In all forms of LFs from both species, the structures
201 at pH 2.0 seems to be similar to that in presence of 6.0 M GuHCl.

202

203 Intrinsic tryptophan fluorescence changes in zinc bound lactoferrin from caprine and bovine induced by pH 2.0 - 7.0

204 Intrinsic fluorescence spectra of ZncLF and ZnbLF at pH 2.0, 4.0, 7.0 and in the unfolded state (3.0 and 6.0
205 M GuHCl) are shown in Fig. 3 and 4, respectively. Apo cLF and apo bLF were used as controls for ZncLF and
206 ZnbLF, respectively. At pH 7.0, for both ZncLF and ZnbLF, λ_{max} value was 334 nm. In case of ZnbLF, at pH 2.0,
207 λ_{max} value was red shifted to 351.1 nm. The spectral changes were very similar to that in apo bLF (Fig. 4A). When
208 the pH was lowered from 7.0 to 2.0, an unfolding of ZnbLF was observed. The unfolded state (6.0 M GuHCl),
209 however, exhibits a larger red shift with λ_{max} at 359.1 nm which indicate that tryptophan residues were maximally
210 exposed to the solvent. A similar trend was observed with respect to ZncLF over the same pH range. λ_{max} value at
211 pH 2.0 was observed to be 348 nm. In presence of 6.0 M GuHCl, this value was shifted to 357.1 nm. Hence the
212 greater exposure of tryptophan residues to the solvent in case of ZnbLF than ZncLF. Further, the red shifts in the
213 λ_{max} values of ZncLF and ZnbLF suggested that a gross conformational alteration in the protein was induced by 6.0
214 M GuHCl. Previously, a comparison among apo and iron saturated forms of cLF and bLF showed that LF unfolds
215 partially due to tryptophan exposure at pH 2.0 (Sreedhara et al. 2010b). The ZncLF and ZnbLF showed similar
216 unfolding patterns at pH 2.0.

217

218 Surface hydrophobicity changes in the zinc bound forms of caprine and bovine lactoferrin at pH 2.0 - 7.0

219 Results of 1-anilino-8-naphthalene sulfonate (ANS) binding experiments with apo and zinc boound LFs
220 from caprine and bovine species in the pH range of 2.0 - 7.0 and in the presence of a denaturant (3.0 and 6.0 M
221 GuHCl) are depicted in the Fig. 5. ANS binding has been used to probe the conformational changes that occur
222 during protein denaturation (Engelhard and Evans 1995). ANS in buffer alone showed a λ_{max} value of about 520 -
223 525 nm in the pH range 2.0-7.0. As evident from the Fig. 5A and B, ANS binding was minimal at pH 7.0 with
224 respect to ZncLF and ZnbLF. A maximal binding of ANS was observed at pH 2.0 with a blue shift of 493 nm and
225 487 nm for ZncLF and ZnbLF, respectively. However, in presence of 6.0 M GuHCl unfolded state these values were
226 shifted to 482 and 480 nm, respectively. Alternatively, from pH 7.0 to 2.0, a concurrent increase in the relative
227 fluorescence intensity was evident in case of apo and zinc bound LFs from both species (Fig. 5B). According to
228 previous reports on native LFs from caprine and bovine, unfolding was observed at pH < 5.0 (Sreedhara et al.
229 2010a). The present data on zinc bound LFs can be correlated with that report. At pH ≤ 4.0, the unfolding of metal
230 bound LF from both species exposed the hydrophobic amino acids to the solvent. At pH 2.0, ZnbLF exhibited higher
231 surface hydrophobicity than ZncLF. The respective apo LF forms from caprine and bovine (controls) showed
232 slightly higher ANS binding as compared to the zinc bound forms. Overall, similar trends in ZncLF and ZnbLF were
233 observed with respect to the ANS binding data. The structural perturbations start at pH ≤ 4.0. At pH 2.0, is the acid
234 denatured state, usually LF molecule opens up, looses almost all the bound zinc. The trend of zinc binding to LF at
235 pH 2.0 was almost similar to that of iron binding at that pH. The results of surface hydrophobicity studies were in a
236 good agreement with the intrinsic tryptophan fluorescence data and the CD structural changes observed.

237

238 Quenching studies of structural changes in zinc bound forms of caprine and bovine lactoferrin at pH 2.0 - 7.0

239 The quenching of tryptophan fluorescence was determined based on the procedure of Eftink and Ghiron
240 using uncharged molecules of acrylamide (Eftink and Ghiron 1981; Eftink and Selvidge 1982; Pawar and
241 Deshpande 2000). Fig. 6 (i) and (ii) shows the Stern-Volmer plots for the fluorescence quenching by acrylamide in
242 apo and zinc bound LFs from caprine and bovine species in the pH range 2.0 - 7.0 and GuHCl denatured states (3.0
243 and 6.0 M GuHCl). Table 3 shows the Stern-Volmer constants (K_{SV}) fitted to the linear parts of the curves. K_{SV}
244 value for ZnbLF at pH 7.0 is 8.25 M^{-1}. Whereas at pH 2.0, the value is 26.4 M^{-1}. Tryptophan exposure was maximal

at acidic pH (2.0).The K_{SV} value is still higher in presence of 6.0 M GuHCl induced unfolded state (45.95 M^{-1}). The 6.0 M GuHCl denatured form of ZnbLF seems to be completely unfolded and hence the molecule opens up with a higher tryptophan exposure to the solvent. These values were compared with that of ZncLF. A similar trend was seen in case of ZncLF. Overall, tryptophan fluorescence was more quenched in ZnbLF than ZncLF. But, the respective apo forms from both species showed more quenching of fluorescence than zinc bound forms. The quenching results of ZncLF and ZnbLF can be correlated with apo and iron saturated LFs from caprine and bovine species at different pH (Sreedhara et al. 2010b).

The results were in a good agreement with intrinsic tryptophan and ANS fluorescence data.

Effect of pH on the thermal denaturation temperature profiles of zinc bound forms of caprine and bovine lactoferrin

The dependence of apparent thermal denaturation temperature (T_m) with respect to the Zn-LF complex was compared among cLF and bLF in the pH range 2.0-7.0. Fig. 7A and B shows the T_m profiles at pH 7.0 and 4.0, respectively. Apo cLF and Apo bLF were used as controls for ZncLF and ZnbLF, respectively. At pH 7.0, ZnbLF exhibited higher T_m (83±1 ^oC) than ZncLF (67±1 ^oC) which was also found between ZnbLF and apo bLF at pH 7.0. However, only a small difference of 3±1 ^oC in T_m was observed between ZncLF and apo cLF at that pH. A reduction in pH from 7.0 to 4.0 suggested that thermal stability was decreased and it was evident with respect to T_m values obtained for apo and ZnLFs from species, caprine and bovine. With respect to ZnLFs from both species, at pH < 4.0, there was a visible aggregation at 25 ^oC and this has made the thermal denaturation measurements difficult. The ZnLFs from both species were unfolded at pH ≤ 3.0 (Surface hydrophobicity experiments) and hence the T_m measurements could not be done. There were reports on the thermal stabilities of native cLF and bLF forms (Hu et al. 2008; Sreedhara et al. 2010a). The native forms of cLF and bLF have most stable forms at pH 7.0 and the thermal stabilities were highest at this pH (Sreedhara et al. 2010a). A comparative study on the thermal stabilities of apo and holo forms of caprine and bovine LFs showed that the thermal stability of LF was dependent of the Fe binding capacity of the protein and pH (Sreedhara et al. 2010b). Therefore, the thermal denaturation of ZncLF and ZnbLF was dependent of the pH and the amount of zinc bound to protein.

Conclusions

pH plays an important role in the thermal stabilities and conformational changes of ZncLF and ZnbLF. The thermal denaturation temperature studies revealed the higher stability of ZnbLF than ZncLF in the pH range used. The zinc bound holo LF forms from both species were showed to be more stable than the respective apo LF forms, indicating that the zinc ion plays an important role in the thermal stability of the protein. The CD structures of ZncLF and ZnbLF showed an increased unfolded structure with reduced pH values. The zinc bound forms of cLF and bLF were compared with the corresponding iron bound LF forms at different pH. The study shows that simulated pH conditions to the gastrointestinal tract influence the zinc availability from LF by structural changes.

This study could contribute as as a model for studying other proteins interacting with zinc.

Abbreviations

LF, lactoferrin; TF, transferrin; apo LF, apo lactoferrin; holo LF, holo lactoferrin; cLF, caprine lactoferrin; bLF, bovine lactoferrin; ZnLF, Zinc bound lactoferrin; Trp, tryptophan; CD, circular dichroism; far UV-CD, far ultra-violet circular dichroism; T_m, thermal denaturation temperature; ANS, 8-anilino-1-naphthalene-sulfonate

Acknowledgements

This work is a part of a joint cooperation project between the Department of Protein Chemistry and Technology, Central Food Technological Research Institute (CFTRI), Mysore (A Constituent Laboratory of CSIR, Delhi), India and the Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Aas, Norway. The authors gratefully acknowledge the financial support from Norwegian Agency for Development Cooperation (NORAD), directorate under Norwegian Ministry of Foreign Affairs (MFA) and Norwegian State Educational Loan Fund. The authors would like to thank Dr. Sridevi Annapurna Singh and Dr. Purnima Kaul, Central Food Technological Research Institute (CFTRI), Mysore, India for providing technical assistance during CD, fluorescence and thermal stability experiments.

295 **References**
296
297 Ainscough EW, Brodie AM, Plowman JE (1979) The chromium, manganese, cobalt and copper complexes of
298 human lactoferrin. Inorg Chim Acta 33: 149-153

299 Anderson BF, Baker HM, Norris GE, Rumball SV, Baker EN (1990) Apolactoferrin structure demonstrates ligand-
300 induced conformational change in transferrins. Nature 344: 784-787

301 Arnaud J, Bouillet MC, Alary J, Favier A (1992) Zinc determination in human milk by flameless atomic absorption
302 spectrometry after dry ashing. Food Chem 44: 213-219

303 Bagshaw CR, Harris DA (1987) Measurement of ligand binding to proteins. In: Harris DA, Bashford CL (eds)
304 Spectrophotometry and Spectrofluorimetry: A practical approach, IRL Press, Oxford, U.K, pp 91-113

305 Baker EN (1994) Structure and reactivity of transferrins. Adv Inorg Chem 41: 389-463

306 Baker EN, Anderson BF, Baker HM, Day CL, Haridas M, Norris GE, Rumball SV, Smith CA, Thomas DH (1994)
307 Three-dimensional structure of lactoferrin in various functional states. Adv Exp Med Biol 357: 1-12

308 Baker HM, Baker CJ, Smith CA, Baker EN (2000) Metal substitution in transferrins: specific binding of cerium (IV)
309 revealed by the crystal structure of cerium-substituted human lactoferrin. J Biol Inorg Chem 5: 692-698

310 Baker EN, Baker HM, Kidd RD (2002) Lactoferrin and transferrin: functional variations on a common structural
311 framework. Biochem Cell Biol 80: 27-34

312 Baker EN, Baker HM (2009) A structural framework for understanding the multifunctional character of lactoferrin.
313 Biochimie 91(1):3-10

314 Baker JF, Ghio AJ (2009) Iron homoeostasis in rheumatic disease. Rheumatol 2009: 48:1339–1344

315 Crichton RR, Charloteaux-Wauters M (1987) Iron transport and storage. Eur J Biochem 164: 485-506

316 Eftink MR, Ghiron CA (1981) Fluorescence quenching studies with proteins. Anal Biochem 114: 199-227

317 Eftink MR, Selvidge LA (1982) Fluorescence quenching of liver alcohol dehydrogenase by acrylamide.
318 Biochemistry 21 (1): 117-125

319 Engelhard M, Evans PA (1995) Kinetics of interaction of partially folded proteins with a hydrophobic dye: Evidence
320 that molten globule character is maximal in early folding intermediates. Protein Sci 4: 1553-1562

321 Harris WR, Stenback JZ (1988) The Bicarbonate-Dependence of Zinc (II)-Transferrin Binding. J Inorg Biochem 33:
322 211-223

323 Hu F, Pan F, Sawano Y, Makino T, Kakehi Y, Komiyama M, Kawakami H, Tanokura M (2008) Studies of the
324 structure of multiferric ion-bound lactoferrin: A new antianemic edible material. Int Dairy J 18: 1051-1056

325 Jabeen T, Sharma S, Singh N, Bhushan A, Singh TP (2005) Structure of the zinc-saturated C-terminal half of bovine
326 lactoferrin at 2.0Å resolution. Acta Cryst D61: 1107-1115

327 Karthikeyan S, Sharma S, Sharma AK, Paramasivam M, Yadav S, Srinivasan A, Singh TP (1999) Structural
328 variability and functional converegence in lactoferrins. Curr Sci 77 (2): 241-255

329 Kato A, Nakai S (1980) Hydrophobicity determined by a fluorescence probe method and its correlation with surface
330 properties of proteins. Biochimica et Biophysica Acta 624: 13-20

331 Khan JA, Kumar P, Srinivasan A, Singh TP (2001) Protein intermediate trapped by the simultaneous crystallization
332 process. Crystal structure of an iron-saturated intermediate in the Fe^{3+} binding pathway of camel lactoferrin
333 at 2.7 a resolution, J Biol Chem 276: 36817–36823

334 Korhonen H, Pihlanto A (2006) Bioactive peptides: Production and functionality. Int Dairy J 16: 945–960

335 Kozlowski H, Janicka-Klos A, Brasun J, Gaggelli E, Valensin D, Valensin G (2009) Copper, iron, and zinc ions
336 homeostasis and their role in neurodegenerative disorders (metal uptake, transport, distribution and regulation).
337 Coord Chem Rev 253: 2665 - 2685

338 Kraulis PJ, Raine ARC, Ghadavi PL, Laue ED (1992) Structure of the DNA-binding domain of zinc GAL4. Nature
339 356: 448-450

340 Kumar S, Sharma AK, Singh TP (2000) Metal substitution in lactoferrins: the crystal structure of manganese
341 lactoferrin at 3.4Å resolution. Ind J Phys 74B: 103-108

342 Marchetti M, Superti F, Ammendolia MG, Valenti PRP, Seganti L (1999) Inhibition of poliovirus type 1 infection
343 by iron-, manganese- and zinc-saturated lactoferrin. Med Microbiol Immunol 187: 199-204

344 Masson PL, Heremans JF, Dive C (1966) An iron-binding protein common to many external secretions. Clin Chim
345 Acta 14: 735-739

346 McClain C, Soutor C, Zieve L (1980) Zinc deficiency: A complication of Crohn's disease. Gastroenterol 78: 272-
347 279

348 Nam MS, Shimazaki K, Kumura H, Lee KK, Yu DY (1999) Characterization of Korean native goat lactoferrin.
349 Comp Biochem Physiol B 123:201-208

350 Pace CN, Scholtz JM (1997) Measuring the conformational stability of a protein. In: Creighton TE (ed) Protein
351 Structure: A Practical Approach, 2nd edn. IRL Press: Oxford, U.K, pp 299-321

352 Pawar SA, Deshpande VV (2000) Characterization of acid-induced unfolding intermediates of glucose/xylose
353 isomerase. Eur J Biochem 267: 6331-6338

354 Recio I, Visser S (1999) Two ion-exchange chromatographic methods for the isolation of antibacterial peptides from
355 lactoferrin: in situ enzymatic hydrolysis on an ion-exchange membrane. J Chrom A 831: 191-201

356 Recio I, Visser S (2000) Antibacterial and binding characteristics of bovine, ovine and caprine lactoferrins: a
357 comparative study. Int Dairy J 10: 597-605

358 Sreedhara A, Flengsrud R, Prakash V, Krowarsch D, Langsrud T, Kaul P, Devold TG, Vegarud GE (2010) A
359 comparison of effects of pH on the thermal stability and conformation of caprine and bovine lactoferrin. Int
360 Dairy J 20:487-494

361 Sreedhara A, Flengsrud R, Langsrud T, Kaul P, Prakash V, Vegarud GE (2010) Structural characteristic, pH and
362 thermal stabilities of apo and holo forms of caprine and bovine lactoferrins. Biometals ‘‘in press’’ (DOI:
363 10.1007/s10534-010-9366-5)

364 Stillman MJ, Presta A (2000) Characterizing metal ion interactions with biological molecules: The spectroscopy of
365 metallothionein. In: Zalups RK, Koropatnick J (eds) Molecular Biology and Toxicology of Metals, Taylor &
366 Francis., London, pp 1-33

367 Swarts JC, Swarts PJ, Baker EN, Baker HM (2000) Spectroscopic studies on metal complexes of Aspergillus
368 awamori-derived recombinant human lactoferrin. Inorg Chim Acta 298: 187-194

369 Underwood EJ (1977) Trace elements in human and animal nutrition. 4th edn. Academic Press, New York, pp 196-
370 242

371 Wally J, Buchanan SK (2007) A structural comparison of human serum transferrin and human lactoferrin. Biometals
372 20: 249–262

373 Yang JT, Wu CS, Martinez HM (1986) Calculation of protein conformation from circular dichroism. In: Hirs CH,
374 Timasheff SN (ed) Methods in Enzymology, vol 130. Academic Press, New York, pp 208-69

375

Figure captions

Fig. 1. Effects of pH 2.0 - 7.0 and GuHCl on the CD secondary structures of **(A)** apo and **(B)** zinc bound forms of caprine lactoferrin. Buffers used are given under Materials and Methods.

Fig. 2. Effects of pH 2.0 - 7.0 and GuHCl on the CD secondary structures of **(A)** apo and **(B)** zinc bound forms of bovine lactoferrin. Buffers used are given under Materials and Methods.

Fig. 3. Fluorescence emission spectra of **(A)** apo and **(B)** zinc bound forms of caprine lactoferrin at pH 2.0 - 7.0 and in presence of GuHCl in the range 300 - 400 nm upon excitation at 290 nm. Slit width was 5/5 nm. Buffers used are given under Materials and Methods.

Fig. 4. Fluorescence emission spectra of **(A)** apo and **(B)** zinc bound forms of bovine lactoferrin at pH 2.0 - 7.0 and in presence of GuHCl in the range 300 - 400 nm upon excitation at 290 nm. Slit width was 5/5 nm. Buffers used are given under Materials and Methods.

Fig. 5. (A) The 8-anilino-1-naphthalene-sulfonate (ANS) fluorescence emission maxima of apo and zinc bound forms of lactoferrin from caprine and bovine species at pH 2.0 - 7.0 and in presence of GuHCl. **(B)** ANS fluorescence intensity of apo and zinc bound forms of lactoferrin from caprine and bovine species at pH 2.0 - 7.0 and in presence of GuHCl. Buffers used are given under Materials and Methods.

Fig. 6 (i). Fluorescence acrylamide quenching of **(A)** apo and **(B)** zinc bound forms of caprine lactoferrin at pH 2.0-7.0 and in presence of GuHCl. **(ii).** Fluorescence acrylamide quenching of **(A)** apo and **(B)** zinc bound forms of bovine lactoferrin at pH 2.0-7.0 and in presence of GuHCl. Buffers used are given under Materials and Methods.

Fig. 7. Thermal denaturation profiles of apo and zinc bound forms of caprine and bovine lactoferrins. **(A)** at pH 7.0 and **(B)** at pH 4.0. The UV-visible spectra were recorded at 287 nm over a temperature range 35 - 90 ^{o}C with 1 ^{o}C increment per min. Buffers used are given under Materials and Methods.

Fig. 1

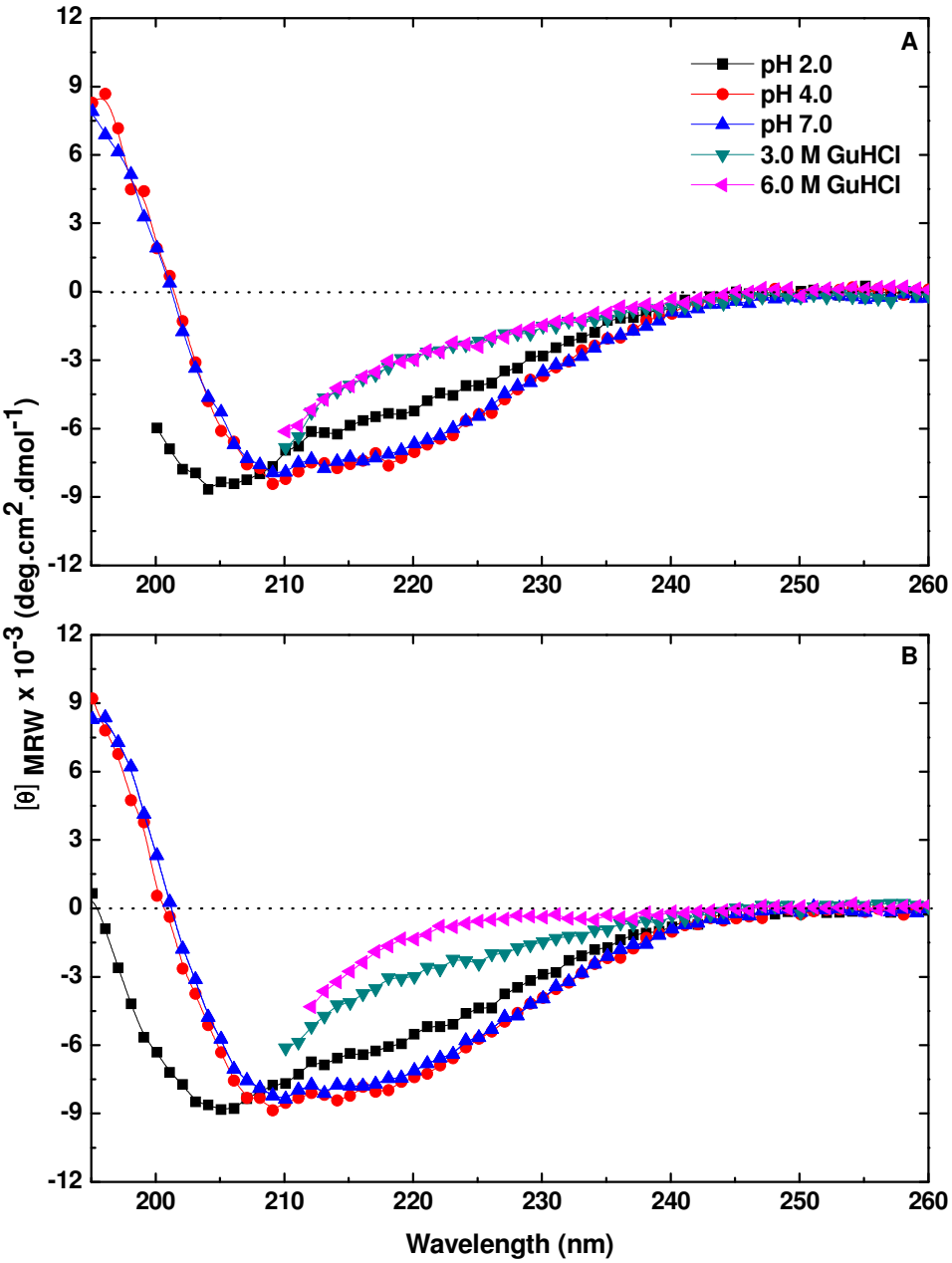
A
B
pH 2.0
pH 4.0
pH 7.0
3.0 M GuHCl
6.0 M GuHCl
[θ] MRW x 10^-3 (deg.cm^2.dmol^-1)
Wavelength (nm)
12
9
6
3
0
-3
-6
-9
-12
200
210
220
230
240
250
260

Fig. 2

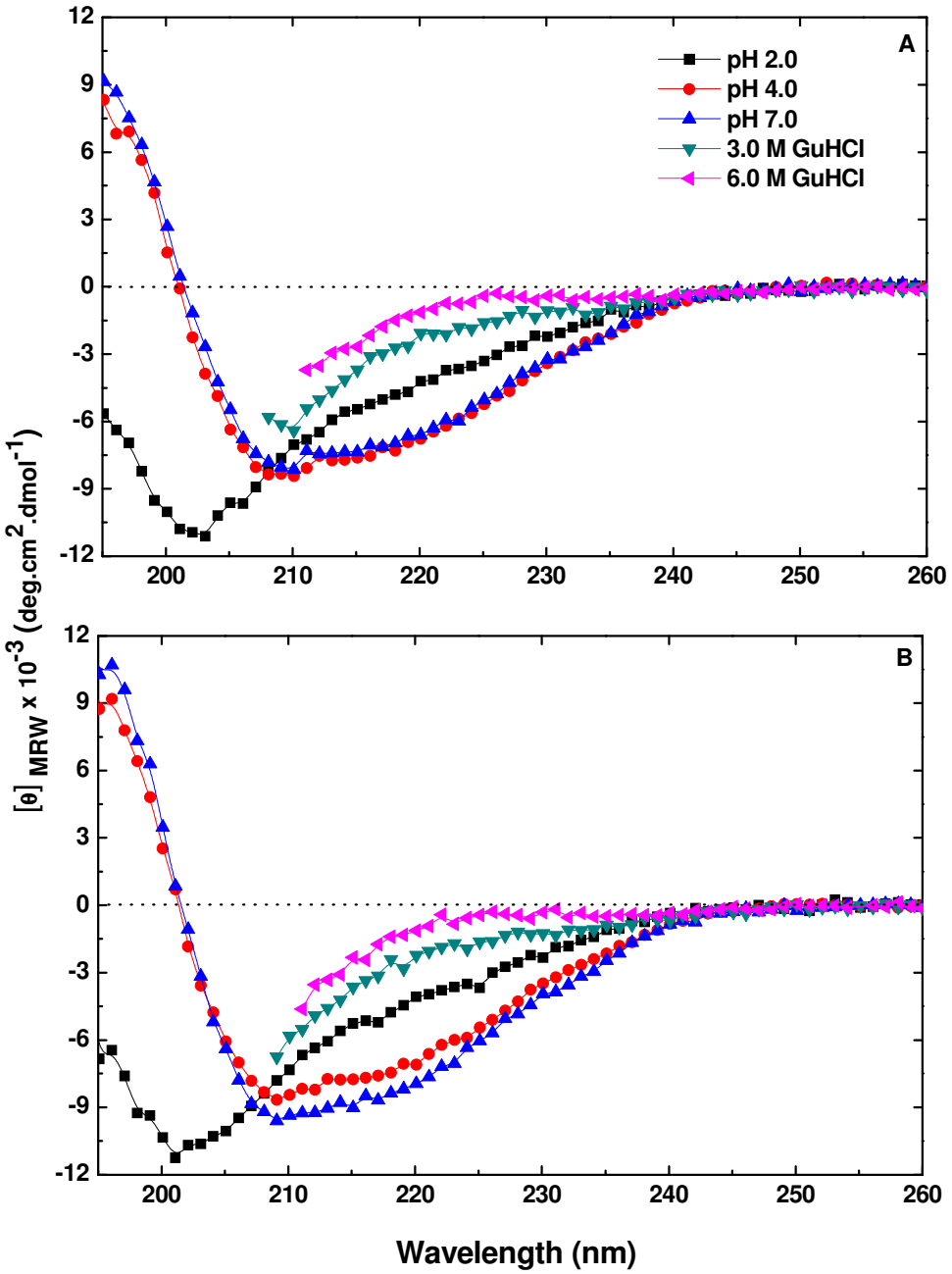
A
B
pH 2.0
pH 4.0
pH 7.0
3.0 M GuHCl
6.0 M GuHCl
[θ] MRW x 10-3 (deg.cm2.dmol-1)
Wavelength (nm)
12
9
6
3
0
-3
-6
-9
-12
200
210
220
230
240
250
260

Fig. 3

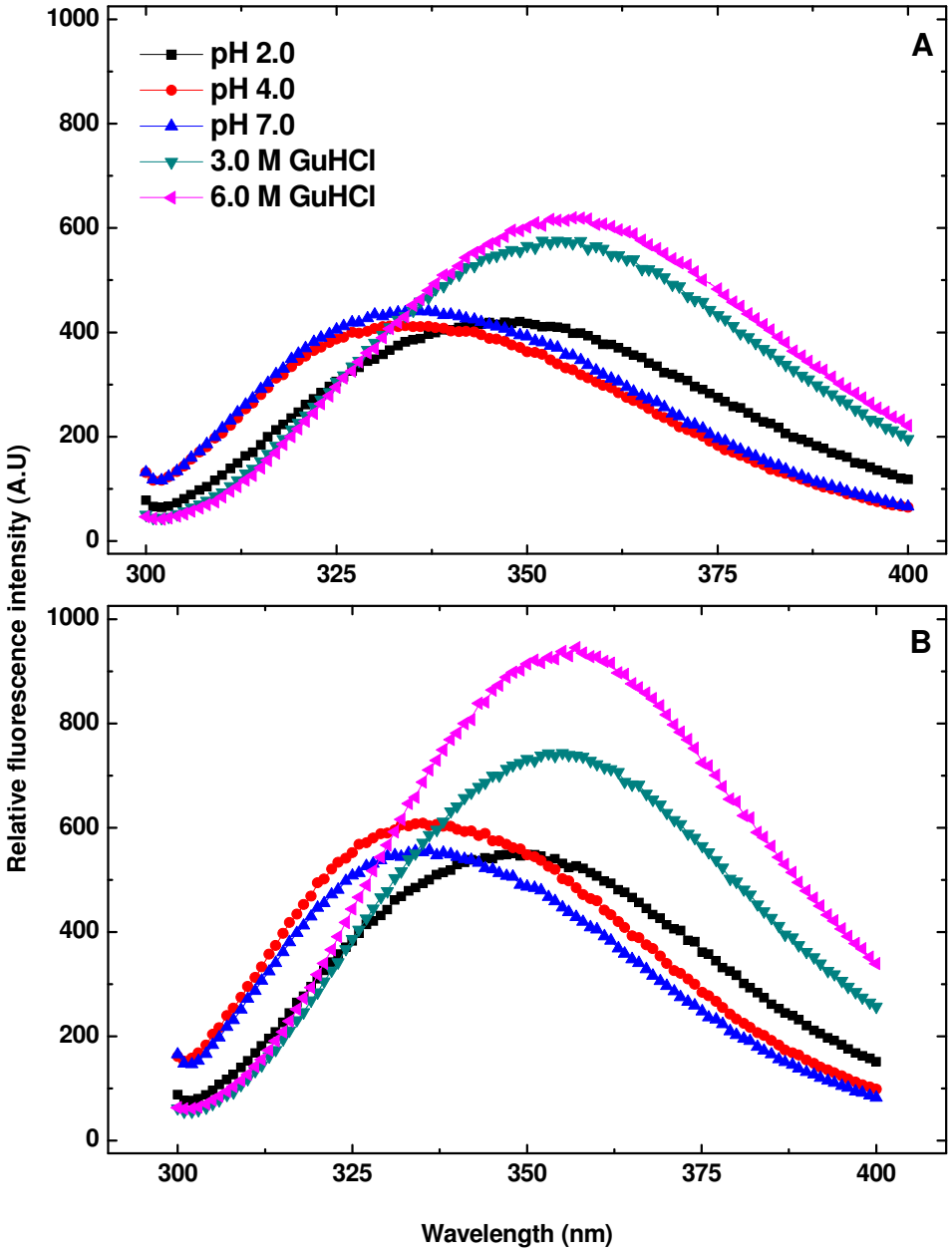
A
B
pH 2.0
pH 4.0
pH 7.0
3.0 M GuHCl
6.0 M GuHCl
Relative fluorescence intensity (A.U)
Wavelength (nm)
0
200
400
600
800
1000
300
325
350
375
400

Fig. 4

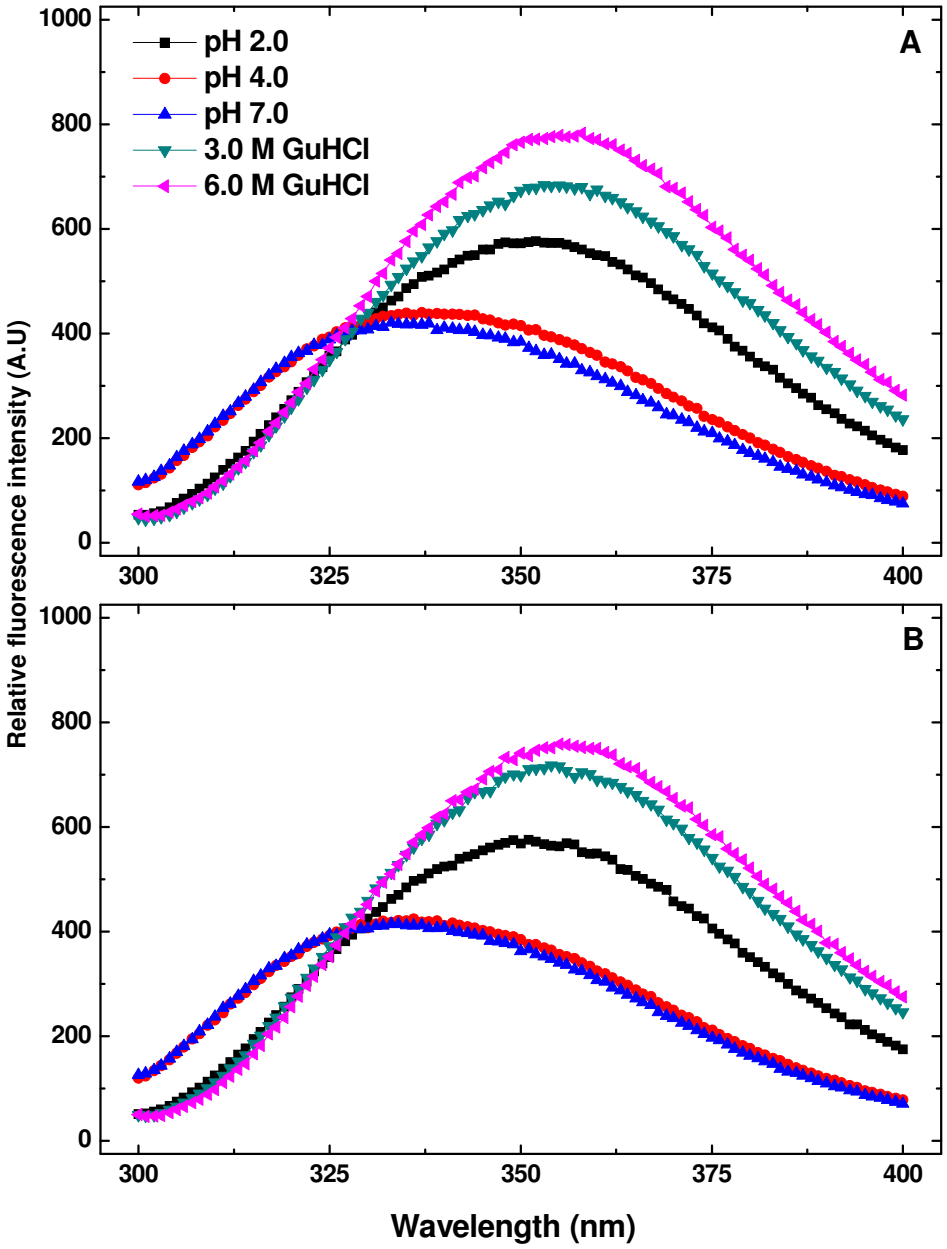
A
B
pH 2.0
pH 4.0
pH 7.0
3.0 M GuHCl
6.0 M GuHCl
Relative fluorescence intensity (A.U)
Wavelength (nm)
0
200
400
600
800
1000
300
325
350
375
400

Fig. 5

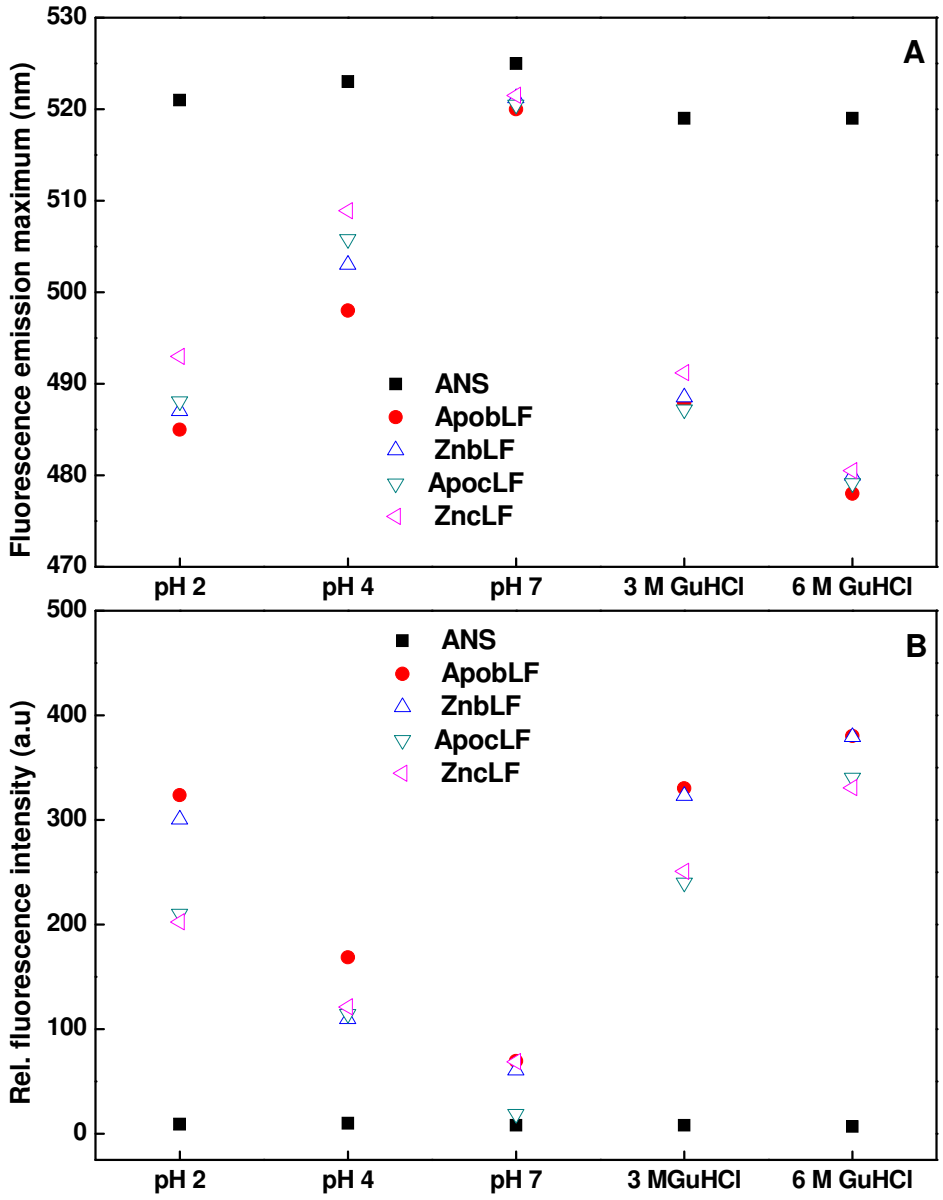
A
Fluorescence emission maximum (nm)
530
520
510
500
490
480
470
ANS
ApobLF
ZnbLF
ApocLF
ZncLF
pH 2
pH 4
pH 7
3 M GuHCl
6 M GuHCl
B
Rel. fluorescence intensity (a.u)
500
400
300
200
100
0
ANS
ApobLF
ZnbLF
ApocLF
ZncLF
pH 2
pH 4
pH 7
3 MGuHCl
6 M GuHCl

Fig.6 (i)

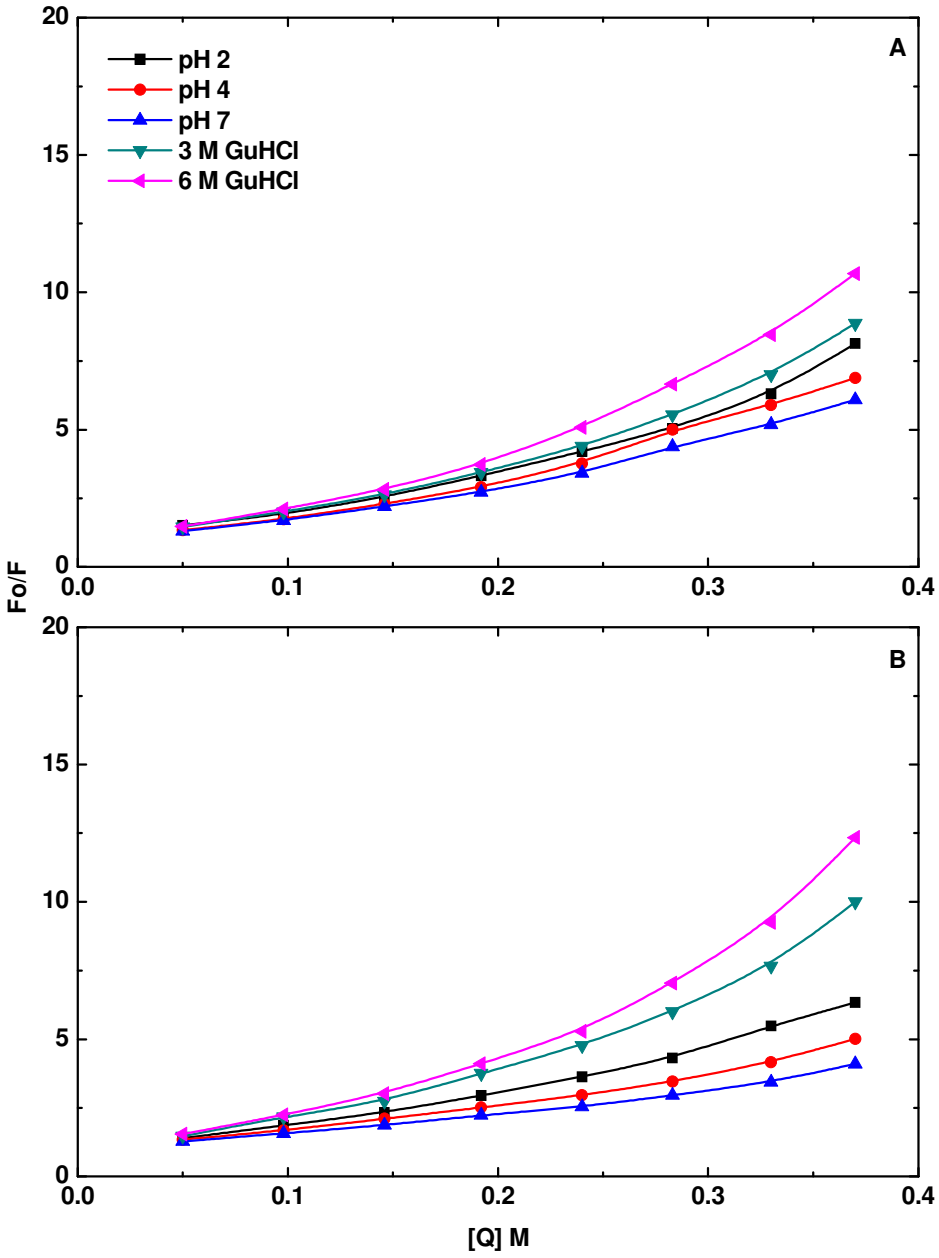

Fig. 6(ii)

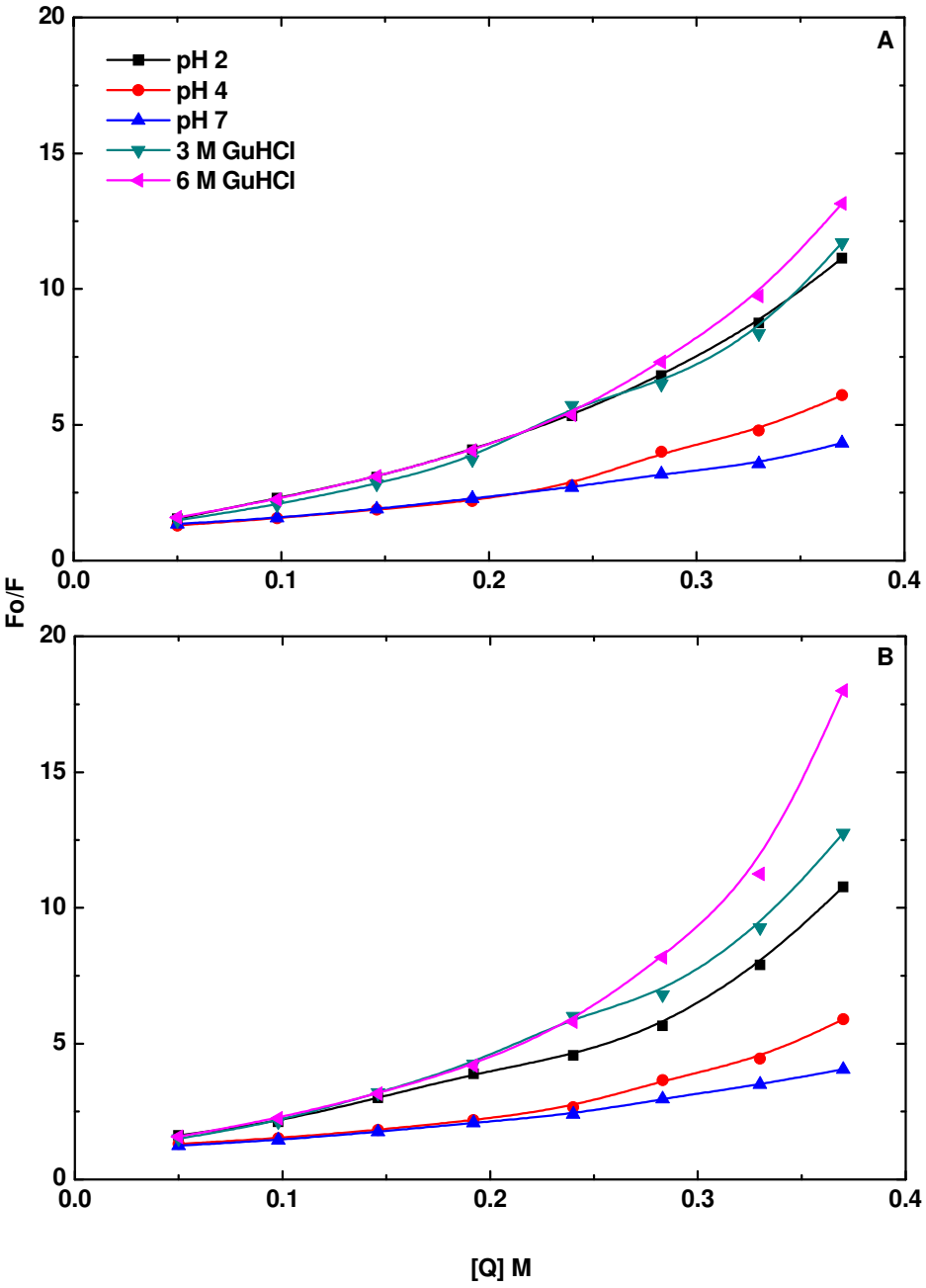

Fig. 7

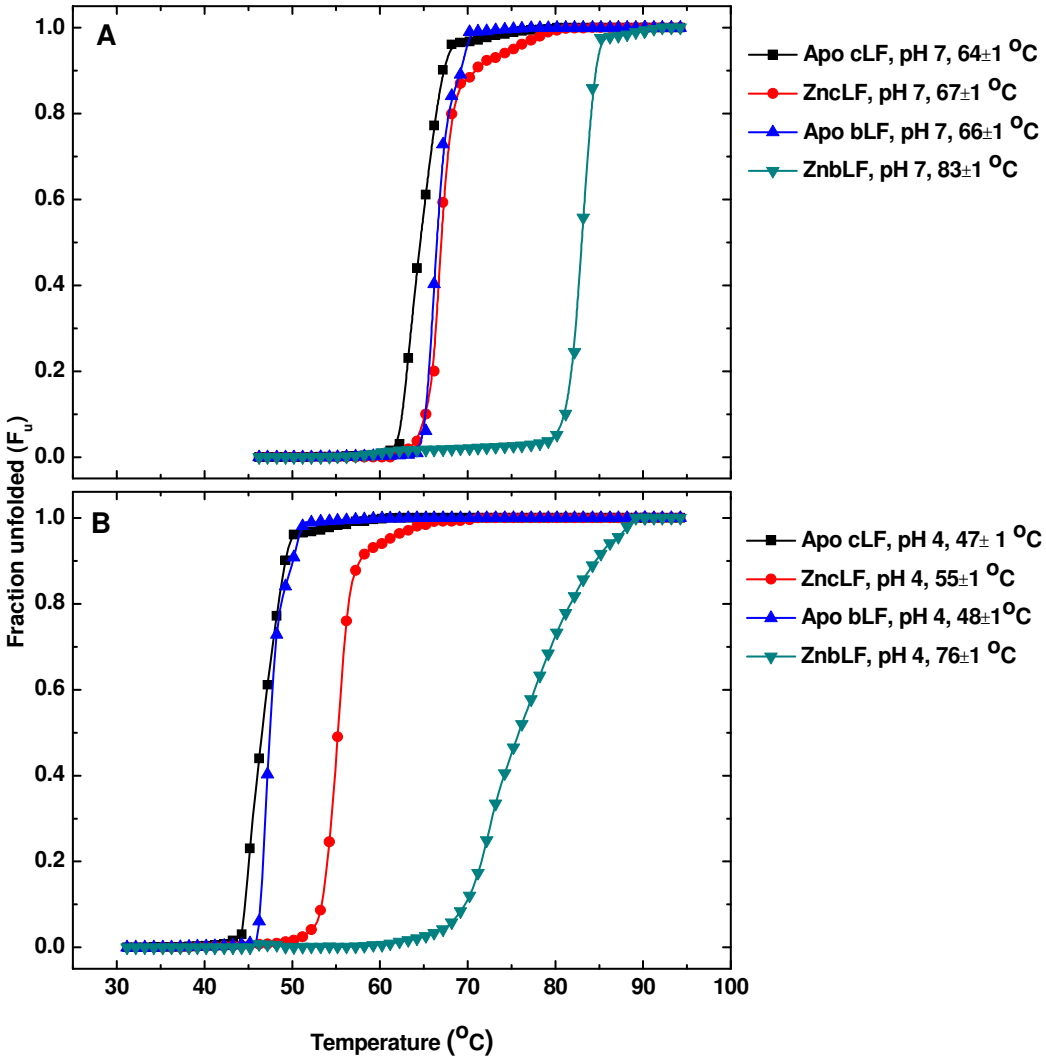
A
B
Fraction unfolded (F_u)
Temperature (^{O}C)
Apo cLF, pH 7, 64±1 ^{O}C
ZncLF, pH 7, 67±1 ^{O}C
Apo bLF, pH 7, 66±1 ^{O}C
ZnbLF, pH 7, 83±1 ^{O}C
Apo cLF, pH 4, 47± 1 ^{O}C
ZncLF, pH 4, 55±1 ^{O}C
Apo bLF, pH 4, 48±1^{O}C
ZnbLF, pH 4, 76±1 ^{O}C

Table 1 Zinc saturation (±0.5%) in ZncLF and ZnbLF at pH 2.0, 4.0, 7.0 and in presence of denaturants (3.0 and 6.0 M GuHCl). The values are given as a mean of three parallels

pH	ZncLF	ZnbLF
2.0	18.0	22.3
4.0	41.3	45.6
7.0	75.8	90.1
3.0 M GuHCl	15.4	16.0
6.0 M GuHCl	9.2	10.5

Table 2 Secondary structural contents (±0.25%) of zinc saturated forms of caprine lactoferrin (ZncLF) and bovine lactoferrin (ZnbLF) as measured by circular dichroism. The values given are a mean of three parallel scans

		α helix	β structure	aperiodic
Apo cLF	pH 2.0	7.0	55.0	38.0
	pH 4.0	21.0	55.0	24.0
	pH 7.0	18.0	57.0	25.0
	3.0 M GuHCl	14.0	55.0	31.0
	6.0 M GuHCl	8.5	56.5	35.0
Zn cLF	pH 2.0	6.0	57.5	36.5
	pH 4.0	21.0	53.0	26.0
	pH 7.0	22.0	53.0	25.0
	3.0 M GuHCl	15.0	54.0	31.0
	6.0 M GuHCl	5.0	57.0	38.0
Apo bLF	pH 2.0	0.0	58.5	41.5
	pH 4.0	17.0	60.0	23.0
	pH 7.0	17.0	62.0	21.0
	3.0 M GuHCl	18.0	54.0	28.0
	6.0 M GuHCl	0.0	54.0	46.0
Zn bLF	pH 2.0	1.0	54.0	45.0
	pH 4.0	18.0	60.0	22.0
	pH 7.0	22.0	56.0	22.0
	3.0 M GuHCl	18.0	53.0	29.0
	6.0 M GuHCl	0.0	55.0	45.0

Table 3 Stern-Volmer constants (K_{SV}) for fluorescence quenching of apo and zinc saturated forms of LFs from caprine and bovine at pH 2.0 - 7.0 and in presence of 3.0 and 6.0 M GuHCl denaturant

	Apo cLF	ZncLF	Apo bLF	ZnbLF
pH 2.0	19.27	14.43	27.41	26.40
pH 4.0	15.92	10.84	13.75	13.21
pH 7.0	13.78	8.37	8.97	8.25
3.0 M GuHCl	21.35	24.3	28.92	31.76
6.0 M GuHCl	26.22	30.65	32.84	45.95

Paper IV

Peptides generated by the *in vitro* digestion of bovine and caprine lactoferrin

Camilla B. Furlund[1*], Ashoka Sreedhara[1,2], Tove G. Devold[1], Ragnar Flengsrud[1], Camilla Sekse[3], Halvor Holm[4], Morten Jacobsen[1, 5], Gerd E. Vegarud[1]

[1]Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, N-1432 Aas, Norway

[2]Department of Protein Chemistry and Technology, Central Food Technological Research Institute (A Constituent Laboratory of CSIR), Mysore, India, 570 020

[3]Section for Bacteriology, Department for Animal Health, National Veterinary Institute, Oslo

[4]Institute of Nutrition, University of Oslo, Rikshospitalet and Radiumhospitalet University Hospital, 0027 Oslo, Norway

[5]Oestfold Hospital Trust, Fredrikstad, Norway

*Corresponding author: Camilla B. Furlund, fax +47 64 96 59 01, email Camilla.furlund@umb.no

Key words: Human gastrointestinal enzymes: model digestion: lactoferrin: bioactive peptides

Abstract

The present investigation was undertaken to identify peptides generated from bovine lactoferrin (bLF) and caprine lactoferrin (cLF) during *in vitro* digestion with human gastrointestinal enzymes, and to examine factors known to influence the outcome of protein degradation, 1) different concentrations of human gastric juice (HGJ) and human duodenal juice (HDJ), 2) different concentrations of bLF and 3) two different gastric pH values. Protein profiles of undigested and digested LF were obtained by SDS-PAGE. The degree of hydrolysis was assayed by the o-phthaldialdehyde (OPA) method. Peptides generated were identified by nano LC-MS. Protein degradation was highly dependent on gastric pH (2.5 and 4.0). At pH 2.5 lower content of intact LF and higher degrees of hydrolysis (~ 10.5) were observed. The peptide profiles from these samples revealed higher number of peptides at pH 2.5 than at pH 4.0. Identical protein degradation patterns were seen in caprine and bovine LF samples. However, their peptide patterns showed differences with regard to number of different peptides and different sequence lengths. At pH 2.5 and 4.0, the apo and holo forms of bLF showed similar degradation patterns. More than 90% peptides were originated from the N-terminal part of bLF (native, apo and holo) or cLF (native) at pH 2.5 and 4.0. During the pH reduction to 2.5 or 4.0, the digested bLF with fast pH reduction generated more peptides when compared to that of slow pH reduction. After the action of HGJ and HDJ, more peptide fragments were detected in native bLF than that of native cLF at both pH values 2.5 and 4.0. The multiple sequence alignment of peptides from LF digests showed the presence of proline and leucine patterns at both pH values, 2.5 and 4.0. This study showed that LF degradation and peptide formation in the stomach and duodenum after digestion is highly dependent on LF concentration in the sample, buffering gastric pH and the dosage of human gastrointestinal enzymes. These findings may be of importance when comparing peptides from LF with non-human enzymes that have previously been proposed to have physiological effects.

Abbrevitations: LF, lactoferrin; BAP, bioactive peptides; LFampin, lactoferrampin; LFcin, lactoferricin; Slow reduction of pH to pH 2.5 or 4.0, HGJ added at pH 7.0; Fast reduction of pH to pH 2.5 or 4.0, HGJ added at pH 2.5 or 4.0.

Introduction

Lactoferrin (LF) is a cationic 80 kDa iron binding glycoprotein, present in exocrine secretions like bile, pancreatic juice and small intestinal fluids and in mucosal secretions like milk, tears, saliva, vaginal fluids, semen, nasal and bronchial secretion and urine [1-4]. Due to the high pI (~ 9.0) it can undergo nonspecific receptor binding to many anionic target cells and proteins [5]. Lactoferrin receptors are found in the gastrointestinal tract, on leukocytes and macrophages, platelets and on bacteria, which makes it important in the first line of defence against microbial infections [4]. LF has bacteriostatic and bactericidal activity against gram positive and gram negative bacteria, antifungal effect and antiviral effect on both RNA and DNA viruses. In addition LF has a role as an iron-transporter and is of importance for infants, because it can bind and release metal atoms, and can deliver essential metals in the gut of the infant [3;4;6].

Lactoferrin in bovine milk consists of 689 amino acid residues with high homology among species. The polypeptide chain is folded into two lobes, an N- and a C-lobe. Each lobe contains one iron (Fe) binding site, that can bind Fe^{3+} reversibly [1-4]. The binding of Fe^{3+} is dependent on a synergistic binding of carbonate anion [1;3;4]. With respect to iron binding, LF exists in two different forms, an iron free (apo LF) form and an iron saturated form (holo LF)[3;4]. LF has the ability to retain bound iron in a broad pH range 2.0-7.0. The holo bLF shows higher content of iron even at pH 2.0 when compared with apo bLF. The apo and holo forms of caprine lactoferrin (cLF) bind less iron than bLF in the pH range 2.0-7.0 [7].

Lactoferrin is an important protein in milk of nearly all mammalian species; the exception is in dogs and rats, where lactoferrin cannot be found. The concentration varies greatly among different species, with a higher concentration in human milk (1.0 g/l) than in many other species like cow and goat (0.1 g/l) [2;4;8]. Lactoferrin is known to be partly digested in the newborns and absorbed in intact form from the gut of infants [2;4;9]. Due to the limited proteolysis in the stomach, LF may have beneficial effects with the release of fragments from lactoferrin that seems to have even more potent bactericidal activity [8].

Many bioactive peptides (BAP) have been identified by the hydrolysis of purified bLF from milk with commercial enzymes. These BAP may act as physiological modulators of metabolism during the intestinal digestion. The BAP usually contain 3-20 amino acid residues, and their activity is based on their amino acid sequence and composition. Some of these BAP fragments have been reported to be related to nutrient uptake

and, immune defence and in activities like opioid, antioxidant and antihypertensive properties [10-16]. In addition potential antimicrobial properties were reported for the peptide fragment lactoferricin (LFcin) f (17-41), being released by the hydrolysis from human LF with commercial pepsin [17]. Another antimicrobial peptide detected in the N1-domain of LF, is lactoferrampin (LFampin) f (268-284), which is close to the LFcin site of the 3 dim. structure of the molecule [18]. However, reports differ with regard to LF resistance to degradation by enzymes and also influenced by iron content as apo, native and holo forms of LF [3;19-21]. A study of the digestion of human milk by newborn infants showed that many milk proteins resisted degradation including LF, however, this was pH and time dependent [9]. Previous studies have identified differences in the protein and peptide profiles from caprine milk and whey when comparing commercial and human gastrointestinal enzymes [22;23]. Kimura et al. identified sixteen antimicrobial peptides from a pepsin digested hydrolysate of caprine lactoferrin (cLF) [24].

Only a very few *in vivo* studies have been reported so far, one of these identified only two peptides (fragment 382-389 and 442-447) from the C terminal part of bLF after milk ingestion[25]. Troost et al. showed that a major proportion (60-80%) of orally administered bLF survived passage through the stomach in adults and that the LF solution was emptying the stomach within 20-30 min after the ingestion. This study showed that pH gradually decreased after ingestion of the test meal and therefore the buffering capacity of food is very important to take into consideration when *in vivo* digestion is performed [26].

Most *in vitro* digestion studies have been done with fast reduction of pH to 2 or 2.5 before adding the pepsin and this may have an important effect on the generation of peptides, both with regard to hydrolytic peptide bonds, sequences and quantity.

Human gastrointestinal enzymes will vary in activity, volume and pH according to age, fasted or fed stage and other physiological conditions as reviewed by Ekmekcioglu [27]. To simulate digestion represented increasing secretion of enzymes, acid and various pH values that correspond to the situations in the human gastrointestinal system in adults or infants is a challenge [27;28].

Today, information about peptides generated from gastrointestinal digestion of lactoferrin is limited. In the present study bovine and caprine lactoferrin were *in vitro* digested with human gastrointestinal enzymes varying the following factors: 1) different activities of HGJ and HDJ added to bLF, 2) different concentrations of

79 bLF 3) mimicking two different levels of gastric pH (2.5 and 4.0). Moreover, the effect of buffering gastric pH,
80 LF concentration and various dosages of the gastrointestinal enzymes on the formation of peptides was studied.

Materials and methods

Lactoferrin

Bovine LF with 95 % purity was supplied by DMV International (Veghel, Netherlands) and was stored at -20 ºC. Caprine LF was purified (> 95 %) using the cation exchange membrane chromatography method [7]. Apo (iron deprived) LF from bovine (bLF) was prepared according to modified of Khan et al.[29;30]. Holo (iron saturated) LF from bovine (bLF) was prepared according to a modified method of Karthikeyan et al. [30;31]. The iron content in the different samples of bovine LF was 5 % in apo-, 15 % in native and 88 % in holo- bLF, Caprine lactoferrin had 5 % iron content.

Aspiration of human gastric and duodenal juices

Human enzymes were obtained by collecting human gastric and duodenal juices according to Holm *et al*. [32]. In brief, a three-lumen tube (Maxter Catheters, Marseille, France) enabled simultaneous installation of stimulation solution and aspiration of gastric and duodenal juices. The stimulation solution consisted of 7 0g/l sucrose, 1.8 g/l NaCl, 3.2 g/l L-phenylalanine and 2.3 g/l L-valine in water, and was instilled close to the papilla of Vater (100 ml/h) to stimulate the production of pancreatic enzymes. The human duodenal juices was aspirated some 18 cm distally. Aspirates were collected on ice, centrifuged (4500 g for 10 min) and frozen in aliquots at -20°C before use. Pooled HGJ and HDJ collected from 20 healthy donors that were pooled in one large batch. The procedure was approved by the ethical committee.

Enzymatic activities of gastric (HGJ) and duodenal (HDJ) enzymes

Pepsin activity in the HGJ was analysed according to Sànchez-Chiang [33]. Total proteolytic activity of HDJ was measured according to Krogdal et al. [34]. One unit of enzyme activity (U) was defined as the amount of enzymes (ml or mg) giving an absorbance of 1.0 at 280 nm with in 10 min at 37°C. All enzyme assays were run in triplicates or more.

105 ***In vitro* digestion of lactoferrin**

106 A two step in vitro digestion model with batches of HGJ and HDJ, used varying enzyme activities
107 (Table 1) [23]. In the gastric phase/step samples were acidified by addition of 1M HCl to either pH 2.5 or 4.0. To
108 simulate buffering and nonbuffering conditions of two different methods for reaching end-pH was used; 1) fast
109 reduction or 2) slow reduction as illustrated in Figure 2. Gastric digestion proceeded for 30 min in total. Before
110 adding HDJ to the samples the pH was adjusted to 7.0, and the duodenal digestion lasted for 30 min. During the
111 digestion samples was taken at different time points and frozen immediately for every step.

112 Bovine LF (native, apo and holo) or native cLF 10 mg/ml, 1 mg/ml or 0.1 mg/ml was dissolved in
113 Millipore water, pH was adjusted and HGJ (5 U/g or 20 U/g) was added according to Figure 1. After 30 minutes
114 1 ml sample were taken out for analysis, and the LF sample were further digested after pH adjusted to pH 7.0
115 and in step 2, the samples were incubated at 37°C for 30 min before the samples were put on ice to stop the
116 enzymatic reaction. All digestion models were run in duplicates.

117 **Protein degradation profiles by SDS-PAGE**

118 Protein degradation profiles of bLF were studied by SDS-PAGE using 15 % acrylamide gels (Bio-Rad
119 Mini-PROTEAN 3 cell system, Bio-Rad Laboratories Ltd, Hemel Hempsted, Herts, UK) [35]. 2 µl of each
120 samples were mixed with 2x SDS sample buffer (0.125M Tris-Cl, 4 % SDS, 2 % glycerol, 2 % 2-
121 mercaptoethanol and 0.03 mM bromophenol blue) and heated at 95 ^{o}C for 5 min before applied on to the gel. 2.0
122 µl of each sample was loaded into the gel. The electrophoresis was performed at 50 V for 20 min, and then the
123 voltage was raised to 150 V for 50 min (Powerpac basic, Biorad). Proteins were visualized by Coomassie
124 Brilliant Blue R-250.

125

126 **Degree of proteolysis/hydrolysis**

127 The spectrophotometric o-phthaldialdehyde (OPA) assay by Chrunch et al with some modifications was
128 used to measure protein hydrolysis in HGJ/HDJ digests [36]. In brief; 100 to 200 µl of sample (diluted 1:10 in
129 100 mM sodium tetraborate and 2 % (wt/wt) SDS to stop proteolysis) was added to 1.0 ml of OPA reagent, (100

mM sodium tetraborate, 2 % (wt/wt) SDS, 6 mM OPA (28.5 mM β-mercaptoethanol). After 2 min of incubation at ambient temperature absorbance was read at 340 nm. Unhydrolyzed LF was used as "blank". Protein concentrations in unhydrolysed and hydrolysed samples were assayed using the absorbance at 280 nm against a bLF standard curve. Protein hydrolysis is expressed as degree of hydrolysis (DH) defined as the percentage peptide bonds hydrolysed by the action of gastric and duodenal proteinases. All samples were run in triplicates.

Preparation of LF peptides <10 kDa

The LF digests were fractionated by centrifugation in a 10 kDa Millipore Amicon Ultra-15 centrifugal cut-off filter (Amicon, Millipore, Bedford, MA). The peptide mix/ fraction <10 kDa was freeze-dried. The peptides were dissolved in 0.1 % (v/v) formic acid. C18 columns were packed, by using Eppendorf GELoader micropipette tips (Eppendorf, Hamburg, Germany) with the insertion of C18 column material (3M Empore C18 extraction disks; 3M Bioanalytical Technologies, St Paul, MN, USA). The peptides were eluted using 3 µl of 70 % acetonitrile and 0.1 % formic acid (v/v).

Nano-LC MS of peptides

Peptide fraction <10 kDa diluted in 10 µl 1% (v/v) formic acid were loaded onto a nanoACQUITY ™ Ultra Performance LC system (Walters Corp., Milford, MA, USA), equipped with a Symmetry C18 (180 µm x 22mm; Waters) in front of a 3 µm Atlantis™ C18 analytical column (100 µm x 100mm; Waters). Peptides were separated at a flow of 0.4 µl/min with a gradient of 5-90 % (v/v) acetonitrile, 0.1 % (v/v) formic acid, eluted to a Q-TOF Ultima Global mass spectrometer (Micromass/Waters) and subjected to data-dependent tandem MS analysis. Peak lists were generated by the Protein-Lynx Global server software (version 2.1), and the resulting pkl files were searched against National Center for Biotechnology Information (NCBI) non-redundant protein sequence database using the MASCOT search engine (http://www.matrixscience.com). Peptide mass tolerances used in the search were 100 parts per million (ppm), and fragment mass tolerance was 0.1 Da. The taxonomy used in the search was mammalian. Data were acquired over a mass:charge ratio of 400-1500 Da, detecting

peptides with two or three charges. Thus, only peptides with mass above 800 and below 4500 Da were subjected to collision-induced fragmentation and further processing.

The peptides produced by LF digestion were studied by multiple sequence alignment using Clustal 2.0.12 and Jalview (http://www.ebi.ac.uk/Tools/clustalw2/). The sequences used here were chosen taking care to include all detected residues with a minimal overlap.

Results

Digestion with varying activity of human gastrointestinal enzymes

Two different concentrations of bLF, 10 mg/ml and 1 mg/ml, were used in this study with slow reduction of pH to 2.5(Table 1). bLF concentration (10 mg/ml) and low activity of HGJ (5 U/g) and HDJ (15 U/g) showed very little protein degradation (Fig. 3, line 3 and 4). Increasing the activity of HGJ (20 U/g) and HDJ (62.5 U/g) resulted in increased degradation of bLF showing many fragments in the M_r range of 35-65 kDa and <20 kDa (Figure 3), however still high amount of undigested LF was observed. When bLF was reduced to 1mg/ml much higher degradation was shown at both low and high activity of HGJ and HDJ (Fig. 3, lines 7-10). No intact bLF was observed after digestion with the highest activity of gastrointestinal enzymes HGJ and HDJ. In addition one study was performed with bLF at 0.1 mg/ml, as comparative concentration to the LF concentration in milk. Lactoferrin was totally degraded with both low and high activities of the HGJ and HDJ (data not shown).

Digestion using varying gastric pH

Two different gastric pH was used, pH 2.5 and pH 4.0, and in addition the simulated effect of slow and fast reduction of pH on the degradation of LF (10 mg/ml) was studied (Fig 2).

Gastric digestion at pH 2.5 and 4.0 resulted in different bLF degradation. Highest degradation was always shown at low gastric pH 2.5 compared to pH 4.0, independent of fast or slow pH reduction (Fig 4, lane 3,

179 5, 7 and 9). More LF was degraded after step 2 with HDJ giving rise to oligopeptides of lower molecular size.
180 Fast reduction in pH down to 2.5, resulted in complete digestion of bLF by HGJ and further degradation of the
181 peptides (MW < 25 kDa) occurred when HDJ was added (Fig 4, lane 7 and 8).

182 A comparison of the digestion between apo and holo forms of bLF resulted in similar protein
183 degradation pattern as the native bLF form (data not shown).

184 Digestion of native caprine LF (10 mg/ml) with HGJ and HDJ resulted in a protein degradation pattern
185 very similar as shown for native bLF (Fig 4).

186

187 **Degree of hydrolysis**

188 The degree of hydrolysis (HD) of digested bLF samples (10 mg/ml) are given in Fig 5. The highest DH was
189 obtained for the samples digested at pH 2.5 as compared to pH 4.0, whereas smaller differences were seen
190 between fast and slow pH approaches.

191

192 **Identification of peptides after digestion of caprine and bovine lactoferrin**

193 Many peptides were generated after digestion of bLF (10 mg/ml) and cLF (10 mg/ml) using the highest
194 activities of HGJ and HDJ (Fig 6 and 7). More peptides were observed at pH 2.5 than at pH 4.0, ranging from
195 short f (237-242) residues to long f (267-299) residues (Table 2). The results also showed that fewer peptides
196 were generated when gastric pH was slowly reduced compared with fast acidification. Thereafter addition of
197 HDJ degraded longer peptides into shorter peptides. However, two very different peptide patterns from fast and
198 slow gastric pH reduction were observed and this is in accordance with the protein degradation profiles in Fig 4
199 for bLF.

200 Most peptides originated from the N-terminal region of lactoferrin. Some peptides within the same
201 region sometimes overlapped and are not seen as individual peptides in the protein sequence figures (Fig 6 and
202 7). Some of the peptides that were found in more than one sample are highlighted in Table 2.

When comparing holoLF with apo and native LF redundant, the digestion of holoLF generated fewer peptides compared with apo and native LF form (Fig 6 and supplemented Figure 1).

There are some differences in the amino acid sequence between bLF and cLF (Figure 6 and 7). When comparing peptides generated after digestion with HGJ and HDJ of cLF and bLF, less peptides from cLF was observed at both pH 2.5 and pH 4.0. The peptides also differed in length and sequence. Two new peptides occurred in the C-terminal end after digestion of cLF; f (618-631) and f (618-640). Also a peptide f (267-288) bLFampin and f (271-288) cLFampin, was found in bovine holo LF f (267-288) after HGJ digestion at pH4, but no longer observed after further HDJ digestion. This peptide sequence was also found as part of a longer peptide f (267-299) for holo bLF after HGJ digestion and in cLF f (267-288) after further digestion with HDJ digestion, both in pH 2.5.

The peptide lactoferricin (bLFcin) f (17-41) was not detected after bLF digestion in our study neither was the peptide lactoferricin cLFcin f (14-42) from cLF.

The results from the multiple sequence alignment of the peptides from lactoferrin digestion are presented in Fig 8. Consensus residues are given and those with highest quality in the consensus analyses are marked.

Discussion

An extensive degradation of bLF and cLF was observed showing the effective action of HGJ and HDJ at gastric pH values. Eriksen et al. have shown that porcine pepsin degraded caprine whey proteins better than human gastrointestinal enzymes at different pH values mimicking the pH range in adult's and infant's stomach [23]. There were reports on bLF digestion using commercial enzymes [9;24;37-41]. These studies have identified many peptides, of which several of these were reported to be bioactive.

In the present study more intact LF was observed at pH 4.0 when compared with pH 2.0 after digestion with HGJ and HDJ. Troost et al. showed that buffering capacity and slow pH reduction in the stomach affected the digestion of LF (15mg/ml) in humans. Up to 60-80% LF resisted gastric digestion in this study dependent on

228 the apo or holo LF form [26]. We observed only differences in apo and holo LF at the peptide level obtained by
229 LC-MS, a much more sensitive analysis than SDS-PAGE. We also observed that different concentration of LF
230 (10mg/ml, 1mg/ml and 0.1mg/ml) affected the degradation pattern. At low concentrations of LF, 1mg/ml and
231 0.1mg/ml, that is representative with the LF content in human and bovine milk, the degradation seemed to vary.

232 Resistance of milk proteins to degradation was also shown by Chatterton et al. [9]. *In vitro* digestion of
233 human milk with infant gastrointestinal enzymes showed considerable amounts of intact proteins after one hour
234 of digestion. They also observed that human milk was more resistant to digestion than bovine milk, and that
235 human LF was partially resistant to the effects of gastric digestion even at pH 2.0 [9].

236 Our study also showed that using two different activities of the gastrointestinal enzymes of HGJ and
237 HDJ affected the degradation of LF.

238 According to Ekmekcioglu individuals produces gastrointestinal enzymes in a range of 1900IU/15 min
239 basal pepsin, 39kU/h amylase, 5-10kU/30min trypsin, 4kU/min lipase and 20mM/min bile salt for middle aged
240 humans [27]. Our enzyme activities lie within the range of individuals observed in other studies [42;43]. In an *in*
241 *vitro* digestion study of bLF, Brines and Brock have digested bLF with trypsin or chymotrypsin to give
242 200µg/ml of the active enzyme. This amount of enzyme is comparable to the level of duodenal juice used in the
243 present study [20].

244 Elbarbary et al [38] identified a peptide in bovine lactoferrin that showed antibacterial activity f (69-77)
245 in addition to the bLFcin. In our study a longer peptide sequence was observed for this peptide in all samples
246 except for native bLF in slow reduction of pH to pH 4.0. Another characterized peptide from the N-terminal is
247 LFampin, characterized by Reico and Visser for both cLF and bLF [41;44]. The peptide sequence for bLF have
248 later been produced synthesised f (265-288) by van der Kraan [18]. In our study we found this peptide as part of
249 longer peptides.

250 The analysis of the generated lactoferrin peptides showed that the scores varied considerable, in part
251 reflecting the number of short peptides. The short peptides aligned all with the central part of the longer peptides.
252 The analysis of digestion generated lactoferrin peptides shows that at pH 2.5 the presence of proline with
253 neighboring hydrophobic residues, preferable leucines could be a possible motif here. This is in accordance with

254 the analysis of the peptides generated from β-lactoglobulin, β-casein and κ-casein glycomacropeptide at pH 2.5
255 using HGJ (unpublished data). The peptides generated at pH 4.0 show a different pattern where lysine, alanine
256 and multiple leucine residues show highest probability of a reliable pattern and indicating a different proteolytic
257 mechanism. At high 2.5 and 4.0 digestion the proline and leucine pattern is present, in addition to glycine
258 residues.

259 The current study has provided a detailed comparison between the digestion patterns of cLF and bLF
260 under different conditions such as human gastrointestinal pH and different enzyme/ substrate ratios. The use of
261 *in vitro* digestion could contribute to a better knowledge about the generation of peptides during gastrointestinal
262 digestion *in vivo*.

263

264 **Acknowledgements**

265 The present study was supported by the Norwegian University of Life Sciences. The authors would like to thank
266 the Masters students Adam Pomorski and Davide Capelli. A part of *in vitro* digestion work used in this study
267 was done by them. Best technical assistance for mass spectrometry analyses was offered by Toril A. Grønset at
268 The Tromsø University Proteomic Platform. Ph.D students Camilla B. Furlund and Ashoka Sreedhara have
269 contributed equally in terms of laboratory experiments and manuscript preparation. Tove G. Devold did degree
270 of hydrolysis experiments. Ragnar Flengsrud has assisted in the peptide alignment program. Camilla Sekse
271 partly involved in digestion work. Halvor Holm and Morten Jacobsen have participated in the research planning.
272 Gerd E. Vegarud coordinated the research and designed the experiments. All authors contributed during the
273 research planning, discussion of the results in the manuscript. The authors report no conflict of interest.

References

[1] Baker EN, Baker HM. A structural framework for understanding the multifunctional character of lactoferrin. Biochimie 2009;91(1):3-10.

[2] Sanchez L, Calvo M, Brock JH. Biological Role of Lactoferrin. Archives of Disease in Childhood 1992;67(5):657-61.

[3] Gonzalez-Chavez SA, Arevalo-Gallegos S, Rascon-Cruz Q. Lactoferrin: structure, function and applications. International Journal of Antimicrobial Agents 2009;33(4).

[4] Levay PF, Viljoen M. Lactoferrin - A General-Review. Haematologica 1995;80(3):252-67.

[5] Steijns JM, van Hooijdonk AC. Occurrence, structure, biochemical properties and technological characteristics of lactoferrin. Br J Nutr 2000;84 Suppl 1:S11-S17.

[6] van der Strate BWA, Beljaars L, Molema G, Harmsen MC, Meijer DKF. Antiviral activities of lactoferrin. Antiviral Research 2001;52(3):225-39.

[7] Sreedhara A, Flengsrud R, Prakash V, Krowarsch D, Langsrud T, Kaul P, et al. A comparison of effects of pH on the thermal stability and conformation of caprine and bovine lactoferrin. International Dairy Journal 2010;20(7):487-94.

[8] Hamosh M. Protective function of proteins and lipids in human milk. Biology of the Neonate 1998;74(2):163-76.

[9] Chatterton DEW, Rasmussen JT, Heegaard CW, Sorensen ES, Petersen TE. In vitro digestion of novel milk protein ingredients for use in infant formulas: Research on biological functions. Trends in Food Science & Technology 2004;15(7-8):373-83.

[10] Chen JR, Suetsuna K, Yamauchi F. Isolation and Characterization of Immunostimulative Peptides from Soybean. Journal of Nutritional Biochemistry 1995;6(6):310-3.

[11] Mendis E, Rajapakse N, Byun HG, Kim SK. Investigation of jumbo squid (Dosidicus gigas) skin gelatin peptides for their in vitro antioxidant effects. Life Sciences 2005;77(17):2166-78.

[12] Pihlanto-Leppala A, Koskinen P, Piilola K, Tupasela T, Korhonen H. Angiotensin I-converting enzyme inhibitory properties of whey protein digests: concentration and characterization of active peptides. Journal of Dairy Research 2000;67(1):53-64.

[13] Pihlanto-Leppala A. Bioactive peptides derived from bovine whey proteins: opioid and ace-inhibitory peptides. Trends in Food Science & Technology 2000;11(9-10):347-56.

[14] Suetsuna K. Isolation and characterization of angiotensin I-converting enzyme inhibitor dipeptides derived from Allium sativum L (garlic). Journal of Nutritional Biochemistry 1998;9(7):415-9.

[15] Suetsuna K, Maekawa K, Chen JR. Antihypertensive effects of Undaria pinnatifida (wakame) peptide on blood pressure in spontaneously hypertensive rats. Journal of Nutritional Biochemistry 2004;15(5):267-72.

[16] Tsuruki T, Kishi K, Takahashi M, Tanaka M, Matsukawa T, Yoshikawa M. Soymetide, an immunostimulating peptide derived from soybean beta-conglycinin, is an fMLP agonist. Febs Letters 2003;540(1-3):206-10.

[17] Bellamy W, Takase M, Wakabayashi H, Kawase K, Tomita M. Antibacterial Spectrum of Lactoferricin-B, A Potent Bactericidal Peptide Derived from the N-Terminal Region of Bovine Lactoferrin. Journal of Applied Bacteriology 1992;73(6):472-9.

[18] van der Kraan MIA, Groenink J, Nazmi K, Veerman ECI, Bolscher JGM, Amerongen AVN. Lactoferrampin: a novel antimicrobial peptide in the N1-domain of bovine lactoferrin. Peptides 2004;25(2):177-83.

[19] Baldi A, Ioannis P, Chiara P, Eleonora F, Roubini C, Vittorio D. Biological effects of milk proteins and their peptides with emphasis on those related to the gastrointestinal ecosystem. J Dairy Res 2005;72 Spec No:66-72.

[20] Brines RD, Brock JH. The effect of trypsin and chymotrypsin on the in vitro antimicrobial and iron-binding properties of lactoferrin in human milk and bovine colostrum. Unusual resistance of human apolactoferrin to proteolytic digestion. Biochim Biophys Acta 1983;759(3):229-35.

[21] Tomita M, Bellamy W, Takase M, Yamauchi K, Wakabayashi H, Kawase K. Potent Antibacterial Peptides Generated by Pepsin Digestion of Bovine Lactoferrin. Journal of Dairy Science 1991;74(12):4137-42.

[22] Almaas H, Holm H, Langsrud T, Flengsrud R, Vegarud GE. In vitro studies of the digestion of caprine whey proteins by human gastric and duodenal juice and the effects on selected microorganisms. Br J Nutr 2006;96(3):562-9.

[23] Eriksen EK, Holm H, Jensen E, Aaboe R, Devold TG, Jacobsen M, et al. Different digestion of caprine whey proteins by human and porcine gastrointestinal enzymes. Br J Nutr 2010;1-8.

[24] Kimura M, Nam MS, Ohkouchi Y, Kumura H, Shimazaki K, Yu DY. Antimicrobial peptide of Korean native goat lactoferrin and identification of the part essential for this activity. Biochemical and Biophysical Research Communications 2000;268(2):333-6.

[25] Chabance B, Marteau P, Rambaud JC, Migliore-Samour D, Boynard M, Perrotin P, et al. Casein peptide release and passage to the blood in humans during digestion of milk or yogurt. Biochimie 1998;80(2):155-65.

[26] Troost FJ, Steijns J, Saris WH, Brummer RJ. Gastric digestion of bovine lactoferrin in vivo in adults. J Nutr 2001;131(8):2101-4.

[27] Ekmekcioglu C. A physiological approach for preparing and conducting intestinal bioavailability studies using experimental systems. Food Chemistry 2002;76(2):225-30.

[28] Kalantzi L, Goumas K, Kalioras V, Abrahamsson B, Dressman JB, Reppas C. Characterization of the human upper gastrointestinal contents under conditions simulating bioavailability/bioequivalence studies. Pharm Res 2006;23(1):165-76.

[29] Khan JA, Kumar P, Paramasivam M, Yadav RS, Sahani MS, Sharma S, et al. Camel lactoferrin, a transferrin-cum-lactoferrin: Crystal structure of camel apolactoferrin at 2.6 angstrom resolution and structural basis of its dual role. Journal of Molecular Biology 2001;309(3):751-61.

[30] Sreedhara A, Flengsrud R, Langsrud T, Kaul P, Prakash V, Vegarud GE. Structural characteristic, pH and thermal stabilities of apo and holo forms of caprine and bovine lactoferrins. Biometals 2010;23(6):1159-70.

[31] Karthikeyan S, Sharma S, Sharma AK, Paramasivam M, Yadav S, Srinivasan A, et al. Structural variability and functional convergence in lactoferrins. Current Science 1999;77(2):241-55.

[32] Holm H, Hanssen LE, Krogdahl A, Florholmen J. High and Low Inhibitor Soybean Meals Affect Human Duodenal Proteinase Activity Differently - Invivo Comparison with Bovine Serum-Albumin. Journal of Nutrition 1988;118(4):515-20.

[33] Sanchez-Chiang L, Cisternas E, Ponce O. Partial purification of pepsins from adult and juvenile salmon fish Oncorhynchus keta. Effect of NaCl on proteolytic activities. Comp Biochem Physiol B 1987;87(4):793-7.

[34] Krogdahl A, Holm H. Inhibition of Human and Rat Pancreatic Proteinases by Crude and Purified Soybean Proteinase-Inhibitors. Journal of Nutrition 1979;109(4):551-8.

[35] Laemmli UK. Cleavage of Structural Proteins During Assembly of Head of Bacteriophage-T4. Nature 1970;227(5259):680-&.

[36] Church FC, Swaisgood HE, Porter DH, Catignani GL. Spectrophotometric Assay Using Ortho-Phthaldialdehyde for Determination of Proteolysis in Milk and Isolated Milk-Proteins. Journal of Dairy Science 1983;66(6):1219-27.

[37] Dionysius DA, Milne JM. Antibacterial peptides of bovine lactoferrin: purification and characterization. J Dairy Sci 1997;80(4):667-74.

[38] Elbarbary HA, Abdou AM, Park EY, Nakamura Y, Mohamed HA, Sato K. Novel antibacterial lactoferrin peptides generated by rennet digestion and autofocusing technique. International Dairy Journal 2010;20(9):646-51.

[39] Hoek KS, Milne JM, Grieve PA, Dionysius DA, Smith R. Antibacterial activity of bovine lactoferrin-derived peptides. Antimicrobial Agents and Chemotherapy 1997;41(1):54-9.

[40] Liepke C, Adermann K, Raida M, Magert HJ, Forssmann WG, Zucht HD. Human milk provides peptides highly stimulating the growth of bifidobacteria. European Journal of Biochemistry 2002;269(2):712-8.

[41] Recio I, Visser S. Antibacterial and binding characteristics of bovine, ovine and caprine lactoferrins: a comparative study. International Dairy Journal 2000;10(9):597-605.

[42] Feldman M, Cryer B, McArthur KE, Huet BA, Lee E. Effects of aging and gastritis on gastric acid and pepsin secretion in humans: a prospective study. Gastroenterology 1996;110(4):1043-52.

[43] Keller J, Layer P. Human pancreatic exocrine response to nutrients in health and disease. Gut 2005;54:1-28.

[44] Recio I, Visser S. Two ion-exchange chromatographic methods for the isolation of antibacterial peptides from lactoferrin. In situ enzymatic hydrolysis on an ion-exchange membrane. J Chromatogr A 1999;831(2):191-201.

Figure captions

Figure 1. Intragastric pH in humans after ingestion of 4.5 g bovine apo LF with citrate buffer (drink 1), 4.5 g bovine apo LF without buffer (drink 2) and 4.5 g bovine holoLF without buffer (drink 3). All drinks contain 80 g/L maltodextrin in 300 mL water. Values are means + SD, n = 12. *P < 0.05, drink 1 vs. drink 2; #P < 0.05, drink 1 vs. drink 3. (Copied with permission from the authors Troost et al (2001).

Figure 2. pH reduction during the course of LF digestion by human gastric juice (HGJ).

Figure 3. SDS-PAGE electrophoretograms of the digestion of 10mg/ml and 1mg/ml native bovine lactoferrin (bLF) by human gastric juice (HGJ) and human duodenal juice (HDJ) of low and high activities. 1. Broad range molecular weight marker; 2. 10 mg/ml bLF, 3. 10 mg/ml bLF added HGJ (5U/g), 4. 10 mg/ml bLF added HGJ (5U/g) + HDJ (15U/g), 5. 10 mg/ml bLF added HGJ (20U/g), 6. 10 mg/ml bLF added HGJ (20U/g) + HDJ (62U/g), 7. 1 mg/ml bLF added HGJ (5U/g), 8. 1mg/ml bLF added HGJ (5U/g) + HDJ (15U/g), 9. 1 mg/ml bLF added HGJ (20U/g), 10. 1 mg/ml bLF added HGJ (20U/g) + HDJ (62U/g).

Figure 4. SDS-PAGE electrophoretograms of the different digestion of native form of bLF by human gastric juice (HGJ) and human duodenal juice (HDJ). 1. LMW marker, 2. 10 mg/ml bLF, 3. Slow reduction of pH to pH 2.5, HGJ, 30 min, 4. Slow reduction of pH to pH 2.5, HGJ + HDJ, 30 min, 5. Slow reduction of pH to pH 4.0, HGJ, 6. Slow reduction of pH to pH 4.0, HGJ + HDJ, 7. Fast reduction of pH to pH 2.5, HGJ, 8. Fast reduction of pH to pH 2.5, HGJ + HDJ, 9. Fast reduction of pH to pH 4.0, HGJ, 10. Fast reduction of pH to pH 4.0, HGJ + HDJ.

Figure 5. Degree of hydrolysis of bLF samples after the gastro-duodenal digestion at pH values 2.5 and 4.0.

Figure 6. Peptide regions (in red) for bLF generated by the fast reduction of pH to pH 2.5. A. Native LF HGJ, B. Apo LF HGJ, C. Holo LF HGJ, D. Native LF HGJ + HDJ, E. Apo LF HGJ + HDJ and F. Holo LF HGJ + HDJ.

Figure 7. Peptide regions (in red) for cLF (10 mg/ml) produced by the fast reduction of pH to pH 2.5 and pH 4.0. A. Native LF HGJ pH 2.5, B. Native LF HGJ pH 4.0, C. Native LF HGJ + HDJ pH 2.5, D. Native LF HGJ + HDJ pH 4.0.

Figure 8. CLUSTAL 2.0.12 multiple sequence alignment (MSA) of the peptides generated after the gastro-duodenal digestion of LF at (1) pH 2.5 and (2) pH 4.0.

Figure 1

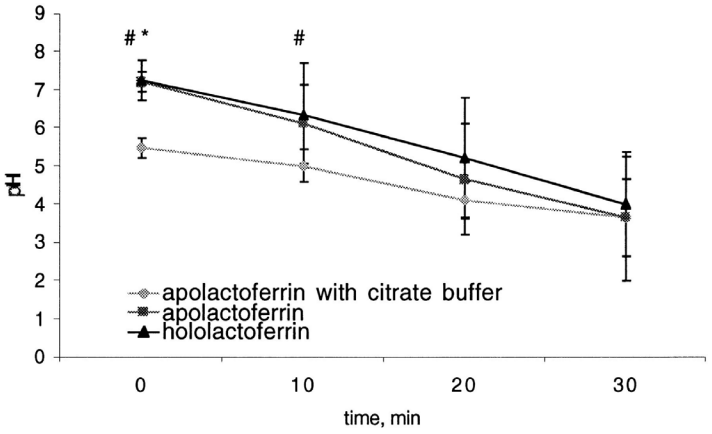
9
8
7
6
5
4
3
2
1
0
pH
*
#
apolactoferrin with citrate buffer
apolactoferrin
hololactoferrin
0
10
20
30
time, min

Figure 2

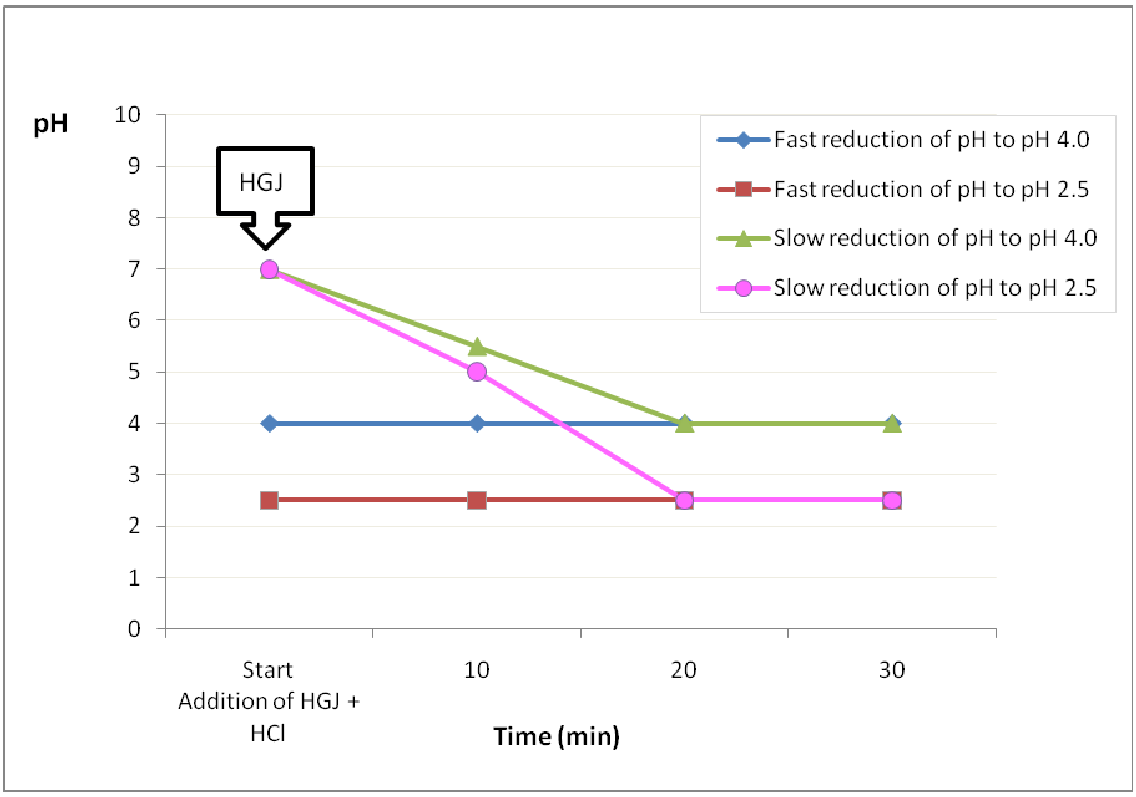
pH
10
9
8
7
6
5
4
3
2
1
0
HGJ
Fast reduction of pH to pH 4.0
Fast reduction of pH to pH 2.5
Slow reduction of pH to pH 4.0
Slow reduction of pH to pH 2.5
Start
Addition of HGJ + HCl
10
20
30
Time (min)

Figure 3

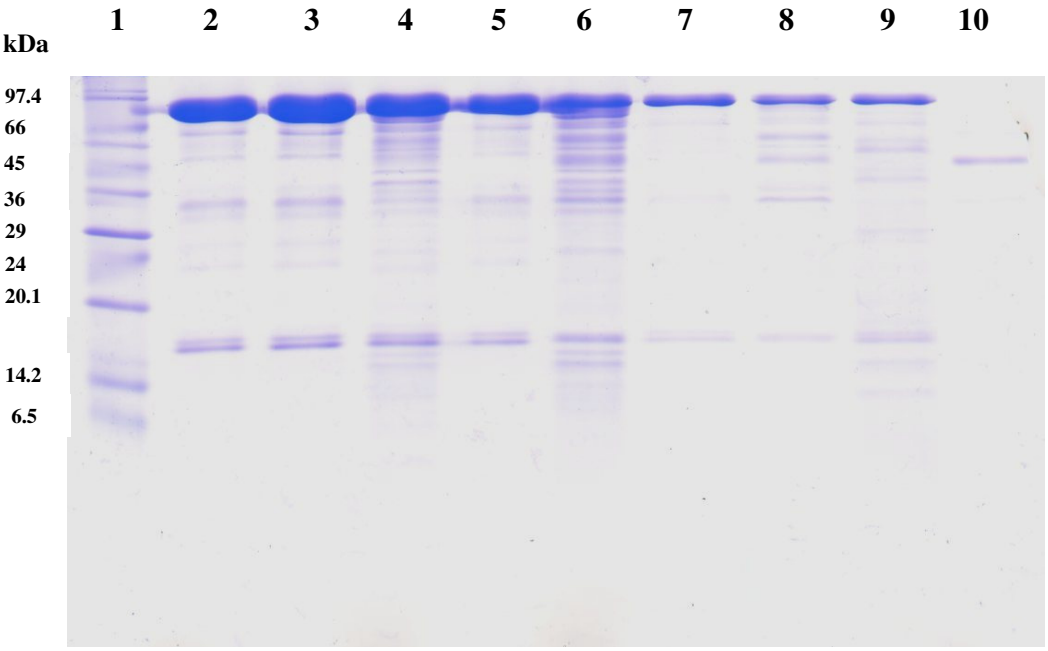

Figure 4

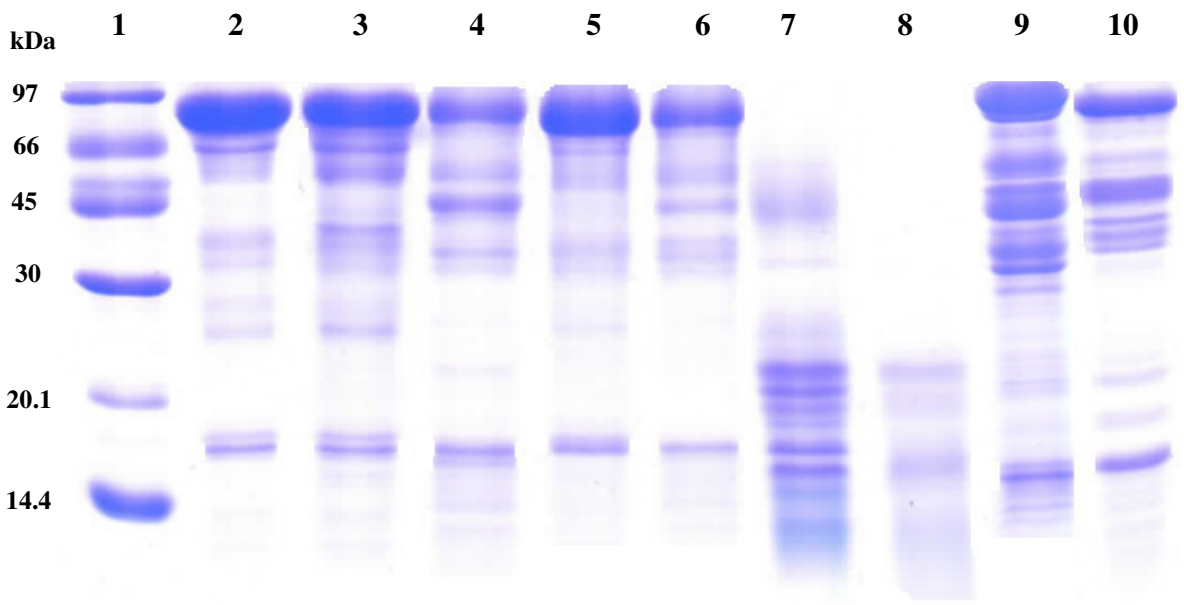

Figure 5

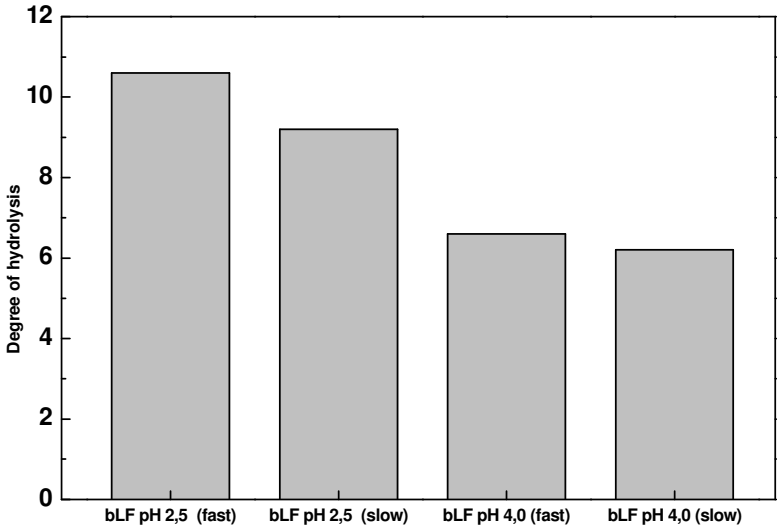
Degree of hydrolysis
0
2
4
6
8
10
12
bLF pH 2,5 (fast)
bLF pH 2,5 (slow)
bLF pH 4,0 (fast)
bLF pH 4,0 (slow)

Figure 6

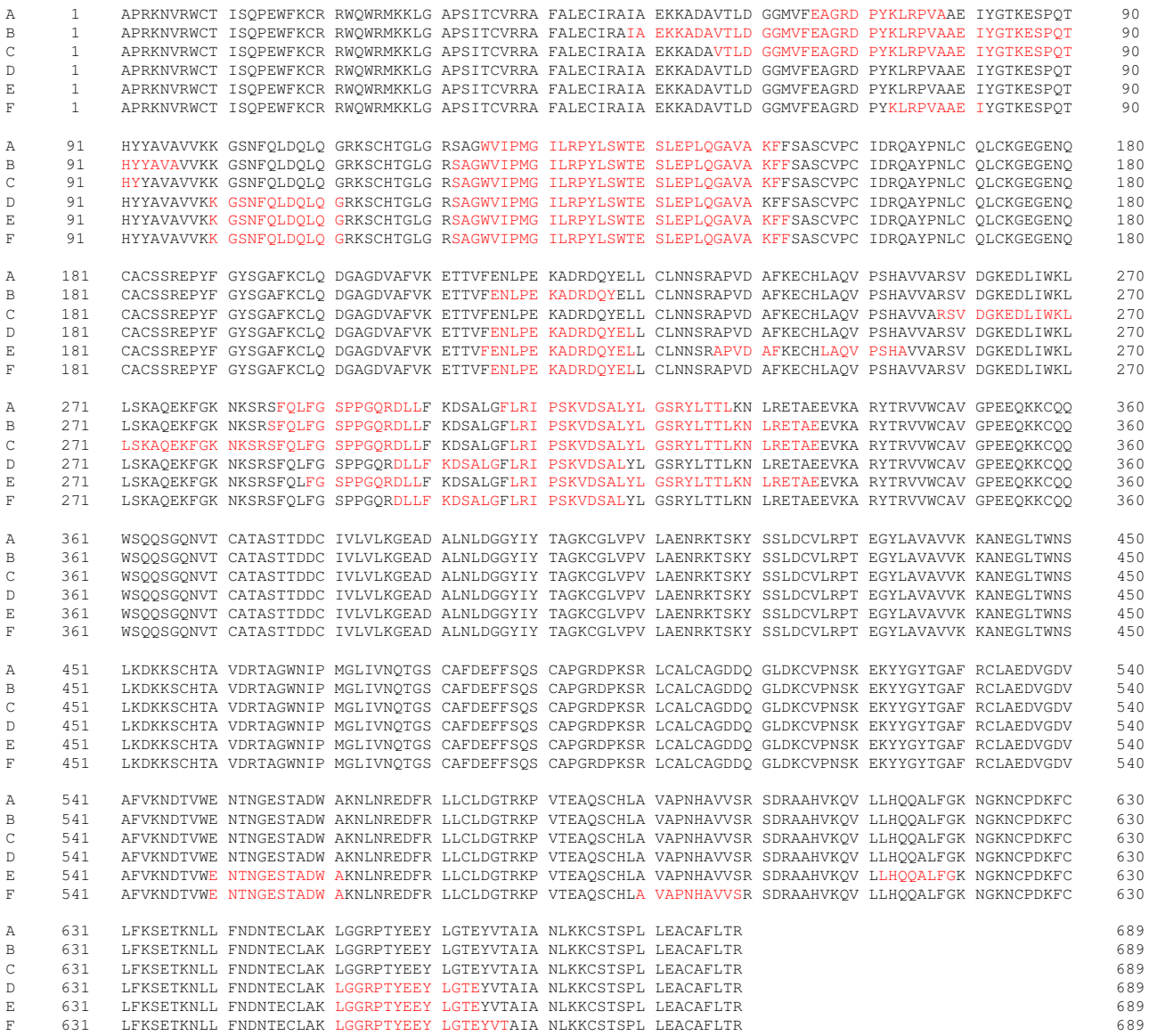
A 1 APRKNVRWCT ISQPEWFKCR RWQWRMKKLG APSITCVRRA FALECIRAIA EKKADAVTLD GGMVFEAGRD PYKLRPVAAE IYGTKESPQT 90
B 1 APRKNVRWCT ISQPEWFKCR RWQWRMKKLG APSITCVRRA FALECIRAIA EKKADAVTLD GGMVFEAGRD PYKLRPVAAE IYGTKESPQT 90
C 1 APRKNVRWCT ISQPEWFKCR RWQWRMKKLG APSITCVRRA FALECIRAIA EKKADAVTLD GGMVFEAGRD PYKLRPVAAE IYGTKESPQT 90
D 1 APRKNVRWCT ISQPEWFKCR RWQWRMKKLG APSITCVRRA FALECIRAIA EKKADAVTLD GGMVFEAGRD PYKLRPVAAE IYGTKESPQT 90
E 1 APRKNVRWCT ISQPEWFKCR RWQWRMKKLG APSITCVRRA FALECIRAIA EKKADAVTLD GGMVFEAGRD PYKLRPVAAE IYGTKESPQT 90
F 1 APRKNVRWCT ISQPEWFKCR RWQWRMKKLG APSITCVRRA FALECIRAIA EKKADAVTLD GGMVFEAGRD PYKLRPVAAE IYGTKESPQT 90
A 91 HYYAVAVVKK GSNFQLDQLQ GRKSCHTGLG RSAGWVIPMG ILRPYLSWTE SLEPLQGAVA KFFSASCVPC IDRQAYPNLC QLCKGEGENQ 180
B 91 HYYAVAVVKK GSNFQLDQLQ GRKSCHTGLG RSAGWVIPMG ILRPYLSWTE SLEPLQGAVA KFFSASCVPC IDRQAYPNLC QLCKGEGENQ 180
C 91 HYYAVAVVKK GSNFQLDQLQ GRKSCHTGLG RSAGWVIPMG ILRPYLSWTE SLEPLQGAVA KFFSASCVPC IDRQAYPNLC QLCKGEGENQ 180
D 91 HYYAVAVVKK GSNFQLDQLQ GRKSCHTGLG RSAGWVIPMG ILRPYLSWTE SLEPLQGAVA KFFSASCVPC IDRQAYPNLC QLCKGEGENQ 180
E 91 HYYAVAVVKK GSNFQLDQLQ GRKSCHTGLG RSAGWVIPMG ILRPYLSWTE SLEPLQGAVA KFFSASCVPC IDRQAYPNLC QLCKGEGENQ 180
F 91 HYYAVAVVKK GSNFQLDQLQ GRKSCHTGLG RSAGWVIPMG ILRPYLSWTE SLEPLQGAVA KFFSASCVPC IDRQAYPNLC QLCKGEGENQ 180
A 181 CACSSREPYF GYSGAFKCLQ DGAGDVAFVK ETTVFENLPE KADRDQYELL CLNNSRAPVD AFKECHLAQV PSHAVVARSV DGKEDLIWKL 270
B 181 CACSSREPYF GYSGAFKCLQ DGAGDVAFVK ETTVFENLPE KADRDQYELL CLNNSRAPVD AFKECHLAQV PSHAVVARSV DGKEDLIWKL 270
C 181 CACSSREPYF GYSGAFKCLQ DGAGDVAFVK ETTVFENLPE KADRDQYELL CLNNSRAPVD AFKECHLAQV PSHAVVARSV DGKEDLIWKL 270
D 181 CACSSREPYF GYSGAFKCLQ DGAGDVAFVK ETTVFENLPE KADRDQYELL CLNNSRAPVD AFKECHLAQV PSHAVVARSV DGKEDLIWKL 270
E 181 CACSSREPYF GYSGAFKCLQ DGAGDVAFVK ETTVFENLPE KADRDQYELL CLNNSRAPVD AFKECHLAQV PSHAVVARSV DGKEDLIWKL 270
F 181 CACSSREPYF GYSGAFKCLQ DGAGDVAFVK ETTVFENLPE KADRDQYELL CLNNSRAPVD AFKECHLAQV PSHAVVARSV DGKEDLIWKL 270
A 271 LSKAQEKFGK NKSRSFQLFG SPPGQRDLLF KDSALGFLRI PSKVDSALYL GSRYLTTLKN LRETAEEVKA RYTRVVWCAV GPEEQKKCQQ 360
B 271 LSKAQEKFGK NKSRSFQLFG SPPGQRDLLF KDSALGFLRI PSKVDSALYL GSRYLTTLKN LRETAEEVKA RYTRVVWCAV GPEEQKKCQQ 360
C 271 LSKAQEKFGK NKSRSFQLFG SPPGQRDLLF KDSALGFLRI PSKVDSALYL GSRYLTTLKN LRETAEEVKA RYTRVVWCAV GPEEQKKCQQ 360
D 271 LSKAQEKFGK NKSRSFQLFG SPPGQRDLLF KDSALGFLRI PSKVDSALYL GSRYLTTLKN LRETAEEVKA RYTRVVWCAV GPEEQKKCQQ 360
E 271 LSKAQEKFGK NKSRSFQLFG SPPGQRDLLF KDSALGFLRI PSKVDSALYL GSRYLTTLKN LRETAEEVKA RYTRVVWCAV GPEEQKKCQQ 360
F 271 LSKAQEKFGK NKSRSFQLFG SPPGQRDLLF KDSALGFLRI PSKVDSALYL GSRYLTTLKN LRETAEEVKA RYTRVVWCAV GPEEQKKCQQ 360
A 361 WSQQSGQNVT CATASTTDDC IVLVLKGEAD ALNLDGGYIY TAGKCGLVPV LAENRKTSKY SSLDCVLRPT EGYLAVAVVK KANEGLTWNS 450
B 361 WSQQSGQNVT CATASTTDDC IVLVLKGEAD ALNLDGGYIY TAGKCGLVPV LAENRKTSKY SSLDCVLRPT EGYLAVAVVK KANEGLTWNS 450
C 361 WSQQSGQNVT CATASTTDDC IVLVLKGEAD ALNLDGGYIY TAGKCGLVPV LAENRKTSKY SSLDCVLRPT EGYLAVAVVK KANEGLTWNS 450
D 361 WSQQSGQNVT CATASTTDDC IVLVLKGEAD ALNLDGGYIY TAGKCGLVPV LAENRKTSKY SSLDCVLRPT EGYLAVAVVK KANEGLTWNS 450
E 361 WSQQSGQNVT CATASTTDDC IVLVLKGEAD ALNLDGGYIY TAGKCGLVPV LAENRKTSKY SSLDCVLRPT EGYLAVAVVK KANEGLTWNS 450
F 361 WSQQSGQNVT CATASTTDDC IVLVLKGEAD ALNLDGGYIY TAGKCGLVPV LAENRKTSKY SSLDCVLRPT EGYLAVAVVK KANEGLTWNS 450
A 451 LKDKKSCHTA VDRTAGWNIP MGLIVNQTGS CAFDEFFSQS CAPGRDPKSR LCALCAGDDQ GLDKCVPNSK EKYYGYTGAF RCLAEDVGDV 540
B 451 LKDKKSCHTA VDRTAGWNIP MGLIVNQTGS CAFDEFFSQS CAPGRDPKSR LCALCAGDDQ GLDKCVPNSK EKYYGYTGAF RCLAEDVGDV 540
C 451 LKDKKSCHTA VDRTAGWNIP MGLIVNQTGS CAFDEFFSQS CAPGRDPKSR LCALCAGDDQ GLDKCVPNSK EKYYGYTGAF RCLAEDVGDV 540
D 451 LKDKKSCHTA VDRTAGWNIP MGLIVNQTGS CAFDEFFSQS CAPGRDPKSR LCALCAGDDQ GLDKCVPNSK EKYYGYTGAF RCLAEDVGDV 540
E 451 LKDKKSCHTA VDRTAGWNIP MGLIVNQTGS CAFDEFFSQS CAPGRDPKSR LCALCAGDDQ GLDKCVPNSK EKYYGYTGAF RCLAEDVGDV 540
F 451 LKDKKSCHTA VDRTAGWNIP MGLIVNQTGS CAFDEFFSQS CAPGRDPKSR LCALCAGDDQ GLDKCVPNSK EKYYGYTGAF RCLAEDVGDV 540
A 541 AFVKNDTVWE NTNGESTADW AKNLNREDFR LLCLDGTRKP VTEAQSCHLA VAPNHAVVSR SDRAAHVKQV LLHQQALFGK NGKNCPDKFC 630
B 541 AFVKNDTVWE NTNGESTADW AKNLNREDFR LLCLDGTRKP VTEAQSCHLA VAPNHAVVSR SDRAAHVKQV LLHQQALFGK NGKNCPDKFC 630
C 541 AFVKNDTVWE NTNGESTADW AKNLNREDFR LLCLDGTRKP VTEAQSCHLA VAPNHAVVSR SDRAAHVKQV LLHQQALFGK NGKNCPDKFC 630
D 541 AFVKNDTVWE NTNGESTADW AKNLNREDFR LLCLDGTRKP VTEAQSCHLA VAPNHAVVSR SDRAAHVKQV LLHQQALFGK NGKNCPDKFC 630
E 541 AFVKNDTVWE NTNGESTADW AKNLNREDFR LLCLDGTRKP VTEAQSCHLA VAPNHAVVSR SDRAAHVKQV LLHQQALFGK NGKNCPDKFC 630
F 541 AFVKNDTVWE NTNGESTADW AKNLNREDFR LLCLDGTRKP VTEAQSCHLA VAPNHAVVSR SDRAAHVKQV LLHQQALFGK NGKNCPDKFC 630
A 631 LFKSETKNLL FNDNTECLAK LGGRPTYEEY LGTEYVTAIA NLKKCSTSPL LEACAFLTR 689
B 631 LFKSETKNLL FNDNTECLAK LGGRPTYEEY LGTEYVTAIA NLKKCSTSPL LEACAFLTR 689
C 631 LFKSETKNLL FNDNTECLAK LGGRPTYEEY LGTEYVTAIA NLKKCSTSPL LEACAFLTR 689
D 631 LFKSETKNLL FNDNTECLAK LGGRPTYEEY LGTEYVTAIA NLKKCSTSPL LEACAFLTR 689
E 631 LFKSETKNLL FNDNTECLAK LGGRPTYEEY LGTEYVTAIA NLKKCSTSPL LEACAFLTR 689
F 631 LFKSETKNLL FNDNTECLAK LGGRPTYEEY LGTEYVTAIA NLKKCSTSPL LEACAFLTR 689

Figure 7

```
A    1     APRKNVRWCA ISLPEWSKCY QWQRRMRKLG APSITCVRRT SALECIRAIA GKNADAVTLD SGMVFEAGRD PYKLRPVAAE IYGTEKSPQT   90
B    1     APRKNVRWCA ISLPEWSKCY QWQRRMRKLG APSITCVRRT SALECIRAIA GKNADAVTLD SGMVFEAGRD PYKLRPVAAE IYGTEKSPQT   90
C    1     APRKNVRWCA ISLPEWSKCY QWQRRMRKLG APSITCVRRT SALECIRAIA GKNADAVTLD SGMVFEAGRD PYKLRPVAAE IYGTEKSPQT   90
D    1     APRKNVRWCA ISLPEWSKCY QWQRRMRKLG APSITCVRRT SALECIRAIA GKNADAVTLD SGMVFEAGRD PYKLRPVAAE IYGTEKSPQT   90

A    91    HYYAVAVVKK GSNFKLDQLQ GQKSCHMGLG RSAGWNIPVG ILRPPLSWTE SAEPLQGAVA RFFSASCVPC VDGKAYPNLC QLCKGVGENK   180
B    91    HYYAVAVVKK GSNFKLDQLQ GQKSCHMGLG RSAGWNIPVG ILRPPLSWTE SAEPLQGAVA RFFSASCVPC VDGKAYPNLC QLCKGVGENK   180
C    91    HYYAVAVVKK GSNFKLDQLQ GQKSCHMGLG RSAGWNIPVG ILRPPLSWTE SAEPLQGAVA RFFSASCVPC VDGKAYPNLC QLCKGVGENK   180
D    91    HYYAVAVVKK GSNFKLDQLQ GQKSCHMGLG RSAGWNIPVG ILRPPLSWTE SAEPLQGAVA RFFSASCVPC VDGKAYPNLC QLCKGVGENK   180

A    181   CACSSQEPYF GYSGAFKCLQ DGAGDVAFVK ETTVFENLPE KADRDQYELL CLNNTRAPVD AFKECHLAQV PSHAVVARSV DGKENLIWEL   270
B    181   CACSSQEPYF GYSGAFKCLQ DGAGDVAFVK ETTVFENLPE KADRDQYELL CLNNTRAPVD AFKECHLAQV PSHAVVARSV DGKENLIWEL   270
C    181   CACSSQEPYF GYSGAFKCLQ DGAGDVAFVK ETTVFENLPE KADRDQYELL CLNNTRAPVD AFKECHLAQV PSHAVVARSV DGKENLIWEL   270
D    181   CACSSQEPYF GYSGAFKCLQ DGAGDVAFVK ETTVFENLPE KADRDQYELL CLNNTRAPVD AFKECHLAQV PSHAVVARSV DGKENLIWEL   270

A    271   LRKAQEKFGK NKSQRFQLFG SPEGRRDLLF KDSALGFVRI PSKVDSALYL GSRYLTALKN LRETAEELKA RCTRVVWCAV GPEEQSKCQQ   360
B    271   LRKAQEKFGK NKSQRFQLFG SPEGRRDLLF KDSALGFVRI PSKVDSALYL GSRYLTALKN LRETAEELKA RCTRVVWCAV GPEEQSKCQQ   360
C    271   LRKAQEKFGK NKSQRFQLFG SPEGRRDLLF KDSALGFVRI PSKVDSALYL GSRYLTALKN LRETAEELKA RCTRVVWCAV GPEEQSKCQQ   360
D    271   LRKAQEKFGK NKSQRFQLFG SPEGRRDLLF KDSALGFVRI PSKVDSALYL GSRYLTALKN LRETAEELKA RCTRVVWCAV GPEEQSKCQQ   360

A    361   WSEQSGQNVT CATASTTDDC IALVLKGEAD ALSLGGGYIY TAGKCGLVPV MAENRKSSKY SSLDCVLRPT EGYLAVAVVK KANEGLTWNS   450
B    361   WSEQSGQNVT CATASTTDDC IALVLKGEAD ALSLGGGYIY TAGKCGLVPV MAENRKSSKY SSLDCVLRPT EGYLAVAVVK KANEGLTWNS   450
C    361   WSEQSGQNVT CATASTTDDC IALVLKGEAD ALSLGGGYIY TAGKCGLVPV MAENRKSSKY SSLDCVLRPT EGYLAVAVVK KANEGLTWNS   450
D    361   WSEQSGQNVT CATASTTDDC IALVLKGEAD ALSLGGGYIY TAGKCGLVPV MAENRKSSKY SSLDCVLRPT EGYLAVAVVK KANEGLTWNS   450

A    451   LKGKKSCHTA VDRTAGWNIP MGLIANQTGS CAFDEFFSQS CAPGADPKSS LCALCAGDDQ GLDKCVPNSK EKYYGYTGAF RCLAEDVGDV   540
B    451   LKGKKSCHTA VDRTAGWNIP MGLIANQTGS CAFDEFFSQS CAPGADPKSS LCALCAGDDQ GLDKCVPNSK EKYYGYTGAF RCLAEDVGDV   540
C    451   LKGKKSCHTA VDRTAGWNIP MGLIANQTGS CAFDEFFSQS CAPGADPKSS LCALCAGDDQ GLDKCVPNSK EKYYGYTGAF RCLAEDVGDV   540
D    451   LKGKKSCHTA VDRTAGWNIP MGLIANQTGS CAFDEFFSQS CAPGADPKSS LCALCAGDDQ GLDKCVPNSK EKYYGYTGAF RCLAEDVGDV   540

A    541   AFVKNDTVWE NTNGESSADW AKNLNREDFR LLCLDGTTKP VTEAQSCYLA VAPNHAVVSR SDRAAHVEQV LLHQQALFGK NGKNCPDQFC   630
B    541   AFVKNDTVWE NTNGESSADW AKNLNREDFR LLCLDGTTKP VTEAQSCYLA VAPNHAVVSR SDRAAHVEQV LLHQQALFGK NGKNCPDQFC   630
C    541   AFVKNDTVWE NTNGESSADW AKNLNREDFR LLCLDGTTKP VTEAQSCYLA VAPNHAVVSR SDRAAHVEQV LLHQQALFGK NGKNCPDQFC   630
D    541   AFVKNDTVWE NTNGESSADW AKNLNREDFR LLCLDGTTKP VTEAQSCYLA VAPNHAVVSR SDRAAHVEQV LLHQQALFGK NGKNCPDQFC   630

A    631   LFKSETKNLL FNDNTECLAK LGGRPTYEKY LGTEYVTAIA NLKKCSTSPL LEACAFLTR                                      689
B    631   LFKSETKNLL FNDNTECLAK LGGRPTYEKY LGTEYVTAIA NLKKCSTSPL LEACAFLTR                                      689
C    631   LFKSETKNLL FNDNTECLAK LGGRPTYEKY LGTEYVTAIA NLKKCSTSPL LEACAFLTR                                      689
D    631   LFKSETKNLL FNDNTECLAK LGGRPTYEKY LGTEYVTAIA NLKKCSTSPL LEACAFLTR                                      689
```

Figure 8

```
7    ARSVDGKEDLIWKLL--SKAQEKFGKNKSRSFQLFGSPPGQRDLL------ 43
14   ------------QVL--LHQQALFG---------------------------- 11
11   -----------ENTN--GESTADWAK-------------------------- 13
12   ------------WAK--NLNRED---------------------------- 9
5    -----------------APVDAF---------------------------- 6
13   -------------AV--APNHAVVS-------------------------- 10
6    ------------LAQ--VPSHA------------------------------ 8
1    ---IAEKKADAVTLD--GGMVFEAGRDPYKLRPVAAEIYGTKESPQTHYYA 46
10   ----------LNLD--GGYI-------------------------------  8
9    ------------YLG--SRYLTTLKN----LRETAEE-------------- 19
16   ---LGGRPTYEEYLG--TEYVTAIAN----LKK------------------ 24
15   ----------FGKNG--KNCPDQFCLFKSETKNLL---------------- 23
4    ---VKETTVFENLPE--KADRDQYEL-------------------------- 21
8    --DLLFKDSALGFLR--IPSKVDSAL-------------------------- 22
2    ------------VVK--KGSNFQLDQLQG---------------------- 15
3    ---SAGWVIPMGILRPYLSWTESLEPLQGAVAKFF----------------- 32

C        AGK    EG  LG    PS A L L     L KL    E  G
Q                   *     ** *
```

A

Q >15 is marked with *
Score: 5-50

```
14   AVVSRSDRAAHVKQVLLHQQAL----------------- 22
15   -------------QVLLHQQALFG--------------- 11
4    -------SAGWVIPMGILRPYLSWTESLEPLQGAVAKFF 32
11   ------RTAGWNIPMGL----------------------- 11
8    --SFQLFGSPPGQRDLLFKDSALGFLRIPSKVDSA---- 33
9    ------VRIPSKVDSALYLGSRY-LTALKNLRETAE--- 29
3    --------KGSNFKLDQLQGQ------------------- 13
7    -------LSKAQEKFGKNKSQRFQL--------------- 18
10   --------VVKKANEGLTW--------------------- 11
6    -------ARSVDGKEDLIWKL------------------- 14
12   --------ENTNGESTADWAKNLNREDFRL---------- 22
13   --------LAVAPNHAVVS--------------------- 11
1    --IAEKKADAVTLDGGMVFEAGRDPYKLRPVAA------ 31
5    -------VKETTVFENLPEKADRDQYEL------------ 21
16   --------LGGRPTYEEYLGTEY----------------- 15
2    ---------AEIYGTEKSPQTHY----------------- 14
C           A VN KEGL    ALY L  LRPL A
Q            *   *  *    **
```

B

Q >20 is marked with *
Score: 5-63

Table 1. Different concentrations of bovine lactoferrin (bLF), human gastric juice (HGJ) and human duodenal juice (HDJ) used during the digestion.

	Concentration of bLF (mg/ml)	10.0	1.0	0.1
Step 1	Low concentration HGJ (U/g)	5.0	50.0	500.0
Step 2	Low concentration HDJ (U/g)	15.6	156.0	1560.0
Step 1	High concentration HGJ (U/g)	20.0	200.0	2000.0
Step 2	High concentration HDJ (U/g)	62.4	624.0	6240.0

Table 2. Common peptides in bLF (native, apo and holo) and cLF (native) after digestion with HGJ and HGJ + HDJ

		Native bLF								Apo bLF				Holo bLF				Native cLF			
		2.5 S**		4.0 S**		2.5 F**		4.0 F**		2.5 F**		4.0 F**		2.5 F**		4.0 F**		2.5 F**		4.0 F**	
Fragment	Peptide regions	G*	D*	G*	D*	G*	D*	G*	D*	G*	D*	G*	D*	G*	D*	G*	D*	G*	D*	G*	D*
49-65	IAEKKADAVTLDGGMVF							O		O											
57-79	VTLDGGMVFEAGRDPYKLRPVAA											O		O							
57-78	VTLDGGMVFEAGRDPYKLRPVA									O		O				O					
66-78	EAGRDPYKLPVA		O			O				O				O		O		O		O	
66-92	EAGRDPYKLPVAAEIYGTKESPQTHY	O								O						O					
79-92	AEIGTKESPQTHY								O	O				O		O		O	O	O	O
100-111	KGSNFQLDQLQG						O				O		O		O		O				
101-111	GSNFQLDQLQG	O	O													O					
122-135	SAGWVIPMGILRPY		O		O		O				O		O		O		O				
125-135	WVIPMGILPY													O	O		O				
125-139	WVIPMGILRPYLSWT	O				O		O	O	O		O				O					
126-135	VIPMGILPY				O		O				O		O		O						
137-152	SWTESL/AEPLQGAVAK/RF	O				O		O		O		O		O		O			O	O	
140-152	ESLEPLQGAVAKF							O	O	O		O		O		O					
141-152	SL/AEPLQGAVAK/RF									O	O	O		O		O			O	O	
209-229	VKETTVFENLPEKADRDQYEL							O				O						O			
216-228	ENLPEKADRDQYE	O					O				O	O	O		O			O			O
237-242	APVDAF		O								O										
247-254	LAQVPSHA										O							O			
267-288	IWKLLSKAQEKFGKNKSRSFQL															O			O		
267-299	IWKLLSKAQEKFGKNKSRSFQLFGSPPGRDLL													O							
285-296	SFQLFGSPPGQR	O		O					O												
286-299	FQLFGSPPGQRDLL					O		O		O		O		O							
289-299	FGSPPGQRDLL		O			O			O	O	O										
297-306	DLLFKDSALG				O		O		O				O		O		O				
297-317	DLLFKDSALGFLRIPSKVDSA		O		O												O				
308-317	LRIPSKVDSA	O			O		O				O		O		O	O	O				
308-318	L/VRIPSKVDSAL	O					O	O				O	O		O						O
325-336	LTTLKNLRETAE							O		O				O							
550-561	ENTNGSTADWA							O	O		O		O		O						
618-641	FGKNGKNCPDQFCLFKSETKNLL																	O			
618-631	FGKNGKNCPDQFCL																		O		
651-660	LGGRPTYEEY						O				O	O	O		O	O	O				
651-664	LGGRPTYEEYLGTE	O					O				O		O		O	O	O				
651-665	LGGRPTYEEYLGTEY		O					O					O								

*** G: Human gastric juice, D: Human duodenal juice**

**** S: slow reduction of pH, F: fast reduction of pH**

Supplementary material

```
A     1    APRKNVRWCT ISQPEWFKCR RWQWRMKKLG APSITCVRRA FALECIRAIA EKKADAVTLD GGMVFEAGRD PYKLRPVAAE IYGTKESPQT    90
B     1    APRKNVRWCT ISQPEWFKCR RWQWRMKKLG APSITCVRRA FALECIRAIA EKKADAVTLD GGMVFEAGRD PYKLRPVAAE IYGTKESPQT    90
C     1    APRKNVRWCT ISQPEWFKCR RWQWRMKKLG APSITCVRRA FALECIRAIA EKKADAVTLD GGMVFEAGRD PYKLRPVAAE IYGTKESPQT    90
D     1    APRKNVRWCT ISQPEWFKCR RWQWRMKKLG APSITCVRRA FALECIRAIA EKKADAVTLD GGMVFEAGRD PYKLRPVAAE IYGTKESPQT    90
E     1    APRKNVRWCT ISQPEWFKCR RWQWRMKKLG APSITCVRRA FALECIRAIA EKKADAVTLD GGMVFEAGRD PYKLRPVAAE IYGTKESPQT    90
F     1    APRKNVRWCT ISQPEWFKCR RWQWRMKKLG APSITCVRRA FALECIRAIA EKKADAVTLD GGMVFEAGRD PYKLRPVAAE IYGTKESPQT    90

A    91    HYYAVAVVKK GSNFQLDQLQ GRKSCHTGLG RSAGWVIPMG ILRPYLSWTE SLEPLQGAVA KFFSASCVPC IDRQAYPNLC QLCKGEGENQ   180
B    91    HYYAVAVVKK GSNFQLDQLQ GRKSCHTGLG RSAGWVIPMG ILRPYLSWTE SLEPLQGAVA KFFSASCVPC IDRQAYPNLC QLCKGEGENQ   180
C    91    HYYAVAVVKK GSNFQLDQLQ GRKSCHTGLG RSAGWVIPMG ILRPYLSWTE SLEPLQGAVA KFFSASCVPC IDRQAYPNLC QLCKGEGENQ   180
D    91    HYYAVAVVKK GSNFQLDQLQ GRKSCHTGLG RSAGWVIPMG ILRPYLSWTE SLEPLQGAVA KFFSASCVPC IDRQAYPNLC QLCKGEGENQ   180
E    91    HYYAVAVVKK GSNFQLDQLQ GRKSCHTGLG RSAGWVIPMG ILRPYLSWTE SLEPLQGAVA KFFSASCVPC IDRQAYPNLC QLCKGEGENQ   180
F    91    HYYAVAVVKK GSNFQLDQLQ GRKSCHTGLG RSAGWVIPMG ILRPYLSWTE SLEPLQGAVA KFFSASCVPC IDRQAYPNLC QLCKGEGENQ   180

A   181    CACSSREPYF GYSGAFKCLQ DGAGDVAFVK ETTVFENLPE KADRDQYELL CLNNSRAPVD AFKECHLAQV PSHAVVARSV DGKEDLIWKL   270
B   181    CACSSREPYF GYSGAFKCLQ DGAGDVAFVK ETTVFENLPE KADRDQYELL CLNNSRAPVD AFKECHLAQV PSHAVVARSV DGKEDLIWKL   270
C   181    CACSSREPYF GYSGAFKCLQ DGAGDVAFVK ETTVFENLPE KADRDQYELL CLNNSRAPVD AFKECHLAQV PSHAVVARSV DGKEDLIWKL   270
D   181    CACSSREPYF GYSGAFKCLQ DGAGDVAFVK ETTVFENLPE KADRDQYELL CLNNSRAPVD AFKECHLAQV PSHAVVARSV DGKEDLIWKL   270
E   181    CACSSREPYF GYSGAFKCLQ DGAGDVAFVK ETTVFENLPE KADRDQYELL CLNNSRAPVD AFKECHLAQV PSHAVVARSV DGKEDLIWKL   270
F   181    CACSSREPYF GYSGAFKCLQ DGAGDVAFVK ETTVFENLPE KADRDQYELL CLNNSRAPVD AFKECHLAQV PSHAVVARSV DGKEDLIWKL   270

A   271    LSKAQEKFGK NKSRSFQLFG SPPGQRDLLF KDSALGFLRI PSKVDSALYL GSRYLTTLKN LRETAEEVKA RYTRVVWCAV GPEEQKKCQQ   360
B   271    LSKAQEKFGK NKSRSFQLFG SPPGQRDLLF KDSALGFLRI PSKVDSALYL GSRYLTTLKN LRETAEEVKA RYTRVVWCAV GPEEQKKCQQ   360
C   271    LSKAQEKFGK NKSRSFQLFG SPPGQRDLLF KDSALGFLRI PSKVDSALYL GSRYLTTLKN LRETAEEVKA RYTRVVWCAV GPEEQKKCQQ   360
D   271    LSKAQEKFGK NKSRSFQLFG SPPGQRDLLF KDSALGFLRI PSKVDSALYL GSRYLTTLKN LRETAEEVKA RYTRVVWCAV GPEEQKKCQQ   360
E   271    LSKAQEKFGK NKSRSFQLFG SPPGQRDLLF KDSALGFLRI PSKVDSALYL GSRYLTTLKN LRETAEEVKA RYTRVVWCAV GPEEQKKCQQ   360
F   271    LSKAQEKFGK NKSRSFQLFG SPPGQRDLLF KDSALGFLRI PSKVDSALYL GSRYLTTLKN LRETAEEVKA RYTRVVWCAV GPEEQKKCQQ   360

A   361    WSQQSGQNVT CATASTTDDC IVLVLKGEAD ALNLDGGYIY TAGKCGLVPV LAENRKTSKY SSLDCVLRPT EGYLAVAVVK KANEGLTWNS   450
B   361    WSQQSGQNVT CATASTTDDC IVLVLKGEAD ALNLDGGYIY TAGKCGLVPV LAENRKTSKY SSLDCVLRPT EGYLAVAVVK KANEGLTWNS   450
C   361    WSQQSGQNVT CATASTTDDC IVLVLKGEAD ALNLDGGYIY TAGKCGLVPV LAENRKTSKY SSLDCVLRPT EGYLAVAVVK KANEGLTWNS   450
D   361    WSQQSGQNVT CATASTTDDC IVLVLKGEAD ALNLDGGYIY TAGKCGLVPV LAENRKTSKY SSLDCVLRPT EGYLAVAVVK KANEGLTWNS   450
E   361    WSQQSGQNVT CATASTTDDC IVLVLKGEAD ALNLDGGYIY TAGKCGLVPV LAENRKTSKY SSLDCVLRPT EGYLAVAVVK KANEGLTWNS   450
F   361    WSQQSGQNVT CATASTTDDC IVLVLKGEAD ALNLDGGYIY TAGKCGLVPV LAENRKTSKY SSLDCVLRPT EGYLAVAVVK KANEGLTWNS   450

A   451    LKDKKSCHTA VDRTAGWNIP MGLIVNQTGS CAFDEFFSQS CAPGRDPKSR LCALCAGDDQ GLDKCVPNSK EKYYGYTGAF RCLAEDVGDV   540
B   451    LKDKKSCHTA VDRTAGWNIP MGLIVNQTGS CAFDEFFSQS CAPGRDPKSR LCALCAGDDQ GLDKCVPNSK EKYYGYTGAF RCLAEDVGDV   540
C   451    LKDKKSCHTA VDRTAGWNIP MGLIVNQTGS CAFDEFFSQS CAPGRDPKSR LCALCAGDDQ GLDKCVPNSK EKYYGYTGAF RCLAEDVGDV   540
D   451    LKDKKSCHTA VDRTAGWNIP MGLIVNQTGS CAFDEFFSQS CAPGRDPKSR LCALCAGDDQ GLDKCVPNSK EKYYGYTGAF RCLAEDVGDV   540
E   451    LKDKKSCHTA VDRTAGWNIP MGLIVNQTGS CAFDEFFSQS CAPGRDPKSR LCALCAGDDQ GLDKCVPNSK EKYYGYTGAF RCLAEDVGDV   540
F   451    LKDKKSCHTA VDRTAGWNIP MGLIVNQTGS CAFDEFFSQS CAPGRDPKSR LCALCAGDDQ GLDKCVPNSK EKYYGYTGAF RCLAEDVGDV   540

A   541    AFVKNDTVWE NTNGESTADW AKNLNREDFR LLCLDGTRKP VTEAQSCHLA VAPNHAVVSR SDRAAHVKQV LLHQQALFGK NGKNCPDKFC   630
B   541    AFVKNDTVWE NTNGESTADW AKNLNREDFR LLCLDGTRKP VTEAQSCHLA VAPNHAVVSR SDRAAHVKQV LLHQQALFGK NGKNCPDKFC   630
C   541    AFVKNDTVWE NTNGESTADW AKNLNREDFR LLCLDGTRKP VTEAQSCHLA VAPNHAVVSR SDRAAHVKQV LLHQQALFGK NGKNCPDKFC   630
D   541    AFVKNDTVWE NTNGESTADW AKNLNREDFR LLCLDGTRKP VTEAQSCHLA VAPNHAVVSR SDRAAHVKQV LLHQQALFGK NGKNCPDKFC   630
E   541    AFVKNDTVWE NTNGESTADW AKNLNREDFR LLCLDGTRKP VTEAQSCHLA VAPNHAVVSR SDRAAHVKQV LLHQQALFGK NGKNCPDKFC   630
F   541    AFVKNDTVWE NTNGESTADW AKNLNREDFR LLCLDGTRKP VTEAQSCHLA VAPNHAVVSR SDRAAHVKQV LLHQQALFGK NGKNCPDKFC   630

A   631    LFKSETKNLL FNDNTECLAK LGGRPTYEEY LGTEYVTAIA NLKKCSTSPL LEACAFLTR                                    689
B   631    LFKSETKNLL FNDNTECLAK LGGRPTYEEY LGTEYVTAIA NLKKCSTSPL LEACAFLTR                                    689
C   631    LFKSETKNLL FNDNTECLAK LGGRPTYEEY LGTEYVTAIA NLKKCSTSPL LEACAFLTR                                    689
D   631    LFKSETKNLL FNDNTECLAK LGGRPTYEEY LGTEYVTAIA NLKKCSTSPL LEACAFLTR                                    689
E   631    LFKSETKNLL FNDNTECLAK LGGRPTYEEY LGTEYVTAIA NLKKCSTSPL LEACAFLTR                                    689
F   631    LFKSETKNLL FNDNTECLAK LGGRPTYEEY LGTEYVTAIA NLKKCSTSPL LEACAFLTR                                    689
```

Figure S1. Peptide regions (in red) from bovine lactoferrin generated by the fast reduction of pH to 4.0. A. Native LF HGJ, B. apo LF HGJ, C. holo LF HGJ, D. Native LF HGJ + HDJ, E. apo LF HGJ + HDJ and F. holo LF HGJ + HDJ.

```
A    1      APRKNVRWCT ISQPEWFKCR RWQWRMKKLG APSITCVRRA FALECIRAIA EKKADAVTLD GGMVFEAGRD PYKLRPVAAE IYGTKESPQT    90
B    1      APRKNVRWCT ISQPEWFKCR RWQWRMKKLG APSITCVRRA FALECIRAIA EKKADAVTLD GGMVFEAGRD PYKLRPVAAE IYGTKESPQT    90
C    1      APRKNVRWCT ISQPEWFKCR RWQWRMKKLG APSITCVRRA FALECIRAIA EKKADAVTLD GGMVFEAGRD PYKLRPVAAE IYGTKESPQT    90
D    1      APRKNVRWCT ISQPEWFKCR RWQWRMKKLG APSITCVRRA FALECIRAIA EKKADAVTLD GGMVFEAGRD PYKLRPVAAE IYGTKESPQT    90

A    91     HYYAVAVVKK GSNFQLDQLQ GRKSCHTGLG RSAGWVIPMG ILRPYLSWTE SLEPLQGAVA KFFSASCVPC IDRQAYPNLC QLCKGEGENQ    180
B    91     HYYAVAVVKK GSNFQLDQLQ GRKSCHTGLG RSAGWVIPMG ILRPYLSWTE SLEPLQGAVA KFFSASCVPC IDRQAYPNLC QLCKGEGENQ    180
C    91     HYYAVAVVKK GSNFQLDQLQ GRKSCHTGLG RSAGWVIPMG ILRPYLSWTE SLEPLQGAVA KFFSASCVPC IDRQAYPNLC QLCKGEGENQ    180
D    91     HYYAVAVVKK GSNFQLDQLQ GRKSCHTGLG RSAGWVIPMG ILRPYLSWTE SLEPLQGAVA KFFSASCVPC IDRQAYPNLC QLCKGEGENQ    180

A    181    CACSSREPYF GYSGAFKCLQ DGAGDVAFVK ETTVFENLPE KADRDQYELL CLNNSRAPVD AFKECHLAQV PSHAVVARSV DGKEDLIWKL    270
B    181    CACSSREPYF GYSGAFKCLQ DGAGDVAFVK ETTVFENLPE KADRDQYELL CLNNSRAPVD AFKECHLAQV PSHAVVARSV DGKEDLIWKL    270
C    181    CACSSREPYF GYSGAFKCLQ DGAGDVAFVK ETTVFENLPE KADRDQYELL CLNNSRAPVD AFKECHLAQV PSHAVVARSV DGKEDLIWKL    270
D    181    CACSSREPYF GYSGAFKCLQ DGAGDVAFVK ETTVFENLPE KADRDQYELL CLNNSRAPVD AFKECHLAQV PSHAVVARSV DGKEDLIWKL    270

A    271    LSKAQEKFGK NKSRSFQLFG SPPGQRDLLF KDSALGFLRI PSKVDSALYL GSRYLTTLKN LRETAEEVKA RYTRVVWCAV GPEEQKKCQQ    360
B    271    LSKAQEKFGK NKSRSFQLFG SPPGQRDLLF KDSALGFLRI PSKVDSALYL GSRYLTTLKN LRETAEEVKA RYTRVVWCAV GPEEQKKCQQ    360
C    271    LSKAQEKFGK NKSRSFQLFG SPPGQRDLLF KDSALGFLRI PSKVDSALYL GSRYLTALKN LRETAEELKA RCTRVVWCAV GPEEQSKCQQ    360
D    271    LSKAQEKFGK NKSRSFQLFG SPPGQRDLLF KDSALGFLRI PSKVDSALYL GSRYLTTLKN LRETAEEVKA RYTRVVWCAV GPEEQKKCQQ    360

A    361    WSQQSGQNVT CATASTTDDC IVLVLKGEAD ALNLDGGYIY TAGKCGLVPV LAENRKTSKY SSLDCVLRPT EGYLAVAVVK KANEGLTWNS    450
B    361    WSQQSGQNVT CATASTTDDC IVLVLKGEAD ALNLDGGYIY TAGKCGLVPV LAENRKTSKY SSLDCVLRPT EGYLAVAVVK KANEGLTWNS    450
C    361    WSEQSGQNVT CATASTTDDC IALVLKGEAD ALNLDGGYIY TAGKCGLVPV LAENRKTSKY SSLDCVLRPT EGYLAVAVVK KANEGLTWNS    450
D    361    WSQQSGQNVT CATASTTDDC IVLVLKGEAD ALNLDGGYIY TAGKCGLVPV LAENRKTSKY SSLDCVLRPT EGYLAVAVVK KANEGLTWNS    450

A    451    LKDKKSCHTA VDRTAGWNIP MGLIVNQTGS CAFDEFFSQS CAPGRDPKSR LCALCAGDDQ GLDKCVPNSK EKYYGYTGAF RCLAEDVGDV    540
B    451    LKDKKSCHTA VDRTAGWNIP MGLIVNQTGS CAFDEFFSQS CAPGRDPKSR LCALCAGDDQ GLDKCVPNSK EKYYGYTGAF RCLAEDVGDV    540
C    451    LKDKKSCHTA VDRTAGWNIP MGLIVNQTGS CAFDEFFSQS CAPGRDPKSR LCALCAGDDQ GLDKCVPNSK EKYYGYTGAF RCLAEDVGDV    540
D    451    LKDKKSCHTA VDRTAGWNIP MGLIVNQTGS CAFDEFFSQS CAPGRDPKSR LCALCAGDDQ GLDKCVPNSK EKYYGYTGAF RCLAEDVGDV    540

A    541    AFVKNDTVWE NTNGESTADW AKNLNREDFR LLCLDGTRKP VTEAQSCHLA VAPNHAVVSR SDRAAHVKQV LLHQQALFGK NGKNCPDKFC    630
B    541    AFVKNDTVWE NTNGESTADW AKNLNREDFR LLCLDGTRKP VTEAQSCHLA VAPNHAVVSR SDRAAHVKQV LLHQQALFGK NGKNCPDKFC    630
C    541    AFVKNDTVWE NTNGESTADW AKNLNREDFR LLCLDGTRKP VTEAQSCHLA VAPNHAVVSR SDRAAHVKQV LLHQQALFGK NGKNCPDKFC    630
D    541    AFVKNDTVWE NTNGESTADW AKNLNREDFR LLCLDGTRKP VTEAQSCHLA VAPNHAVVSR SDRAAHVKQV LLHQQALFGK NGKNCPDKFC    630

A    631    LFKSETKNLL FNDNTECLAK LGGRPTYEEY LGTEYVTAIA NLKKCSTSPL LEACAFLTR                                      689
B    631    LFKSETKNLL FNDNTECLAK LGGRPTYEEY LGTEYVTAIA NLKKCSTSPL LEACAFLTR                                      689
C    631    LFKSETKNLL FNDNTECLAK LGGRPTYEEY LGTEYVTAIA NLKKCSTSPL LEACAFLTR                                      689
D    631    LFKSETKNLL FNDNTECLAK LGGRPTYEEY LGTEYVTAIA NLKKCSTSPL LEACAFLTR                                      689
```

Figure S2. Peptide regions for bovine lactoferrin at slow reduction of pH to 2.5 and 4.0. A. Native LF HGJ pH 2.5, B. Native LF HGJ pH 4.0, C. Native LF HGJ + HDJ pH 2.5, D. Native LF HGJ + HDJ pH 4.

Table S1. All peptides in bLF (native, apo and holo) and cLF (native) after digestion with HGJ and HGJ + HDJ

		Native bLF								Apo bLF				Holo bLF				Native cLF			
		2.5 S**		4.0 S**		2.5 F**		4.0 F**		2.5 F**		4.0 F**		2.5 F**		4.0 F**		2.5 F**		4.0 F**	
Fragmn	Peptides	G*	D*	G*	D*	G*	D*	G*	D*	G*	D*	G*	D*	G*	D*	G*	D*	G*	D*	G*	D*
49-65	IAEKKADAVTLDGGMVF							O		O											
52-65	KKADAVTLDGGMVF							O													
57-63	VTLDSGM																		O		
57-70	VTLDGGMVFEAGRD									O											
57-78	VTLDGGMVFEAGRDPYKLRPVA									O		O				O					
57-79	VTLDGGMVFEAGRDPYKLRPVAA											O		O							
58-63	TLDGGM				O																
60-66	DSGMVFE																		O		
60-78	DGGMVFEAGRDPYKLRPVA									O											
66-77	EAGRDPYKLRPV								O												
66-78	EAGRDPYKLRPVA		O			O				O				O				O			
66-79	EAGRDPYKLRPVAA							O						O		O		O			
66-92	EAGRDPYKLRPVAAEIYGTKESPQTHY	O								O						O					
66-96	EAGRDPYKLRPVAAEIYGTEKSPQTHYYAVA																	O			
67-78	AGRDPYKLRPVA							O						O						O	
68-78	GRDPYKLRPVA							O													
69-93	RDPYKLRPVAAEIYGTEKSPQTHYY																		O		
71-78	PYKLRPVA																			O	
73-81	KLRPVAAEI														O						
74-82	LRPVAAEIY														O						
74-84	LRPVAAEIYGT														O						
79-86	AEIYGTEK																	O			O
79-92	AEIYGTKESPQTHY								O					O		O			O	O	
79-96	AEIYGTKESPQTHYYAVA									O								O			
80-92	EIYGTKESPQTHY									O		O									
81-92	IYGTEKSPQTHY											O							O	O	
82-92	YGTKESPQTHY											O									
100-109	KGSNFQLDQL		O				O								O						
100-111	KGSNFQLDQLQG										O		O		O						
100-112	KGSNFKLDQLQGQ																		O		
101-111	GSNFQLDQLQG	O	O				O									O	O				
104-112	FQLDQLQGQ							O													

122-134	SAGWVIPMGILRP		O						O						O						
122-135	SAGWVIPMGILRPY				O		O				O		O		O		O				
122-139	SAGWVIPMGILRPYLSWT							O		O				O		O					
125-135	WVIPMGILRPY														O						
125-136	WVIPMGILRPYL	O														O					
125-137	WVIPMGILRPYLS							O													
125-138	WVIPMGILRPYLSW					O						O									
125-139	WVIPMGILRPYLSWT	O				O			O	O											
125-140	WVIPMGILRPYLSWTE					O		O		O				O		O					
126-135	VIPMGILRPY				O		O				O		O								
126-138	VIPMGILRPYLSW														O						
126-139	VIPMGILRPYLSWT							O													
128-135	PMGILRPY										O		O		O						
130-136	GILRPYL														O						
130-138	GILRPYLSW														O						
130-145	GILRPYLSWTESLEPL										O				O						
130-151	GILRPYLSWTESLEPLQGAVAK														O						
131-139	ILRPYLSWT							O													
131-140	ILRPYLSWTE							O						O							
131-152	ILRPYLSWTESLEPLQGAVAKF											O				O					
136-149	LSWTESLEPLQGAV												O		O						
136-150	LSWTESLEPLQGAVA	O	O				O						O		O						
136-152	LSWTESLEPLQGAVAKF										O				O						
136-153	LSWTESLEPLQGAVAKFF		O		O						O				O		O				
137-152	SWTESLEPLQGAVAKF	O				O		O		O		O		O		O					
137-152	SWTESAEPLQGAVARF																		O	O	
139-152	TESLEPLQGAVAKF					O		O						O							
139-153	TESLEPLQGAVAKFF										O				O						
140-150	ESLEPLQGAVA										O										
140-152	ESLEPLQGAVAKF							O	O	O				O		O					
140-152	ESAEPLQGAVARF																			O	
140-153	ESLEPLQGAVAKFF									O		O									
141-152	SAEPLQGAVARF																		O		
141-152	SLEPLQGAVAKF							O		O	O	O		O		O					
141-152	SAEPLQGAVARF																			O	

141-153	SLEPLQGAVAKFF														O					
206-215	VAFVKETTVF									O										
209-227	VKETTVFENLPEKADRDQY																	O	O	
209-229	VKETTVFENLPEKADRDQYEL							O				O						O		
215-228	FENLPEKADRDQYE	O									O		O							
215-229	FENLPEKADRDQYEL																	O		
216-224	ENLPEKADR							O					O							
216-226	ENLPEKADRDQ				O						O		O							
216-227	ENLPEKADRDQY							O		O					O				O	
216-228	ENLPEKADRDQYE	O					O					O	O		O					
216-229	ENLPEKADRDQYEL							O			O							O			O
222-229	ADRDQYEL	O	O																	
237-242	APVDAF		O								O									
247-254	LAQVPSHA										O							O		
257-270	ARSVDGKEDLIWKL							O				O								
258-266	RSVDGKEDL												O	O			O			
258-270	RSVDGKEDLIWKL							O												
259-272	SVDGKEDLIWKLLS																O			
259-277	SVDGKEDLIWKLLSKAQEK								O											
267-288	IWKLLSKAQEKFGKNKSRSFQL															O				
267-299	IWKLLSKAQEKFGKNKSRSFQLFGSPPGQRDLL													O						
267-288	IWELLRKAQEKFGKNKSQRFQL																		O	
270-288	LLRKAQEKFGKNKSQRFQL																		O	
271-288	LRKAQEKFGKNKSQRFQL																		O	O
271-288	LSKAQEKFGKNKSRSFQL	O										O		O		O				
283-289	SRSFQLF												O				O			
285-296	SFQLFGSPPGQR	O		O					O											
285-299	SFQLFGSPPGQRDLL							O		O		O		O						
285-301	SFQLFGSPPGQRDLLFK	O																		
285-307	SFQLFGSPPGQRDLLFKDSALGF								O			O				O				
286-299	FQLFGSPPGQRDLL					O		O		O		O		O						
286-307	FQLFGSPPGQRDLLFKDSALGF															O				
289-299	FGSPPGQRDLL		O			O			O	O	O			O							
289-300	FGSPPGQRDLLF											O		O						
289-307	FGSPPGQRDLLFKDSALGF			O				O				O				O				

290-314	GSPPGQRDLLFKDSALGFLRIPSKV		O																	
297-306	DLLFKDSALG						O		O				O		O					
297-307	DLLFKDSALGF												O							
297-208	DLLFKDSALGFL															O				
297-314	DLLFKDSALGFLRIPSKV			O									O				O			
297-317	DLLFKDSALGFLRIPSKVDSA		O		O												O			
301-317	KDSALGFLRIPSKVDSA								O											
302-312	DSALGFLRIPS				O															
302-314	DSALGFLRIPSKV		O		O												O			
302-316	DSALGFLRIPSKVDS								O											
302-317	DSALGFLRIPSKVDSA		O										O							
306-314	GFLRIPSKV		O																	
308-315	VRIPSKVD																		O	
308-316	LRIPSKVDS												O		O					
308-317	LRIPSKVDSA				O						O		O		O	O				
308-318	LRIPSKVDSAL	O					O	O				O	O		O					
308-320	LRIPSKVDSALYL													O						
308-324	LRIPSKVDSALYLGSRY			O		O					O	O		O						
308-328	LRIPSKVDSALYLGSRYLTTL					O				O						O				
308-336	VRIPSKVDSALYLGSRYLTALKNLRETAE																		O	
309-318	RIPSKVDSAL																			O
312-317	SKVDSA						O													
318-328	LYLGSRYLTAL																		O	
318-328	LYLGSRYLTTL													O						
319-328	YLGSRYLTTL											O								
319-336	YLGSRYLTTLKNLRETAE										O			O		O				
319-337	YLGSRYLTALKNLRETAEE																	O		
325-336	LTTLKNLRETAE							O		O				O						
325-337	LTALKNLRETAEE																	O		
329-338	KNLRETAEEV														O					
438-448	VVKKANEGLTW							O												
463-273	RTAGWNIPMGL							O												
550-560	ENTNGESTADW							O	O		O		O		O					
550-562	ENTNGESTADWAK							O	O											
563-571	NLNREDFRL							O												

589-599	LAVAPNHAVVS								O												
590-599	AVAPNHAVVS														O						
596-617	AVVSRSDRAAHVKQVLLHQQAL											O									
604-619	AAHVKQVLLHQQALFG		O																		
604-620	AAHVKQVLLHQQALFGK								O												
609-619	QVLLHQQALFG				O								O				O				
611-619	LLHQQALFG	O																			
612-619	LHQQALFG		O								O						O				
618-631	FGKNGKNCPDQFCL																		O		
618-640	FGKNGKNCPDQFCLFKSETKNLL																	O			
651-659	LGGRPTYEE				O																
651-660	LGGRPTYEEY										O	O	O		O						
651-661	LGGRPTYEEYL						O								O						
651-664	LGGRPTYEEYLGTE						O				O		O		O	O	O				
651-665	LGGRPTYEEYLGTEY	O	O				O	O					O								
651-667	LGGRPTYEEYLGTEYVT														O						
652-660	GGRPTYEEY										O										
666-674	VTAIANLKK						O														

Curriculum Vitae

I was born on the 13th of August 1977 in Thirthahalli, Karnataka, India. After completing my intermediate college studies in Natural Sciences in 1995, I joined Kuvempu University (Karnataka, India) and got my B.Sc degree (Chemistry, Physics, Mathematics) in 1998. After that I started my M.Sc degree (Chemistry) in 1998 in Kuvempu University. I got my M.Sc degree in 2000. In the beginning of 2001, I joined as a Project Assistant under the guidance and supervision of Dr. V. Prakash, Director, Central Food Technological Research Institute (CFTRI) Mysore (A Constituent Laboratory of CSIR Delhi), India. During 2001-2002, I worked on a project 'purification and physicochemical/biophysical characterization of proteins and enzymes' in the Department of Protein Chemistry and Technology (CFTRI). Thereafter in 2003 I joined the Laboratory of Biophysics at Wageningen University, The Nethelands where under the supervision of Prof. Marcus Hemminga I worked as a M.Sc student on the European Union Project MIVase - New Therapeutic Approaches to Osteoporosis: targeting the osteoclast V-ATPase. In 2005, I worked as a Lecturer in the Department of Chemistry at Goutham Institute of Medical Sciences and Technology, Bangalore, India. In 2006, I got a Senior Research Fellowship (SRF) from CSIR Delhi, India and worked in CFTRI on a project 'purification and characterization of fish actomyosin'. The project was collaborated with Mangalore Fisheries College, Mangalore, India. During my SRF period for 6 months, I worked under the guidance and supervision of Dr. V. Prakash (CFTRI) and Prof. B. A. Shamasundar (Mangalore Fisheries College). I enrolled as a Ph.D scholar (2006-2010) in Food Science under the guidance and supervision of Prof. Gerd E. Vegarud (Principal supervisor), Prof. Ragnar Flengsrud (Co-supervisor) and Prof. Thor Langsrud (Co-supervisor) in the Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Norway. My Ph.D project was carried out in collaboration with the Department of Protein Chemistry and Technology, CFTRI, Mysore, India. Dr. V. Prakash was the guide and supervisor during my project work in CFTRI. Dr. Purnima Kaul has co-supervised me during the project work in CFTRI. The title of my PhD project is 'Structure, Stability and Digestion of Caprine and Bovine Lactoferrin. A Comparative Study'. My doctoral degree defense will take place on 18th March 2011 in Norwegian University of Life Sciences, Norway. Currently looking forward for a suitable postdoctoral level scientist position.