Diss. ETH No. 12947

Compositional and Structural Analysis of Camel Milk Proteins with Emphasis on Protective Proteins

A DISSERTATION
For the Degree of
DOCTOR OF TECHNICAL SCIENCES
Submitted to the
EIDGENÖSSISCHE TECHNISCHE HOCHSCHULE
SWISS FEDERAL INSTITUTE OF TECHNOLOGY
Zurich

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Zurich 1998

To my family, especially my parents,
much appreciation

ACKNOWLEDGEMENTS

I would like to thank Prof. Z. Puhan and Dr. Z. Farah for giving me the chance to carry out this thesis and for their guidance and advise in the course of the work.

I thank Prof. Stranzinger for taking over the external examination of this work.

I am in debt to Prof. Teuber and the Laboratory of Food Microbiology, who allowed me to carry out all molecular biological experiments having unrestricted access to their laboratories and equipment.

I wish to thank Prof. Brückner of the University Giessen, and Dr. Greiling and Mr M. Leitenberger, of the University Hohenheim, Stuttgart, who helped me in many ways in the course of the thesis.

Doing research about an animal, which lives in arid regions, but not in Switzerland, I was happy and thankful, that I could do part of my research at the Central Veterinary Research Laboratory, Dubai, under assistance of Dr. Dr. habil. U. Wernery and his group. Especially I would like to thank Dr W. Hölzer and Dr. J. Kinne, who helped me to carry out my experiments.

I also would like to thank the Evans family and Ms D. Atkins, for giving me the possibility to do research at Ol Maisor Farm, Rumuruti, Kenya, as well as to Dr M. Younan and Dr E. Beretta for doing biopsy of udder tissue.

I wish to thank Dr. M. Hässig of Tierspital Zürich, who often advised me in questions about anatomy and veterinary medicine.

I am also very grateful to Mr and Mrs Breitling of the Kamelfarm Fatamorgana in Rotfelden, Germany, for giving me a hearty welcome every time I went there and for providing me with blood and milk samples of excellent quality.

Last but not least, I wish to thank my colleagues at the Laboratory of Dairy Science, for providing a sociable working atmosphere and assisting me in numerous ways.

ABBREVIATIONS

aa	Amino Acid
Ab	Antibody
ABTS	2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonic acid
AS	Aminosäure(n)
α_{s1}-CN	α-Casein, calcium sensitive type 1
α_{s2}-CN	α-Casein, calcium sensitive type 2
β-CN	β-Casein
κ-CN	κ-Casein
kDa	Kilo Dalton
HPLC	High Pressure Liquid Chromatography
LINE	Long Interspersed Repetitive Element
LP-s	Lactoperoxidase-system
MALDI-MS	Matrix Assisted Laser Desorption/Ionization Mass Spectrometry
MFG	Milk Fat Globule
MFGM	Milk Fat Globule Membrane
ORF	Open Reading Frame of a cDNA or genomic DNA sequence
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
pfu	Plaque Forming Units
PGRP	Peptidoglycan Recognition Protein
PP3	Proteose Peptone Component 3
SDS	SodiumDodecyl Sulphate
SINE	Short Interspersed Repetitive Element
TFA	Trifluoroacetic Acid
WAP	Whey Acidic Protein

SUMMARY

The present study aims to contribute in characterisation of the camel milk protein fraction.

Proteins from milk of Somali and Arabian camel breeds *(Camelus dromedarius)* were separated by acid precipitation into a casein and a whey fraction. Both fractions were further separated by reversed-phase HPLC chromatography, whey proteins also by Heparin-Sepharose chromatography. Purity of isolated proteins was verified by SDS-PAGE. Proteins were quantified by absorption spectrometry at 280 nm, or by peak integration at 220 nm. The structures of the isolated proteins, as well as of gastric aspartic proteases, were characterised by mass spectrometry, N-terminal sequence analysis, and cDNA sequence analysis. It was concluded, that camel and cow milk strongly differ in the composition of both, casein and whey protein fraction, and that camel and bovine milk proteins exhibit distinct structural variations.

Tryptic digests of α_{s1}-, α_{s2}-, β- and κ-CN, and cDNA clones corresponding to the four caseins, were sequenced. The number of residues were α_{s1}-CN 207, α_{s2}-CN 178, β-CN 217, κ-CN 162. Similarity to bovine proteins was α_{s1}-CN A 60.3%, α_{s2}-CN 73.0%, β-CN 80.4%, κ-CN 73.8%. Acid precipitated casein of pooled milk was separated by reversed-phase HPLC and monitored at 220 nm, and its composition, estimated from peak integration, was α_{s1}-CN 22%, α_{s2}-CN 9.5%, β-CN 65%, κ-CN 3.5%. Degrees of phosphorylation and glycosylation were determined by mass spectrometry and sequence pattern analysis. Molecular masses determined were α_{s1}-CN A, 24.7 kDa and 24.8 kDa; α_{s1}-CN B, 25.3 kDa; α_{s2}-CN, 22.0 kDa; β-CN, 24.9 kDa; κ-CN, 22.3-23.0 kDa. The pH values of the most probable isoelectric points were: α_{s1}-CN A 6P, 4.41; α_{s1}-CN B 6P, 4.40; α_{s2}-CN 9P, 4.58; β-CN 4P, 4.66; κ-CN 1P, with 10 sialic acid residues bound, 4.10. It was concluded, that the low gel firmness of renneted camel milk, as compared to renneted milk of true ruminants, is a result of the different composition of the casein fraction, and marked variations in the κ-CN primary structure.

The structure of chymosin and pepsin mRNA from gastric mucosa of camels was determined, to understand specificity of interaction with κ-CN. Chymosin was 323 aa residues long, with a molecular weight of 35.6 kDa and an isoelectric point at pH 4.71. It shared 96.9% sequence similarity with

bovine chymosin. Specificity pockets for interaction with the chymosin sensitive region in κ-CN were found to have more pronounced hydrophobic or hydrophilic characteristics than those in bovine chymosin, and were thus probably better suited for hydrolysis of the scissile bond in camel κ-CN. Pepsin was 327 aa residues long, with a molecular weight of 34.9 kDa and an isoelectric point at pH 3.16. It shared 98.5% sequence similarity with porcine pepsin and was supposed to have a similar activity. It was concluded, that the higher activity of camel chymosin in milk renneting, compared to bovine chymosin, was based on higher specificity towards its natural substrate, which is camel milk κ-CN, and that large-scale production should be envisaged.

Whey proteins of camel milk were examined with focus on their relative distribution, as compared to whey proteins of cow milk, and with special interest in proteins with possible antimicrobial activity.

A novel milk protein was isolated by heparin-sepharose affinity chromatography from camel whey, which was found at a high concentration of 370 mg l^{-1} and had 91.2% similarity with human peptidoglycan recognition protein (PGRP). The protein was 172 aa residues long, with a molecular mass of 19.117 kDa, and an isoelectric point at pH 8.73. In analogy to homologous proteins from the immune system of vertebrates and invertebrates, activity against gram-positive, and probably against gram-negative bacteria, and a potential anti-tumour activity was proposed.

A camel whey protein with 83.6% amino acid sequence similarity to lactophorin from bovine whey and 67.9% similarity to the mouse *(Mus musculus)* glycosylation dependent cell adhesion molecule GlyCAM-1, was found to be a product of a alternatively spliced gene. About 75% of the protein was expressed as a long variant A with 137 aa residues and a molecular weight of 15.7 kDa, about 25% was expressed as a short variant B with 122 aa residues and a molecular weight of 13.8 kDa. Both proteins were probably three-fold phosphorylated. In contrast to bovine lactophorin and mouse and rat GlyCAM-1, no glycosylation was found in the camel whey protein. Due to this difference, specific interaction with carbohydrate binding proteins, as reported for GlyCAM-1, was excluded, and a function of the protein other than cell recognition or rotaviral inhibition was proposed. Higher amounts of the protein were found in camel milk, with about 954 mg l^{-1}, than in cow milk, with about 300 mg l^{-1}. In analogy to bovine lactophorin, the protein was proposed to have a function in prevention of fat globule aggregation, e.g. during secretion of fat globules into the alveolar

lumen of the lactating udder, and to be a natural inhibitor of spontaneous lipolysis by lipoprotein lipase.

Lactoferrin of camel whey was isolated by heparin affinity chromatography. The protein sequence, as deduced from cDNA, had 91.6% similarity to bovine lactoferrin, the same length of 689 aa residues, a molecular mass of about 80.5 kDa and an isoelectric point at pH 8.14. The protein was probably two-fold glycosylated, and was found to have two centres for binding of Fe^{3+} and CO_3^{2-}. An antimicrobial N-terminal proteolytic breakdown product was proposed in analogy to bovine lactoferricin. The protein was found in camel milk at a higher concentration of 220 mg l^{-1}, than in cow milk with 140 mg l^{-1}.

Lactoperoxidase, as deduced from cDNA, was 612 aa residues long, with a molecular mass of 69.5 kDa for the unmodified protein and an isoelectric point at pH 8.63. Analysis of the primary structure, which had 94.9% homology to cow lactoperoxidase, showed potential for four sites of glycosylation, covalent binding of a ferric heme and strong binding of a calcium ion. Similar activity in a lactoperoxidase system as reported for bovine lactoperoxidase, was proposed.

The different composition of the camel milk whey and casein fractions, as compared to milk of ruminants, was suggested to be a result of genetic and environmental factors.

ZUSAMMENFASSUNG

Mit der vorliegenden Arbeit möchten wir zum besseren Verständnis der Proteinfraktion von Kamelmilch beitragen.

Milch von Somalischen und Arabischen Kamelen wurde durch Säurefällung in eine Kasein- und eine Molkenfraktion aufgetrennt. Beide Fraktionen wurden mit Hilfe der Phasenumkehr-Chromatographie weiter aufgetrennt. Ausserdem wurden Molkenproteine mittels Heparin-Affinitätschromatographie isoliert. Die Reinheit der Fraktionen wurde mit SDS-PAGE überprüft. Proteinkonzentrationen wurden mittels Absorptionsspektrometrie bei 280 nm gemessen, oder durch Integration der Signale, die während der Phasenumkehr-Chromatographie bei 220 nm aufgezeichnet wurden. Die Struktur der isolierten Milchproteine und der Labenzyme wurde durch Massenspektrometrie, Edman-Abbau und cDNA-Sequenzierung bestimmt. Sowohl die Zusammensetzung der Kasein- und Molkenfraktionen, als auch strukturelle Eigenschaften der einzelnen Proteine, unterschieden sich stark von Resultaten, die aus der Untersuchung von Kuhmilch bekannt waren.

Die Primärstruktur von α_{s1}-, α_{s2}-, β- und κ-CN wurde durch Edman-Abbau von tryptischen Fragmenten und durch cDNA-Sequenzierung bestimmt. α_{s1}-CN hatte 207, α_{s2}-CN 178, β-CN 217 und κ-CN 162 AS. Die Ähnlichkeit zu Kuhmilchproteinen war 60.3% für α_{s1}-CN, 73.0% für α_{s2}-CN, 80.4% für β-CN und 73.8% für κ-CN. Säuregefälltes Kasein von Mischmilch wurde ausserdem mittels Phasenumkehr-Chromatographie getrennt und bei 220 nm aufgezeichnet. Aufgrund der Signalintensität wurde der relative Gehalt von α_{s1}-CN auf 22%, von α_{s2}-CN auf 9.5%, von β-CN auf 65% und von κ-CN auf 3.5% des Gesamtkaseins geschätzt. Seitenketten der Proteine, die möglicherweise posttranslational phosphoryliert oder glykosyliert waren, wurden durch Massenspektrometrie und durch die Analyse von Erkennungsmustern in der Primärstruktur bestimmt. Für α_{s1}-CN A wurden Massen von 24.7 und 24.8 kDa gemessen, α_{s1}-CN B, α_{s2}-CN, β-CN und κ-CN hatte Massen von 25.3 kDa, 22.0 kDa, 24.9 kDa und 22.3-23.0 kDa. Der isoelektrische Punkt von α_{s1}-CN A 6P war bei pH 4.41, von α_{s1}-CN B 6P bei 4.40, von α_{s2}-CN 9P bei 4.58, von β-CN 4P bei 4.66 und von κ-CN 1P mit 10 gebundenen Salicilsäureresten bei 4.10. Die geringe Gallertfestigkeit von labbehandelter Kamelmilch, verglichen mit labbehandelter Milch von echten Wiederkäuern, wurde auf die verschiedenartige Zusammensetzung

der Kaseinfraktion, und auf deutliche Unterschiede in der κ-CN-Struktur zurückgeführt.
Die mRNA von Chymosin und Pepsin aus der Schleimhaut des Labmagens eines Kamels wurde sequenziert, um die spezifische Interaktion der Enzyme mit κ-CN zu analysieren. Kamel-Chymosin hatte eine Länge von 323 AS, ein Molekulargewicht von 35.6 kDa und einen isoelektrischen Punkt bei pH 4.71. Die Ähnlichkeit zu bovinem Chymosin war 96.9%. Sogenannte Spezifizitätstaschen für die Interaktion mit der chymosin-sensitiven Region von κ-CN wurden gefunden, mit hydrophilen oder hydrophoben Eigenschaften, die ausgeprägter waren, als diejenigen in bovinem Chymosin. Daraus wurde geschlossen, dass Kamel-Chymosin zur Hydrolyse von Kamel-κ-CN wahrscheinlich besser geeignet ist als bovines Chymosin. Kamel-Pepsin hatte eine Länge von 327 AS, ein Molekulargewicht von 34.9 kDa und einen isoelektrischen Punkt bei pH 3.16. Die Ähnlichkeit zu Schweine-Pepsin war 98.5%. Es wurde angenommen, dass die Aktivität beider Enzyme ähnlich war. Die höhere Aktivität von Kamel-Chymosin, die für die Gerinnung von Kamelmilch im Vergleich zur Aktivität von bovinem Chymosin nachgewiesen worden war (Wangoh, 1993), wurde auf die hohe Enzym-Substrat-Spezifität von Chymosin und κ-CN zurückgeführt, und es wurde angeregt zu prüfen, ob sich das Enzym für die Käseproduktion eignen würde.

Die Molkenproteine der Kamelmilch wurden im Hinblick auf ihre Konzentration, im Vergleich zu Molkenproteinen der Kuhmilch, untersucht, mit besonderem Interesse an Proteinen mit möglicher antimikrobieller Aktivität.
Ein Protein wurde mittels Heparin-Sepharose-Affinitätschromatographie aus Kamelmolke isoliert, das keine Ähnlichkeit zu in der Literatur beschriebenen Molkenproteinen hatte. Die Konzentration in Molke wurde auf 370 mg l^{-1} geschätzt. Das Protein hatte 91.2% strukturelle Ähnlichkeit zum menschlichen Peptidoglycan Erkennungsprotein (PGRP), eine Länge von 172 AS, eine Masse von 19.117 kDa und einen isoelektrischen Punkt bei pH 8.73. Aufgrund der Wirkungsweise von strukturell verwandten Proteinen von Wirbeltieren und Invertebraten wurde vorgeschlagen, dass das Protein gegen grampositive, und wahrscheinlich auch gramnegative Bakterien wirksam ist.

Kamel-Lactophorin, ein Molkenprotein mit 83.6% Ähnlichkeit zu bovinem Lactophorin und 67.9% zum murinen Zelladhäsions-Molekül GlyCAM-1, wurde zu ca. 75% in einer langen Variante A mit 137 AS und einer Masse von 15.7 kDa, und zu ca. 25% in einer kurzen Variante B mit 122 AS und

einer Masse von 13.8 kDa gefunden. Die Heterogenität war die Folge von differenziellem Splicing und Verlust des zweiten Exons in Variante B. Beide Proteine waren vermutlich dreifach phosphoryliert. Im Gegensatz zu bovinem Lactophorin, und zu den Maus- und Rattenvarianten von GlyCAM-1, war das Kamelprotein nicht glycosyliert. Spezifische Interaktion mit zuckerbindenden Proteinen, wie sie bei GlyCAM-1 berichtet wurden, konnten deshalb ausgeschlossen werden, und eine Funktion des Proteins in der spezifischen Erkennung von Zelltypen oder in der Suppression von Rotaviren ist nicht wahrscheinlich. Die Konzentration in Kamelmilch war etwa 954 mg l^{-1}, während die Konzentration von bovinem Lactophorin in Kuhmilch lediglich etwa 300 mg l^{-1} betrug. Es wurde vermutet, dass das Protein eine ähnliche Funktion in Milch wie sein bovines Pendant ausübte, das die Zusammenballung der Fettkügelchen unterdrückt, z.B. während der Ausscheidung in die Milchdrüsen, und das die spontane Lipolyse des Milchfetts durch Lipoprotein Lipase hemmt.

Lactoferrin wurde aus Kamelmolke mit Hilfe der Heparin-Affinitätschromatographie isoliert. Die Proteinsequenz, die von der cDNA-Sequenz abgeleitet wurde, hatte 91.6% Ähnlichkeit mit bovinem Lactoferrin, hatte ebenso wie dieses Protein eine Länge von 689 AS, eine Masse von 80.5 kDa und einen isoelektrischen Punkt bei pH 8.41. Das Protein war wahrscheinlich zweifach glycosyliert und hatte zwei mögliche Zentren für die Bindung von Eisen und Karbonat. Analog zu bovinem Lactoferricin wurde ein N-terminales proteolytisches Abbauprodukt mit antimikrobiellem Potential vorgeschlagen. Die Konzentration des Proteins betrug 220 mg l^{-1}, und war höher als in Kuhmilch mit 140 mg l^{-1}.

Die Primärstruktur von Lactoperoxidase wurde aus ihrer cDNA-Sequenz abgeleitet, umfasste 612 AS, hatte eine Masse von 69.5 kDa und einen isoelektrischen Punkt bei pH 8.63. Die Ähnlichkeit zum bovinen Homolog war 94.9%. Ähnlich wie dieses Protein war Lactoperoxidase aus Kamelmilch vermutlich vierfach glycosyliert, hatte eine Häm-Gruppe kovalent und ein Kalziumion elektrostatisch gebunden. Eine vergleichbare Aktivität im Lactoperoxidase-System wurde vermutet.

Die unterschiedliche Zusammensetzung von Molke und Kasein, im Vergleich zu Milch von Wiederkäuern, ist vermutlich eine Folge von erblichen Faktoren und Umwelteinflüssen.

1 INTRODUCTION

The Arabian camel (*Camelus dromedarius*) is the most important livestock animal in the semi-arid areas of Northern and Eastern African countries. Of 19.6 million camels world-wide (dromedaries and bactrians), 14.6 million are held in Africa, 6.1 million of which in Somalia alone. Camels are multipurpose animals, they are used for milk, meat and hide supply, as well as for transport and for field cropping.

Whereas world-wide production of camel milk merely contributes to 0.23% of total milk production, it accounts for 38% of total milk production in Somalia. World-wide production of camel milk exceeds production of camel meat 5.3 times. Compared to world milk production of all livestock, which surpasses world meat production only 2.6 times, it is obvious, that camels are mainly held as a milk supplying livestock. Total milk yield of the 19.6 million camels was 1.3 billion litre in 1997. Average milk yield per animal was therefore 0.18 litre per day. Total milk yield of the 1.333 billion cattle was 471 billion litre, average milk yield per animal was therefore 0.97 litre per day. This figures implicate, that milk productivity of camels is more than five times lower than milk productivity of cattle. On the other hand, average milk yield of Somali camels was 0.38 litre per day, of Somali cattle only 0.30 litre per day (FAO, 1998). This figures illustrate, that camel milk yield is higher than the milk yield of cattle in arid countries. Milk yield per hectare is even higher in a mixed herd of camels and small ruminants, due to complementary grazing patterns. The Arabian camel is the livestock, which has the best chance to survive a prolonged drought period, and helps the camel keeping societies to survive one ore more dry seasons. Furthermore, due to their instinct for deferred grazing, camel herds help to keep the ecological balance intact.

Research on camels is scarce, especially on camel milk. Of a total of more than 150,000 publications indexed by CAB International, Oxon, UK, every year, an average of 210 publications was dedicated to camel research in general, and an average of merely 24 studies focused on camel milk research. As Fig. 1.1 illustrates, research with focus on camel milk was rare, compared to the much greater efforts in research about cow milk, but relatively continuous over the years. Nevertheless, only 0.33% of all research done about milk focused on camels in the years 1984 to 1998. Given the impact, this animal has on the social and economical situation in

arid countries, it is without doubt, that there should be envisaged a serious step up in camel milk research.

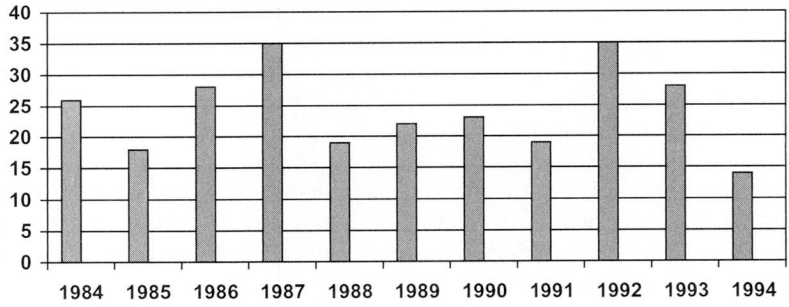

Fig. 1.1 Research studies published about camel milk in the years 1984 to 1994.

Due to the importance of camels as milk suppliers, research about camel milk should tackle technological problems first, in order to understand the technological properties of the milk. Main questions which arise when trying to make products from camel milk, and which cannot be answered satisfactorily until now, are:

- Why is the curd formed by fermentation or rennet coagulation of camel milk much weaker than the curd formed by coagulation of milk of ruminants?
- Why are proteins of camel whey more resistant to prolonged heat treatment than proteins of bovine whey? Due to this, proof of pasteurisation by the most common methods, e.g. denaturation of alkaline phosphatase, is not feasible with camel milk.
- Why is camel milk at high heat temperature less heat stable than cow milk (Farah & Atkins, 1992)?
- Why is growth of many strains of lactic acid bacteria retarded (Abu Tarboush, 1994 and 1996; Kamoun, 1995)?

The reasons for the different properties of camel milk and cow milk may be manifold. The survival of the offspring in a given environment depends on the immunological situation with regard to the placenta type, the colostrum, the development and stimulation of the immune system in the calf, and the nutritional properties in general. Certainly, the long period of the

paleontological development may have caused noticeable differentiation. The family of the Arabian and the Bactrian camels belong to the suborder of *Tylopoda*, together with the llama and vicuna families. Because all members of the *Camelidae* suborder ruminate, they were put into a close paleontological relationship with true ruminants. Most animals used for milk production are members of the *Bovidae* family of *Ruminantae*, e.g. cattle, sheep, buffaloes and goats. Recent research using morphological and molecular biological methods showed, that true ruminants are closer related to *Cetacea* (baleen and tooth whales) than to *Tylopoda* and *Suiformae*, which are more distantly related. For this reason, the order of *Cetacea* and the order of *Artiodactyla* (even toed ungulates) were put into one order *Cetartiodactyla* (Graur and Higgins, 1994). *Tylopoda* and *Suiformae* are probably closer related to each other than to *Ruminantae* (Martinat *et al.* 1991).

Differences in the immunological situation as a result of the different habitats, in which the animals live, adaptation to a sub-optimal food supply and quality, and differences in the way, the offspring is raised, as well as the more distant paleontological relationship, can give some explanations to generally observed differences between camel milk and milk of ruminants, in terms of chemical characteristics and technological properties. Furthermore, it has to be considered, that systematic selection for productive traits has never been done in camels (Schwartz & Dioli, 1992).

Most research done for characterisation of camel milk focused on the study of gross components, such as total protein, fat or minerals. Many properties of milk depend on the protein fraction. To understand the true nature of the different proteins, modern analytical methods for isolation and characterisation have to be applied. There is a need to know the distribution of the individual proteins in the casein and whey fraction, and to obtain insight into the primary structure and the way of modifications of the different proteins, which finally will lead to a better understanding of the processing quality of camel milk. In the course of this thesis, we tried to throw light on different aspects of the camel milk protein fraction.

Among these were:

- Quantification and structural analysis of camel milk caseins.
- Quantification of the main whey proteins α-lactalbumin and lactophorin.
- Structural analysis of different functional whey proteins: lactoferrin, lactoperoxidase, and peptidoglycan recognition protein.

- Structural analysis of renneting enzymes chymosin and pepsin, and comparison to the homologous proteins from cattle.

As there is little research already done in this area, and the milk protein fraction is a complex subject to investigate, the results of the present work can only give limited insight into the nature of camel milk proteins, but will give hints, which questions have to be addressed in further research on this subject.

In the present thesis, only *Camelus dromedarius* proteins were studied. Camel proteins described are named without annotation of species in the following text, to facilitate ease of reading.

2 LITERATURE REVIEW

This review was written with the intention to cover some general aspects with regard to camel milk, and not to give background information about the proteins studied. Literature concerning individual proteins will be discussed in the respective chapters of "Results and Discussion".

Taxonomy
The *Camelidae* belong to the order of *Artiodactyla* (even toed ungulates), and the suborder *Tylopoda* (pad footed animals). They are divided into the genus Camelus, with the two old-world species *Camelus dromedarius* (Arabian camel) and *Camelus bactrianus* (Bactrian camel), the genus *Lama*, with the new-world species *Lama glama* (llama), *Lama guanicoe* (guanaco), and *Lama pacos* (alpaca), and the genus *Vicugna* with the new-world species *Vicugna vicugna* (vicuna). Camelids spread from North America, where their ancestors originated, to South America, Asia and Africa in the late tertiary age. In North America they became extinct. The old-world species *Camelus dromedarius* and *Camelus bactrianus* can be crossed and the female offspring is most likely fertile in the first generation, but the males F_1 seem to be sterile. The Arabian camel was probably domesticated in the region of today's Yemen and Oman about 3,000 to 4,000 years ago and then introduced with the spice trade into North and East Africa, Persia and India. The wild Arabian camel became extinct (Schwartz & Dioli, 1992).

Camel breeds are not as much differentiated as breeds from other livestock. Systematic selection for productive traits has never been done in camels, with the exception of racing camels. There is a rough classification into the slender type, which is used for riding purposes, and the stout type, which is used for transport and milk production purposes, and which dominates in Eastern Africa. The weights of former type rarely exceed 400 kg in females and 550 kg in males, whereas females of the milk type have weights up to 650 kg and males up to 800 kg. The typical Somali dairy breed, which is the "Benadir" type, belongs to the heavy baggage type. It can be assumed, that there is a genetic variability , which is about as high as in cattle, upon study of body constitution, milk and meat production (Wilson, 1990).

Anatomy and Physiology
Camels are ruminating, but do not belong to the suborder *Ruminantia*. They differ from true ruminants in their foot anatomy, the absence of horns

or antlers and their stomach system. A similar system of multi-compartmented stomach digestion of fibrous food by rumination and by microbial fermentation evolved in *Ruminantia* and *Tylopoda* independently. Whereas the stomach of ruminants consists of four compartments, the stomach of camels is merely divided into three compartments. The compartment, where the renneting enzymes chymosin and pepsin are secreted, is the largest one, and is subdivided by a strong muscular ridge into a cranial and a caudal portion. The mechanism of digestion is also different in ruminants and camels. Whereas all digested food is mixed in the rumen and reticulum of ruminants, and transported in the organ homogeneously some hours after feed intake, the camels have developed a suction pressure rhythm to separate particles and fluids, whereby fluids are pressed into the glandular sacs for absorption, and feed particles are retained in the forestomach for prolonged microbial degradation (Schwartz & Dioli, 1992).

Arabian camels are especially adapted to a hot and arid environment. The hump of the camel serves to survive seasons of limited forage supply. Other livestock in arid areas, such as zebus and fat tailed sheep developed a similar organ. Even more important for survival in arid regions is short time water deprivation. Whereas cattle death occurs 3 to 4 days after water deprivation, healthy camels can survive up to 20 days. The mechanisms, which help to keep water requirement at a low level for a prolonged period of time are multiple. Water losses through urine are minimised by a superior system of urine concentration, faecal dehydration and retention of metabolites in the body fluids. Unlike other mammals, Arabian camels can tolerate body temperature fluctuations from 34 °C to 42 °C, thus saving a considerable amount of water during the daytime, mainly in the afternoon, in contrast to other animals, which regulate their body temperature by reinforced sweating. The body temperature is lowered in the night time beneath a level, which is physiologically tolerable for other mammals. (Schwartz & Dioli, 1992). Camels have developed a system of increased and diluted milk delivery in times of water deprivation, to prevent the newborn from dehydration (Yagil & Etzion, 1980).

Gross Composition of Camel Milk

There are several studies concerning the content of proteins, fat, lactose, minerals and vitamins in camel milk. Table 2.1 gives an overview of these data.

Table 2.1. Gross composition of camel milk and camel colostrum, compared to cow and human milks. (Abu-Lehia, 1987; Gorban & Izzeldin, 1997; Farah, 1996).

	Camel milk	**Cow milk**
Protein, g l^{-1}	27-40	27-47
Fat, g l^{-1}	32-38	average 38
Lactose, g l^{-1}	39-56	average 47
Minerals, mg l^{-1}		
Calcium	1,060-1,570	1,000-1,400
Copper	1.3-1.8	0.1-0.2
Inorganic Phosphate	580-1,040	650-1,100
Iron	1.3-2.5	0.3-0.8
Kalium	600-2,100	1,350-1,550
Magnesium	75-160	100-150
Mangane	0.08-0.2	0.04-0.2
Sodium	360-620	350-600
Zinc	4.0-5.0	3.5-5.5
Vitamins, mg kg^{-1}		
Ascorbic acid (C)	24-36	3-23
Cobalamin (B12)	0.002	0.002-0.007
Folic Acid	0.004	0.01-0.10
Niacin (B)	4.6	0.5-0.8
Pantothenic Acid	0.88	2.6-4.9
Pyridoxin (B6)	0.52	0.40-0.63
Retinol (A)	0.10-0.15	0.17-0.38
Riboflavin (B2)	0.42-0.80	1.2-2.0
Thiamin (B1)	0.33-0.60	0.28-0.90
Tocopherol (E)	0.53	0.2-1.0
Total Solids, g l^{-1}	10-11.5	12.5

The average concentration of total solids is slightly lower than in cow milk, but the relative amount of the main components protein, fat and lactose is similar to cow milk.

Main mineral salts in camel and cow milk are sodium chloride, calcium and magnesium phosphate, and citrate. The ionic strength of milk is low compared to body fluids. Nevertheless, the physical state of milk components, and the stability of proteins are strongly influenced by mineral salts, particularly by the phospho-caseinate complex. The reported levels for calcium and phosphate are similar to cow milk, the casein stabilisation potential is therefore similar in camel and cow milk. It has to be considered, that mineral and vitamin content of milk depend mainly on feeding, health status of the udder and stage of lactation.

Camel milk contains less vitamin A, B_1, B_2, E, folic acid and pantothenic acid. The opaque white appearance of camel milk may be a consequence of the low vitamin A level. The content of niacin and vitamin C is substantially higher than in cow milk. A higher level was also reported for carnitin (vitamin B_T). Whereas only 235-290 nmol l^{-1} were measured in fresh cow milk, 410 nmol l^{-1} were detected in camel milk (Alhomida, 1996). The higher proportions of these vitamins were considered to be an adaptation to the arid environment.

Physico-chemical Properties of Camel Milk

The chemical and technological characteristics of raw camel milk are different from those of raw cow milk. Basic parameters, which influence the suitability of the milk for processing, such as acidity, specific gravity and freezing point, were reported by several authors and usually compared to values of cow milk. The data collected show great variations and should be interpreted with care. Systematic errors may have arisen from experimental implementation and from the small sample sizes, which were studied. Some values may vary due to differences in animal feeding and husbandry. An additional constraint for interpretation is, that breeds are poorly characterised, which applies also to the camels chosen for investigation. There would be a need for a more systematic approach for determination of compositional values.

The value for the actual acidity of camel milk is similarly to cow milk between pH 6.5 and 6.75. The maximal buffering capacity of skim camel milk is at pH 4.95, compared to pH 5.65 for skim cow milk (Al-Saleh & Hammad, 1992). This gives indication that the composition of constituents

with buffering capacity is different between camel and cow milk. Titratable acidity is between an equivalent of 0.13-0.16% lactic acid in fresh milk, which is slightly lower than the mean value of 0.17% for cow milk, and seems to depend on the breed (Wangoh, 1997).

Whereas cow milk possesses a pronounced heat stability maximum at pH 6.7 and a minimum at pH 6.9, when determined at 130 °C, with stability decreasing at pH lower than 6.7 and increasing at pH higher than 6.9, camel milk does not show an increased stability at pH 6.7. Heat stability of camel milk is much lower than of cow milk. Heat coagulation time for cow milk at 130 °C is about 40 min at pH 6.7, whereas camel milk coagulates at this temperature and pH in 2 to 3 min (Farah & Atkins, 1992). There is no information available concerning the ethanol stability of camel milk.

The freezing point of camel milk was found to be between -0.57 °C and -0.61 °C (Wangoh, 1997). It is lower than the freezing point of cow milk, which is between -0.51 °C and -0.56 °C. A higher salt or lactose concentration in the camel milk, which was studied, as compared to cow milk, may have contributed to this result.

Values for specific gravity measured by Kamoun (1990) and Wangoh (1997) depended on the breed chosen and varied between 1.028 kg l^{-1} and 1.033 kg l^{-1}. These values are similar to values for cow milk, which are between 1.026 kg l^{-1} and 1.034 kg l^{-1}. Other authors reported lower viscosity and density for camel milk than for cow milk (Kamoun, 1995). A mean value for viscosity of Egyptian camel milk was 2.2 mPa s, which is higher than the mean value of 1.8 mPa s for cow milk (Hassan *et al.* 1987). These contradictory results may be explained by differences in husbandry, mainly in water supply. Hassan *et al.* (1987) also observed strong seasonal variations in milk viscosity, specific gravity and titratable acidity. Milk of heifers deprived from water for several days was reported to be more dilute, probably to protect the calf from dehydration during dry periods (Yagil & Etzion, 1980).

Composition and Characteristics of the Protein Fraction

The total protein content of camel milk is similar to cow milk. Values are in the range of 27 g l^{-1} to 40 g l^{-1} (Farah, 1996). The ratio of whey proteins to caseins is about 0.4, and thus higher than in cow milk, where it is about 0.2. Camel milk seems to have a slightly higher amount of non protein nitrogen than cow milk (Farah, 1996). There are no statistically significant data available to the average amino acid composition of the camel milk protein fraction.

The size distribution of casein micelles is in the range of 15 nm to 500 nm with a maximum in volume frequency between 260 nm and 300 nm (Farah & Rüegg, 1989). The distribution is significantly broader than that of cow milk, where it is in the range of 15 nm to 300 nm, with a maximum between 100 nm and 140 nm. The number of large micelles is significantly higher, which is unfavourable for formation of a firm coagulum in milk processing. A negative correlation between mean micelle size and κ-CN content of cow milk was reported (Schlimme, 1990). Small micelles of about 60 nm contained 12% κ-CN, large micelles of about 200 nm contained mere 2% κ-CN. Since these data also correlate in human milk, which has a mean micelle diameter of 20 nm to 60 nm and a rather high κ-CN content, it was assumed, that camel milk was low in κ-CN or devoid of this protein (Farah, 1996).

A markedly higher resistance of whey proteins towards heat treatment was reported (Farah, 1986). Degree of denaturation varied in camel milk from 32% to 35% at 80 °C. In cow milk, 70% to 75% of whey proteins were denatured at this temperature.

Activity of plasmin and plasminogen in camel milk of early lactation was shown to be below the level in cow milk (Baer *et al.* 1994). Furthermore, activation of plasminogen into plasmin by urokinase was threefold lower than activation of bovine plasminogen. Cross-reactivity of bovine and camel plasminogen, using antibodies against bovine plasminogen, was not detected, which indicated, that the two plasminogen types were structurally different. Slow activation and low activity may be a consequence of serine protease inhibitors in camel milk. The activity of camel and cow plasmin on camel casein were similar. Other authors reported higher proteolytic activity in camel milk than in cow milk (Abu-Tarboush, 1994).

Milk of non-ruminants, such as human, horse or pig milks, revealed high proportions of glycoproteins, which were connected to the milk fat globule membrane. These glycoproteins were similar in structure and function to intestinal mucins, with molecular masses up to 500 kDa and with up to 80% carbohydrate content (Schlimme, 1990). The main function of these proteins may be in prevention of fat globule aggregation and of lipolysis. They may also have an antiviral and antimicrobial effect. A high proportion of these proteins is also likely to occur in camel milk, since a far thicker MFGM was observed than in milk of other animals (Knoess *et al.* 1986).

Technology applied for Processing of Camel Milk

The far greatest amount of camel milk is consumed as a fresh or as a naturally fermented product. "Susa", a product consumed in North-Eastern Africa, is made by incubation of milk in smoke sanitised wooden buckets for about one to three days. The consistency of fermented camel milk is thin. A flocculent precipitate is formed, rather than a firm coagulum. Studies carried out in Kenya showed that the quality of "Susa" can be improved using selected, mesophilic starter cultures rather than spontaneous bacterial contamination for fermentation. The Somali consumers preferred this novel product to the traditional product (Farah *et al.* 1990). Growth of bacterial strains used for cow milk fermentation may be inhibited by the natural antimicrobial activity of camel milk (Elagamy *et al.* 1992). Stronger initial growth was reported for *Lactobacillus acidophilus* (Abu-Tarboush, 1994). This could be due to a higher content of non protein nitrogen in camel milk (Bayoumi, 1990).

Butter is traditionally produced by skimming of creamed up fat, and subsequent churning. This technique cannot be applied to camel milk fat, since the milk shows little tendency to cream up. Butter was produced by heating the milk at 65 °C for 30 min and separating the cream by centrifugation. To obtain a reasonable butter yield, camel cream was churned at temperature between 22 °C and 25 °C. The corresponding temperature for cow milk cream are between 8 °C to 14 °C. The reason for this difference is the high melting point of camel milk fat, which is at 40 °C to 41 °C. This seems to shift the ideal ratio of solid to liquid fat at given temperature towards a point higher than that of cow milk fat (Farah & Rüegg, 1989). It was shown, that creaming of camel milk fat was markedly improved by dissolution in skim cow milk. Cow milk fat dissolved in skim camel milk, on the other hand, showed a sharp decrease in the ability to cream up. It can therefore be concluded, that agglutinin (immunoglobulin M), the factor, which promotes creaming of cow milk fat, is low or devoid in camel milk (Farah & Rüegg, 1989). Churning of camel milk fat may be aggravated by the much lower ratio of lipid droplet to MFGM in camel milk fat globules. The average moisture content of camel butter is 12.65%, and thus much lower than the content in cow milk butter, which is 15.56% (Hagrass *et al.* 1987). This may explain the sticky texture of camel milk butter. Camel milk butter may be more susceptible to light oxidation, due to the higher amount of non saturated triglycerides. It would be well worthy to study the sensitivity of camel milk fat towards lipolysis and oxidation. Having in mind, that insolation in camel keeping countries is high and the total surface of milk fat is larger, since the volume to surface ratio of camel

milk fat globules is only 4.40 µm (Farah & Rüegg, 1989; Mehaia, 1995), compared to a value of 5.32 µm for cow milk fat globules, light oxidation of fresh camel milk may be a concern, since milk is often stored in transparent containers.

Similarly to horse milk, the renneting capability of camel milk is poor (Bayoumi, 1990). Addition of 2% $CaCl_2$ increased curd firmness slightly, whereas addition of higher percent amounts decreased coagulation time without further improvement of curd firmness. Renneting is probably low, because the mean size of casein micelles is about double of cow milk casein micelles. Electron micrographs showed, that the network formed at the coagulation point was less compact than in renneted cow milk, and the micelles were linked merely by contact adhesion, with little change in the original micellar structure, whereas the network formed in cow milk consisted of fused micelles (Farah & Bachmann, 1987). Cheese yield is in the range of 35% of milk dry mass, compared to about 85% for cow milk. This result may be explained by the lower amount of total solids, the poor rennetability, the smaller fat globules, the sodium concentration, which is often higher than in cow milk, and the higher proportion of whey proteins. Higher cheese yield was obtained with sophisticated technology, addition of $CaCl_2$ and fourfold higher chymosin concentrations than used in cow milk (Ramet, 1987). Higher cheese yields were also obtained, when the milk was blended with milk from ruminants (Ramet, 1991). Most studies on cheese production from camel milk report the production of a low fat cheese with slightly bitter taste (Farah, 1996). It can be assumed, that this type of cheese finds little consumer acceptability in camel keeping countries, where cheese has to be introduced as a novel product.

Different studies showed that seasonal variations in camel milk production are great and much of surplus milk is collected during the rainy season. Processing camel milk into pasteurised and fermented products will therefore be of great advantage, allowing the camel small-holder to commercialise his milk (Farah, 1996). Camel milk is commercially pasteurised in Saudi-Arabia and Mauritania. Problems may arise from the low heat coagulation time of camel milk and a tendency to flocculate. There are also environmental and socio-economical factors which make milk processing by pasteurisation a difficult task in arid countries (Abeiderrahmane & Reed, 1993).

A problem, which arises from the higher general heat stability of camel whey proteins is, that the most commonly used methods for determination

of pasteurisation all fail. The inactivation of phosphatase and lactoperoxidase do not occur to the same extent. Positive reaction of the former by short time heat denaturation is able to detect 0.1% raw milk in pasteurised cow milk. The latter is able to detect 5% raw milk in high temperature treated cow milk. Both reactions do not work in camel milk (Montet, 1997), even not when modified in a way similar to the pasteurisation proof of goats milk by alkaline phospatase.

Nutritional Quality and Therapeutic Use of Camel Milk
The gross composition of camel milk is similar to cow milk. Camel milk is therefore supposed to be nutritionally equivalent to cow milk.

The low proportion of vitamins A, B_1, B_2, E, folic acid and pantothenic acid is a disadvantage in the composition of camel milk. A balanced diet with camel milk as basic foodstuff should consider this aspect. Especially a problem is the low level of vitamin A, since green vegetables are a minor part of the diet in arid areas. Lack of vitamin A leads to a higher child mortality rate and, in extreme cases, to blindness. There is no information available about the vitamin D (calciferol) content of camel milk. Vitamin D is important for bone formation of children. A high amount in camel milk would therefore be desirable. A higher level of vitamin A and D could be achieved by appropriate feeding, whereas the vitamins of the B-group are mainly provided by the microflora of the rumen. The high content of vitamin C, niacin and carnitin is nutritionally important for camel milk consumers, since fruits and vegetables are scarce in arid areas (Farah, 1996).

The lipid fraction in camel milk is characterised by a high proportion of long chain fatty acids, which accounts for 96.4%, compared to 85.3% in bovine milk (Abu-Lehia, 1989). A higher proportion of short chain fatty acids would be favourable for consummation, since short chain fatty acids alleviate digestion of the triglycerides. The higher proportion of 43.1% non saturated fatty acids, compared to 38.8% in cow milk fat, is favourable for the body metabolism. Most prominent is palmitoleic acid with 10.4%, compared to 3.6% in cow milk fat, whereas the proportion of the essential linoleic acid is slightly lower in camel milk fat, with 2.9% compared to 3.2% in cow milk fat. There is no information about the amount of cholesterol in camel milk fat, which is about 0.4% in cow milk fat (Schlimme, 1990).

Camel milk is used in the traditional medicine of Northern and Eastern African countries for treatment of inflammation and wounds (Yagil, 1987).

Raw milk is also used in the therapy of diarrhea, mainly of newborn children, and of peptic ulcers. Complete healing of 57.5 % of human patients suffering from gastrointestinal ulcers, and treated with camel milk was reported, compared to 34.5 % after treatment with cow milk (Lozovich, 1995). A higher antimicrobial potential of raw camel milk compared to raw cow milk has been reported (Farah, 1996). There are also reports of camel milk used in non-conventional cancer therapy and treatment of neurodermitis and diabetes. Fermented camel milk (shubat) is sucessfully used in the treatment of peptic ulcers in Russia (Sukhov *et al.* 1986).

3 MATERIALS AND METHODS

3.1 Protein Analysis

Sample Preparation
Milk of individual Somali and Arabian camels was collected during milking at Ol Maisor Ranch, Rumuruti, Kenya, and at Kamelfarm Fatamorgana, Rotfelden, Germany, immediately frozen at -20 °C for transport and stored at -70 °C until analysis. After thawing, the milk, which had a pH of about 6.6, was skimmed at 1000 g, 4 °C for 15 min. The casein fraction was isolated by acid precipitation of 1 l milk at pH 4.6 and 37 °C for 20 min, using 0.1% acetic acid, followed by addition of 10 mM sodium acetate for neutralisation, and centrifugation at 4000 g for 5 min. Casein pellet and whey were frozen and stored at -70 °C. For crude preparation of an α- and a β-CN fraction, the casein pellet was dissolved in 200 ml 10 M urea, diluted with 460 ml double distilled water and the pH adjusted to 7.5 with 1 M sodium hydroxide. The solution was diluted with 200 ml double distilled water and adjusted to pH 5.0 with 1 M-HCl (Hipp *et al.* 1952). The firm precipitate consisted mainly of α- and κ- CN. After centrifugation at 600 g for 5 min, the supernatant was saturated with ammonium sulphate for precipitation of β-CN. Both precipitates were lyophilised. 1 g acid precipitated casein or lyophilisate were dissolved in 5 ml sample buffer with 10 M urea, 140 mM sodium citrate, 35 mM 1,3-Bis[tris(hydroxy-methyl)-methylamino]-propane, 780 mM β-mercaptoethanol, and 200 mM Tris-(hydroxymethyl)-aminomethan/HCl at pH 8.0, and stirred for 1 h. Whey was dialysed twice against double distilled water for 5 h at 4 °C, and once against 10 mM sodium phosphate buffer at pH 7.4 for 14 h, using an autoclaved SPECTRA/POR membrane tubing with a molecular cutoff of 6 to 8 kDa (Spectrum Medical Industries, Inc., Los Angeles, CA 90060 USA). Prior to chromatography, samples were filtered through a hydrophilic 0.45 µm membrane (ME25; Schleicher & Schuell, 37586 Dassel, Germany).

RP-C_{18} HPLC Chromatography
Individual caseins were separated by HPLC (LaChrom; Merck, D-64293 Darmstadt, Germany) on a silica-coated, analytical reversed-phase C_8 column (GromSil 200 ODS-4 HE, 5µ, 250 x 4.6 mm; Grom, D-71083 Herrenberg, Germany). Solvent A was 0.1% (v/v) TFA in double distilled, nanofiltered water, solvent B was 0.1% (v/v) TFA in acetonitrile. After injection of 10 µl to 50 µl casein filtrate, elution was performed by a linear

gradient from 0 to 35% solvent B over 15 min, followed by a linear gradient from 35 to 45% B over 35 min. The flow rate was 1 ml min^{-1} and runs were performed at ambient temperature. Whey proteins were separated by injection of 40 µl filtrate. Elution was performed by a 5 min hold with 0% solvent B, a linear gradient from 0 to 30% solvent B over 5 min, followed by a linear gradient from 30 to 70% B over 40 min. The flow rate was 1 ml min^{-1} and runs were performed at ambient temperature. For large scale isolation of individual caseins, a silica-coated, semi-preparative reversed-phase C_{18} column (GromSil 300 ODS-5 ST, 5 µ, 250 x 20 mm; Grom) was used to separate the proteins of the crude α- and β-CN fractions. After injection of 1 ml filtrate, elution was performed by a 9 min hold at 0% solvent B, followed by a linear gradient from 0 to 40% B over 3 min and a linear gradient from 40 to 43% B over 28 min. The flow rate was 9.5 ml min^{-1}, and runs were performed at 30 °C. The column effluent was monitored with a diode array detector (L-7450; Merck) from 200 to 300 nm. Proteins eluted were collected manually and lyophilised.

Heparin-Sepharose Affinity Chromatography
40 ml whey were loaded on a 1 ml Heparin Sepharose HiTrap column (Amersham Pharmacia, 751 25 Uppsala, Sweden). The column was washed with 10 ml of 10 mM sodium phosphate, 20 mM sodium chloride buffer at pH 7.4. Elution was performed at ambient temperature by a linear gradient from 0.02 to 1 M sodium chloride over 40 min. The column effluent was monitored with an UV detector (L-7300; Merck) at 280 nm. Proteins eluted were collected manually and lyophilised. Fractions were further purified, prior to micro-sequencing and molecular mass determination, by reversed-phase C_{18} HPLC. Elution was performed by a linear gradient from 0.1% TFA in double distilled, nanofiltered water, to 0.1% TFA in acetonitrile, over 60 min.

Amino Acid Sequencing
Proteins collected from the effluent of the semi-preparative column were used directly for N-terminal sequencing. Eluted proteins were applied on a TFA-treated cartridge filter and dried under continuous nitrogen flow. Automated Edman degradation (Matsudaira, 1989) was performed using an ABI 471A sequencer (PE Applied Biosystems, Foster City, CA 94404 USA), equipped with a 120A HPLC, for on-line reversed-phase C_{18} HPLC analysis of phenylthiohydantoinyl aa derivatives.

From each of the peaks corresponding to α_{s1}-, α_{s2}-, β- and κ-CN, 1 mg lyophilisate was dissolved in 1 ml of a buffer with 40% (v/v) acetonitrile,

60% (v/v) double distilled water, containing 400 mM ammonium carbonate, pH 9 and digested overnight at 37 °C with 25 µg trypsin (Sequencing grade, Boehringer, D-68305 Mannheim, Germany). Peptides were separated using the same analytical column and a linear gradient from 0 to 100% solvent B over 180 min. The flow rate was 1 ml min^{-1} and runs were performed at ambient temperature. Peptides eluted from the column were collected manually and dried by vacuum-centrifugation with a speed-vac SVC100 (Savant Instruments, New York, 11741-4306 USA). Samples were dissolved in 100 µl of 50% (v/v) acetonitrile, 50% (v/v) water. 20-100 µl were applied on a TFA treated cartridge filter and dried under a continuous nitrogen flow, and used for automated Edman degradation.

Evaluation of Protein Purity

About 1 µg sample was loaded on a 12.32% T, 2.7% C_{bis} sodium dodecylsulfate (SDS) polyacrylamide 1.5 mm vertical slab gel with a pH at 8.8, which was overlaid by a 4.62% T, 2.7% C_{bis} SDS stacking gel at pH 6.8. Samples were separated in a MiniProtean II™ apparatus (BioRad, Hercules, CA 94547 USA) at 40 mA. Proteins were stained with 1% Coomassie G-250 in 40% methanol, 10% acetic acid for one hour, followed by destaining in 40% methanol, 10% acetic acid overnight. A 1:10 diluted protein marker (V5231; Promega, Madison, WI 53711-5399 USA) was used for mass determination.

Mass Determination of HPLC Separated Proteins

Molecular masses of proteins were measured by matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS). Vacuum-dried casein and whey protein samples were dissolved in 39.5% (v/v) acetonitrile, 59.5% (v/v) double distilled water, 1% TFA. Samples were co-crystallised with an equal volume amount of α-cyano-4-hydroxy-cinnamic acid (5 g l^{-1}) in 0.2% TFA. 1-3 pmol sample was applied to the target, and air-dried at ambient temperature. For analysis, to a time-of-flight mass spectrometer in linear mode was used (Voyager Elite, PerSeptive Biosystems, Framingham, MA 01701 USA). Spectra were recorded using a nitrogen ultraviolet laser at 337.1 nm and an acceleration voltage of 25 kV. The instrument was calibrated with porcine myoglobin, a monomeric protein of 16,953 kDa.

Quantification

Protein peaks of the HPLC runs described in "Isolation of individual caseins" were integrated at 220 nm. Relative amounts of peaks corresponding to the different caseins and whey proteins were calculated

and the results were compared with weights of the lyophilized fractions and literature values (Farah, 1996).

3.2 DNA Analysis

PolyA-mRNA Isolation
Udder tissue of a lactating Somali camel (1 g) was taken in the morning after milking and immediately homogenised with a rotor-stator homogeniser (Kinematica, CH-6014 Littau, Switzerland). PolyA-mRNA was isolated with the Oligotex™ Direct mRNA Kit (Quiagen, D-40724 Hilden, Germany) according to the manufacturer's instruction for large scale preparation of mRNA. Total yield was 21.6 µg mRNA and the A_{260}:A_{280} ratio was 2.4. In the same way, polyA-mRNA was prepared from 500 µg of mucosa tissue from the rough and the smooth part of the rennet stomach of a 1.5 years old Arabian camel. Yield was 68 µg mRNA, and 60µg mRNA, respectively, with an A_{260}:A_{280} ratio of 1.9.

Construction of a cDNA Library
Single stranded cDNA, for use in PCR, was synthesised with a reverse transcription system (A3500; Promega), according to the manufacturer's recommendations.

A sample of mRNA (2 µg) was used for synthesis of cDNA using the Universal RiboClone® cDNA Synthesis System (Promega) with an oligo$(dT)_{15}$ primer and EcoR I adapters. One-fifth of the resulting cDNA was ligated to 1 µg dephosphorylated λ-gt11 arms (Promega). The ligated DNA was in-vitro packaged using an E. coli C Packagene® λ DNA extract (Promega). All work was done according to the manufacturer's instructions. Phages were plated on E. coli LE 392 (Promega). The titre of the library was estimated at $2.6x10^5$ pfu ml^{-1}. 100 µl of the library were amplified and produced a lysate with a titre of $1x10^8$ pfu ml^{-1}.

Sequence Analysis
The cDNA library was screened for cDNA corresponding to α_{s1}-, α_{s2}-, β- and κ-CN by nucleic acid hybridisation (Maniatis *et al.* 1989). Plaque lifts, hybridisation and signal detection were done with the digoxigenin (DIG) system of Boehringer, using uncharged nylon membranes, DIG EasyHyb solution, anti-DIG-AP Fab fragments and CSPD®, according to the manufacturer's instructions. Specific probes were synthesised by the

polymerase chain reaction (PCR) with DIG-11-dUTP to screen for cDNA corresponding to α_{s1}-, α_{s2}-, β- and κ-CN. Degenerate PCR primers were designed with the help of amino acid sequences obtained from sequencing the N-terminus and tryptic digests of the caseins (see above). The following primer pairs were used (IUB code for mixed base sites):

α_{s1}-CN
5'-TAYCCNGARGTNTTYCARAAY-3', derived from the sequence Tyr-Pro-Glu-Val-Phe-Gln-Asn at the N-terminus of α_{s1}-CN, and
5'-NGGRTGNGCDATRTAYTGCAT-3', derived from the sequence Met-Gln-Tyr-Ile-Ala-His-Pro, part of a prominent fragment of the α_{s1}-CN tryptic digest eluted at 123 min.

α_{s2}-CN
5'-AARCAYGARATGGAYCA-3', derived from the sequence Lys-His-Glu-Met-Asp-Gln, at the N-terminus of α_{s2}-CN, and
5'-TGRTCCCANGGRTTCAT-3', derived from the sequence Met-Asn-Pro-Trp-Asp-Gln, part of a major fragment of the α_{s2}-CN tryptic digest eluted at 101.5 min.

β-CN
5'-GARAARGARGARTTYAARACN-3', derived from the sequence Glu-Lys-Glu-Glu-Phe-Lys-Trp at the N-terminus of β-CN, and
5'-RTCNGGNACNGGYTCYTGRAA-3', derived from the sequence Phe-Gln-Glu-Pro-Val-Pro-Asp, part of a major fragment of the β-CN tryptic digest eluted at 53 min.

κ-CN
5'-GARGTNCARAAYCARGARCAR-3', derived from the sequence Glu-Val-Gln-Asn-Gln-Glu-Gln at the N-terminus of κ-CN, and
5'-GATCTCAGTCGAAGTAATTTG-3', derived from a sequenced PCR fragment of 320 bp, which was synthesised using genomic DNA of a Bactrian camel and bovine primers JK 501 and JK 302 (Schlee & Rottmann, 1992).

The base lengths of the probes against α_{s1}-, α_{s2}-, β- and κ-CN cDNAs were 528, 271, 597, and 486 respectively. Positive plaques were picked and verified by PCR, using the appropriate primer pairs from before. For each protein, the cDNA insert of one positive plaque was excised with partial

EcoR I-digest, ligated into a pGEM-7Z vector (Promega), dialysed, and transformed into *E. coli* XL1-Blue (Stratagene, La Jolla, CA 92037 USA) by electroporation with a Gene-Pulser® (BioRad) at 2.5 kV, 25 µFD, and 200 Ω in 0.2 cm cuvettes. The transformed bacteria were plated overnight at 37 °C on IPTG/X-Gal/Ampicillin-selective agar. White colonies were picked and grown overnight in 20 ml LB-Ampicillin 100 (Maniatis, 1989). Plasmid DNA was purified for fluorescent sequencing with the Wizard Plus SV Minipreps DNA Purification System (Promega).

Fluorescent sequencing of the cDNA was carried out using an ALF automated device (Amersham Pharmacia), internal Cy5™-dATP labelling (Amersham Pharmacia) and primer walking starting from commercial SP6 and T7 primers (Promega).

Overlapping fragments, which were produced by polymerase chain reaction (PCR), were used for sequence analysis of renneting enzymes and whey proteins. The following protocol was applied to most of the reactions: 2 µl of the λ-cDNA library or 0.5 µl of single stranded cDNA were taken as templates in 50 µl PCR assays with 2.5 units Taq Polymerase (Amersham Pharmacia), which was blended with 0.05 units Pfu Polymerase (Stratagene), and 5 µl 10x TaqPlus Precision incubation buffer (Stratagene), 20 nmol of each dNTP (Amersham Pharmacia) and 50 pmol of specific primers. 30 cycles were run with initial 2 min denaturation at 94 °C, followed by 10 sec denaturation at 94 °C, 30 sec annealing at 55 °C and 2 min 30 sec elongation at 68 °C. Elongation prolongation was 20 sec per cycle. A final 10 min incubation step at 72 °C was added to increase the concentration of full length products. Each PCR product was generated twice and ligated into a pGEM®-T Easy vector (Promega) according to the manufacturers instructions. In case of base reading ambiguities, a third PCR product was generated. Two λ-gt11 vector specific general primers were constructed for sequence analysis of full-length cDNA products of the respective clones:

λ-gt11 forward: 5'-GACGACTCCTGGAGCCCGTCAGTA-3'
λ-gt11 reverse: 5'-CACCAGACCAACTGGTAATGGTAG-3'

The following PCR products were generated, mostly with the help of highly conserved regions in the cDNA sequences of other species (mixed base sites according to IUB code):

Chymosin
A 0.15 kbp PCR product of chymosin exon 1 was generated with
5'-GTGGGCCCTGGCTACAGCAG-3', and
5'-TGGTGATCYCASYGCCYTGGGAGAG-3', and genomic camel DNA. This sequence was used to generate a 1.2 kbp PCR product of the chymosin ORF with
5'-TGACCAGGTCCAGGTCCAGGATGC-3', and
5'-GGSGACAGYGAGGTTYKTRGTCAGSG-3', and cDNA from stomach mucosa.

Pepsin
A 1.2 kbp PCR product was generated with
5'-KRGASTTGGGASCCRGGAAGAACC-3', and
5'-RGATCTTCCTGGGAGGTGGCTGGA-3', and cDNA from stomach mucosa.

Peptidoglycan Recognition Protein
A 0.3 kbp PCR product was generated with
5'-CCCGCCTGCGGTTCNATHGTNCC-3', and
5'-TGATGTTCCAGCCTCGGCCTTCAT-3', and cDNA from mammary gland tissue. This sequence was used to generate a 0.65 kbp PCR product with
5'-CCGAGTGCAGAGAAAGGCTAACAC-3', and λ-gt11 reverse, and a 0.4 kbp PCR product with
5'-CCATCTTCTCCGATCAGGAAGTTG-3', and λ-gt11 forward, and cDNA from mammary gland tissue.

Lactophorin
A 0.28 kbp and a 0.32 kbp PCR product were generated with
5'-GCCAGCTTGGCCGCCACCTCTCTC-3', and
5'-GGCATGAGGGAATAGGCTTTTCAG-3', and cDNA from mammary gland tissue. This sequence was used to generate a 0.6 kbp PCR product with
5'-CCACCTCTCTCGCCAGCCTTAATG-3', and λ-gt11 forward,
and a 0.55 kbp PCR product with
5'-AAAGTCCATGGTTTCTCTCATGGT-3', and λ-gt11 forward, and cDNA from mammary gland tissue.

To obtain the intron sequences of lactophorin, the PCR reaction, as described before, was applied, and 1 µl genomic DNA was used as a template, which was isolated from Arabian camels using a QIAamp Blood Kit (Qiagen GmbH, 40724 Hilden, Germany) according to the manufacturers' instructions.

A PCR product, which contained intron 1 was generated with
5'-ATGAAATTCTTCGCTGTCCTGCTG-3' and
5'-CTGAGACTCCATGTAGATTTCATC-3'.
A PCR product, which contained intron 2 was generated with
5'-GATGAAATCTACATGGAGTCTCAG-3'
5'-GACCTGATGGTTGCTCATGATGAC-3'
A PCR product, which contained intron 3 was generated with
5'-CAATCAGAAGAGACCAAAGAACTC-3'
5'-TATGATTTTATGAGTGAGCTCCAC-3'

Lactoferrin
A 0.4 kbp PCR product was generated with
5'-CTGTCCCATAGACCTCTGCCGCTA-3', λ-gt11 reverse, and cDNA from mammary gland tissue. A 0.8 kbp PCR product was generated with
5'-GTTCRRTGGTGTRCCRTMTCCMMA-3', and
5'-GTCTTTGAACAGCAGGTCCTTCTG-3', and cDNA from mammary gland tissue. A 1 kbp PCR product was generated with
5'-TTCCAGCTCTTTGGCTCYCC-3', and
5'-TTGAACAGAAGGTTTTTGGT-3', and cDNA from mammary gland tissue. A 0.4 kbp PCR product was generated with
5'-CCAGGCAAGTTTTGCTTGTTCCAG-3', λ-gt11 reverse, and cDNA from mammary gland tissue.

Lactoperoxidase
A 1.35 kbp PCR product was generated with
5'-CTTCTGCATCTCATCACCTAGCAC-3', λ-gt11 reverse, and cDNA from mammary gland tissue. A 0.24 kbp PCR product was generated with
5'-GGAGCAYAACCGGCTGGCCAGAGAA-3', and
5'-GTGGCCAAAGCGGAAGGCRAAGGTG-3', and cDNA from mammary gland tissue. A 1.35 kbp PCR product was constructed with
5'-GTGCTAGGTGATGAGATGCAGAAG-3', λ-gt11 reverse, and cDNA from mammary gland tissue.

Transformation, blue/white screening, bacterial culture and plasmid purification was done in the same way as with the pGEM-7Z vector. Fluorescent sequencing was carried out using an ALF automated device (Amersham Pharmacia) with standard operating procedures. Sequencing samples were prepared, using the Cy5™-dATP labelled, vector specific primers:

Cy5-SP6: 5'-TACTCAAGCTATGCATCCAACGCG-3', and
Cy5-T7: 5'-ACTCACTATAGGGCGAATTGGGCC-3',

and the Thermo Sequenase cycle sequencing kit RPN 2438 (Amersham Pharmacia) according to the manufacturer's instructions. The following 25 cycles were applied: 95 °C, 30 sec, 50 °C, 30 sec, 72 °C, 50 sec. Where sequencing results differed, a third PCR product was sequenced. Overlapping sequences were detected using the FASTA module of the gcg/egcg programme package (Genetics Computer Group, Madison, WI 53711 USA). Consecutive sequences were joined and vector specific sequences removed. In this way, complete cDNA sequences were obtained.

3.3 Computational Sequence Analysis

Alignments of DNA and protein sequences and DNA similarity searches were performed using the gcg/egcg programme package (Genetics Computer Group).

Genomic DNA was screened for interspersed elements using RepeatMasker (Smit, 1996).

Probability calculations for intron-exon junctions were made by a combined linear discriminant recognition function, using information about significant triplet frequencies in various functional parts of splicing site regions, and preferences of octanucleotides in protein coding and intron regions (Solovyev *et al.* 1994).

Protein sequence similarity searches against the Swissprot database (Swiss Institute of Bioinformatics, Geneva, Switzerland) were made using a Smith & Waterman algorithm with default values (Barton, 1997).

Secondary structure predictions were made using nearest neighbour analysis with local alignments (Salamov & Solovyev, 1997).

Potential for O-glycosylation was analysed, using the NetOGlyc 2.0. Prediction Server trained on mucin type O-glycosylation sites in mammalian proteins, which are glycosylated by the UDP-GalNAc-polypeptide N-acetylgalactosaminyltransferase family (Hansen *et al.* 1995).

Low resolution models of the tertiary structures of camel milk proteins were obtained by comparative modelling (Guex & Peitsch 1997). The primary structures, which were revealed by amino acid and cDNA sequencing, were threaded over tertiary structures of proteins with high similarity in primary structure. Multiple sequence alignments were made for improvement of modelling reliability. Energy minimisation of the model was done with force field computation by GROMOS96.

4 RESULTS AND DISCUSSION

4.1 Caseins

Literature

The protein fraction of cow milk consists to about 80% of caseins. Four different gene products are designated as α_{s1}-, α_{s2}-, β-, and κ-caseins, which together form micellar structures of 20 nm to 500 nm by non-covalent aggregation. α_{s1}-CN, α_{s2}-CN, and β-CN are structurally related and contribute to 38%, 10% and 36% of total casein. γ-CN, a term used for breakdown products of β-CN, contributes to about 3% of the casein fraction, and κ-CN, a protein, which is structurally not related to the other caseins, contributes to about 13% of total casein. All caseins share a distinct amphiphilic nature. The N-terminal region of κ-CN, and the C-terminal regions of α_{s1}-, α_{s2}-, and β-CN are pronounced hydrophobic. The N-terminal regions of α_{s1}-, α_{s2}-, and β-CN are hydrophilic and contain clustered phosphoserines, which bind inorganic calcium phosphate with extremely low dissociation rates. The C-terminal region of κ-CN is hydrophilic and poly-O-glycosylated. All caseins have a high proline content, which prevents formation of secondary structures, mainly of α-helices, in the intact protein. Cysteine was not found in α_{s1}-, α_{s2}-, and β-CN of any species analysed. The two cysteines found in bovine κ-CN were shown to form intermolecular crosslinks in micelles, which may support the rigidity of the structure and help in growth termination of micelles (Swaisgood, 1992). In camel milk, large micelles, which usually contain low amounts of κ-CN, were found in higher amounts than in cow milk (Farah & Rüegg, 1989). Due to the ability to bind inorganic calcium phosphate, casein is a rich source of this mineral, which is a prerequisite in bone formation of the suckling.

The colloidal-dispersed structure of caseins contributes to the low viscosity of the milk. Casein micelles are held together mainly by hydrophobic forces between the hydrophobic parts of the proteins, which are found in the core of the micelles, and by phosphoserine clusters, which bind calcium phosphate crystals with high affinity. Milk casein micelles are protected against hydrophobic forces, which promote aggregation and coagulum formation, by a hydrophilic, negatively charged layer of oligosaccharides, which cover the entire surface, and which are attached to C-terminal threonines of κ-CN. Proteolytic cleavage of the glycomacropeptide, which is the C-terminal part of κ-CN, by the renneting enzymes chymosin and

pepsin, leads to aggregation of casein micelles, which subsequently form a micellar network, observed as a firm coagulum. The coagulation process is promoted by acidification of the milk to pH 4.7, which leads to charge neutralisation, and by addition of calcium salts. Camel milk shows little tendency to coagulate upon rennet treatment and acidification, only a weak curd is obtained (Farah, 1996). The milk tends to flocculate, which is of disadvantage for manufacturing of cheese and fermented milk products. Addition of up to 2% $CaCl_2$ increases the strength of the coagulum slightly (Bayoumi, 1990).

With the present study, we intended to learn about the molecular organisation of the caseins found in camel milk micelles for comparison with cow milk caseins. Low rigidity of the coagulum may be a result of total casein concentration in camel milk, of the proportion of individual caseins, and of the protein properties, which are defined by primary structure and by post-translational modifications. We expected, that these properties are inherited by the breed, and independent of husbandry and feeding parameters. We therefore decided to choose randomly selected camels of the Somali breed, which is the most common breed world-wide with about 6 million animals, for investigation of milk and cDNA. The nucleotide sequences described in Figs 4.1 – 4.4 were therefore considered to represent the most frequent of presumed genetic variants of camel milk caseins. These sequences have been deposited to the GenBank™/EBI Data Bank with accession numbers AJ012628 (α_{s1}-CN A), AJ012629 (α_{s2}-CN), AJ012630 (β-CN), Y10082 (κ-CN).

We decided to present and discuss the results of camel milk casein analysis within one chapter, to take respect to the manifold interactions between the four caseins.

Table 4.1. Physicochemical characteristics of camel milk caseins compared to cow milk caseins[a]

Species	Casein	Amino acid residues	Molecular mass [kDa] based on		Isoelectric point[b] based on		Charged modifications of amino acid residues	Relative amount in total casein	Similarity to corresponding bovine proteins[c]
			Amino acid sequence	Mass spectrometry	Amino acid sequence	Amino acid sequence with modifications			
Camel	α_{s1}-CN A	207	24.275	24.755 24.668	4.78	4.41	6 Ser-P	22%	60.3%
Cow	α_{s1}-CN B	199	22.975		4.76	4.26	8 Ser-P	38%	
Camel	α_{s2}-CN	178	21.266	21.993	5.81	4.58	9 Ser-P	9.5%	73.0%
Cow	α_{s2}-CN A	207	24.348		8.68	4.78	11 Ser-P	10%	
Camel	β-CN	217	24.651	24.900	5.17	4.76	3 Ser-P	65%	80.4%
Cow	β-CN A2	209	23.583		5.01	4.49	5 Ser-P	39%	
Camel	κ-CN	162	18.254	22.294- 22.987	8.27	4.11	1 Ser-P, 10 Thr-NANA	3.5%	73.8%
Cow	κ-CN A	169	18.974		5.97	3.97	1 Ser-P, 12 Thr-NANA	13%	

NANA, *N*-acetylneuraminic (sialic) acid.
a Data on cow caseins after Eigel *et al.* (1984).
b Calculated with the gcg programme (Genetics Computer Group, Madison, WI 53711 USA).
c Similar and identical residues, as aligned in Fig. 4.9.

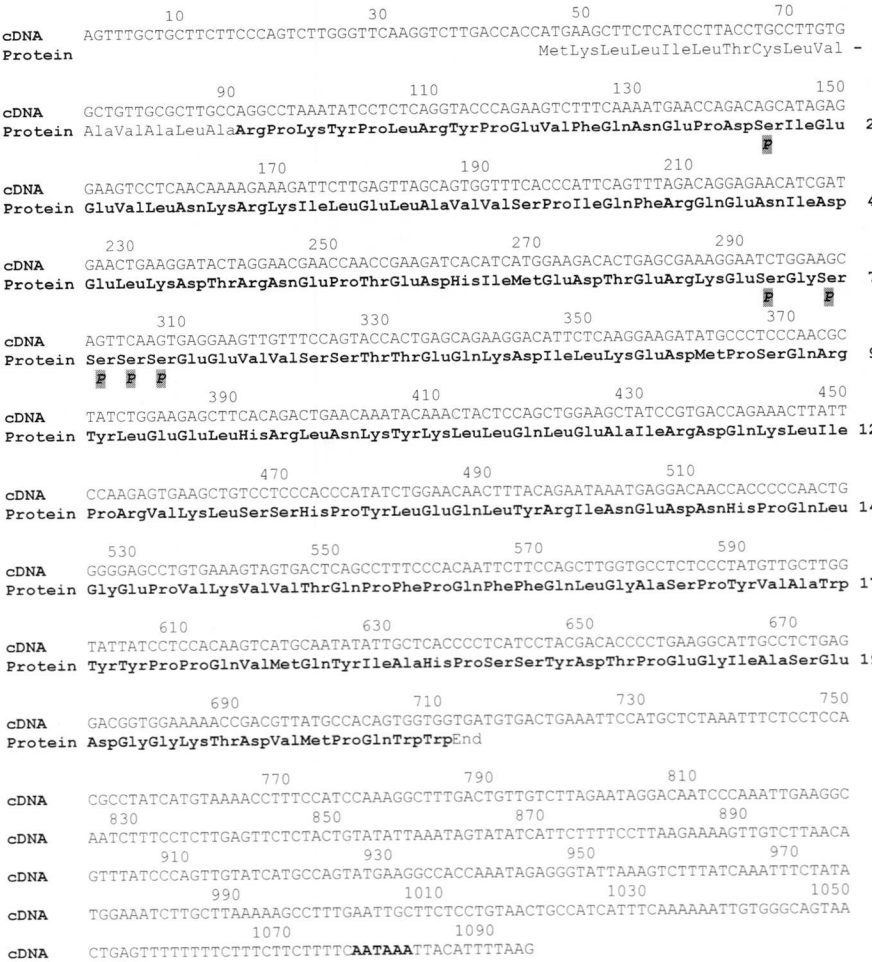

Fig. 4.1. cDNA sequence of camel milk α_{s1}-CN A and corresponding protein, mature protein in bold. The open reading frame of the cDNA sequence is from A^{46} to G^{711} and the polyadenylation signal in bold from A^{1077} to A^{1082}. Numbering of the amino acid chain starts from the first residue of the mature protein. ***P***, potentially phosphorylated serine residues.

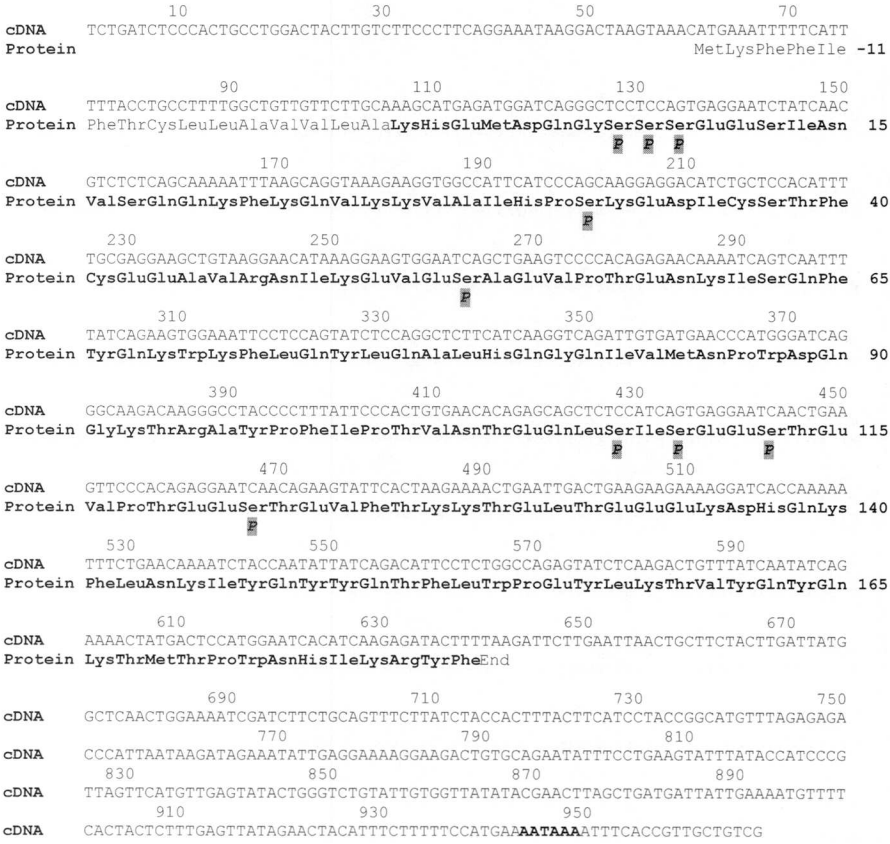

Fig. 4.2. cDNA sequence of camel milk α_{s2}-CN and corresponding protein, mature protein in bold. The open reading frame of the cDNA sequence is from A^{61} to T^{639} and the polyadenylation signal in bold from A^{944} to A^{949}. Numbering of the amino acid chain starts from the first residue of the mature protein. ***P***, potentially phosphorylated serine residues.

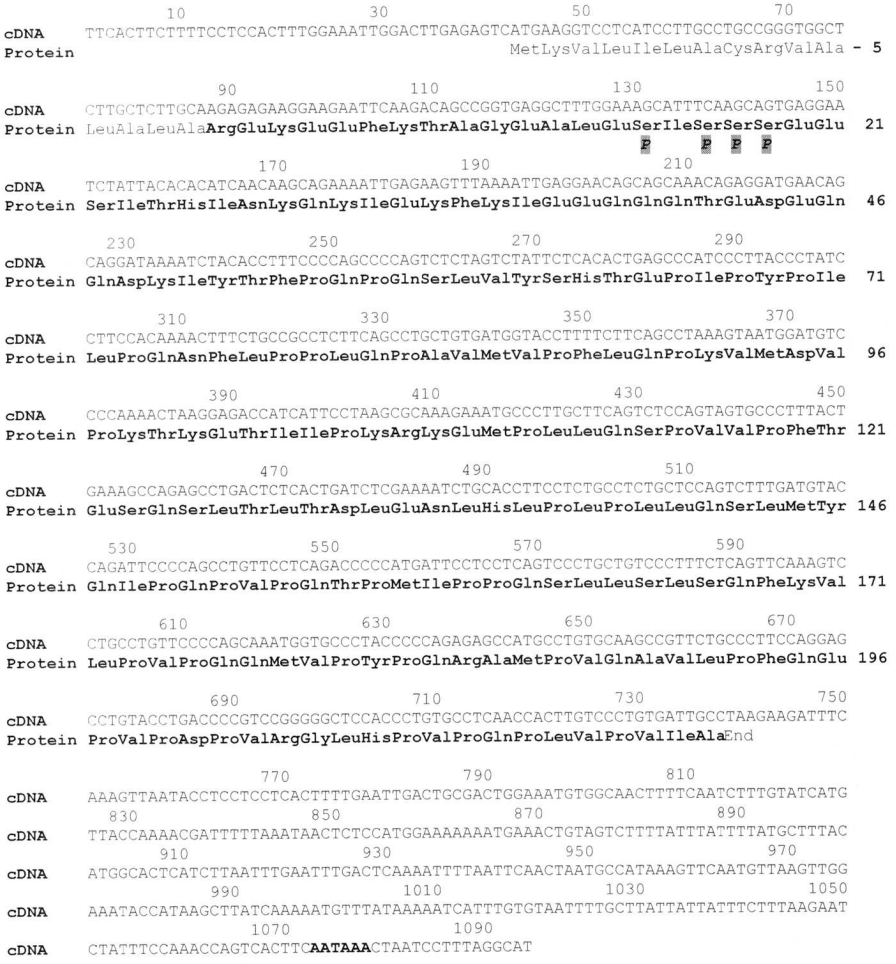

Fig. 4.3. cDNA sequence of camel milk β-CN and corresponding protein, mature protein in bold. The open reading frame of the cDNA sequence is from A^{43} to C^{738} and the polyadenylation signal in bold from A^{1073} to A^{1078}. Numbering of the amino acid chain starts from the first residue of the mature protein. ***P***, potentially phosphorylated serine residues.

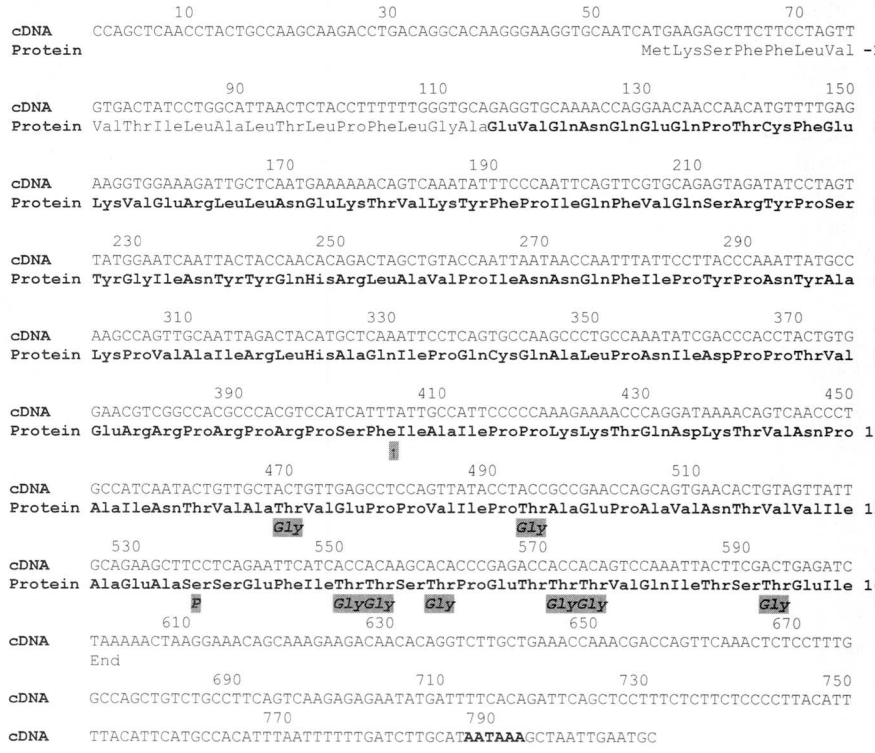

Fig. 4.4. cDNA sequence of camel κ-CN and corresponding protein, mature protein in bold. The open reading frame of the cDNA sequence is from A^{55} to C^{600} and the polyadenylation signal in bold from A^{788} to A^{793}. Numbering of amino acid chain starts from the first residue of the mature protein. Chymosin cleavage site, Phe^{97}- Ile^{98}; ***P***, potentially phosphorylated serine; ***Gly***, glycosylated threonines. Arrow indicates chymosin cleavage site.

Elution Pattern of Caseins in Reversed-Phase Chromatography
Proteins of fractions I, II, III, V and VII (Fig. 4.5) were partially sequenced. Fraction I consisted of κ-CN, II and III of α_{s1}-CN, V of α_{s2}-CN and VII of β-CN. Amino acid sequencing of the fragment eluted at 123 min from the tryptic digest of fraction III revealed a major insert, Glu-Gln-Ala-Tyr-Phe-His-Leu-Glu, (Fig. 4.6) between Gln^{154} and Pro^{155} of the mature protein (Fig. 4.1). Measured protein mass was about 25.7 kDa. From these results, we propose an α_{s1}-CN B 6P with 215 amino acids, a molecular mass of 25.773 kDa and an isoelectric point at pH 4.40 (Fig. 4.6). Long and short variants of α_{s1}-CN also occur in ovine milk (Ferranti *et al.* 1995).

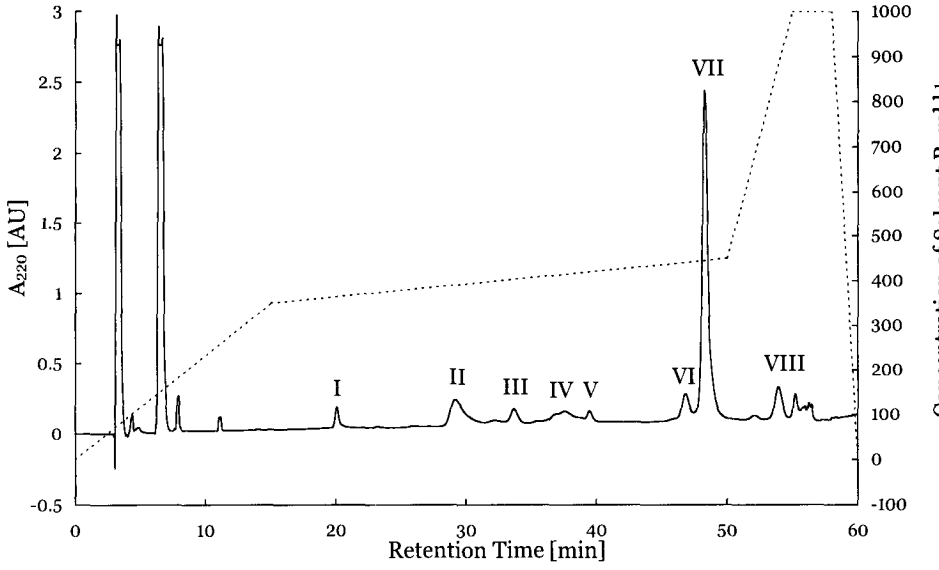

Fig. 4.5. Reversed-phase C_{18} HPLC chromatogram of acid precipitated camel milk casein. Peaks I, II, III, V, VII and VIII were collected for further analysis. Peaks IV and VI were not identified. Gradient of solvent B as dashed line.

The authors suggested, that those variants were a result of alternative splicing of the heterogeneous nuclear RNA transcribed from the α_{s1}-CN gene rather than gene products of two different alleles.

```
RPKYPLRYPEVFQNEPDSIEEVLNKRKILDLAVVSPIQFRQENIDELKDT   50
                  P
RNEPTEDHIMEDTERKESGSSSSEEVVSSTTEQKDILKEDMPSQRYLEEL  100
                    P PPPP
HRLNKYKLLQLEAIRDQKLIPRVKLSSHPYLEQLYRINEDNHPQLGEPVK  150

VVTQEQAYFHLEPFPQFFQLGASPYVAWYYPPQVMQYIAHPSSYDTPEGI  200

ASEDGGKTDVMPQWW                                      215
```

Fig. 4.6 Proposed amino acid sequence of camel milk α_{s1}-CN B 6P. Insert shaded and amino acid residues in bold. ***P***, potentially phosphorylated serines.

The minor peak VI to the left of peak VII (Fig. 4.5) is suggested to represent a variant of β-CN. Comparing the γ-CN sequence of camel milk obtained by Beg *et al.* (1986 *a*) with the β-CN sequence shown here revealed a single exchange: Glu^{195} for Gln^{195}. The fragment sequenced by Beg may therefore belong to a novel β-CN variant B. Molecular masses of fraction VIII were 13.9, 15.7, 15.75 and 15.9 kDa and presumably belonged to hydrophobic γ-CN.

Species	Casein	Sequence of signal peptide
Camel	α_{s1}-CN	MKLLILTCLVAVALA
Cow	αs1-CN	MKLLILTCLVAVALA
Camel	αs2-CN	MKFFIFTCLLAVVLA
Cow	αs1-CN	MKFFIFTCLLAVALA
Camel	β-CN	MKVLILACRVALALA
Cow	β-CN	MKVLILACLVALALA
Camel	κ-CN	MKSFFLVVTILALTLPFLGA
Cow	κ-CN	MMKSFFLVVTILALTLPFLGA

Fig. 4.7. Sequence comparison of signal peptides from camel and cows' milk caseins. Conserved residues are shaded.

Signal Sequences

cDNA sequences were translated into casein precursor proteins containing signal peptides (Figs 4.1 – 4.4), which lead proteins into the rough endoplasmic reticulum and are subsequently cleaved off (Burgess & Kelly, 1987). Signal peptide sequences of all examined proteins were highly

conserved between camel and cow (Fig. 4.7). The signal peptides of the calcium-sensitive caseins α_{s1}-, α_{s2}- and β-CN were all 15 amino acids in length and similar in amino acid sequence. We assume that this similarity is a result of the common evolutionary origin of the respective genes (Rosen, 1987) and of the similar protein target and function.

Phosphorylation

Caseins are preferentially phosphorylated by mammary gland casein kinase, which recognises the pattern [Ser, (Thr)]–Xaa-[SerP, Glu, (Asp)]–Xaa, with Glu or Asp at position +1 and +3 enhancing phosphorylation (Swaisgood, 1992). The motif was found six times in α_{s1}-CN (Ser^{18}, Ser^{68}, Ser^{70}, Ser^{71}, Ser^{72} and Ser^{73}), nine times in α_{s2}-CN (Ser^{8}, Ser^{9}, Ser^{10}, Ser^{32}, Ser^{53}, Ser^{108}, Ser^{110}, Ser^{113} and Ser^{121}), four times in β-CN (Ser^{15}, Ser^{17}, Ser^{18} and Ser^{19}) and twice in κ-CN (Ser^{141} and Ser^{159}) (Figs 4.1 – 4.4). Ser^{159} in κ-CN was found towards the end of the protein, a position that is less frequently phosphorylated. Modification of the proposed sites is in agreement with the molecular masses measured by matrix assisted laser desorption/ionisation mass spectrometry. The measured molecular masses of α_{s1}-, α_{s2}- and β-CN were a multiple of one phosphate group, which accounts for 80 Da, higher than the molecular masses calculated from the unmodified amino acid chain (Table 4.1). The most frequent form of β-CN had only three phosphate groups bound instead of the four groups predicted. The degree of phosphorylation of κ-CN could not be determined by this method because this protein was also glycosylated. Respective pH values of isoelectric points for camel milk proteins compared to the most frequent variants of respective bovine milk proteins are given in Table 4.1. Isoelectric focusing of camel milk caseins revealed a narrower pH range than for cow milk caseins, within which the major bands appeared. Focusing of the different bands near pH 4.6 was in good accordance with the calculated values. Although camel milk caseins were less phosphorylated than cow milk caseins, the pH values of their isoelectric points were similar. However the amount of micellar calcium phosphate may be lower than in cow milk.

Glycosylation of κ-Casein

Another posttranslational modification found in caseins is glycosylation of Thr residues in κ-CN. This occurs for Thr near Arg/Lys, Thr or Pro, and is likely to be inhibited by Ile (Pisano *et al.* 1994). Prediction of O-glycosylation was done by the method of Hansen *et al.* (1995). Whereas bovine κ-CN had high glycosylation probability at Thr^{121}, Thr^{124}, Thr^{131},

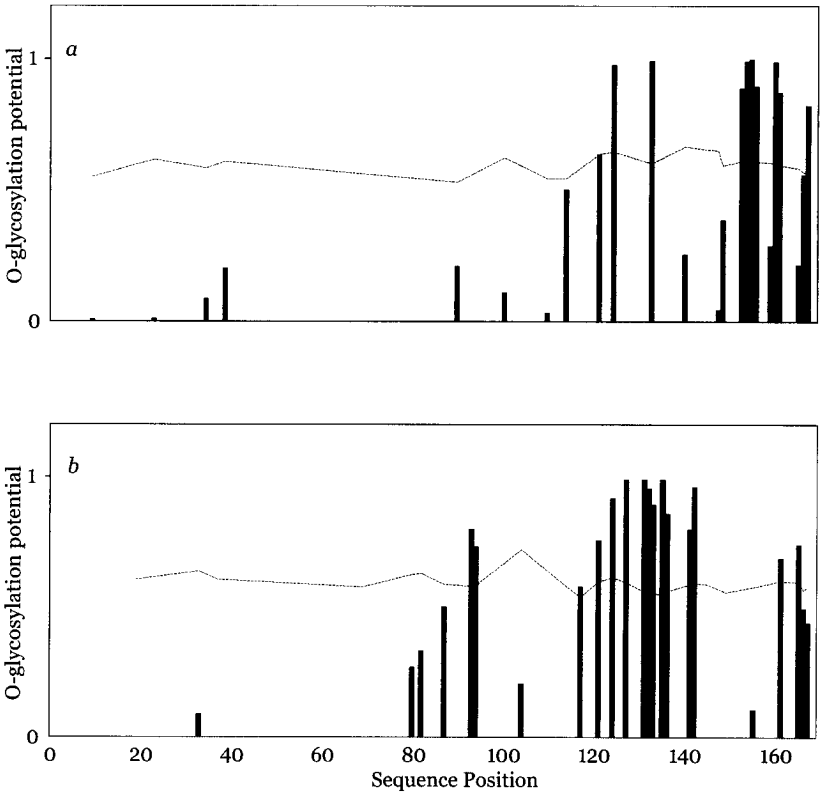

Fig. 4.8. O-glycosylation potential of threonines in κ-casein. The potential of the residues is shown as a solid bar with a value from 0 (no potential) to 1 (high potential). The threshold, which depends on the primary structure of the protein, is shown as a dashed line. The probability of glycosylation is the difference between the potential and the threshold. (a) Camel κ-casein. (b) Bovine κ-casein.

Thr^{133}, Thr^{135}, Thr^{136}, Thr^{142}, and Thr^{165}, glycosylation potential of camel κ-CN was high at Thr^{119}, Thr^{127}, Thr^{146}, Thr^{147}, Thr^{149}, Thr^{153}, Thr^{154}, and Thr^{160} (Fig. 4.8). Ser^{148} in camel κ-CN, and Ser^{127}, Ser^{132}, and Ser^{141} of bovine κ-CN also had a high glycosylation potential and the bovine κ-CN serine residues were reported to be glycosylated sometimes (Swaisgood, 1992). The positions in camel κ-CN were predominantly towards the C-terminal end of the glycomacropeptide, whereas glycosylation potential of bovine κ-CN was high towards the N-terminal end. This difference is suggested to be a structural consequence of a deletion, which makes camel κ-CN seven amino acids shorter than the bovine counterpart (Fig. 4.9). It is assumed, that bovine κ-CN and camel κ-CN have different affinities towards camel and bovine chymosin. These differences between camel and bovine κ-CN may also have implications on κ-CN tertiary structure, micelle assembly and structure, hydrophilic shielding of the micelle core by the glycosylated part of κ-CN, and charge neutralisation by calcium salts. Total glycosylation potential was similar in bovine and camel κ-CN. Micro-heterogeneity found in κ-CN of other species due to differences in pattern and composition of carbohydrate moieties is also expected in camel κ-CN. The carbohydrates attached in bovine κ-CN are trisaccharides of the type NANA-α(2→3)Gal-β(1→3)NAGA-β(1), or tetrasaccharides, which have an additional α(2→6)NAGA linked NANA residue. If five of the threonines with glycosylation potential have two sialic acid residues bound, and one serine is phosphorylated, the isoelectric point will be lowered to 4.11. SDS-PAGE and mass spectroscopic studies revealed that most of the κ-CN analysed was of relatively low molecular mass, making the low glycosylated form predominant and the protein very basic. This finding disagrees with the high sialic acid content reported by Mehaia (1987). It is assumed, that peaks in the MALDI spectrum with higher molecular masses were not detected, due to peak broadening by κ-CN micro-heterogeneity.

Primary Structure

Similarly to bovine caseins, camel α_{s1}-CN and β-CN were devoid of cysteine residues, and α_{s2}-CN and κ-CN both contained only two cysteines. The proline content in camel caseins was slightly higher than in bovine caseins, with 9.2% in α_{s1}-CN, 4.5% in α_{s2}-CN, 17.1% in β-CN, and 13.6% in κ-CN, compared to 8.5% in bovine α_{s1}-CN B, 4.8% in bovine α_{s2}-CN A, 16.7% in bovine β-CN A2, and 11.8% in bovine κ-CN A. This higher proline content in camel caseins may lead to destabilisation of secondary structures in a more pronounced way than it occurs in bovine caseins.

The gene structure of α_{s1}-, α_{s2}-, and β-CN is characterised by many short exon sequences, which are usually interrupted by long introns. The mRNA of bovine α_{s1}-CN is only 1172 bp long, the related hnRNA comprises 17,508 bp. The inserted sequence Asn^{14} to Glu^{20} in both variants of camel α_{s1}-CN was probably the result of an additionally expressed exon between bovine exon 3 and exon 4, and the deletion of bovine Glu^{141} to Glu^{148} in camel α_{s1}-CN A referred to bovine exon 16. The insert of Gln^{117} to His^{128} in camel α_{s1}-CN A and B was not at an intron/exon junction and therefore likely the result of an insertion in the camel α_{s1}-CN gene. The intron/exon structure of the bovine α_{s2}-CN gene is not yet resolved. The mRNA of bovine β-CN is 1089 bp long, the heterogeneous nuclear RNA precursor 8498 bp. Camel and bovine β-CN had a similar length with few insertions in camel β-CN (Fig. 4.9). The short insertions were a result of mutations in the camel β-CN gene, rather than a consequence of alternative splicing. Camel κ-CN was seven residues shorter than the bovine counterpart. Deletion of Val^{83} to Ala^{90} is suggested to be the result of a mutation in the third exon of the κ-CN gene, which codes for the largest part of the mature protein in both species. The bovine κ-CN mRNA is 850 bp long, the corresponding hnRNA more than 6,000 bp. In contrast to the other casein genes, the gene does not consist of short exon sequences, which partially may have evolved by exon duplication in tandem direction. The deletion of Val^{83} to Ala^{90} is also found in porcine κ-CN, and the cleavage site Phe^{97}-Ile^{98} also corresponds to the porcine cleavage site. Nonetheless, porcine κ-CN contains an insert in the glycomacropeptide, which was not found in the camel protein. The glycosylation potential of porcine κ-CN was more evenly distributed along the whole glycomacropeptide sequence than in camel and bovine κ-CN.

Secondary Structure

Sequence comparison of cow and camel milk caseins are shown in Fig. 4.9. Few pronounced structural differences were found, when camel and cow milk caseins were compared. Although α_{s1}-CN of camel and cow milk had a low percentage similarity in primary structure, similarities in the secondary structure, (a series of α-helical regions followed by a C-terminus with little defined secondary structure), predominated. In camel milk α_{s1}-CN, hydrophilicity of the N-terminal end was slightly more pronounced. The deletions that shortened camel milk α_{s2}-CN compared with cow milk α_{s2}-CN A occurred in an α-helical region between bovine Glu^{49} and Asn^{83} (Fig. 4.9). They went along with the loss of the phosphorylated serine cluster Ser^{56}, Ser^{57} and Ser^{58}). This loss may have implications in micelle

Phosphorylation Clusters

	αααααα ββ ββ αααααααααα ααααα	
Camel α_{s1}-CN A	RPKYPLRYPEVFQNEPD**S**IEEVLNKRKILELAVVSPIQFRQENIDEL KDTRNEPTEDHIMEDTERKE**S**G**S SSS**	73
Bovine α_{s1}-CN B	RPKHPIKHQGLPQ EVLNENLLRFFVAPFPEVFGKEKVNELSKDIG**S**E**S**TEDQAMEDIKQMEAE**S**I**SSS**	68
	ααααααααααααα αααααααααα αααααααα ββ ααααα	
Camel α_{s1}-CN A	EEVVSSTTEQKDILKEDMPSQR YLEELHRLNKYKLLQLEAIRDQKLIPRVKLSSHPYLEQLYRINEDNHPQL	145
Bovine α_{s1}-CN B	EEIVPN**S**VEQKHIQKEDVPSERYLGYLEQLLRLKKYKVPQLEIVPN **S**AEERLHSMKEGIHAQQ	131
	Deletion in Camel α_{s1}-CN A	
	ββββ ββββ ααααα	
Camel α_{s1}-CN A	GEPVKVVTQ PFPQFFQLGASPYVAWYYPPQVMQYIAHPSSYDTPEGIASEDGGKTDVMPQWW	207
Bovine α_{s1}-CN B	KEPMIGVNQELAYFYPELFRQFYQLDAYPSGAWYYVPLGTQYTDAPSFSDIPNPIGSENSEKT TMPLW	199
	Phosphorylation Cluster **Cysteines** **Phosphorylation Cluster, lost in**	
	αααααααααααααααα αααααααααααααααααααααααα ααααααα	
Camel α_{s2}-CN	KHEMDQG**SSS**EESINVSQQKFKQVKKVAIHP**S**KEDI**C**STF**C**EEAVRNIKE VE**S**AEVPTE	59
Bovine α_{s2}-CN A	KNTMEHV**SSS**EESI I**S**QETYKQEKNMAINP**S**KENL**C**STF**C**KEVVRNANEEEYSIG**SSS**EE**S**AEVATEEVKITVD	74
	Camel α_{s2}-CN **Phosphorylation Cluster**	
	αααααααααααααααααααααααααα βββ ββββ ααααα	
Camel α_{s2}-CN	NKISQFYQKWKFLQYLQALHQGQIVMNPWDQGKTRAYPFIPTVNTEQL**S**I**S**EE **S**TEVPTEE**S**TEVF	125
Bovine α_{s2}-CN A	DKHYQKALNEINQFYQKFP QYLQYLYQGPIVLNPWDQVKRNAVPITPTLNREQL**S**T**S**EENSKKTVDME**S**TEVF	147
	αα ααααααααααααααα αααααααα ααααααααααα	
Camel αs2-CN	TKKTELTEEEKDHQKFLNKIYQYYQTFLWPEYLKTVYQYQKTMTPWNHIK RYF	178
Bovine αs2-CN A	TKKTKLTEEEKNRLNFLKKISQRYQKFALPQYLKTVYQHQKAMKPWIQPKTKVIPYVRYL	207

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                         Phosphorylation Cluster
                 αααααααααααα        αα      ααααα                  ββ        βββ
Camel β-CN       REKEEFKTAGEALESISSSEESITHINKQKIEKFKIEEQQQTEDEQQDKIYTFPQPQSLVYSHTEPIPYPILPQN   75
Bovine β-CN A2   RELEELNVPGEIVESLSSSEESITRINK KIEKFQSEEQQQTEDELQDKIHPFAQTQSLVYPFPGPIPNS LPQN   73

                                       Hydrophobic C-terminal Domain
                                                                     ββββ           ααααα
Camel β-CN       FLPPLQPAVMV PFLQPKVMDVPKTKETIIPKRKEMPLLQSPVVPFTESQSLTLTDLENLHLPLPLLQSLMYQIP  149
Bovine β-CN A2   IPPLTQTPVVVPPFLQPEVMGVSKVKEAMAPKHKEMPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPH  148

                                                                                ββ
Camel β-CN       QPVPQTPMIPPQSLLSLSQFKVLPVPQQMVPYPQRAMPVQAVLPFQEPVPDPVRGLHPVPQPLVPVIA         217
Bovine β-CN A2   QPLPPTVMFPPQSVLSLSQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPVRG  PFPII V           209


                     Cysteine
                   αααααααα        ββ  βββββ          ββββββ                    ββββ
Camel κ-CN       EVQNQEQPTCFEKVERLLNEKTVKYFPIQFVQSRYPSYGINYYQHRLAVPINNQFIPYPNYAKPVAIRLHAQIPQ   75
Bovine κ-CN A    QEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYGLNYYQQKPVALINNQFLPYPYYAKPAAVRSPAQILQ   75

           Cysteine    Deletion            ◇             Thr/Ser Glycosylation Potential
                                         βββββ                                ββββ
Camel κ-CN       CQALPNI        DPPTVERRPRPRPSFIAIPPKKTQDKTVNPAINTVATVEPPVIPTAEPAVNTVVIAEASS  142
Bovine κ-CN A    WQVLSNTVPAKSCQAQPTTMARHPHPHLSFMAIPPKKNQDKTEIPTINTIASGEPTSTPTTEAVESTVATLEDSP  150

                  ββββ     ββββββ
Camel κ-CN       EFITTSTPETTTVQITSTEI                                                         162
Bovine κ-CN A    EVI ESPPEINTVQVTSTAV                                                         169
```

Fig. 4.9 Sequence comparison of cow and camel milk caseins. Bovine caseins according to the Swissprot database. Modified residues and cysteines in bold. ◇ Site of proteolytic cleavage by aspartic proteases in κ-casein.

assemblage and stability, as well as in the nutritional behaviour of the caseins (Ferranti *et al.* 1995). Similarly to cow milk, camel milk α_{s2}-CN was the most hydrophilic among the four caseins and had a high potential for secondary structures, mainly α-helices (Fig. 4.9). The two cysteine residues also occurred at about position 40.

Chymosin Sensitive Site of κ-Casein

The site of cleavage of camel milk κ-CN by chymosin is Phe^{97}-Ile^{98} (Fig. 4.4), leaving a macropeptide of 6.774 kDa, 65 amino acids in length with an isoelectric point of the unmodified peptide at pH 4.13. In bovine κ-CN, the site of cleavage is Phe^{105}-Met^{106}, leaving a macropeptide of 6.707 kDa, 64 amino acids in length with an isoelectric point of the unmodified peptide at pH 3.87. The amino acid sequence from His^{98} to Lys^{112} is involved in binding and cleavage of bovine κ-CN by chymosin (Visser, 1987). It is assumed, that the proline residues in this sequence stabilise the correct conformation of κ-CN in the active site cleft of chymosin, and the basic residues are thought to bind to acidic residues at either end of the active side cleft (Plowman, 1995). All proline residues were conserved in camel milk κ-CN as shown in Fig. 4.10 and the bovine residue Leu^{103} was replaced by Pro^{95}. This additional proline residue may help to stabilise a conformation of κ-CN in the active site cleft of camel chymosin different to the conformation of cow milk κ-CN in the cleft of bovine chymosin. Histidine residues in the sequence His^{98}-Pro-His-Pro-His^{102} of cow milk κ-CN were replaced by more basic arginine residues in camel milk κ-CN (Fig. 4.10). Since arginine remains protonised at higher pH values and has a longer and more flexible side chain, it can be speculated that these residues will still bind to the acidic centres of chymosin at a higher local pH and that the camel milk κ-CN backbone does not need to be bound as tightly to chymosin as it was shown for cow milk κ-CN (Plowman, 1995).

Models for secondary structure patterns were similar in bovine and camel milk κ-CN, with an N-terminal α-helix containing one Cys followed by β-pleated sheets and a second Cys (Fig. 4.9). Both Cys residues were at positions similar to those in bovine milk κ-CN, and are suggested to be involved in intermolecular crosslinks (Richardson *et al.* 1992). Of κ-caseins already sequenced, porcine κ-CN was most similar in overall structure, revealing a cleavage site highly similar in primary and secondary structure. Porcine chymosin acting on porcine milk was shown to have 6-8 times higher proteolytic specificity compared with bovine chymosin (Houen *et al.*

1996). A similar ability to cleave camel milk κ-CN with very high specificity is assumed rennet coagulation of camel milk with camel chymosin.

Camel:
Arg^{90}-Pro-Arg-Pro-Arg-Pro-Ser-Phe-Ile-Ala-Ile-Pro-Pro-Lys-Lys^{104}

Cow:
His^{98}-Pro-His-Pro-His-Leu-Ser-Phe-Met-Ala-Ile-Pro-Pro-Lys-Lys^{112}

Fig. 4.10. Sequence comparison of the chymosin-sensitive region of κ-CN from camel and cow milk. Conserved residues are shaded.

Rennet Coagulation

The quantitative distribution of individual caseins in total casein, as calculated from Fig. 4.5, were α_{s1}-CN 22%, α_{s2}-CN 9.5%, β-CN 65%, κ-CN 3.5%. κ-CN was considered to have a terminating function in micelle growth (Horne *et al.* 1989), as well as a stabilizing effect on the micelle by its net charge and by steric hindrance of aggregation by its hydrophilic C-terminal end (Holt *et al.* 1996). The glycosylated forms may have a stronger impact on both these functions than the non-glycosylated form, which seemed to be predominant in camel milk, owing to steric repulsion of charged sialic acid groups and to increased hydrophilicity. After cleavage of the macropeptide, hydrophobic forces and electrostatic interactions of bivalent cations with negatively charged groups promote coagulation and stabilise the curd (Dalgleish, 1983, Mora-Gutierrez *et al.* 1993). Since β-CN predominated in the camel milk studied and phosphorylation of α_{s1}- and α_{s2}-CN was lower than in cow milk, we assume that hydrophobicity is the driving force in coagulation of camel milk. It has been shown that camel milk casein is less stable at elevated temperature than cow milk (Farah & Atkins, 1992). This may be an effect of the high β-CN content. On the other hand, in milks with amounts of κ-CN higher than in cow milk, e.g. in buffalo milk with 130-200 g κ-CN/kg total casein (El-Din & Aoki, 1993), curd firmness was found to be higher than in cow milk (Bayoumi, 1990). Moreover, milks of transgenic mice producing bovine κ-CN were shown to have a linear correlation between the amount of κ-CN and curd firmness (Gutiérrez-Adán *et al.* 1996), and an inverse correlation with micelle size. The mean diameter of camel milk casein micelles is larger than that of casein micelles in bovine

milk (Buchheim *et al.* 1989). Milk of animals traditionally used for processing to cheese has higher amounts of κ-CN and lower amounts of β-CN than camel milk. It may be assumed that lack of selective breeding of camels for milk with favourable cheese-making properties is responsible for the high β-CN and the low κ-CN content.

Considerations for Camel Milk Processing
Technological difficulties in processing camel milk are probably due to different proportions of the individual caseins compared with cow milk, rather than to structural variations within the proteins. It has been shown that a high degree of β-CN and a low degree of κ-CN adversely affects some of the processing characteristics of casein micelles (Schmidt & Koops, 1977), such as stability towards ethanol, homogenisation and heat treatment. It must be assumed that the differences in protein composition between the ruminant milks and camel milk will have a major impact on technological properties, and that a lower ratio of β-CN to κ-CN would be favourable for curd coagulation and heat sterilisation.

4.2 Renneting enzymes

Literature
Chymosin (EC 3.4.23.4) and pepsin (EC 3.4.23.1), the renneting enzymes of the main stomach, are aspartic proteases, and thus belong to the broad AA clan of peptidases (Rawlings & Barrett, 1995). Aspartic proteases are found in eukaryotes, retroviruses and some plant viruses. Eukaryotic aspartic proteases are monomers of about 35 kDa, which are folded into a pair of tandemly arranged domains, with, e.g. in camel chymosin, about 20.4% similarity. It is assumed, that the two domains evolved from duplication of an ancestral gene encoding a primordial domain. The overall secondary structure consists almost entirely of pleated sheet, and is low in α-helices. Each domain contains an active site, centred on a catalytic aspartyl residue, with a consensus sequence [hydrophobic]-Asp-Thr-Gly-[Ser/Thr], which helps to maintain the correct φ-loop conformation of the site, and with multiple hydrophobic residues near the aspartic residue. The two catalytic centres are arranged face-to-face in the tertiary structure of correctly folded proteins. The distance between the aspartic side-chains is about 3.5 Å in bovine chymosin. The residues were reported to be extensively hydrogen bonded, concomitantly with the adjacent threonine residues, to the corresponding residues of the other domain, or to neighbouring atoms of the own domain, to fix the correct position. Optimal activity of an aspartic

protease is achieved, when one of the aspartic residues is protonated, and the other negatively charged. Thus, the pH optimum depends on the electrostatic micro-environment created by the residues surrounding the aspartic residues. The active sites are embedded, with low accessibility, in the middle of a cleft, about 40 Å in length, which separates the two domains, and which is covered by a flap that extends about from Leu^{73} to Ile^{85} in the N-terminal domain of bovine and camel chymosin. The B-factor of this flap is low, which indicates high flexibility. In crystallographic studies, oligopeptide protease inhibitors were shown to bind within the cleft. The flap was found to close down over the inhibitors, excluding solvent, while becoming considerably less flexible (Davies, 1990).

A short N-terminal peptide of about 45 aa is cleaved from the proenzyme of eukaryotic aspartate proteases by an autocatalytic process at acidic pH (Fig. 4.13). This peptide is of distinctly basic nature, with isoelectric points at pH 10.37 for camel chymosin, and at pH 10.17 for camel pepsin. In porcine pepsin, the peptide was shown to be buried along the substrate binding cleft in the proenzyme, thereby inhibiting the enzyme by blocking the active site with Ser^{-34} and Leu^{-1}, and by displacing a water molecule, which is bound in the active enzyme, with the highly conserved Lys^{-9} (Sielecki *et al.* 1991). Six of the basic aa residues of the porcine peptide were shown to form ion pairs with acidic residues, which are involved in substrate binding in mature porcine pepsin. Lowering the pH possibly protonates these acidic residues, conformational changes and loss of α-helical structure are followed by cleavage of the amide bond between Leu^{-29} and Ile^{-28}, with further structural rearrangement and proteolytic steps leading to the mature enzyme (Davies, 1990). The propeptide was found to be a prerequisite for correct folding of the enzyme (Li *et al.* 1998). It serves for protection of cellular proteins from digestion, before the enzyme is transferred, for example into a lysosomal compartment, or is secreted, which is, in the case of the renneting enzymes chymosin and pepsin, from the mucosa layer of the stomach into the peptic juices.

Aspartic proteases are endopeptidases with high substrate specificity. The peptide substrate is numbered away from the scissile bond, P_1 to P_n on the N-terminal side of the amino acid chain, and P'_1 to P'_n on the C-terminal side. Specificity pockets of the corresponding enzyme subsites are labelled S_1 to S_n, and S'_1 to S'_n, respectively. Substrates, such as κ-CN or angiotensinogen, a protein cleaved by renin, are usually bound in a β-pleated structure with a turn at P'_3 to P'_5. The active site cleft accommodates a hepta- to nonapeptide substrate, and probably also the same target site of

a protein, such as κ-CN in chymosin. P_3 and P'_2 were found to be critical in substrate binding (Davies, 1990). It is assumed, that binding of an appropriate peptide leads to a conformational strain in the scissile peptide bond, which is twisted out of planarity. This twisting reduces the double-bond character of the amide bond, in a way, that the nitrogen atom of P'_1 adopts the pyramidal nature of a secondary amine, which is more likely to accept a proton. A central water molecule, which is bound by hydrogen bonds to two carbonyl groups of the catalytic aspartates, is ready for a nucleophilic attack on the carbonyl group of P_1. The nitrogen atom will be protonated by the C-terminal catalytic aspartic acid, and the original conformation of the enzyme restored by release of the hydrolysed fragments, and binding of a free water molecule by the catalytic aspartates. In this way, and by isotope exchange experiments with ^{18}O-water, the reaction mechanism is supposed to be based on a general base catalysis, without covalent intermediates (Davies, 1990).

Enzymes of the aspartic protease family are of commercial interest in processing of milk, soya and cocoa (Albert *et al.* 1998). Chymosin is the preferred enzyme in the cheese-making process, since specificity for κ-CN is high, general proteolytic activity is low, and optimal activity is achieved at mildly acidic conditions (Williams *et al.* 1997). Zymogens of the natural renneting enzymes chymosin and pepsin are isolated from the mucosa layer of the forth stomach of the unweaned calf, and in the case of pepsin, also of the adult animal. Pepsin exhibits broader proteolytic activity than chymosin, with a lower pH optimum, and is less suitable for cheese production, since bitter, hydrophobic peptides, which are formed by proteolytic action on α_{s1}- and β-CN during ripening, impair the sensory value of the cheese. The low general activity of bovine chymosin was explained by a self-inhibited state of the resting enzyme, which is provoked by occupation of the S_1 substrate binding pocket by Tyr^{77}, a residue at the tip of the flap, which extends from the N-terminal domain, and which is in chemical equilibrium with the active state of the enzyme. It was suggested, that only interaction with a specific substrate, which should be an analogue of the chymosin sensitive region in κ-CN, would be able to shift the equilibrium towards the active state of the enzyme. Binding of the cluster His-Pro-His-Pro-His, which corresponds to bovine chymosin His^{98} to His^{102}, (Fig 4.10) was shown to induce conversion from the self-inhibited to the active state, probably by structural transformation of the enzyme (Gustchina *et al.* 1998). Self-inhibition is suppressed in pepsin. Steric hindrance by Phe^{111}, which corresponds to Val^{113} in bovine chymosin, inhibits interaction of Tyr^{75} with the active site. Pepsin has therefore greater

general proteolytic activity than other mammalian acidic proteases. A site-directed mutation of Val^{113} to Phe^{113} in recombinant calf chymosin led to a two-fold increase in K_M values, as compared to wild-type chymosin, without altering k_{cat} values, and increased the number of residues participating in specificity subsites S_1 and S_3, giving further support for the central role of the side-chain at position Val^{113} in chymosin, respectively Phe^{111} in pepsin, in proteolytic activity (Strop *et al.* 1990).

Enzymatic coagulation of milk by proteolytic enzymes is an apparent two-step process. The first step is characterised by hydrolysis of κ-CN at the surface of casein micelles. κ-CN covers the predominantly hydrophobic core of the micelles by a C-terminal glycomacropeptide, which prevents micellar aggregation by steric hindrance and charge repulsion. This C-terminal part is specifically cleaved during the enzymatic reaction at a hydrophobic cleavage site, which is Phe^{105}-Met^{106} in bovine κ-CN. In the second step of the renneting process, an exponential increase in coagulation of casein micelles is observed. Aggregation starts from the moment, when about 60% to 80% of κ-CN is cleaved. At this time, a sufficient part of the hydrophobic micelle surface is supposed to have the potential to participate in the aggregation process. A substantial reduction in coagulation time and an increase in curd strength is achieved by pH reduction and by addition of free Ca^{2+} (Dalgleish, 1992). Rennet coagulation of camel milk was found to follow a similar mechanism as reported for cow milk (Mehaia, 1988).

Camel κ-CN was found to contain a distinctly different cleavage site for aspartic proteases, compared to bovine κ-CN (Fig. 4.10). The exchange of the amino acid Leu^{103} to the imino acid Pro^{95}, which probably has implications on peptide folding, is most significant. It was of interest to see, if the amino acid substitutions in κ-CN could be correlated with changes in the specificity pockets of aspartic proteases from the camel stomach.

Primary Structures

PCR amplification products of partial cDNA clones of camel chymosin and pepsin were sequenced (Figs 4.11 and 4.12). The clone for camel chymosin (EMBL/GenBank™ accession number AJ131677) was 1173 bp long, and contained a 5'-untranslated region of 21 bp, and a 3'-untranslated region of 9 bp. Only a partial consensus region according to Kozak (1989) was found in front of the translational start, with a purine at −3 bp and cytosines at -4 bp and −5 bp. The open reading frame ranged from A^{22} to C^{1164}. Proprae-

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                10                  30                  50                  70
cDNA    NTGACCAGGTCCAGGTCCAGGATGCGGTGCCTCGTGGTGCTACTTGCAGCCCTCGCTCTCTCCCAGGCCAGTGGG
Protein                       MetArgCysLeuValValLeuLeuAlaAlaLeuAlaLeuSerGlnAlaSerGly -41

                    90                  110                 130                 150
cDNA    ATCACCAGGATCCCTCTGCACAAAGGCAAGACTCTGAGAAAAGCGCTGAAGGAGCGTGGGCTCCTGGAGGACTTT
Protein IleThrArgIleProLeuHisLysGlyLysThrLeuArgLysAlaLeuLysGluArgGlyLeuLeuGluAspPhe -16

                    170                 190                 210
cDNA    CTGCAGAGACAACAGTATGCCGTCAGCAGCAAGTACTCCAGCTTGGGGAAGGTGGCCAGGGAACCCCTGACCAGC
Protein LeuGlnArgGlnGlnTyrAlaValSerSerLysTyrSerSerLeuGlyLysValAlaArgGluProLeuThrSer  10

                230                 250                 270                 290
cDNA    TACCTGGATAGTCAGTACTTTGGGAAGATCTACATCGGGACCCCACCCCAGGAGTTCACCGTGGTGTTTGACACT
Protein TyrLeuAspSerGlnTyrPheGlyLysIleTyrIleGlyThrProProGlnGluPheThrValValPheAspThr  35

                310                 330                 350                 370
cDNA    GGCTCCTCTGACCTGTGGGTGCCCTCTATCTACTGCAAGAGCAATGTCTGCAAAAACCACCACCGCTTTGACCCG
Protein GlySerSerAspLeuTrpValProSerIleTyrCysLysSerAsnValCysLysAsnHisHisArgPheAspPro  60

                    390                 410                 430                 450
cDNA    AGAAAGTCGTCCACCTTCCGGAACCTGGGCAAGCCCCTGTCCATCCATTACGGCACGGGCAGCATGGAGGGCTTT
Protein ArgLysSerSerThrPheArgAsnLeuGlyLysProLeuSerIleHisTyrGlyThrGlySerMetGluGlyPhe  85

                    470                 490                 510
cDNA    CTGGGCTACGACACCGTCACCGTCTCCAACATTGTGGACCCCAACCAGACTGTGGGCCTGAGCACCGAGCAACCT
Protein LeuGlyTyrAspThrValThrValSerAsnIleValAspProAsnGlnThrValGlyLeuSerThrGluGlnPro 110

                530                 550                 570                 590
cDNA    GGCGAGGTCTTCACCTACTCCGAGTTTGACGGGATCCTGGGGCTGGCCTACCCCTCGCTTGCCTCCGAGTACTCG
Protein GlyGluValPheThrTyrSerGluPheAspGlyIleLeuGlyLeuAlaTyrProSerLeuAlaSerGluTyrSer 135

                610                 630                 650                 670
cDNA    GTGCCCGTGTTTGACAATATGATGGACAGACACCTGGTGGCCCGAGACCTGTTCTCGGTTTACATGGACAGGAAT
Protein ValProValPheAspAsnMetMetAspArgHisLeuValAlaArgAspLeuPheSerValTyrMetAspArgAsn 160

                    690                 710                 730                 750
cDNA    GGCCAGGGGAGCATGCTTACACTGGGGGCCATTGACCCGTCCTACTACACCGGCTCCCTGCACTGGGTGCCCGTG
Protein GlyGlnGlySerMetLeuThrLeuGlyAlaIleAspProSerTyrTyrThrGlySerLeuHisTrpValProVal 185

                    770                 790                 810
cDNA    ACCTTGCAGCAGTACTGGCAGTTCACCGTGGACAGTGTCACCATCAACGGGGTGGCAGTGGCCTGTGTTGGTGGC
Protein ThrLeuGlnGlnTyrTrpGlnPheThrValAspSerValThrIleAsnGlyValAlaValAlaCysValGlyGly 210

                830                 850                 870                 890
cDNA    TGTCAGGCCATCCTGGACACGGGTACCTCCGTGCTGTTCGGGGCCCAGCAGCGACATCCTCAAAATTCAGATGGCT
Protein CysGlnAlaIleLeuAspThrGlyThrSerValLeuPheGlyProSerSerAspIleLeuLysIleGlnMetAla 235

                910                 930                 950                 970
cDNA    ATTGGAGCCACAGAGAACCGATATGGTGAGTTTGACGTCAACTGTGGGAACCTGAGGAGCATGCCCACCGTGGTC
Protein IleGlyAlaThrGluAsnArgTyrGlyGluPheAspValAsnCysGlyAsnLeuArgSerMetProThrValVal 260

                    990                 1010                1030                1050
cDNA    TTCGAGATCAATGGCAGAGACTACCCACTGTCCCCCTCCGCCTACACAAGCAAGGACCAGGGCTTCTGCACCAGT
Protein PheGluIleAsnGlyArgAspTyrProLeuSerProSerAlaTyrThrSerLysAspGlnGlyPheCysThrSer 285

                    1070                1090                1110
cDNA    GGCTTTCAAGGTGACAACAATTCCGAGCTGTGGATCCTGGGGGATGTCTTCATCCGGGAGTATTACAGTGTCTTT
Protein GlyPheGlnGlyAspAsnAsnSerGluLeuTrpIleLeuGlyAspValPheIleArgGluTyrTyrSerValPhe 310

                1130                1150                1170
cDNA    GACAGGGCCAACAATCGCGTGGGGCTGGCCAAGGCCATCTGATTGCAC
Protein AspArgAlaAsnAsnArgValGlyLeuAlaLysAlaIleEnd                                  323
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Fig. 4.11. cDNA sequence of camel chymosin and corresponding protein, with mature protein starting from Gly^{1}, in bold. ORF from A^{22} to C^{1164}.

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                10                  30                  50                  70
cDNA    NNTAGTGATTGABGTTDXGAGCCGGGAAGAACCATGAGGTGGCTGTTACTGCTCGGCTTGGTGGCGCTCTCCGAG
Protein                                    MetArgTrpLeuLeuLeuLeuGlyLeuValAlaLeuSerGlu -50

                90                 110                 130                 150
cDNA    TGCATCACCCACAAGGTCCCGCTCGTCAAGAAGAaGTCCTTGagGaAGAaCCTGACTGAGCAAGGCAAACTGAAG
Protein CysIleThrHisLysValProLeuValLysLysLysSerLeuArgLysAsnLeuThrGluGlnGlyLysLeuLys -25

                   170                 190                 210
cDNA    GACTTCCTGAAGATCCACCACCACAACCTAGCCAGCAAGTACTTCCCTGCCACCTCAGAGGCTGCCAACTTCCTG
Protein AspPheLeuLysIleHisHisHisAsnLeuAlaSerLysTyrPheProAlaThrSerGluAlaAlaAsnPheLeu  1

          230                 250                 270                 290
cDNA    GACGAACAGCCCCTTGAGAACTACCTGGATACGGAGTACTTTGGCACCATCAGCATCGGAACCCCGGCTCAGAAC
Protein AspGluGlnProLeuGluAsnTyrLeuAspThrGluTyrPheGlyThrIleSerIleGlyThrProAlaGlnAsn  26

                310                 330                 350                 370
cDNA    TTCACCGTCATCTTTgAcACTGGCTCTTCCAACCTGTGGGTGCCCTCCATCTACTGCTCCAGCTCTGCCTGCACC
Protein PheThrValIlePheAspThrGlySerSerAsnLeuTrpValProSerIleTyrCysSerSerSerAlaCysThr  51

                   390                 410                 430                 450
cDNA    AACCACAACCGCTTCAaCCCTGAGGaATCCTCCACCTACCAGGGCACCGACGAGACGCTCTCCATCACCTATgGC
Protein AsnHisAsnArgPheAsnProGluGluSerSerThrTyrGlnGlyThrAspGluThrLeuSerIleThrTyrGly  76

                   470                 490                 510
cDNA    ACCGGCAGCATGACAGGCATCCTCGGATATGACACTGTCCAGGTTGGAGGCATCAGCGATGTCAACCAGATCTTT
Protein ThrGlySerMetThrGlyIleLeuGlyTyrAspThrValGlnValGlyGlyIleSerAspValAsnGlnIlePhe 101

          530                 550                 570                 590
cDNA    GGCCTGAgTGAGACAGAGCCCGGCTCCTTCCTGTAtTATGccCCCTTCGATGGCATCCTGGGTCTGGCTTACCCC
Protein GlyLeuSerGluThrGluProGlySerPheLeuTyrTyrAlaProPheAspGlyIleLeuGlyLeuAlaTyrPro 126

                610                 630                 650                 670
cDNA    AGCATCTCCTCCTCCGGGGgCAcCCCTGtCTTTGACAACATCTGGGACGAGGGTCTGATTTCCGAAGACCTCTTC
Protein SerIleSerSerSerGlyGlyThrProValPheAspAsnIleTrpAspGluGlyLeuIleSerGluAspLeuPhe 151

                   690                 710                 730                 750
cDNA    TCTGTCTACCTGAGCTCCAATGACGAGAgTGGAAGCGTGGTgATATTTGGTGGCATCGATTCttCTtACTATACA
Protein SerValTyrLeuSerSerAsnAspGluSerGlySerValValIlePheGlyGlyIleAspSerSerTyrTyrThr 176

                   770                 790                 810
cDNA    GGAAGCCTCAACTGGGTGCCtGTtTCTGTTGAGGGTTACTGGCAGATCACCGTGGACAGCATCACCATGGAAGGA
Protein GlySerLeuAsnTrpValProValSerValGluGlyTyrTrpGlnIleThrValAspSerIleThrMetGluGly 201

          830                 850                 870                 890
cDNA    GAGTCCATCGCTTGCAGCAGTGGCTGCCAGGCCATTGTTGACACCGGCACCTCTCTGCTGGCCGGCCCAACCGAC
Protein GluSerIleAlaCysSerSerGlyCysGlnAlaIleValAspThrGlyThrSerLeuLeuAlaGlyProThrAsp 226

                910                 930                 950                 970
cDNA    GCCATtTCTAACATCCAGAGCTACATCGGAGCCAGTGAGGACTCATACGGTGATATGGTGGTCAGCTGCTCCTCC
Protein AlaIleSerAsnIleGlnSerTyrIleGlyAlaSerGluAspSerTyrGlyAspMetValValSerCysSerSer 256

                   990                1010                1030                1050
cDNA    ATCAGCAGCCTGCCCAACATCGTCTTCACCATCAACGGCGTCCAGTACCCTCTGTCCCCCAGTGCCTACATCCTG
Protein IleSerSerLeuProAsnIleValPheThrIleAsnGlyValGlnTyrProLeuSerProSerAlaTyrIleLeu 271

                  1070                1090                1110
cDNA    GAGAGCGACGACAGCTGCACCAGTGGCTTCGAGGGCATGGACCTCTCCAGCTCCGAAGAGCtctggatCCTGGGT
Protein GluSerAspAspSerCysThrSerGlyPheGluGlyMetAspLeuSerSerSerGluGluLeuTrpIleLeuGly 301

          1130                1150                1170                1190
cDNA    GACGTCTTCATCCGCCAGTACTTCACCGTCTTCGACAGGGCAAACAACCAGGTCGGCCTGGCTGCCGTGGCCCAA
Protein AspValPheIleArgGlnTyrPheThrValPheAspArgAlaAsnAsnGlnValGlyLeuAlaAlaValAlaGln 326

               1210
cDNA    GCCTGAGTCTKCCACCACCTCCC
Protein AlaEnd                                                                    328
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Fig. 4.12. cDNA sequence of camel pepsin and corresponding protein, with mature protein from Leu^{1} in bold. Open reading frame from A^{34} to C^{1203}.

chymosin was 381 aa residues long, with a molecular weight of 42.082 kDa and an isoelectric point at pH 6.25. The start site of praechymosin was determined by similarity as Ser^{42}. The 16 aa signal peptide had 100% sequence similarity with the signal sequence of bovine chymosin. Praechymosin was 365 aa resiudes long, with a molecular weight of 40.428 kDa and an isoelectric point at pH 5.99. The start site of mature, activated chymosin was determined by similarity as Gly^{1}. Mature chymosin was 323 aa residues long, with a molecular weight of 35.629 kDa and an isoelectric point at pH 4.71. It shared 96.9% sequence similarity with bovine chymosin, 97.8% with porcine chymosin, 89.8% with camel pepsin, and 89.5% with porcine pepsin. The clone for camel pepsin (EMBL/GenBank™ accession number AJ131678) was 1223 bp long, and contained a 5'-untranslated region of 33 bp, and a 3'-untranslated region of 20 bp. As with chymosin cDNA, only a partial consensus region according to Kozak (1989) was found in front of the translational start, with a purine at −3 bp and cytosines at −1 bp and −2 bp. The open reading frame ranged from A^{34} to C^{1203}. Propraepepsin was 390 aa residues long, with a molecular weight of 42.117 kDa and an isoelectric point at pH 3.95. The start site of praepepsin was determined by similarity as Ile^{-48}. The 15 aa signal peptide had 100% sequence similarity with the signal sequence of human and porcine pepsin. Praepepsin was 375 aa residues long, with a molecular weight of 40.418 kDa and an isoelectric point at pH 3.92. The start site of mature, activated pepsin was determined by similarity as Leu^{1}. Mature pepsin was 327 aa residues long, with a molecular weight of 34.907 kDa and an isoelectric point at pH 3.16. It shared 98.5% sequence similarity with porcine pepsin. Although the sequences contained possible sites for posttranslational modification, comparison to other mammalian acid proteases indicated, that camel chymosin and pepsin were not modified, e.g. by phosphorylation or glycosylation.

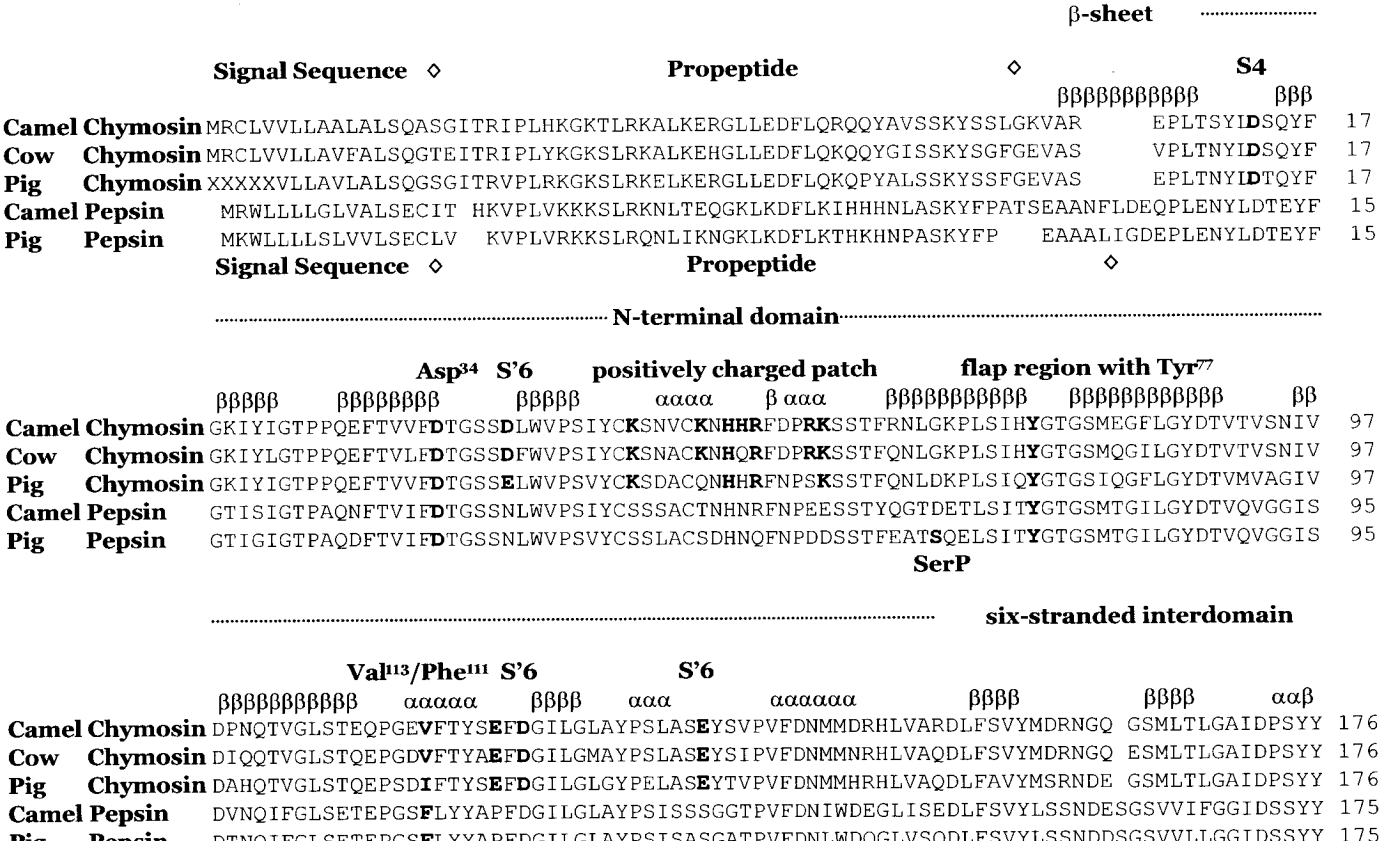
Functional Aspects

Wangoh *et al.* (1993) showed, that the clotting time of camel milk is significantly reduced, when camel rennet is used instead of calf rennet. Isolated chymosin and pepsin fractions from camel and calf stomachs were tested on their respective activity to clot camel milk, and it was found, that the main clotting activity of calf rennet resided in the pepsin fraction, whereas the main clotting activity of camel rennet originated from the chymosin fraction. Renneting of camel milk with the calf chymosin fraction did not result in milk coagulation. We suggest, that the Arg^{90}-Arg^{94} activator region of camel milk κ-CN is not suitable for release of calf chymosin from its self-inhibited state. As mentioned before, a considerable variation in

primary structure was found when camel and bovine κ-CNs were compared. Noticeable modifications in camel κ-CN were the deletion on the N-terminal side of the cleavage site (Fig. 4.9), the different localisation of sites with high probability of glycosylation (Fig. 4.8), the replacement of a leucine, which was reported to be highly flexible (Plowman & Creamer, 1995), with a rigid proline at P_3, and replacement of weakly basic histidine residues with highly basic arginine residues at P_4, P_6, and P_8.

The region around the cleavage site, which is proposed to have a β-sheet structure in extended conformation (Williams *et al.* 1997), is probably less flexible in camel κ-CN, due to the exchange of leucine to proline. We assume, that in general, substrate specificity of chymosin is especially high for κ-CN of the same species. The general proteolytic activity of porcine chymosin, for example, was reported to be about ten times lower than the activity of bovine chymosin, whereas its cow milk clotting activity was only four times lower. When tested against sow milk, the clotting activity was about six to eight times greater than that of calf chymosin (Houen *et al.* 1996). Nevertheless, camel chymosin was found to clot camel and cow milk equally well (Wangoh, 1993). Specificity pockets of camel chymosin, which bind more flexible arginine sidechains, may also be suited to host the more rigid histidine side-chains of bovine κ-CN. It can be assumed, that the protonation status of the histidines will be important for binding to camel chymosin. In this context, it would be of interest to know the pH optimum of camel chymosin for specific hydrolysis of the cleavage sites in camel and bovine κ-CN. High activity of bovine pepsin in camel milk renneting however can be explained by the high general proteolytic activity of pepsin. The primary structure of bovine pepsin is not yet known, but it can be assumed, that the protein does contain a bulky residue, which suppresses self inhibition, as it was found in camel and porcine pepsin with Phe^{111} (Fig. 4.13). Camel chymosin contained a Val^{113} at the corresponding site, in the same way as bovine chymosin. It can be assumed, that camel chymosin is in a chemical equilibrium of an active an a resting state, in a similar way as bovine chymosin.

Several studies tried to unveil the mechanism for specific binding of bovine κ-CN and bovine chymosin. Comparative modelling (Gilliland *et al.* 1991; Plowman, 1995), kinetic studies (Visser *et al.* 1987; Strop *et al.* 1990; Gustchina *et al.* 1998) and structural analysis of inhibitor complexes (Groves *et al.* to be published) showed, that specificity of binding depends on alternating binding of hydrophobic and positively charged residues of


β-sheet
Signal Sequence ◇ Propeptide ◇ S4
βββββββββββ βββ
Camel Chymosin MRCLVVLLAALALSQASGITRIPLHKGKTLRKALKERGLLEDFLQRQQYAVSSKYSSLGKVAR EPLTSYIDSQYF 17
Cow Chymosin MRCLVVLLAVFALSQGTEITRIPLYKGKSLRKALKEHGLLEDFLQKQQYGISSKYSGFGEVAS VPLTNYIDSQYF 17
Pig Chymosin XXXXXVLLAVLALSQGSGITRVPLRKGKSLRKELKERGLLEDFLQKQPYALSSKYSSFGEVAS EPLTNYIDTQYF 17
Camel Pepsin MRWLLLLGLVALSECIT HKVPLVKKKSLRKNLTEQGKLKDFLKIHHHNLASKYFPATSEAANFLDEQPLENYLDTEYF 15
Pig Pepsin MKWLLLLSLVVLSECLV KVPLVRKKSLRQNLIKNGKLKDFLKTHKHNPASKYFP EAAALIGDEPLENYLDTEYF 15
Signal Sequence ◇ Propeptide ◇
N-terminal domain
Asp34 S'6 positively charged patch flap region with Tyr77
βββββ βββββββββ βββββ αααα β ααα βββββββββββ βββββββββββ ββ
Camel Chymosin GKIYIGTPPQEFTVVFDTGSSDLWVPSIYCKSNVCKNHHRFDPRKSSTFRNLGKPLSIHYGTGSMEGFLGYDTVTVSNIV 97
Cow Chymosin GKIYLGTPPQEFTVLFDTGSSDFWVPSIYCKSNACKNHQRFDPRKSSTFQNLGKPLSIHYGTGSMQGILGYDTVTVSNIV 97
Pig Chymosin GKIYIGTPPQEFTVVFDTGSSELWVPSVYCKSDACQNHHRFNPSKSSTFQNLDKPLSIQYGTGSIQGFLGYDTVMVAGIV 97
Camel Pepsin GTISIGTPAQNFTVIFDTGSSNLWVPSIYCSSSACTNHNRFNPEESSTYQGTDETLSITYGTGSMTGILGYDTVQVGGIS 95
Pig Pepsin GTIGIGTPAQDFTVIFDTGSSNLWVPSVYCSSLACSDHNQFNPDDSSTFEATSQELSITYGTGSMTGILGYDTVQVGGIS 95
SerP
six-stranded interdomain
Val113/Phe111 S'6 S'6
βββββββββββ ααααα ββββ ααα αααααα ββββ ββββ ααβ
Camel Chymosin DPNQTVGLSTEQPGEVFTYSEFDGILGLAYPSLASEYSVPVFDNMMDRHLVARDLFSVYMDRNGQ GSMLTLGAIDPSYY 176
Cow Chymosin DIQQTVGLSTQEPGDVFTYAEFDGILGMAYPSLASEYSIPVFDNMMNRHLVAQDLFSVYMDRNGQ ESMLTLGAIDPSYY 176
Pig Chymosin DAHQTVGLSTQEPSDIFTYSEFDGILGLGYPELASEYTVPVFDNMMHRHLVAQDLFAVYMSRNDE GSMLTLGAIDPSYY 176
Camel Pepsin DVNQIFGLSETEPGSFLYYAPFDGILGLAYPSISSSGGTPVFDNIWDEGLISEDLFSVYLSSNDESGSVVIFGGIDSSYY 175
Pig Pepsin DTNQIFGLSETEPGSFLYYAPFDGILGLAYPSISASGATPVFDNLWDQGLVSQDLFSVYLSSNDDSGSVVLLGGIDSSYY 175

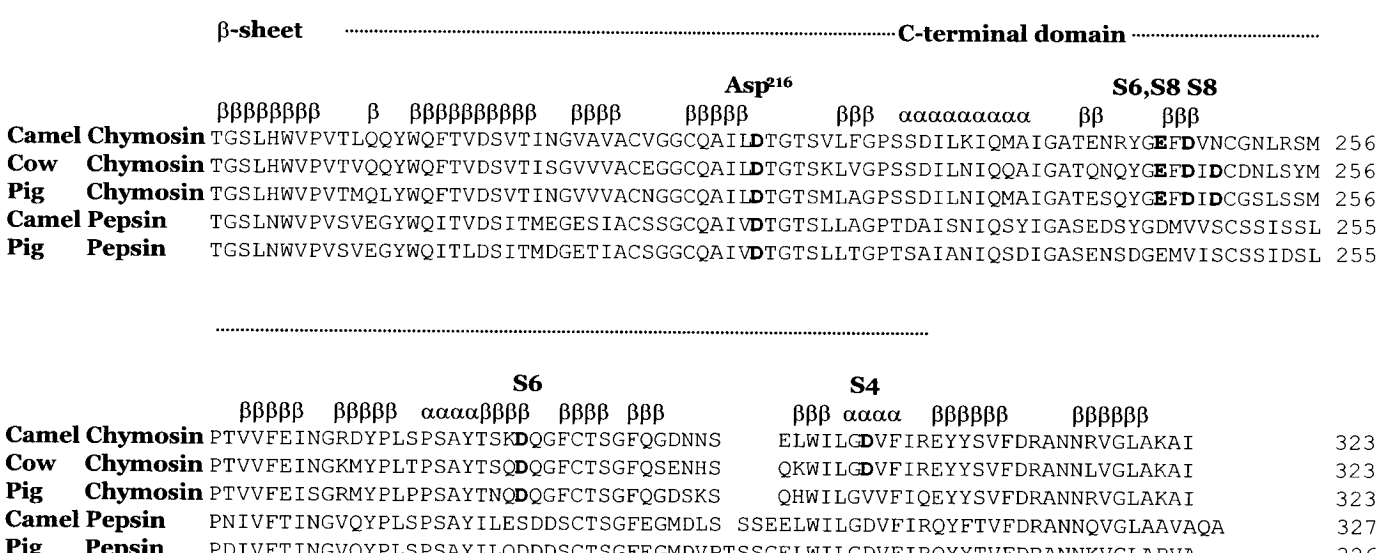


Fig. 4.13. Schematic drawing of the relationship between structure and function in camel, bovine and porcine chymosin, and camel pepsin. Numbering starts from the first residue of the mature proteins. Functional residues in bold. α-helical regions designated as "α", β-pleated regions as "β". ◇ Cleavage sites of the signal peptide and propeptide. Numbering of specificity pockets as found in camel chymosin.

the chymosin sensitive region in κ-CN by hydrophobic and negatively charged specificity pockets in chymosin. This pattern is also true, and even pronounced, for the binding region of camel κ-CN, which is on the N- and C-terminal side of the cleavage site Phe^{97}-Ile^{98}. The rigid structure of the region, which is supposed to be a prerequisite for catalytic action of chymosin by distortion of an amide bond and subsequent hydrolysis, is even more pronounced in the extended chymosin sensitive region of camel κ-CN, by replacement of Thr^{93}, Leu^{103}, and Thr^{131}, with Pro^{85}, Pro^{95}, and Pro^{123}. In an attempt to find possible specificity pockets for camel κ-CN, we modelled the tertiary structure of camel chymosin with the help of the resolved 1CMS structure of bovine chymosin (Gilliland *et al.* 1991) by the method of Guex & Peitsch (1997), and superposed the sequence of the chymosin sensitive region of camel κ-CN over the energy minimised structure of the bovine region, as described by Plowman & Creamer (1995). The φ- and ψ-angles of Pro^{95} were turned to the most probable angle of −60° and 150° (Fig. 4.14). The proline residues were found to introduce a kink in the chymosin sensitive region of κ-CN, which may help to present the hydrophobic cleavage site to the catalytic centre of chymosin (Kumosinski *et al.* 1993). The chymosin sensitive regions of camel and bovine κ-CN were laid into the cleft of the respective chymosin variants with the help of the recently resolved chymosin-CP113972 inhibitor complex (Groves *et al.* to be published), as shown in Fig. 4.15. We found, that the binding pockets of the aa residues in the vicinity of the cleavage site were much similar in camel and bovine chymosin, with the exception of S'1, which was deprived from two tyrosine residues in camel chymosin (Table 4.2). This reduction in binding sites for P'1 may be explained by the exchange of Met^{106} to Ile^{98} at P'1, an amino acid, which is not able to interact with the aromatic sidechains of tyrosine residues, in contrast to methionine. Binding of Ile^{98} is supposed to depend largely on hydrophobic interaction. It will be necessary for catalytic activity of the aspartate residues in chymosin, that Ile^{98} is brought into close contact with the S'1 specificity pocket, by electrostatic interactions of other κ-CN sidechains with the respective specificity pockets.

When we examined possible interactions of P'6, P4, P6, and P8 in camel κ-CN, we found more aspartic acids with probability for ion pair binding in camel chymosin, than those found in bovine chymosin, for binding of the respective residues of bovine κ-CN. It is not possible to make an accurate prediction of the residues involved in electrostatic binding of the chymosin sensitive region of camel and bovine κ-CN, as long as an experimentally resolved structure of κ-CN is not available. Nevertheless, distinct differen-

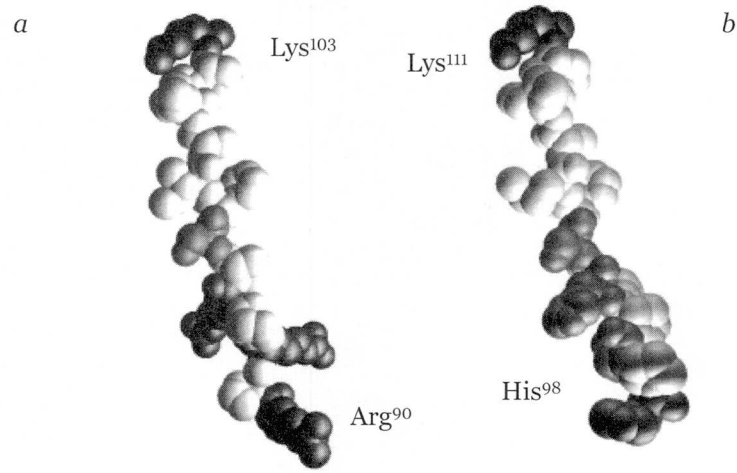


Fig. 4.14. Space-filling model of the chymosin sensitive regions Arg^{90}-Lys^{103} of (a) camel κ-CN, and His^{98}-Lys^{111} of (b) bovine κ-CN. Polar and weakly basic aa residues are light shaded, strongly basic aa residues are dark shaded.

ces in binding of camel and bovine κ-CN can be predicted, due to the higher proline content of the extended chymosin sensitive region in camel κ-CN, and the protonation of the arginine residues at neutral pH, in contrast to the histidine residues of bovine κ-CN. In this context, it would be interesting to understand, why histidine and arginine are found only in the activator region P4-P9 of κ-caseins from different species, and lysine only in P'6, P'7 and P'11 (Fig. 4.16). The compilation demonstrates the high conservation of the chymosin sensitive region beyond various mammalian orders and the importance of arginine and histidine residues on P' positions and lysine on P positions. Only hippopotamus and rodent sequences revealed distinct variations in this region. Gutiérrez-Adán *et al.* (1996) reported long renneting times of 115 to 125 min and a low curd strength for murine milk treated with calf chymosin. Fig. 4.16 shows, that the deletion on the N-terminal side of the cleavage site is found in all orders studied with the exception of ruminants. Camel, horse, human and mouse milk were found to have longer renneting times and low curd strength, when treated with calf chymosin (Bayoumi, 1990).

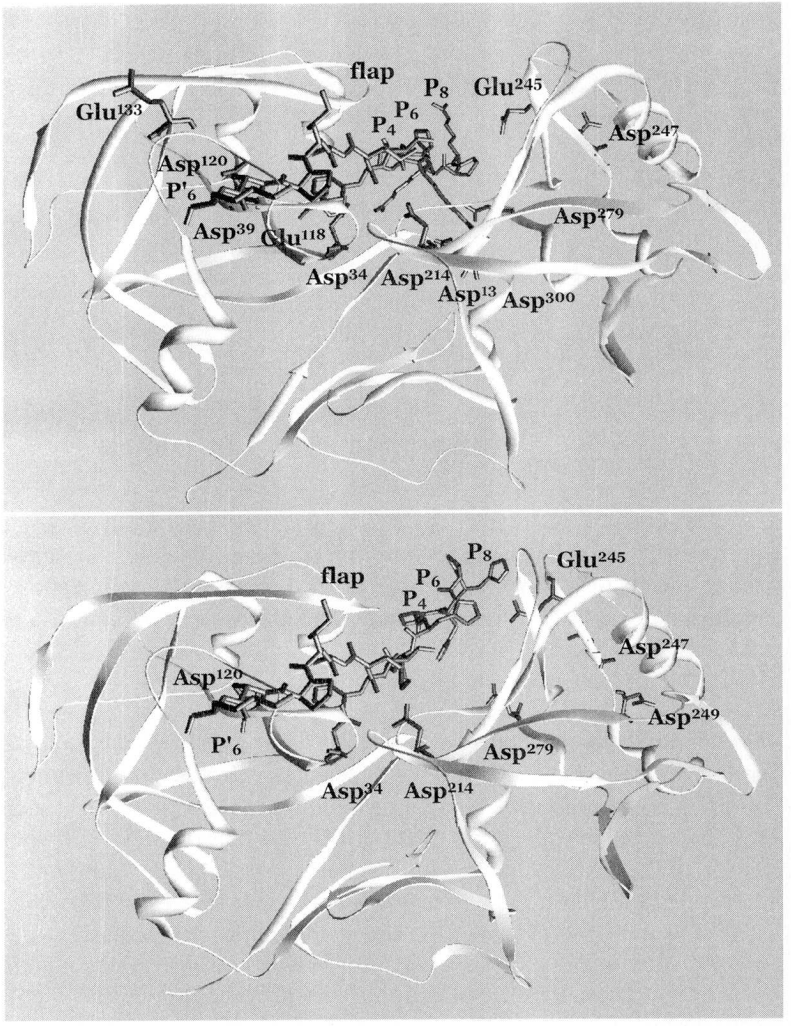


Fig. 4.15. A pictorial view on the interaction of chymosin and the chymosin sensitive site in κ-casein. Chymosin in ribbon-like representation. Chymosin sensitive region of κ-casein and acidic amino acids, which possibly interact with κ-CN are shown as wireframe. (a) Interaction of camel chymosin and Arg90–Lys103 of camel κ-casein. (b) Interaction of bovine chymosin and His98–Lys111 of bovine κ-casein.

Table 4.2. Comparison of chymosin sensitive region in camel and bovine κ-casein and residues of respective specificity pockets in camel and bovine chymosin.

	Camel κ-CN residue	Specificity subsites in camel chymosin	Bovine κ-CN residue	Specificity subsites in bovine chymosin
9	Arg^{89}		Arg^{97}	Asp^{249}
8	Arg^{90}	Glu^{245}, Asp^{247}	His^{98}	
6	Arg^{92}	Asp^{279}, Glu^{245}	His^{100}	Glu^{245}
4	Arg^{94}	(Glu^{118}), Asp^{13}, Asp^{300}	His^{102}	Asp^{279}
3	Pro^{95}	Tyr^{77}, Thr^{219}	Leu^{103}	Gln^{15}, Thr^{79}, Gly^{218}, Thr^{219} Ser^{220}
2	Ser^{96}	Tyr^{77}, Gly^{78}, Thr^{219}, Phe^{223}	Ser^{104}	Tyr^{77}, Gly^{78}, Thr^{79}, Gly^{218}, Thr^{219}, Gln^{288}
1	Phe^{97}	Gly^{36}, Ser^{37}, Tyr^{77}, Ile^{122}, Tyr^{190}, Gly^{218}, Thr^{219}	Phe^{105}	Gly^{36}, Ser^{37}, Tyr^{77}, Ile^{122}, Gly^{218}
1'	Ile^{98}	Gly^{36}, His^{76}, Ile^{297}	Met^{106}	Gly^{36}, His^{76}, Tyr^{77}, Tyr^{190}, Ile^{214}
2'	Ala^{99}	Gly^{36}, Ser^{37}, Leu^{130}, Tyr^{190}	Ala^{107}	Gly^{36}, Ser^{37}, Ile^{75}, Leu^{130}, Tyr^{190}
3'	Ile^{100}	Ser^{129}, Ala^{131}	Ile^{108}	Ser^{129}, Ala^{131}, Gln^{189}
4'	Pro^{101}	Ser^{38}, Tyr^{127}, Pro^{128}, Ser^{129}, Leu^{130}, Ala^{131}, Gln^{189}, Tyr^{190}	Pro^{109}	Ser^{38}, Tyr^{127}, Pro^{128}, Ser^{129}, Leu^{130}, Ala^{131}, Gln^{189}, Tyr^{190}
6'	Lys^{103}	Asp^{39}, Asp^{120}, Glu^{133}	Lys^{111}	Asp^{120}

Residues more distant to the cleavage site in κ-CN were discussed to be involved in binding to peripheral sites in chymosin. It was observed, that the B variant of bovine κ-CN was associated with firmer cheese curds, shorter renneting times, a smaller average micelle size, higher milk casein and κ-CN concentrations, and lower whey protein concentrations, when compared to the A variant (Jakob, 1994), and the C variant exhibited longer rennet coagulation times, than variants A and B (Jakob, 1993). Sequence comparison revealed hydrophobic residues in variant B at positions, where variant A contained a glycosylated Thr^{136} and an acidic Asp^{148}, and a histidine in variant C at the position of Arg^{97}. The effects reported for variant B are likely to result from alterations on the level of gene expression, as well as on enzymatic suitability as a substrate for bovine chymosin. Camel κ-CN contained hydrophobic residues at the corresponding sites, in the same way as bovine κ-CN B. Arg^{97} in bovine κ-CN A and B was proposed to interact with Asp^{249}, in a structural arrangement, where no interaction of His^{98} with an acidic chymosin residue was possible (Plowman *et al.* 1997). Interaction of His^{97} in bovine κ-CN C with Asp^{249} was not possible, which gave explanation for the extended renneting time. It was also shown, that interaction of Arg^{97} was not possible, when Asp^{249} was replaced by Asn^{249}, as it was the case in camel chymosin. It is possible, that interaction of all arginines of the camel κ-CN chymosin sensitive region is brought about by replacement of Leu^{103} with Pro^{95} at position P3. Williams *et al.* (1997) proposed an interaction of Asp^{244} in the less thermostable bovine chymosin variant A with His^{98}. Variant A exhibits a 20% higher clotting ability than chymosin B, which may be explained by stronger interaction of the N-terminal chymosin sensitive region of κ-CN with this variant. In camel chymosin, a glycine was found at this position, as in bovine chymosin B. Arg^{90} at position P8 is likely to interact either with Glu^{245} or with Asp^{247} in camel chymosin, so there is no need for replacement of Gly^{244}. High conservation of the lysine at position P'11 throughout different mammalian orders point to the importance of this residue for optimal binding to chymosin (Fig 4.16), although it was shown, that the peptide His^{98} to Lys^{112} was sufficient for a hydrolysis rate similar to full-length mature bovine κ-CN.

A pronounced dipole moment was found between the N- and C-terminal domains of mammalian aspartic proteases. This dipole was found to be more pronounced and have a different orientation in bovine chymosin, than in other aspartic proteases, including pepsin. A patch of the positively charged residues Lys^{48}, Lys^{53}, His^{55}, Arg^{57}, Arg^{61}, and Lys^{62} on the surface of

Position	PPPPPPPPP PPPPPPPPP 987654321 123456789
Arabian Camel	PNI.....DPPTVE**RR**P**R**P**R**PSF IAIPP**KK**TQD**K**TVNPAINTVATVE
Guanaco	PNI.....DPPTVE**RR**P**R**P**R**PSF IAIPP**KK**TQD**K**TVIPAINTVATVE
Collared peccary	PNV.....YPPTGA**RR**P**R**P**H**ASF IAIPP**KK**NQDTTAIPAINSIATVE
Pig	PNV.....YPPTVA**RR**P**R**P**H**ASF IAIPP**KK**NQD**K**TAIPAINSIATVE
Human	PNS.....**H**PPTVV**RR**PNL**H**PSF IAIPP**KK**IQD**K**IIIPTINTIATVE
Finback whale	PNI.....**H**PPTVA**HH**P**H**P**R**PSF IAIPP**KK**TQD**K**TVIPIINTIATAE
Sperm whale	PNI.....**H**PPTLA**HH**P**H**P**R**PSF TAIPP**KK**TQD**K**TAIPIINTIATVE
Hippopotamus	PDI.....NPPTVPC**RRR**P**H**PSF LAIPPX**K**DQN**K**TVIPIINTIATXE
Panda	PNA.....YPPTVV**RR**P**H**L**H**PSF IAIPP**KK**IQD**K**TSISTINTIVSAE
Snow leopard	PNT.....YTPTVV**RH**P**H**LPASF IVIPP**KK**IQD**K**TGNPTINTIATAE
Grevy zebra	PNI.....YPSTVV**RH**P**R**P**H**PSF IAIPP**K**XLQE**K**TVIP**K**INTIATVE
Tapir	PNI.....YPSTVV**RH**PY**RR**PSF IAIPP**KK**LQD**K**TV**R**PNINTIATVE
Guinea pig	TDI.....**H**QSTMQY**H**QA**KH**PSF MAILS**KK**ILG**K**ATILSTDAIAAPE
Rabit	PNI.....**H**QP**K**VG**RH**..S**H**PFF MAILPN**K**MQD**K**AVTPTTNTIAAVE
Mouse	PNF.....PQSAGVPYAIPNPSF LAMPTNENQDNTAIPTIDPITPIV
Rat	PNF.....PQPVGVP**H**PIPNPSF LAIPTNE**KH**DNTAIPASNTIAPIV
Mountain goat	PNTAPA**K**SCQDQPTTMA**RH**P**H**P**H**LSF MAIPP**KK**DQD**K**TEIPTINTIASAE
Muskox	PNTAPA**K**SCQDQPTTMA**RH**P**H**P**H**LSF MAIPP**KK**DQD**K**TEIPTINTIASAE
Rupicapra	PNTAPA**K**SCQDQPTTMA**HH**P**H**P**H**LSF MAIPP**KK**DQD**K**TEIPTINTIASAE
Goat	PNTVPA**K**SCQDQPTTLA**RH**P**H**P**H**LSF MAIPP**KK**DQD**K**TEVPAINTIASAE
Sheep	PNAVPA**K**SCQDQPTAMA**RH**P**H**P**H**LSF MAIPP**KK**DQD**K**TEIPAINTIASAE
Dall sheep	PNTVPA**K**SCQDQPTTMA**HH**P**H**P**H**LSF MAIPP**KK**DQD**K**TEIPAINTIASVE
Saiga tatarica	PNTVPA**K**SCQDQPTTMA**RH**P**H**P**H**LSF MAIPP**KK**DQD**K**TEIPTINTVASAE
Cattle	SNTVPA**K**SCQAQPTTMA**RH**P**H**P**H**LSF MAIPP**KK**NQD**K**TEIPTINTIASGE
European bison	SNTVPA**K**SCQAQPTTMA**RH**P**H**P**H**LSF MAIPP**KK**NQD**K**TEIPTINTIASGE
Water buffalo	PNTVPA**K**SCQAQPTTMT**RH**P**H**P**H**LSF MAIPP**KK**NQD**K**TEIPTINTIVSVE
Mule deer	PNTVPA**K**SCQAQPTTLA**RH**P**H**P**R**LSF MAIPP**KK**NQD**K**TDIPTINTIATVE
Reindeer	PNTVPA**K**SCQAQPTTLA**RH**P**H**P**R**LSF MAIPP**KK**NQD**K**TDIPTINTIATVE
Red deer	PNTVPA**K**FCQAQPTTMA**RH**P**H**P**R**LSF MAIPP**KK**NQD**K**TDIPSINTIATAE
Elaphurus	PNTVPA**K**FCQPQPTTMA**RH**P**H**P**R**LSF MAIPP**KK**NQD**K**TDIPSINTIATAE
Cervus duvaucelii	PNTVSA**R**SCQAQPTTMA**RH**P**H**P**H**LSF MAIPP**KK**NQD**K**TDIPSINTIATAE
Sambar	PNTVPA**R**SCQPQPTTMA**RH**P**H**P**H**LSF MAIPP**KK**NQD**K**TDIPSINTIATAE
Giraffe	PNTVPA**K**SCQAQPTTMA**RR**P**H**P**R**LSF MAIPP**KK**NQD**K**TDSPTINTIATVE
Chinese muntjak	PNTVPATSCQAQPATVA**RH**P**H**P**R**LSF MAIPP**KK**SQD**K**TD**H**PTINTSATVE
Chevrotain	LNAVSA**K**PCQAPPTTMA**RR**P**R**P**H**LSF MAIPP**KK**DQD**K**TDTPTINTIVTVE

Fig. 4.16. Sequence comparison of the chymosin sensitive region of κ-CN from different species. Basic amino acid residues in bold.

the N-terminal lobe of bovine chymosin was discussed to have favourable electrostatic interactions with the negatively charged residues on the surface of casein micelles (Gilliland *et al.* 1991). This patch is less pronounced on porcine chymosin, which may explain the low clotting activity towards cow milk, but more pronounced in camel chymosin, with an additional His^{56}, which helps to explain the good renneting properties of camel chymosin towards cow milk.

Camel pepsin was similar in structure to porcine pepsin. The isoelectric point of the mature protein was at pH 3.16, and thus slightly higher than that of porcine pepsin, which was at pH 3.10. The protein was not found to contain a consensus sequence Ser-Xaa-Glu for mammary gland protein kinase, whereas the porcine protein was shown to be indeed phosphorylated at Ser^{70}, which decreases the pH of the isoelectric point additionally. Pepsin is mainly expressed in the mucosa of the highly acidic environment of the stomach in adult animals, whereas chymosin is usually found in the stomach of suckling animals. Developmentally regulated expression of aspartic proteases in the mammalian stomach corresponds to the pH optima of enzymatic activities, which are at pH 3.8 for bovine chymosin B and 4.0 for bovine chymosin A, and at a pH below 2.0 for pepsin (Mantafounis & Pitts, 1990; Andreeva & James, 1991). The low pH optimum of pepsin was explained by the ability of pepsin, to maintain negative charges, e.g. of Asp^{11}, Asp^{118}, and Asp^{138}, at the very low pH values found in the gastric lumen of mammals. Camel pepsin contains four basic and 41 acidic aa residues, one acidic residue less than porcine pepsin. Since most charged residues are at similar positions as in porcine pepsin, it seems likely, that optimal activity is only at a slightly higher pH than in porcine pepsin. A pronounced structural stability is expected at the extreme acidic pH, whereas the protein is likely to be denatured at pH greater than 6.5, in a similar way as porcine pepsin, due to the surplus of negatively charged residues at this pH (Andreeva & James, 1991).

Based on structural comparison, we suppose that camel chymosin is better suited for rennet coagulation of camel milk than calf chymosin, which was studied elsewhere (Ramet, 1987; Mehaia, 1988). Although firm coagulation of the milk was reported in some studies, a consistent quality was not obtained in the cheese making process and addition of elevated amounts of $CaCl_2$ was a prerequisite. Use of camel chymosin may help to prevent the formation of bitter peptides during cheese ripening, which impair the taste, and therefore could help to promote consumer acceptability of cheese

products in camel keeping countries. Large-scale production of this enzyme, e.g. by recombinant methods, should be envisaged therefore.

4.3 Major Whey Proteins

Literature
Bovine whey is defined as the supernatant of casein precipitation at pH 4.6. Major protein components of bovine whey are β-lactoglobulin with 55.0% of total whey protein, α-lactalbumin with 20.25% and blood serum albumin with 6.6% (Schlimme, 1990). Although the precipitation point of camel milk casein was shown to be lower than that of cow milk casein (Wangoh, 1997), pH 4.6 was chosen for studies on camel whey for better comparison to bovine whey.

Reversed-Phase Chromatography of Whey Proteins
Whey of acid precipitated camel milk was separated by reversed-phase-C_{18} chromatography (Fig. 4.17). Peaks were identified by N-terminal sequencing. Whey acidic protein (WAP) was eluted at 16.9 min, α-lactalbumin at 23.3 min to 30.0 min, and lactophorin at 36.3 min to 40.0 min. Absorption of lactophorin at 280 nm was weak. The presumed low level in tyrosine, tryptophan and cysteine was confirmed by sequence analysis.

Integration of peak areas was done at 220 nm. α-lactalbumin accounted for 86.6% of total peak area, lactophorin for 11.5%, and whey acidic protein (WAP) for 1.9%. SDS-PAGE revealed, that blood serum albumin and other proteins coeluted with α-lactalbumin as minor fractions. Minor whey proteins were not detected by RP-C_{18} chromatography of total whey. If it is assumed, that camel milk contains an average of 8,300 mg l^{-1} whey proteins, the amount of α-lactalbumin, together with coeluted proteins, is about 7,200 mg l^{-1}, the amount of lactophorin about 954 mg l^{-1}, and of WAP about 157 mg l^{-1}.

The major camel whey proteins α-lactalbumin, lactophorin and WAP were already sequenced by Beg (1985, 1986 *b*, 1987). In this study, we corrected the sequence of lactophorin, as presented by Beg (1987), since this protein may have interesting features to explain some characteristics of camel milk. In a further study, it would also be of interest to characterise the potential

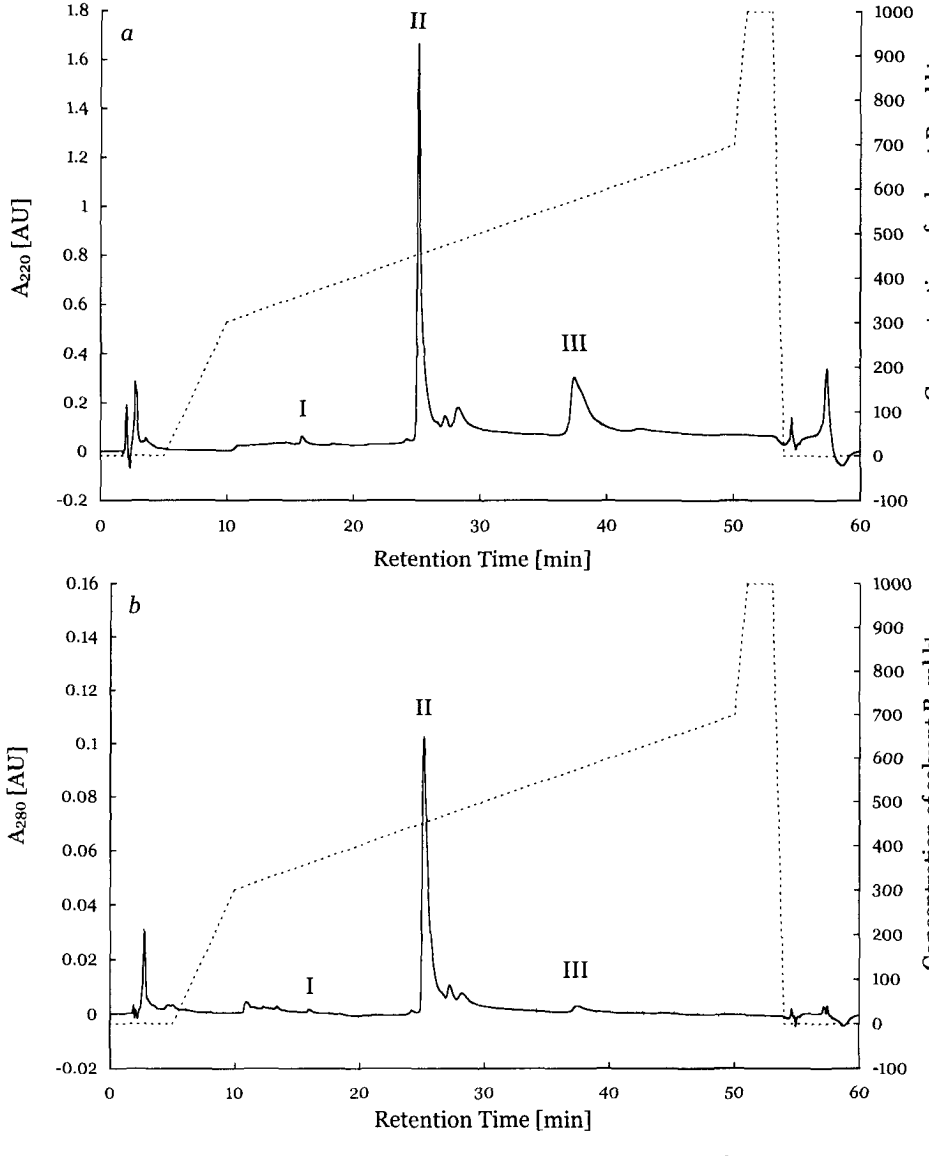


Fig. 4.17. Reversed-phase C_{18} HPLC chromatogram of camel milk whey proteins. Peaks I, II and III were collected for further analysis. Gradient of solvent B as dashed line. (a) Absorption at 220 nm. (b) Absorption at 280 nm.

Table 4.3. Physicochemical characteristics of camel and cow milk whey proteins[a]

Species	Protein	Amino acid residues	Molecular mass [kDa] based on		Isoelectric point[b] basd on		Charged modifications of amino acid residues	Concentration in milk [mg l^{-1}]	Similarity to corresponding bovine proteins[c]
			Amino acid sequence	Mass spectrometry	Amino acid sequence	Amino acid sequence with modifications			
Camel	α-lactalbumin	123	14.430	n.d.	4.87	n.d.	n.d.	>5,000	88.5%
Cow	α-lactalbumin	123	14.186	n.d.	4.65	n.d.	n.d.	600-1700	
Camel	PGRP	172	19.143	19.117	8.73	8.73	none	370	
Camel	lactophorin A	137	15.442	15.706	5.10	4.70	3 SerP	954	83.6%
Cow	lactophorin	135	15.304	18.700	6.03	4.9-6.1	3 SerP	300	
Camel	lactoferrin	689	75.250	80.16-80.73	8.14	n.d.	n.d.	220	91.6%
Cow	lactoferrin	689	76.143	≈80	8.18	n.d.	n.d.	140	
Camel	lactoperoxidase	612	69.460	n.d.	8.63	n.d.	n.d.	n.d.	94.9%
Cow	lactoperoxidase	612	69.569	76.5/78.43	7.90	n.d.	n.d.	30	
Camel	WAP	117	12.564	n.d.	4.70	n.d.	n.d.	157	
Cow	β-lactoglobulin B	162	18.281	n.d.	4.66	n.d.	n.d.	<4000	

a Data on bovine whey proteins after Eigel *et al.* (1984), Hernández *et al.* (1990), Dull *et al.* (1990), De Wit & Hooydonk (1996).
b Calculated with the gcg programme (Genetics Computer Group, Madison, WI 53711 USA).
c Similar and identical residues.

protease-inhibitor WAP in terms of enzymatic activity and tertiary structure.

4.3.1 Lactophorin

Literature
Lactophorin is a major protein component of camel whey, which was first described by Beg *et al.* (1987) and characterised as cysteine free and with N-terminal heterogeneity in the amino acid sequence. It is structurally closely related to bovine lactophorin, which is a minor protein component of bovine whey. Bovine lactophorin was found to be a hydrophobic phosphoglycoprotein with an apparent mass of 28 kDa and a concentration in milk of about 300 mg l^{-1} (Johnsen *et al.* 1996). The protein was shown to have good emulsifying qualities (Courthaudon *et al.* 1995) and to inhibit spontaneous lipolysis by lipoprotein lipase (Girardet *et al.* 1993). Strong sequence similarities were found to mouse and rat glycosylation dependent cell adhesion molecule GlyCAM-1.

The present investigation aimed to determine the correct structures and relative amounts of camel lactophorin variants, to compare the gene structure with the structural organisation of the bovine lactophorin gene and of the murine GlyCAM-1 gene, and to find indications for the function of the protein in camel milk. Since the primary structure was not determined to certainty by Edman sequencing of peptide fragments, we decided to sequence the corresponding cDNA, which was obtained from lactating mammary gland.

Bovine lactophorin is the major protein component of proteose peptone component 3, and is therefore often named proteose peptone component 3 (MPP3, HFPP3). This term was not used for designation of the camel protein, because the protein was not isolated from proteose peptone, and because bovine PP3 consisted of several proteins, of which lactophorin was just the main fraction. Furthermore, the term PP3 is also used for designation of other proteins than the milk protein. The term "lactophorin" was introduced by Kanno (1989 *a*) to describe a whey component with affinity to soluble glycoprotein antiserum. This term was chosen for designation of the protein studied, since it was used particularly for description of this protein family. The term "lactoglycophorin", as proposed by Girardet & Linden (1996), could not be used for camel lactophorin, because the protein was not glycosylated.

Lactophorin Gene Structure

The genomic structure of the lactophorin gene was analysed by sequencing of PCR products, which spanned from exon 1 to exon 4 (EMBL/GenBank™ accession number AJ131714). Similarly to the gene structures of bovine lactophorin and murine GlyCAM-1, the coding sequence of the camel lactophorin gene was interrupted by three intron sequences at G^{106}, T^{151}, and G^{376} (Fig. 4.18, 4.19). These positions corresponded to the positions in the murine GlyCAM-1 gene and the bovine lactophorin gene. Intron I was 686 bp long, intron II 844 bp and intron III 236 bp. The SINE Bov-A2 sequence, which is present in bovine intron I, the microsatellite sequence $(AC)_{19}$, which is present in bovine intron II, and the LINE/L1, which is present in bovine intron III, were not found in the camel gene (Fig. 4.18). Intron I of the camel lactophorin gene contained a LINE/L2. A corresponding interspersed element was found in the bovine lactophorin gene, but not in the murine GlyCAM-1 gene. Camel intron I was 286 bp shorter than bovine intron I, camel intron II was 58 bp longer than bovine intron II and camel intron III was 269 bp shorter than bovine intron III. Compared to the bovine gene, the camel gene contained few interspersed elements. The peptide omitted in variant B of the camel protein conferred to the very short exon II, which was surrounded by two long intron sequences. This peculiarity may promote the probability of alternative splicing at this site. Exon II was 45 bp long, which was a multiple of three bp, and was therefore deleted without frameshift in camel lactophorin B mRNA. Analysis of intron/exon junctions revealed, that camel exon II had a low splicing probability of 0.69 at the acceptor site and 0.77 at the donor site, compared to 0.81 at both corresponding sites of bovine exon II and 0.68 at the acceptor site and 0.82 at the donor site of murine exon II. This twofold low splicing probability in the camel gene may promote exclusion of exon II from the mRNA.

Primary Structure

The N-terminus of RP-C_{18}-HPLC purified camel lactophorin was sequenced and the amino acid heterogeneity reported by Beg *et al.* (1987) was confirmed. N-terminal heterogeneity was found to be the result of alternative splicing. The sequence, which corresponded to exon2, was deleted in a minor fraction of camel lactophorin. PCR amplification products of two full length cDNA clones of 632 bp and 587 bp were sequenced (Fig 4.19). Both cDNA clones contained a 5'-untranslated region of 42 bp and a 3'-untranslated region of 125 bp. The 5'-untranslated region contained a partial Kozak-box (C^{36}CCCACC), with cytosines at -1, -4 and -5 bp, and an adenine at -3 bp in front of the translational start A^{43}TG. The 3'-

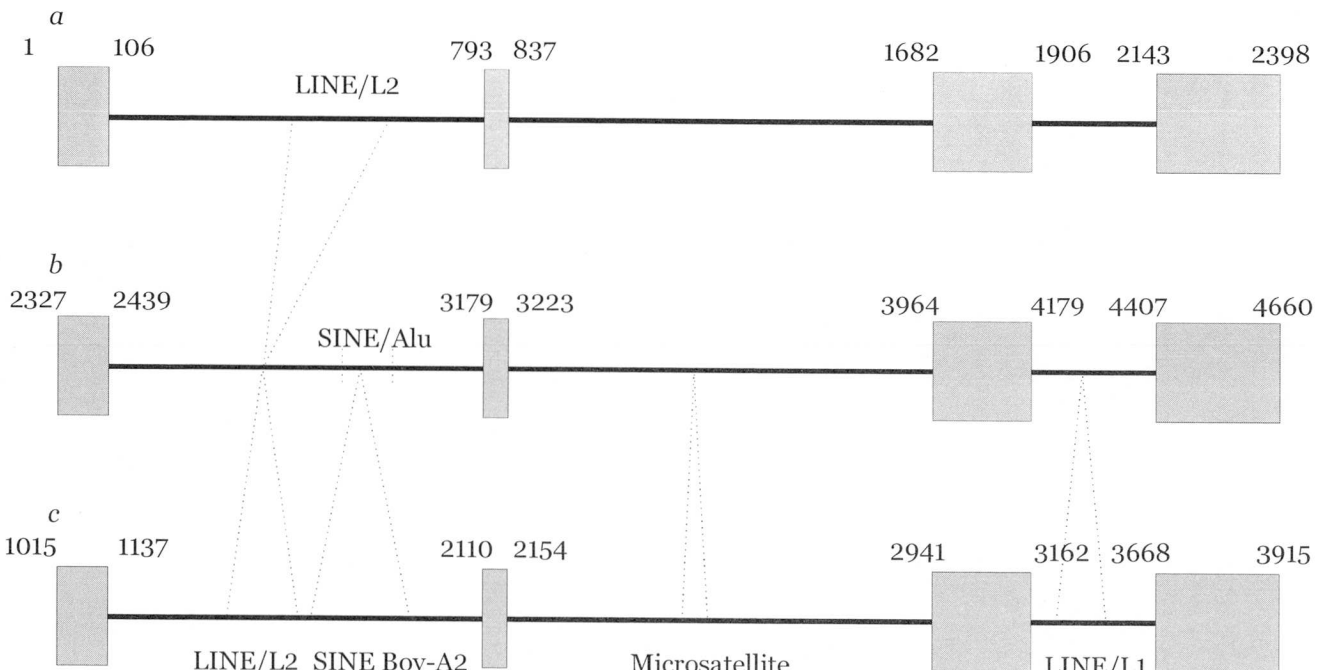


Fig. 4.18. Schematic comparison of the genomic structures of (a) Camelus dromedarius lactophorin gene (EMBL AC AJ131714), (b) Mus musculus GlyCAM-1 gene (EMBL AC D16108), (c) Bos taurus lactophorin gene (EMBL AC X83391). Bars indicate transcribed exon sequences. Interspersed elements are indicated by dashed lines. Numbering of bases according to full length sequences as described in the EMBL/GenBank/DDBJ Nucleotide Sequence Database.

```
                10                  30                  50                  70
cDNA    CGTTGCTGTCGCCAGGAAAACAGATCCTGCTCCAGCCCCACCATGAAATTCTTCGCTGTCCTGCTGCTGGCCAGC
Lph                                                     MetLysPhePheAlaValLeuLeuLeuAlaSer - 8

                    90                  110                 130                 150
cDNA    TTGACCTCCGCCTCTCTTGCCAGCCTTAATGAGCCAAAAGATGAAATCTACATGGAGTCTCAGCCCACAGATACC
Lph A   LeuThrSerAlaSerLeuAlaSerLeuAsnGluProLysAspGluIleTyrMetGluSerGlnProThrAspThr  18
Lph B   LeuThrSerAlaSerLeuAlaSerLeuAsnA

                    170                 190                 210
cDNA    TCTGCCCAGGTCATCATGAGCAACCATCAGGTCTCCAGTGAGGACCTTTCTATGGAGCCTTCCATCTCCAGAGAA
Lph A   SerAlaGlnValIleMetSerAsnHisGlnValSerSerGluAspLeuSerMetGluProSerIleSerArgGlu  43
Lph B    laAlaGlnValIleMetSerAsnHisGlnValSerSerGluAspLeuSerMetGluProSerIleSerArgGlu  28
                                        P                   P           P       P
                230                 250                 270                 290
cDNA    GATCTGGTTTCCAAAGACGATGTTGTGATCAAATCTGCCAGGAGACACCAGAATCAGAATCCCAAGCTGCTTCAC
Lph A   AspLeuValSerLysAspAspValValIleLysSerAlaArgArgHisGlnAsnGlnAsnProLysLeuLeuHis  68
Lph B   AspLeuValSerLysAspAspValValIleLysSerAlaArgArgHisGlnAsnGlnAsnProLysLeuLeuHis  53

                310                 330                 350                 370
cDNA    CCCGTGCCACAGGAGAGCAGTTTCAGAAATACTGCCACTCAATCAGAAGAGACCAAAGAACTCACTCCTGGGGCT
Lph A   ProValProGlnGluSerSerPheArgAsnThrAlaThrGlnSerGluGluThrLysGluLeuThrProGlyAla  93
Lph B   ProValProGlnGluSerSerPheArgAsnThrAlaThrGlnSerGluGluThrLysGluLeuThrProGlyAla  78

                    390                 410                 430                 450
cDNA    GCAACAACCTTAGAGGGAAAACTGGTGGAGCTCACTCATAAAATCATAAAGAATCTGGAAAACACCATGAGAGAA
Lph A   AlaThrThrLeuGluGlyLysLeuValGluLeuThrHisLysIleIleLysAsnLeuGluAsnThrMetArgGlu 118
Lph B   AlaThrThrLeuGluGlyLysLeuValGluLeuThrHisLysIleIleLysAsnLeuGluAsnThrMetArgGlu 103

                    470                 490                 510
cDNA    ACCATGGACTTTCTGAAAAGCCTATTCCCTCATGCCTCTGAAGTCGTGAAGCCCCAATGACGGGGATGCTCACGT
Lph A   ThrMetAspPheLeuLysSerLeuPheProHisAlaSerGluValValLysProGlnEnd                137
Lph B   ThrMetAspPheLeuLysSerLeuPheProHisAlaSerGluValValLysProGlnEnd                122

                530                 550                 570                 590
cDNA    CCCAGGCTGGACCGCAGCAGGTGCCTGCAGCACCCTCACCGCTGGCCTGACCACCGCCGTCTCTCAGCCCCTCGC
                610                 630
cDNA    GTTCCTTATTAAAGCATCGCATCCCAAGCCTG
```

Fig. 4.19. cDNA sequence of camel milk lactophorin A and corresponding protein variants A (Lph A), and B (Lph B), with mature proteins in bold. The open reading frame of the cDNA sequence is from A^{43} to A^{507} and the polyadenylation signal in bold from A^{608} to A^{613}. The sequence from A^{107} to T^{151} corresponds to exon 2 and is deleted in camel milk lactophorin B. Numbering of the amino acid chain starts from the first residue of the mature protein. **P**, potentially phosphorylated serine residues.

untranslated region contained a polyadenylation signal A^{608}TTAAA in the longer clone, which corresponded to A^{563}TTAAA in the shorter clone. The longer clone contained an open reading frame for a peptide of 156 aa residues and the shorter for a peptide of 141 aa residues. The start site of both mature proteins was confirmed, by N-terminal protein sequencing, to be Ser^{1}. The 19 aa signal peptides conformed to the usual pattern for signal peptides (Nielsen *et al.* 1997) and had 100% sequence similarity to the signal peptides of bovine lactophorin and GlyCAM-1 proteins. Two mature lactophorin variants were found and designated as variants A and B. Variant A consisted of 137 aa residues and variant B of 122 aa residues. Computational analysis (Barton, 1997) of camel lactophorin A revealed sequence similarities of 83.6% to bovine lactophorin, and 67.9% to mouse GlyCAM-1. The sequence of Beg *et al.* (1987) was corrected by insertion of Met^{11} to Ser^{35} for variant A and Ser^{10} to Ser^{20} for variant B (Fig. 4.20). The exchange of Leu^{108}, in the sequence of Beg *et al.* to Ile^{108} in variant A, and of Leu^{93} to Ile^{93} in variant B, respectively, could be due to an allelic variant, or to an error in protein sequencing. The inserted sequence was rich in Ser/Thr patterns, which gave indication for protein phosphorylation, and lacked cysteine residues.

Camel Whey protein (Beg, 1987):
SLNEPKDIMY MEPSISRED
Camel Whey protein (Beg, 1987):
SLN AAQVEI MEPSISRED
Camel lactophorin A:
SLNEPKDEIYMESQPTDTSAQVIMSNHQVSSEDLSMEPSISRED
Camel lactophorin B:
SLN AAQVIMSNHQVSSEDLSMEPSISRED

Fig 4.20. Corrected N-terminal sequences of lactophorin A and B compared to the sequences proposed by Beg *et al.* (1987).

The primary structures of camel and bovine lactophorin were highly similar. Percent sequence similarity of camel lactophorin to bovine and caprine lactophorin was much higher than to rat and murine GlyCAM-1 (Table 4.4). This result could partly be due to a closer evolutionary relationship between camels and cattle, but it also could give indication for a closer functional relationship of the camel and bovine lactophorins in milk. Camel

lactophorin B exhibited less sequence similarity, due to a gap in the sequence pile-up, produced by the deletion already mentioned.

Glycosylation accounts for about 17% to 18% of protein mass of bovine lactophorin (Ng *et al.* 1970; Kanno, 1989 b; Girardet *et al.* 1994), phosphorylation for about 0.5% (Ng *et al.* 1970) to 1.1% (Pâquet *et al.* 1988). An average of two to four residues of bovine lactophorin are phosphorylated and two to three residues are glycosylated. Dissimilarly to the camel protein, bovine lactophorin was originally isolated from proteose peptone component 3, and was reported to be N- and O-glycosylated. Nevertheless, high similarity of the corrected camel and the bovine primary structure and common secondary structural features gave indication that both proteins were true homologues and exerted a similar function in milk.

N-terminal Heterogeneity

Glu^{4} to Ser^{18}, the 15 aa peptide which was not found in variant B and corresponded to exon 2, was of acidic nature, with an isoelectric point at pH 3.70, and with distinct hydrophilicity. Bovine and caprine lactophorin, murine and rat GlyCAM-1, which are the only fully sequenced homologues from other species, were not reported to be expressed in different variants due to alternative splicing. N-terminal sequences of ovine and llama lactophorins did not show amino acid heterogeneity either (Fig. 4.21, N-terminal sequence of the sheep homologue identical to the caprine N-terminal sequence). Endoplasmatic signal peptidase cuts llama pre-lactophorin three aa residues prior to the cleavage site of the camel and bovine counterparts, and both of the sequenced GlyCAM-1 proteins are cleaved one amino acid beyond this site. The different cleavage sites additionally demonstrate a high variability of the N-terminal part of the lactophorin/GlyCAM-1 family. If this variability is the result of a variation in functionality between the N-termini, or if it results from a prevalent low N-terminal functionality of the proteins could not be decided, because the tertiary structures are not yet resolved and the functional domains not yet determined. Nevertheless, many residues of the N-terminal part were highly conserved (Fig. 4.21). This gave indication, that this part of the protein was of functional importance. A function, which depended on the N-terminal sequence as found in bovine lactophorin and camel lactophorin A, was expected to be lost or significantly altered in camel lactophorin B.

Table 4.4. Physico-chemical and sequence characteristics of mature, secreted GlyCAM-1 and lactophorin.

Species	Protein	Calculated MW [kDa]	Measured MW [kDa]	Amino acid residues	Isoelectric point (calculated)	Isoelectric point (measured)	Similarity to camel lactophorin A	Concentration (milk/serum) [mg l-1]
Camelus dromedarius	Lactophorin A	15.442	15.706	137	5.10	n.d.	100%	954
Camelus dromedarius	Lactophorin B	13.661	13.822	122	6.01		89.1%	
Bos taurus	Lactophorin	15.304	18.700[a]	135	6.03	4.9-6.1[b]	83.6%	300[c]
Capra hircus	Lactophorin	15.194	n.d.	136	4.98	n.d.	89.8%	n.d.
Rattus norvegicus	GlyCAM-1	13.456	50.000	127	4.45	n.d.	67.6%	n.d.
Mus musculus	GlyCAM-1	14.154	50.000	132	4.27	n.d.	67.9%	1.3-1.6[d]

a Sørensen *et al.* 1997.
b Girardet & Linden, 1991.
c Johnsen *et al.* 1996.
d Singer & Rosen, 1996.

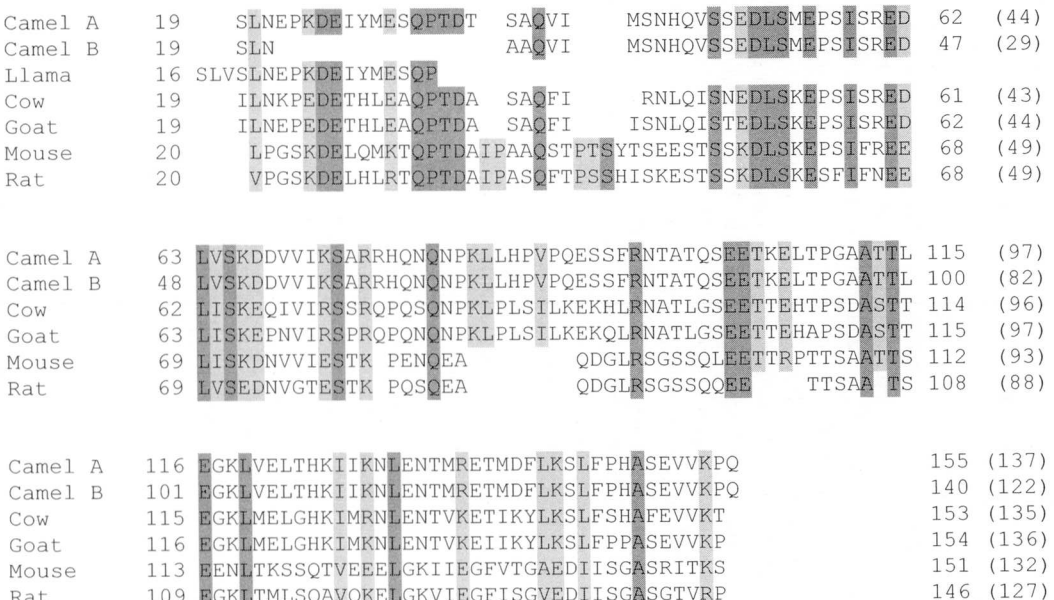


Fig. 4.21. Sequence alignment of mature proteins of the GlyCAM-1/lactophorin family. Numbering starts at the first residue of the signal peptide, to facilitate comparison of the polypeptide chains. Numbering of mature peptide chains in brackets. Positions with conserved amino acids are dark shaded. Positions with similar amino acids are light shaded. Camel A: *Camelus dromedarius* lactophorin A, Camel B: *Camelus dromedarius* lactophorin B, Llama: *Llama llama* lactophorin N-terminal sequence, Cow: *Bos taurus* lactophorin, Goat: *Capra hircus* lactophorin N-terminal sequence, Mouse: *Mus musculus* GlyCAM-1, Rat: *Rattus norvegicus* GlyCAM-1.

Secondary Structure

The amino acid composition of lactophorin was similar to α- and β-caseins, although the proline content was lower (6.6% in camel lactophorin, compared to 17.1% in camel β-casein). Similarly to caseins, camel and bovine lactophorin were characterised by an acidic N-terminal part of the protein, which was rich in Glu, Ser, Thr, and contained clustered phosphoserines, whereas the C-terminal part was rich in hydrophobic residues, with the difference, that lactophorin was considered to form a C-terminal amphiphilic helix, with mixed basic and acidic residues on the polar side (Girardet & Linden, 1996; Fig. 4.22). This structural property was more pronounced in bovine lactophorin and rodents' GlyCAM-1 proteins, than in the camel homologue, since the latter protein contained a helix-breaking Pro^{128} in variant A, and Pro^{113} in variant B, near the C-terminus, which induced a kink towards the C-terminal end of the helix. The recently resolved primary structure of caprine lactophorin (Lister *et al.* 1998) contained Pro^{128} and Pro^{129} in the corresponding region, which indicated, that modification of the primary sequence at this site was not arbitrary. The side chain of Thr^{105} in variant A, and Thr^{90} in variant B, furthermore protruded into the hydrophobic part of the helix. The C-terminal part of bovine lactophorin was discussed to be involved in binding of phospholipids of the milk fat globule membrane (MFGM). Different proteins, such as PAS-6/7, cecropins and magainins, were shown to interact with phospholipid membranes by amphiphilic helices (Sørensen *et al.* 1997).

Although camel lactophorin was isolated from whey, the predicted secondary structure gave indication for binding to MFGM phospholipids. Bovine lactophorin was detected in whey and in MFGM, but not in casein (Kester & Brunner, 1982; Kanno, 1989 a; Sørensen *et al.* 1997). Another MFGM binding protein, bovine PAS-6/7, was shown to interact with phospholipids through the polar side of its C-terminal amphiphilic helix (Andersen *et al.* 1997). Initial binding of lactophorin may occur in the same way, but strong binding to MFGM could be due to transmembrane integration into the phospholipid fat globule micelle. PAS-6/7 was shown to bind phospholipids with different affinities. Blends of phospholipids were bound with much higher affinities. The highest affinity was found for the complex phospholipid mixture extracted from MFGM. The pronounced difference in the structures of the C-terminal parts of bovine and camel lactophorins may be due to a different phospholipid composition in bovine and camel milk fat globules. Camel milk fat globules have a slightly smaller average diameter of 2.61 μm, compared to bovine milk fat globules, and a different composition of phospholipids (Farah, 1996). Major components of

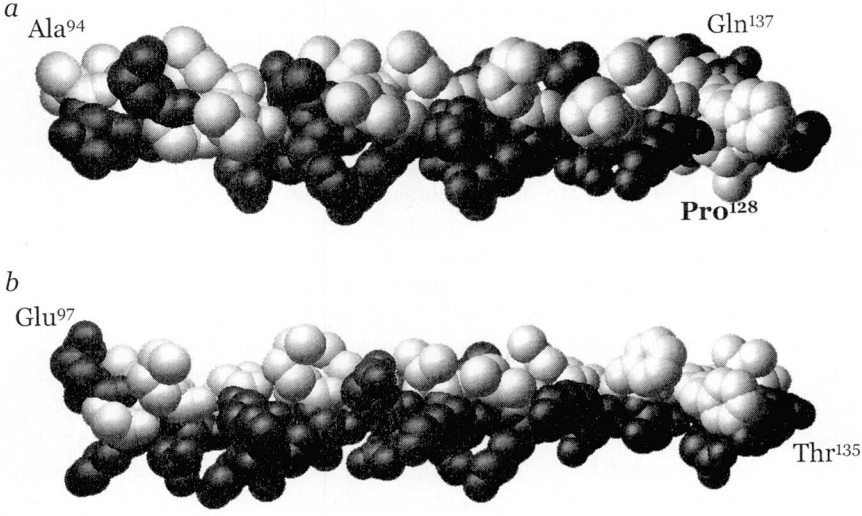


Fig. 4.22. Pictorial view on the α-helical C-terminal part of (a) camel, and (b) bovine lactophorin. The hydrophobic side of the helix is facing up, and the hydrophilic side is facing down. Polar and charged residues are dark shaded. Kinks of Pro[128] and Pro[136] in camel lactophorin A, respectively of Pro[113] and Pro[121] in camel lactophorin B, are towards the C-terminus, which is set to the right hand side, and are not clearly visible in this representation. The amphiphilic character of the helix is more pronounced in bovine lactophorin.

camel MFGM are phosphatidylethanolamine, with 35.9%, phosphatidylcholine, with 24.0%, and sphingomyelin, with 28.3%, (Morrison, 1968), whereas bovine MFGM mainly consists of 31.8% phosphatidylethanolamine, 34.5% phosphatidylcholine and 25.2% sphingomyelin. The proportion of phosphatidylcholine, which does not bind to PAS-6/7, is much smaller in camel milk than in cow milk. The proportion of non saturated 16:1 fatty acid in milk fat is about three to four times higher than in cow milk (Abu-Lehia, 1989, Farah, 1996). There could be a function of the helix-breaking proline to induce a variation in the tertiary structures of camel and bovine lactophorins, which may be important for specific binding to the milks' phospholipid mixture.

Phosphorylation

The molecular mass of the single charged MALDI peak of camel lactophorin A was 15.706 kDa, the molecular mass of the double charged peak was 15.689 kDa. The differences of the peaks to the calculated mass of 15.442 kDa were 264 Da and 247 Da respectively. This could account for a threefold phosphorylated protein. Respective molecular masses of camel lactophorin B were 13.822 kDa, and 13.921 kDa, respectively. The differences of the peaks to the calculated mass of 13.661 kDa were 161 Da and 260 Da respectively. This could account for a two- to threefold phosphorylated protein. Milk proteins with a low isoelectric point are preferentially phosphorylated by mammary gland casein kinase, according to the consensus pattern [Ser, (Thr)]–Xaa-[SerP, Glu, (Asp)]–Xaa, with Glu or Asp at position +1 and +3 enhancing phosphorylation (Swaisgood, 1992). Ser^{30}, Ser^{35}, Ser^{39}, Ser^{41}, and Ser^{83} in variant A, and Ser^{15}, Ser^{20}, Ser^{24}, Ser^{26}, and Ser^{68} in variant B, respectively, fit to the consensus pattern. Ser^{30}, Ser^{35}, Ser^{39} and Ser^{41} in variant A, and Ser^{15}, Ser^{20}, Ser^{24}, and Ser^{26} in variant B, were located in a predicted loop surrounded by α-helical regions and had high phosphorylation potential. Respective Ser^{29}, Ser^{34}, Ser^{38}, and Ser^{40} of bovine lactophorin are partially phosphorylated (Sørensen & Petersen, 1993). Ser^{47} in camel variant A, and Ser^{32} in variant B, respectively, were probably not phosphorylated, because the sequence Ser^{47}-Lys^{48}-Asp^{49} did

Camel Lactophorin A:

```
        10        20        30        40        50        60        70
        ββββ       ααααα                 ααααα   ααααααααααα
SLNEPKDEIYMESQPTDTSAQVIMSNHQVSSEDLSMEPSISREDLVSKDDVVIKSARRHQNQNPKLLHPV

        80        90       100       110       120       130
             αααα      αααααααααααααααααααααααααααααααααα
PQESSFRNTATQSEETKELTPGAATTLEGKLVELTHKIIKNLENTMRETMDFLKSLFPHASEVVKPQ
```

Bovine Lactophorin:

```
        10        20        30        40        50        60        70
αα       ααα      ααααααα               ααααα  βββββ            αααα
ILNKPEDETHLEAQPTDASAQFIRNLQISNEDLSKEPSISREDLISKEQIVIRSSRQPQSQNPKLPLSIL

        80        90       100       110       120       130
αα                        ααααααααααααααααααααααααααααααααααααααα
KEKHLRNATLGSEETTEHTPSDASTTEGKLMELGHKIMRNLENTVKETIKYLKSLFSHAFEVVKT
```

Fig. 4.23. Secondary structure predictions for camel lactophorin A and bovine lactophorin. α-helical regions designated as "α", β-pleated regions as "β". Serine residues with phosphorylation potential in bold.

not fit to the consensus pattern for mammary gland casein kinase, although full phosphorylation of the homologous Ser^{46} in bovine lactophorin was reported. This serine was found in another loop region than the other serine residues with phosphorylation potential (Fig. 4.23), indicating a special function of this serine, e.g. in binding of calcium.

Camel lactophorin A was of a distinct acidic nature, with an isoelectric point at pH 5.10, the isoelectric point of variant B at pH 6.01 was similar to bovine lactophorin (Table 4.4). Threefold phosphorylation would decrease the isoelectric point of variant A to pH 4.70, and of variant B to pH 5.16.

Potential for Binding of free Calcium

The sequence presented by Beg *et al.* (1987) was complemented with 25 amino acids in variant A and with 11 in variant B (Fig. 4.20). The corrected protein sequences were rich in glutamic acid, lysine, serine and leucine, and had only minor amounts of aromatic and sulphated residues. The inserted sequence turned out to have a high potential for phosphoserine clusters. This structure also occurs in caseins and exhibits high binding affinities for Ca^{2+} (Bernos *et al.* 1997). It was suggested, that lactophorins may bind calcium in the milk (Sørensen & Petersen, 1993), thereby controlling the solubility of non casein calcium phosphate. Additional support for this idea is given by the finding, that binding of L-selectin to plasma GlyCAM-1 is calcium-dependent (Suguri *et al.* 1996). Serum GlyCAM-1 of mice may be phosphorylated by casein kinase II at Ser^{4}, Thr^{29}, Ser^{35}, Thr^{61}, Ser^{77}, Thr^{91}, and Thr^{92}. Although GlyCAM-1 is also expressed in milk, it lost most phosphorylation patterns for mammary gland casein kinase. Only Ser^{30}, Ser^{40}, and Ser^{93} of mouse GlyCAM-1 may be phosphorylated in milk. Neither casein kinase will produce phosphorylation clusters in GlyCAM-1. Calcium may therefore bind to GlyCAM-1 in a different way than to lactophorin.

Glycosylation

There was no indication for glycosylation of the two camel lactophorin variants from mass spectometrical analyses. Prediction of O-glycosylation was done by the method of Hansen *et al.* (1995). The sequences were found to have a similarly low potential for O-glycosylation by UDP-GalNAc-polypeptide N-acetylgalactosaminyl Transferase as has bovine lactophorin, in contrast to mouse and rat GlyCAM-1 sequences (Fig. 4.24). Whereas mouse GlyCAM-1 revealed a strong O-glycosylation potential of Ser^{23}, Thr^{24}, Thr^{26}, Ser^{27}, Thr^{29}, Ser^{30}, Thr^{82}, Thr^{83}, Thr^{86}, Thr^{87} and Thr^{91}, there was only a low O-glycosylation potential of Thr^{16}, Thr^{81}, and Thr^{90} found in camel

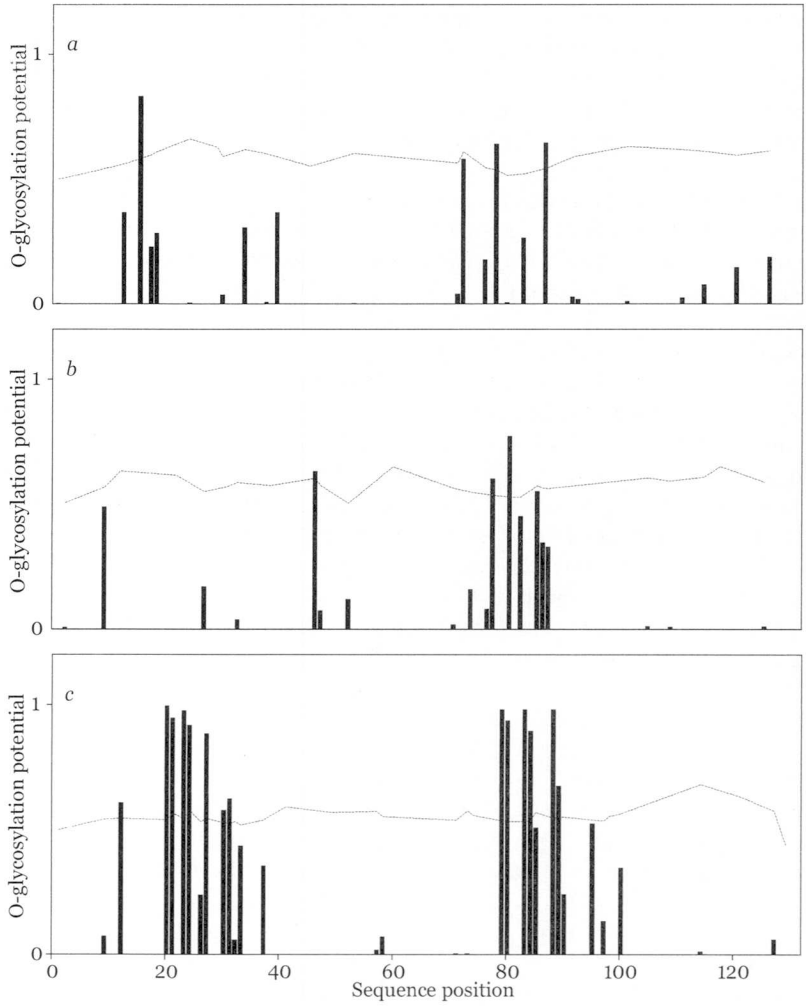


Fig. 4.24. O-glycosylation potential of threonines and serines in proteins of the GlyCAM-1/lactophorin family. (a) Camel Lactophorin A. (b) Bovine Lactophorin. (c) Murine GlyCAM-1. The potential of the residues is shown as a solid bar with a value from 0 (no potential) to 1 (high potential). The threshold, which depends on the primary structure of the protein, is shown as a dashed line. The probability of glycosylation is the difference between the potential and the threshold.

lactophorin A and of Ser^{54}, Thr^{86} and Thr^{89} in bovine lactophorin. The pattern Asn-Xxx-Ser/Thr, which is a prerequisite for N-glycosylation, and which occurs and provokes glycosylation in the bovine variant at Asn^{77} (Girardet *et al.* 1995), was not found in camel lactophorin.

Relation to the Mucin-type Proteins

GlyCAM-1, a glycosylation dependent cell adhesion molecule found in blood serum, is a ligand for lymphocyte L-selectin, which is expressed on high endothelial venules in peripheral lymph nodes and at sites of chronic inflammation (Berg *et al.* 1998). It mediates the recruitment of lymphocytes from blood into these tissues as a soluble, probably multimeric, protein, with highly dynamic adhesion of the cells due to low affinity and rapid kinetics of selectin interactions (Nicholson *et al.* 1998). L-selectin, a protein with a lectin domain, has high affinity for mucins with multiple O-linked carbohydrates of the lactosamin type. Binding depends on sialylation, sulfation and fucosylation. GlyCAM-1 was found to contain sulfated O-linked carbohydrates of the type Sia-α(2→3)Gal-β(1→4)Fuc-α(1→3)GalNac. The same protein is also secreted in mouse milk (Dowbenko et al. 1993; Nishimura *et al.* 1993), but with a different glycosylation structure than in blood serum. GlyCAM-1 has much higher probability of being glycosylated than lactophorins (Fig. 4.24). O-glycosylation of the mouse homologue should be expected at 11 residues. Glycosylation of camel lactophorin A was only expected at Thr^{16}, whereas the bovine protein had medium high probability of glycosylation at Thr^{89}. Implications of secondary and tertiary structures may favour or prevent glycosylation of these sites (Fig 4.23). Bovine lactophorin was reported to be partially O-glycosylated at Thr^{16}, completely N-glycosylated at Asn^{77} and completely O-glycosylated at Thr^{86} (Sørensen & Petersen, 1993). Camel lactophorin did not reveal a recognition pattern Asn-Xaa-Thr for N-glycosylation, in contrast to the bovine homologue. Analysis of mass spectroscopic data did not give indication of glycosylation. Only two sharp peaks were present, which did not allow for glycosylation of either variant. Absence of glycosylation rendered camel lactophorin less hydrophilic than other proteins of the GlyCAM-1/lactophorin family. If lactophorin is bound to MFGM by the C-terminal part, the glycosylated residues of bovine lactophorin are likely to protrude into the milk serum and may help to maintain a hydrophilic covering of the fat globules. This would help to prevent aggregation of fat globules and binding to alveolar epithelial cells in the udder by electric charge repulsion. Absence of glycosylation in camel lactophorin may be compensated by a higher concentration of the protein in the milk (Table 4.4), as compared to bovine lactophorin, to fulfil this

proposed function of lactophorin. Glycosylation of the bovine protein may also be part of infant protection against viral or bacterial infection. In a similar way as the Tamm-Horsfall protein in urine, bovine lactophorin may help to inhibit bacterial adhesion to host tissue (Sørensen & Petersen, 1993). Stimulation of the growth of bifidobacteria by bovine lactophorin was also demonstrated (Girardet & Linden, 1996). Next to bacterial infections, rotaviral infection is an important cause of infant diarrhoea, which can lead to death of the animal. The milk mucin complex was shown to protect the infant by specific interaction with the virus, in a similar way as intestinal mucins (Yolken *et al.* 1992, 1994). Lack of glycosylation in camel milk lactophorin may indicate, that such functionality is not needed in camel milk, perhaps because the danger of infection is lower in the dry climate where camels live. To sum up, we suggest that the common function of camel and bovine lactophorins in milk cannot depend on glycosylation of the proteins. Camel lactophorin is also not likely to be expressed in blood serum and to have a function similar to GlyCAM-1 proteins, since specific binding to L-selectin would depend on glycosylation. It is not known at present, if camels have a GlyCAM-1 related protein in blood serum with high probability of glycosylation.

Protein Expression and Concentration in Camel Milk

Lactophorin was found to be the protein present at the second highest concentration in camel whey. Only α-lactalbumin was found at higher concentrations. The concentration of the proteins was calculated by peak area integration at 220 nm. Total lactophorin was detected at a concentration of about 950 mg l^{-1} throughout the lactation period. The relative amount of variant B in camel milk was about one quarter of lactophorin A, as estimated by data from N-terminal sequencing, mass spectroscopy and PCR products generated from cDNA.

Expression of GlyCAM-1 in milk was shown to depend on regulation by glucocorticoid receptors and anti-sense RNA (Kawamura *et al.* 1987). Furthermore, binding sites for mammary gland specific transcription factors were found on the 5'-flanking region of the bovine lactophorin gene (Johnsen *et al.* 1996, Girardet & Linden, 1996). This indicates, that the expression of bovine lactophorin is regulated in a similar way as caseins. Bovine lactophorin was found to be expressed specifically in the lactating mammary gland (Johnsen *et al.* 1995; Groenen *et al.* 1995). A protein concentration of 300 mg l^{-1} milk was reported (Johnsen *et al.* 1996). The concentration of lactophorin was therefore higher in camel milk than in cow milk.

Oligomerisation

Bovine lactophorin was reported to be a complex of about 190 kDa (Sørensen *et al.* 1997), which segregated to a complex of about 40 kDa upon dissolution in 5 M guanidine hydrochloride (Ng *et al.* 1970). The high molecular complex would account for a decamer, the low molecular complex for a dimer. Camel lactophorin was found to have an apparent mass of 15 kDa by SDS-PAGE and of about 30 kDa by Sephadex G-100 chromatography (Beg *et al.* 1987). A complex with a higher molecular mass was not found. The protein was therefore probably dimerised in whey. A model of GlyCAM-1 forming a dimer by association of the C-terminal helical parts was proposed by Lasky *et al.* (1992). Dimerisation of bovine lactophorin was proposed to occur in a similar way (Girardet & Linden, 1996).

Proteolytic Cleavage

Bovine lactophorin was found to be susceptible for proteolytic cleavage by plasmin at Arg^{53}-Ser^{54}, producing an 11 kDa and an 18 kDa fragment, as judged by SDS-PAGE (Kanno & Ogawa, 1989; Sørensen & Petersen, 1993). Proteolytic fragments of camel lactophorin were found in tiny amounts in camel whey. A minute peak at 13.7 min (Fig. 4.17) probably contained the hydrophilic N-terminal parts of both lactophorin variants, as judged by N-terminal sequencing. Camel milk was shown to be low in plasmin activity (Baer *et al.* 1994). Either plasmin concentration in camel milk is low, or plasmin is repressed by serine protease inhibitors, such as the putative trypsin-type protease inhibitor whey acidic protein (WAP). Camel plasmin may also have a different turnover rate than bovine plasmin. The amino acid sequence at the site, where the bovine protein is cleaved, Lys^{54}-Ser^{55} in variant A, and Lys^{39}-Ser^{40} in variant B, respectively, could be a target for plasmin cleavage, but was found in a α-helical region, whereas the bovine site was at the C-terminal end of a β-folded structure (Fig. 4.23). This difference may render the site of camel lactophorin less susceptible for proteolytic cleavage.

Inhibition of Lipolysis

An important function of lactophorin in milk seems to be the continued maintenance of the fat dispersion. Early indication that proteose peptone was the component responsible for milk foaming was given by Jelen (1973). Strong foaming of whey depleted from main proteins and lactose was observed, the foam quality was negatively affected by addition of calcium hydroxide. Shimizu *et al.* (1989) showed, that the high emulsifying activity of proteose peptone component 3, which mainly consists of lactophorin,

was not negatively affected by extended heat treatment at 100 °C. Emulsions remained stable from pH 3 to pH 9. Addition of 5 mM $CaCl_2$ or 50 mM NaCl increased the emulsifying activity. The amount of adsorbed bovine lactophorin was about 3 mg g^{-1} oil in an experimental lipid-water emulsion. Surface tension was reduced to a level below 25 mN m^{-1}. The C-terminal proteolytic product formed by plasmin, and probably containing an amphiphilic helix, did not adsorb strongly to the hydrophobic interface. β-lactoglobulin was not able to reduce the surface tension to a similar level. Lactophorin showed a much stronger adsorption to the lipid-water interface than β-lactoglobulin (Courthaudon *et al.* 1995). The fact that only intact lactophorin was able to bind strongly to the lipid-water interface, and not its proteolytic breakdown product, gave indication, that the N-terminal part of the protein was important for conformational stabilisation of the amphiphilic helix. It was interesting, that prolonged heat treatment did not deteriorate the emulsifying activity of lactophorin. The absence of cysteines may contribute to the thermostability of the protein. The higher amount of lactophorin in camel milk, compared to cow milk, could partly be due to the greater surface area of 2.29 m^2 for 1 g of camel milk fat, compared to 1.79 m^2 for cow milk fat.

Spontaneous lipolysis by lipoprotein lipase was demonstrated for milk stored for several days at 4 °C (Girardet *et al.* 1993). Bovine lactophorin was found to prevent the lipolytic action of porcine pancreatic lipase (PPL), a serine lipase similar to lipoprotein lipase, by competitive adsorption to the hydrophobic phase in a lipid-water emulsion and displacement of lipase into the aqueous phase. In this model system, the protein did not interact directly with the lipase to exert this function (Girardet *et al.* 1993). PPL cannot adsorb on a lipid-water interface at a surface tension between 20-30 mN m^{-1} in the absence of colipase. 50% inhibition of lipolytic activity was achieved with a 6.7-fold higher (w/v) PP3 concentration compared to the inhibitory effect of β-lactoglobulin. This would correspond to about a 3.4-fold higher lactophorin concentration, if it is assumed, that about half of PP3 consists of this active component. Both adsorptive proteins have similar molecular masses. In the β-lactoglobulin/PPL-system, lipolytic activity was fully restored upon addition of colipase, which binds PPL to the hydrophobic phase, and low concentrations of different bile salts, e.g. 0.5 mM taurodeoxycholate. Lipolytic activity of PPL was only restored in the lactophorin/PPL-system upon addition of colipase and 2 mM taurodeoxycholate, reactivation with other bile salts was not possible. It was concluded, that bovine lactophorin was more difficult to desorb from the oil-water interface than β-lactoglobulin.

Lactophorin was shown to prevent lipolysis, in contrast to β-Cn-5P f(1-105/7) , also named PP5, a proteolytic breakdown product of β-CN, which seemed to activate lipolytic activity (Girardet & Linden, 1996). Since it is known, that lipoprotein lipase is largely associated to casein micelles in fresh milk, it can be considered that lactophorin prevents lipolysis of milk fat in the mammary gland, and looses this function when digested by plasmin and gut proteases, whereas PP5 stimulates lipoprotein lipase, when formed during storage or digestion of milk, and accelerates milk fat uptake in this way. Interestingly, not only the potential lipase inhibitor lactophorin is found at higher levels in camel milk than in bovine milk, but also the potential protease inhibitor whey acidic protein, a 12.5 kDa protein, which is highly stimulated by glucocorticoids (Beg *et al.* 1986). A higher level of natural preserving agents may bring about the longer storage life of raw camel milk compared to raw cow milk (Farah, 1996).

Applications
The camel protein is a glycosylation free and mono- or dimeric member of the lactophorin/GlyCAM-1 family, which is easily purified in large quantities from camel whey. This properties make the protein a good substrate for functional characterisation, crystallisation and X-ray structure analysis. Furthermore, the protein could be studied for its presumed qualities as an emulsifying agent and inhibitor of lipolysis in food products, such as butter or ice cream.

4.4 Minor Whey Proteins

In this study, we focused on minor whey proteins with potential for protection of the milk against microbial spoilage. There is a special interest in the study of these proteins for prolongation of the storage time, and for evaluation of the therapeutic potential of camel milk.

Antimicrobial Proteins in Milk
Milk is a foodstuff of high nutritional value. It serves the new-born as its sole diet at a time when it is rapidly growing. Essential food components, such as vitamins, minerals, fat and proteins are available at high amounts. This composition makes milk an ideal medium for growth of all sorts of micro-organisms. The new-born, which does not have a working immune system yet, has to be protected from infection, but also the udder of the lactating animal. Contaminated milk would also be a good vector for the transmission of disease germs from the heifer to the infant. Components are

thus required, which prevent or regulate the growth of micro-organisms in the milk, the udder and the intestine of the new-born. Among others, these factors may be specific proteins, fats, or minerals. Several antimicrobial proteins were described in milk, such as lysozyme, immunoglobulins, lactoferrin and lactoperoxidase. Interest in whey proteins for commercial use as natural antibacterial or antiviral agents in food and cosmetics has increased during the past years. Different proteins are discussed as prebiotic food supplements, which could regulate the microbial flora of the intestine and activate the immune system. New chromatographic methods, e.g. the Streamline system (Noppe *et al.* 1996), allow for large scale purification of minor whey components. Main focus was thrown onto the already mentioned, well characterised protective proteins. These proteins are predominantly of basic nature, and are purified in a simple and cost-effective procedure by cation exchange chromatography.

Camel milk is usually consumed as a raw or sour milk product, and is not refrigerated or pasteurised. Processing technology, which guarantees a constant hygienic quality of the milk, cannot be applied due to scarce of investment capital. Hygienic problems arise from to the special environment and the living conditions in semi arid areas, where camels are usually kept (Abeiderrahmane & Reed, 1993). Brucellosis, trypanosomiasis and other diseases are latent dangers to the health of the herds, the pastoralists and the consumers. Hence natural inhibition of pathogen growth in milk is of importance.

Camel milk was frequently reported to have high antimicrobial activity, and was shown to inhibit pathogen growth more than bovine milk (Elagamy *et al.* 1992). Antimicrobial properties were partially attributed to well characterised proteins, such as lactoferrin, lactoperoxidase, lysozyme and immunoglobulin A. These proteins were shown to have higher concentrations or higher activity in camel milk compared to bovine milk. In this study we determined the basic physico-chemical and structural parameters of lactoferrin and lactoperoxidase, of which the relationship between structure and function is well characterised in human and bovine counterparts. Lysozyme was not found in the milk studied, which may be due to the fact, that the milk was from the end of the lactation period, and the lysozyme concentration was shown to depend much on the lactation stage (Barbour *et al.* 1984). Furthermore, a cDNA corresponding to lysozyme was not found by screening with oligonucleotides derived from conserved regions of the already known C-type lysozymes. We suppose, that the lysozyme activity measured by other authors (Elagamy *et al.* 1996) has

to be assigned to other enzymes, e.g. to the Peptidoglycan Recognition Protein, which is discussed in this thesis.

4.4.1 Peptidoglycan Recognition Protein

Literature

A novel protein family was described recently, which is involved in primary immune response of vertebrates and invertebrates on gram-positive bacteria and other invading organisms, such as nematodes, and works by non-clonal pattern recognition (Yoshida *et al.* 1996; Kang *et al.* 1998). Inactivation of pathogens probably occurs by binding to peptidoglycan structures in bacterial cell walls, hence the name was given peptidoglycan recognition proteins (PGRP). The protein was first described in pigs challenged with nematodes, and was weakly detected in neutrophils (Fornhem *et al.* 1996). Specific mRNA was detected in organs related to the immune system of the vertebrate species *Homo sapiens* and *Mus musculus*, and of the invertebrate moth *Trichoplusia ni*, the cabbage looper. mRNA was also detected in murine adenocarcinoma cell cultures from mammary gland, whereby a relation to metastasis frequency was reported (Kustikova *et al.* 1996). In a recent study, the murine protein was shown to induce tumour cell apoptosis (Kiselev *et al.* 1998). The assignment *tag7* protein, (tumour associated gene 7 protein), was given in cancer related studies for PGRP. For ease of reading, we will refer to *tag7* protein as PGRP. In this thesis, we describe a closely related homologue of PGRP, which was isolated from camel whey by heparin-sepharose chromatography, and probably serves the same function of specific pathogen inhibition in camel milk. This protein class was not yet found in milk. PGRP was found in higher amounts in camel milk than concentrations reported for other antibacterial proteins, such as lactoferrin, lactoperoxidase, or lysozyme. It possibly helps in inactivation of gram-positive bacteria and other pathogens of camels. Expression of the protein may be an adaptation to the special environment in arid regions.

Primary Structure

A 19.110 kDa protein was eluted from heparin-sepharose. The N-terminal sequence of the reverse phase purified protein was determined as Arg-Glu-Asp-Pro-Pro-Ala-Cys-Gly-Ser-Ile. A full length cDNA clone of 700 bp, corresponding to the N-terminal sequence was obtained (Fig 4.25; EMBL/GenBank™ accession number AJ131676). The length of the 5'-untranslated region was 24 bp, a partial Kozak-box (Kozak, 1989) in front of the

```
            10                  30                  50                  70
cDNA    GTTGCTGTCGGCGATCTGCCTGCCATGACCCGGCACTGCGTGCTTCTCGTCTGGGCTCTCCTCGCCCTCCTCAGC
Protein                          MetThrArgHisCysValLeuLeuValTrpAlaLeuLeuAlaLeuLeuSer - 5

                    90                  110                 130                 150
cDNA    CTCGGAGCGGCTCGAGAAGACCCGCCGGCCTGCGGCTCCATCGTGCCCCGCCGAGAGTGGAGGGCCCTGGCGTCC
Protein LeuGlyAlaAla**ArgGluAspProProAlaCysGlySerIleValProArgArgGluTrpArgAlaLeuAlaSer** 21

                        170                 190                 210
cDNA    GAGTGCAGAGAAAGGCTAACACGGCCGGTGCGCTACGTGGTGGTGTCGCACACTGCGGGCAGCCACTGCGACACC
Protein **GluCysArgGluArgLeuThrArgProValArgTyrValValValSerHisThrAlaGlySerHisCysAspThr** 46

          230                 250                 270                 290
cDNA    CCGGCTTCGTGCGCGCAGCAGGCCCAGAACGTGCAAAGCTACCATGTGCGGAACCTGGGCTGGTGCGACGTGGGC
Protein **ProAlaSerCysAlaGlnGlnAlaGlnAsnValGlnSerTyrHisValArgAsnLeuGlyTrpCysAspValGly** 71

              310                 330                 350                 370
cDNA    TACAACTTCCTGATCGGAGAAGATGGGCTCGTGTACGAAGGCCGAGGCTGGAACATCAAGGGCGCCCACGCAGGT
Protein **TyrAsnPheLeuIleGlyGluAspGlyLeuValTyrGluGlyArgGlyTrpAsnIleLysGlyAlaHisAlaGly** 96

                    390                 410                 430                 450
cDNA    CCCACCTGGAACCCCATATCCATAGGCATCTCCTTCATGGGCAACTATATGAATCGAGTGCCCCCGCCCCGCGCC
Protein **ProThrTrpAsnProIleSerIleGlyIleSerPheMetGlyAsnTyrMetAsnArgValProProProArgAla** 121

                        470                 490                 510
cDNA    CTCCGGGCAGCCCAGAATCTGCTGGCTTGTGGTGTGGCTCTGGGAGCCCTGAGATCCAACTACGAGGTCAAAGGA
Protein **LeuArgAlaAlaGlnAsnLeuLeuAlaCysGlyValAlaLeuGlyAlaLeuArgSerAsnTyrGluValLysGly** 146

          530                 550                 570                 590
cDNA    CACCGGGATGTGCAGCCGACCCTCTCTCCAGGTGACCGGCTCTACGAAATCATCCAGACTTGGTCACACTACCGC
Protein **HisArgAspValGlnProThrLeuSerProGlyAspArgLeuTyrGluIleIleGlnThrTrpSerHisTyrArg** 171

              610                 630                 650                 670
cDNA    GCATGAGGCCCTCTCCGGCTCGGCACACCGCTCCCATCCCCACTCTGTCAGAAACCCCACTGCCTCTCCCCCCCC
Protein **Ala**End                                                                  172

                    690
cDNA    CC**AATAAA**GGTGAAGCTCAAACTGT
```

Fig. 4.25. cDNA sequence of camel milk peptidoglycan recognition protein (PGRP) and corresponding peptide, with mature protein in bold. The open reading frame of the cDNA sequence is from A^{25} to A^{603}, and the polyadenylation signal in bold from A^{678} to A^{683}. Numbering of the amino acid chain starts from the first residue of the mature protein.

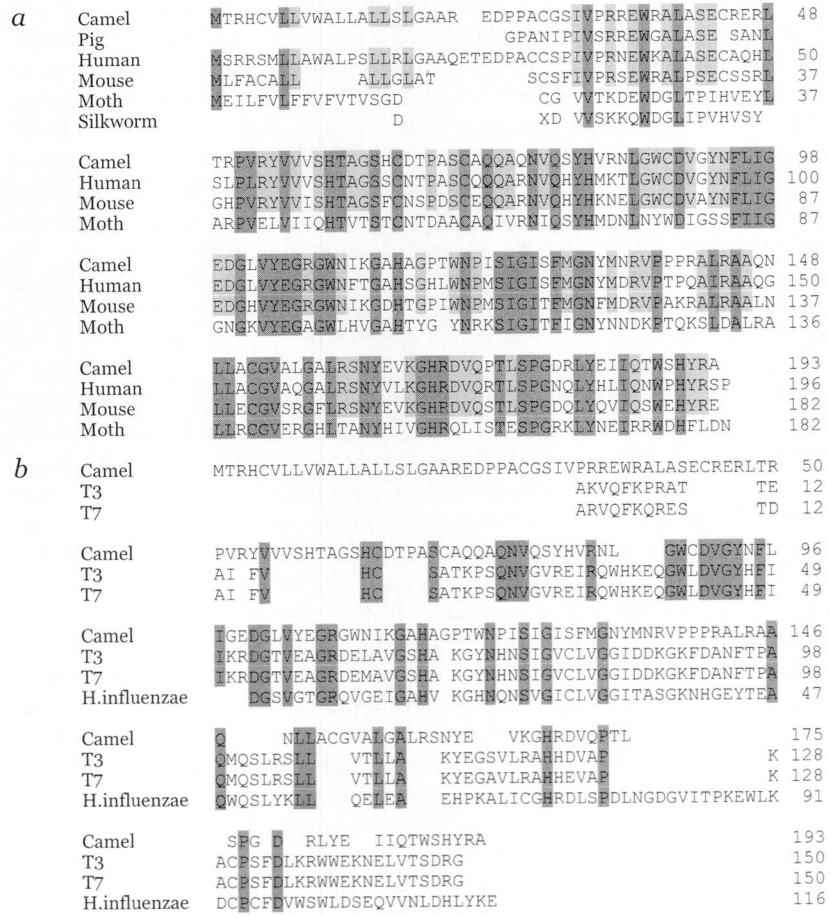


Fig. 4.26. Sequence alignment of peptidoglycan recognition proteins and related proteins. (a) PGRP proteins from Camel (*Camelus dromedarius)*, Pig *(Sus scorfa)*, Man *(Homo sapiens)*, Mouse *(Mus musculus)*, Moth *(Trichoplusia ni)*, and Silkworm (*Bombyx mori*). Positions with conserved amino acids are dark shaded. Positions with amino acids only conserved in vertebrates are light shaded. (b) Similarity of camel PGRP to related N-acetylmuramoyl-L-alanine amidases from T3 and T7 viruses and from *Haemophilus influencae.*

translational start site $A^{25}TG$ consisted of a purine at −3 bp, and cytosines at -1 bp, -2 bp, -5 bp, and -6bp. Expression of the murine homologue of camel PGRP was reported to be regulated at the posttranscriptional level (Kiselev *et al.* 1998). The 3'-untranslated region contained a polyadenylation signal A^{678}ATAAA. The open reading frame from A^{25} to A^{603} coded for a peptide chain of 193 aa residues and a molecular mass of 21.377 kDa. Mature PGRP was 172 aa residues long and had a calculated molecular mass of 19.143 kDa, and a molecular mass of 19.117 kDa, measured by MALDI-MS. Since the masses were nearly identical, it was concluded, that the protein was not modified after translation, e.g. by glycosylation or by phosphorylation, and did not bind a ligand. The isoelectric point of camel PGRP was at pH 8.73, and thus distinctly higher than the isoelectric points of the human, murine, moth and silkworm proteins, which were at pH 7.94, 7.49, 7.25 and 6.5, respectively. Only porcine PGRP was reported to have a higher isoelectric point at pH >10.5 (Fornhem *et al.* 1996). The 19 aa signal sequence had 85.7% signal sequence similarity with the signal sequence of human PGRP, but did not reveal significant similarities to mouse and moth PGRP signal sequences. The mature protein had 91.2% similarity with human PGRP, 87.9% with murine PGRP, and 70.8% with moth PGRP. Weaker similarities were calculated for T3 and T7 N-acetylmuramoyl-L-alanine amidases, which were 57.9%, and 57.0%, respectively. 19 residues were conserved in all proteins studied (Fig. 4.26). Camel PGRP protein had a high content of small aa residues of 39.0% and was rich in arginine, but poor in lysine, although the isoelectric point was highly basic. The arginine residues were weakly conserved among PGRP from different species. Murine PGRP was reported to exist in two major forms, as a monomer, and as trimer (Kiselev *et al.* 1998). A trimeric protein may have higher affinity to target sites on bacterial or eucaryotic cell walls, and even be able to agglutinate cells. The uneven number of cysteines in murine and human PGRP indicates possibility of covalent intermolecular crosslinking. Mass determination of camel PGRP was done by MALDI-MS and SDS-PAGE. Both methods only gave indication for a monomeric protein.

Affinity Chromatography and Quantification

The peptidoglycan recognition protein (PGRP) was isolated from camel whey by heparin-sepharose chromatography, of a milk sample taken at the end of the lactation period, about 360 days after parturition. A single band, as judged by SDS-PAGE, was obtained by elution from 0.35 M NaCl to 0.40 M NaCl (Fig. 4.27, peak I). Heparin binding proteins from bovine whey were eluted under the same conditions. Heparin is a highly sulfated glycosaminoglycan, attached to a core peptide with $(Ser\text{-}Gly)_{10}$, and produ-

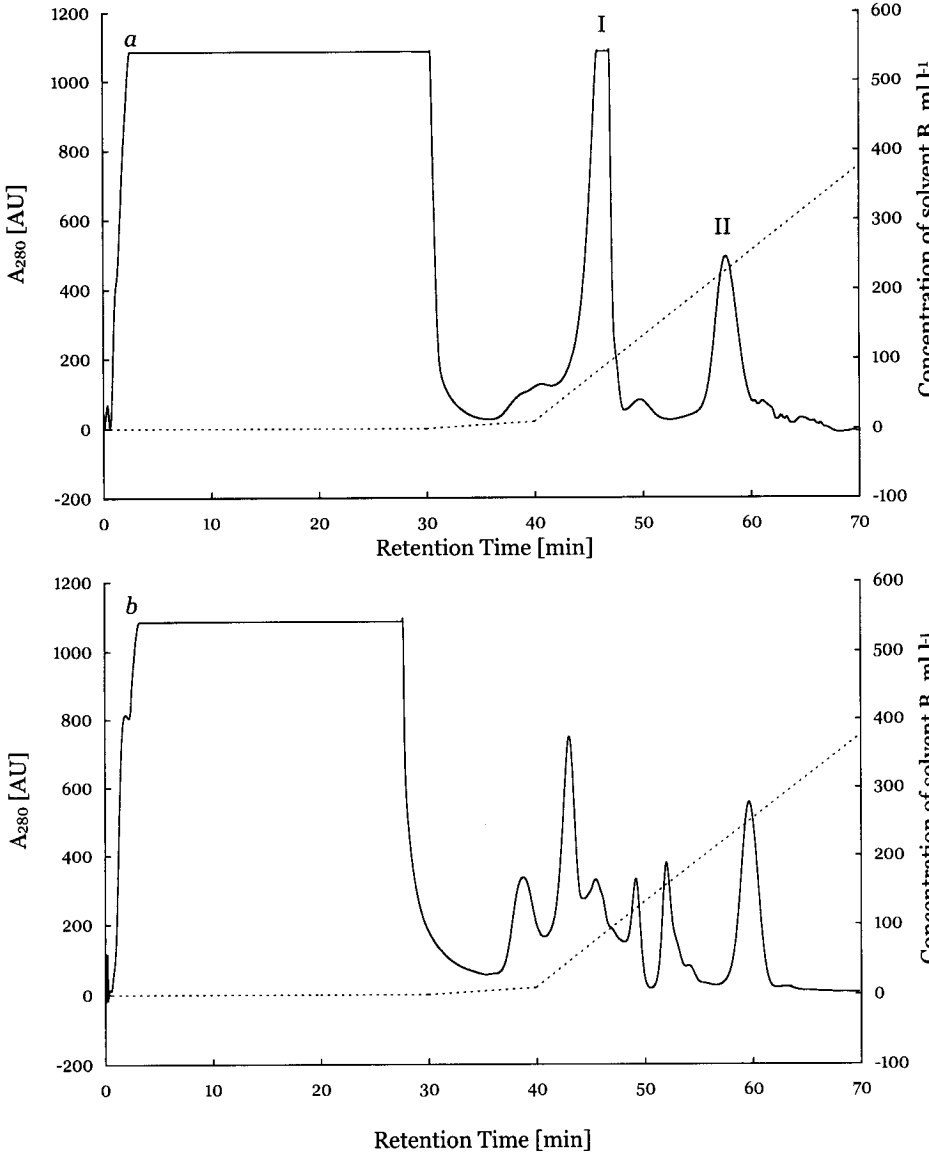


Fig. 4.27. Heparin affinity chromatography of (a) camel and (b) cow milk whey. Peaks I and II were collected for further analysis. Peak I consisted of PGRP and peak II of lactoferrin. Gradient of solvent B as dashed line.

ced by mast cells, where it is stored in secretory granules and released upon immunological stimulation. It is only in a few cases the natural substrate of proteins bound by heparin-sepharose affinity chromatography. Most proteins bind to related glycosaminoglycans, e.g. to extracellular, matrix associated, or membrane bound heparan-sulfate peptidoglycans (Vlodavsky *et al.* 1992). Elution from the heparin-sepharose column was at a ionic strength, which was higher than in a physiological sodium chloride solution (0.15 M NaCl). It was therefore concluded, that PGRP bound specifically to heparin. A protein with similar characteristics as PGRP was not isolated from bovine milk by heparin chromatography in the present study, ant not described in the literature. The extinction of PGRP was recorded at 280 nm, using an extinction coefficient of 35210 M^{-1} cm^{-1}. Calculated concentration of the protein in the milk analysed was 370 mg l^{-1}.

Functional Aspects of PGRP

The antimicrobial system of milk contains components, which are imported from blood serum, such as different types of immunoglobulins, and components produced in the lactating udder, such as MFGM bound mucins, lactoferrin, lactoperoxidase and lysozyme. Whereas the concentration of blood serum derived antimicrobial proteins is elevated in milk from infected udder, concomitantly with an elevated leukocyte level, the concentration of antimicrobial proteins, which originate from alveolar cells in the mammary gland, is hormonally regulated and usually depends on the stage of lactation. The concentrations of lysozyme and lactoferrin in camel milk were found to decrease rapidly within the first months of lactation (Barbour *et al.* 1984; Abd El-Gawad *et al.* 1996). In contrast, PGRP was isolated in major amounts from end-lactational milk. This indicated constant expression of the protein in camel milk in the course of lactation. It was found, that some antimicrobial milk proteins, such as lactoferrin and lysozyme, also participate in the action of the primary immune system. This system is usually based on targeting of structures common to invading pathogens, such as negatively charged lipopolysaccharides on the surface of gram-negative bacteria, and peptidoglycans, which are predominant in the cell wall of gram-positive bacteria, and consist of alternating GlcNac and MurNAc, cross-linked by short peptides (Dziarski *et al.* 1998). Proteins, which participate in the innate, humoral immune system, are supposed to recognise core structures of the molecules, which are highly conserved between species. The identification of non-self molecules by common structures, typical for the molecule class, was termed pattern-recognition (Kang *et al.* 1998). PGRP of silkworm was shown to recognise multiple repeating units of the glycan portion of peptidoglycans, but not β1,3-glucan,

chitin, and the diglycosidic core structure of peptidoglycans (Yoshida *et al.* 1996). High homology between vertebrate and invertebrate PGRP gives indication, that a similar binding specificity will be found for camel PGRP.

The pattern of PGRP expression is different between species. PGRP was strongly induced after infection of the moth *Trichlopusia ni* with *Enterobacter cloacae*, a gram-negative bacterium. Constitutive expression of PGRP was observed in the silkworm *Bombyx mori* (Yoshida *et al.* 1996). In two mammalian species, pig (*Sus scorfa*), and mongolian jird (*Meriones unguiculatus*), PGRP was induced upon infection with the nematodes *Ascaris suum*, and *Brugia malayi*, respectively (Fornhem *et al.* 1996; Williams, EMBL-ID BMAA28200, unpublished). The porcine protein was probably isolated from neutrophil granules (Fornhem *et al.* 1996), and strong expression of murine PGRP was detected in different leukocyte types (Kiselev *et al.* 1998). A strong signal of human PGRP mRNA was detected in human bone marrow, and of murine PGRP mRNA in spleen and lung. Weak expression of human PGRP was found in lymphoid organs, such as spleen, thymus and peripheral leukocytes, but also in human kidney, liver, small intestine, and lung. It was concluded, that the expression profile was consistent with a function in the mammalian immune system. However, the camel is the first species, where PGRP was found to be secreted into milk.

In the traditional medicine, camel milk is used in the treatment of peptic ulcer and tumours. There is a possibility, that PGRP could favourably be used in the therapy of these illnesses. Murine PGRP cDNA was rescued by a PCR based differential display of a cDNA library from VMR-Liv, a neoplastic cell line from murine mammary gland with high frequency of metastasis in liver, against a cDNA library from VMR-0, a neoplastic cell line from murine mammary gland with low metastatic potential. The cDNA did not hybridise to total RNA from VMR-0 cells, but strongly to total RNA from VMR-Liv cells. Hybridisation to tumour-free murine liver tissue was not detected. The cDNA was found in different other murine adenocarcinoma cell lines, such as VMR-Ov, a cell line with high metastatic potential in ovular tissue. Based on these and other data, PGRP was suggested to have a function in control of breast cancer metastasis (Kustikova *et al.* 1996). It might be, that the upregulated expression of PGRP in metastatic mammary cells helps in the invasion of endothelial tissue. Extravasation of metastasising cells involves adhesive interactions with endothelial cells and mast cells of the immune system (Vlodavsky *et al.* 1992). The invading cells must degrade the subendothelial glycoproteins and proteoglycans in order to migrate out of the vascular compartment into

the underlying basal lamina. Based on the finding, that camel PGRP specifically bound to heparin, we suggest that PGRP may adhere to the extracellular matrix of endothelial cells. The main components of this matrix are collagens, laminin, fibronectin, elastin, heparan sulfate proteoglycans, dermatan sulfate proteoglycans and smaller amounts of chondroitin sulfate proteoglycans (Vlodavsky *et al.* 1992). It is suggested, that PGRP can bind to some types of proteoglycans. By disruption, the peptidoglycan network, which has interconnecting and cell signalling functions, may be broken. This would result in disassembly of the subendothelial extracellular matrix and open the way for intrusion, finally resulting in metastasis of the primary tumour. Another intriguing feature of PGRP is its ability to induce regulated cell death. Murine PGRP was able to induce apoptosis in L929 cells, but also in the human breast adenocarcinoma cell line MCF7 (Kiselev *et al.* 1998). The protein probably was recognised by a specific receptor. Induction of cell death was independent of TNF-induced apoptosis and resulted in considerable DNA fragmentation. The functions of PGRP as a cytokine and as an apoptosis inducing factor may be related, and an imminent role of the protein in tumour growth was reported (Kiselev *et al.* 1998).

PGRP cDNA was also found in irradiated colon tissue (Marra *et al.* EMBL-ID AA734993, unpublished). PGRP is secreted from cells of the lymph system and from exocrine glands in a similar fashion as lysozyme C, which is an N-acetylglucosamine-β(1-4)-N-acetylmuramic acid hydrolase with broad antimicrobial activity. Expression of PGRP in metastatic adenocarcinoma and in irradiated tissue may be caused by a similar mechanism as expression of lysozyme, another protective milk protein, in these tissues. The protein may induce apoptosis of damaged cells, and stimulate the host immune response.

Since the protein was found to bind strongly to gram-positive bacteria, as well as to isolated peptidoglycan from *Micrococcus luteus*, but was not able to exert hydrolytic activity on the peptidoglycan heteropolymers of the gram-negative bacterium *Escherichia coli*, it was concluded, that the protein was able to bind peptidoglycan without cleaving it (Kang *et al.* 1998). Sequence similarity between PGRP, and a goup of three N-acetylmuramoyl-L-alanine amidases designated as T3-, T7-, and *Haemophilus influenzae* lysozyme, demonstrated a common origin of these proteins. In contrast to the PGRP family, this viral and bacterial lysozyme family proved to hydrolyse muramoyl-peptide bonds (Inouye *et al.* 1973). The reaction centre contained a zinc ion and was highly conserved. Zinc

binding residues His^{17}, Tyr^{46}, His^{122}, and Cys^{130} were only partially conserved in PGRP (Fig. 4.26). Nevertheless, porcine PGRP was isolated by zinc affinity chromatography, and the camel protein proved to have all residues conserved, except Cys^{130}, which was exchanged against Ser^{177}, a related aa residue with higher partial charge density. It is suggested, that amidase activity of camel PGRP could be activated in a microenvironment, where Zn^{2+} is provided in high amounts.

The affinity to heparin also suggests a function of the protein in angiogenesis, which is an initial process in wound healing and healing of duodenal and gastric peptic ulcer (Folkman & Shing, 1992). Synergistic action of PGRP with lactoperoxidase and lactoferrin may inhibit the growth of gram-negative bacteria. Duodenal and gastric peptic ulcer are often caused by *Helicobacter pylori*, a gram-negative bacterium. The antimicrobial activity of PGRP could even potentiate the possible beneficial effect of PGRP, which may result from a proposed binding to the extracellular matrix. Camel PGRP is suggested to have a beneficial influence on establishing a favourable gut microflora in the new-born animal. The protein is supposed mainly to inhibit growth of gram-positive bacteria, among which pathogens, e.g. *Bacillus* strains are found, but also lactic acid bacteria, such as bifidobacteria, lactobacilli, lactococci, streptococci and leukonostoc strains. There are reports of retarded growth of lactic acid bacteria in camel milk (Abu Tarboush, 1994 and 1996; Kamoun, 1995). On the other hand, *Bifidobacterium longum* 15707 was reported to grow faster in camel milk than in cow milk, whereas growth of other *Bifidobacterium* strains was retarded (Abu Tarboush, 1998). It would be of interest to know the inhibitory effect on these different bacteria, and to find starter cultures for camel milk, which are not much inhibited by PGRP. Possible activity of PGRP, which can be isolated from camel milk in large amounts, and by a cheap, single-step procedure, on peptic ulcers and on tumour growth inhibition should also be studied.

4.4.2 Lactoferrin

Literature

Among the protective milk proteins, structure and function of lactoferrin, also named lactotransferrin, are best studied. Industrial scale purification from whey is carried out by cation exchange, and use as a preserving agent in food, drugs and cosmetics has been proposed (Saito *et al.* 1994).

```
            10                  30                  50                  70
cDNA    AGTCGCCTCAGGACCCCAGACATGAAGCTCTTCTTCCCCGCCCTGCTGTCCCTCGGGGCCCTTGGACTGTGTCTG
Protein                    MetLysLeuPhePheProAlaLeuLeuSerLeuGlyAlaLeuGlyLeuCysLeu - 2

                90                  110                 130                 150
cDNA    GCTGCCTCTAAGAAAAGTGTTCGATGGTGCACCACATCACCAGCAGAGTCGTCAAAATGTGCCCAATGGCAACGG
Protein AlaAlaSerLysLysSerValArgTrpCysThrThrSerProAlaGluSerSerLysCysAlaGlnTrpGlnArg  24

                170                 190                 210
cDNA    AGGATGAAAAAAGTGCGTGGTCCCTCTGTCACCTGCGTAAAGAAGACATCTCGCTTTGAATGCATCCAGGCCATC
Protein ArgMetLysLysValArgGlyProSerValThrCysValLysLysThrSerArgPheGluCysIleGlnAlaIle  49

            230                 250                 270                 290
cDNA    TCGACAGAAAAGGCAGATGCTGTGACCCTTGACGGTGGTTTGGTGTATGACGCAGGCCTGGACCCCTACAAGCTG
Protein SerThrGluLysAlaAspAlaValThrLeuAspGlyGlyLeuValTyrAspAlaGlyLeuAspProTyrLysLeu  74

                310                 330                 350                 370
cDNA    CGGCCGATAGCGGCAGAGGTCTATGGGACAGAAAACAATCCCCAAACCCACTATTATGCCGTTGCCATTGCCAAA
Protein ArgProIleAlaAlaGluValTyrGlyThrGluAsnAsnProGlnThrHisTyrTyrAlaValAlaIleAlaLys  99

                390                 410                 430                 450
cDNA    AAGGGCACCAACTTTCAGCTGAACCAGCTACAAGGCCTGAAGTCCTGCCATACCGGCCTTGGCAGGTCCGCTGGG
Protein LysGlyThrAsnPheGlnLeuAsnGlnLeuGlnGlyLeuLysSerCysHisThrGlyLeuGlyArgSerAlaGly 124

                470                 490                 510
cDNA    TGGAACATCCCTATGGGGCTACTTCGTCCATTCTTGGACTGGACAGGGCCTCCTGAGCCCCTCCAGAAAGCTGTG
Protein TrpAsnIleProMetGlyLeuLeuArgProPheLeuAspTrpThrGlyProProGluProLeuGlnLysAlaVal 149

            530                 550                 570                 590
cDNA    GCCAAATTCTTCTCTGCCAGCTGTGTTCCCTGCGTGGATGGAAAAGAGTACCCCAACCTGTGTCAGCTGTGTGCA
Protein AlaLysPhePheSerAlaSerCysValProCysValAspGlyLysGluTyrProAsnLeuCysGlnLeuCysAla 174

                610                 630                 650                 670
cDNA    GGGACGGGGGAAAATAAATGTGCCTGCTCCTCCCAGGAACCATATTTTGGCTACTCTGGTGCCTTCAAGTGTCTG
Protein GlyThrGlyGluAsnLysCysAlaCysSerSerGlnGluProTyrPheGlyTyrSerGlyAlaPheLysCysLeu 199

                690                 710                 730                 750
cDNA    CAAGATGGGGCTGGAGATGTGGCCTTTGTCAAGGACAGTACAGTGTTTGAGAGCCTGCCAGCGAAGGCGGACAGG
Protein GlnAspGlyAlaGlyAspValAlaPheValLysAspSerThrValPheGluSerLeuProAlaLysAlaAspArg 224

                770                 790                 810
cDNA    GACCAGTATGAGCTGCTCTGCCCAAACAATACTCGGAAACCAGTGGATGCATTCCAGGAGTGTCATCTAGCCCGG
Protein AspGlnTyrGluLeuLeuCysProAsnAsnThrArgLysProValAspAlaPheGlnGluCysHisLeuAlaArg 249
                                Gly
            830                 850                 870                 890
cDNA    GTCCCTTCTCATGCTGTTGTGGCCCGAAGTGTGAATGGCAAGGAGGACTTGATCTGGAAACTTCTCGTCAAGGCA
Protein ValProSerHisAlaValValAlaArgSerValAsnGlyLysGluAspLeuIleTrpLysLeuLeuValLysAla 274

                910                 930                 950                 970
cDNA    CAGGAAAAGTTTGGAAGAGGCAAGCCATCAGGATTCCAGCTCTTTGGCTCTCCTGCTGGGCAGAAGGACCTGCTG
Protein GlnGluLysPheGlyArgGlyLysProSerGlyPheGlnLeuPheGlySerProAlaGlyGlnLysAspLeuLeu 299

                990                 1010                1030                1050
cDNA    TTCAAAGACTCTGCCCTTGGGTTGTTGAGGATCTCCTCAAAGATAGATTCTGGGCTGTACCTGGGCTCCAACTAC
Protein PheLysAspSerAlaLeuGlyLeuLeuArgIleSerSerLysIleAspSerGlyLeuTyrLeuGlySerAsnTyr 324

                1070                1090                1110
cDNA    ATCACTGCCATCCGAGGCCTGAGGGAAACGGCGGCGGAGGTGGAGTTGAGGCGCGCGCAGGTCGTGTGGTGCGCG
Protein IleThrAlaIleArgGlyLeuArgGluThrAlaAlaGluValGluLeuArgArgAlaGlnValValTrpCysAla 349

            1130                1150                1170                1190
cDNA    GTGGGCTCCGACGAGCAGCTCAAGTGCCAGGAGTGGAGCCGCCAGAGCAACCAAAGCGTGGTCTGTGCCACGGCC
Protein ValGlySerAspGluGlnLeuLysCysGlnGluTrpSerArgGlnSerAsnGlnSerValValCysAlaThrAla 374
                                                    Gly
                1210                1230                1250                1270
cDNA    TCCACCACCGAGGACTGCATCGCCCTGGTGCTGAAAGGAGAAGCTGATGCTTTGAGCTTGGATGGAGGATATATC
Protein SerThrThrGluAspCysIleAlaLeuValLeuLysGlyGluAlaAspAlaLeuSerLeuAspGlyGlyTyrIle 399
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                  1290                1310                1330                1350
cDNA     TACATTGCGGGCAAGTGTGGCTTGGTGCCTGTCTTGGCGGAGAGCCAACAATCCCCCGAAAGCAGTGGCTTAGAT
Protein  TyrIleAlaGlyLysCysGlyLeuValProValLeuAlaGluSerGlnGlnSerProGluSerSerGlyLeuAsp 424

                       1370                1390                1410
cDNA     TGTGTGCATCGACCGGTAAAAGGGTATCTTGCCGTGGCGGTTGTCAGGAAAGCAAATGACAAGATCACCTGGAAT
Protein  CysValHisArgProValLysGlyTyrLeuAlaValAlaValValArgLysAlaAsnAspLysIleThrTrpAsn 449

          1430                1450                1470                1490
cDNA     TCTCTGAGAGGCAAGAAGTCCTGCCACACCGCCGTGGACAGGACCGCAGGCTGGAACATCCCCATGGGCCTGCTC
Protein  SerLeuArgGlyLysLysSerCysHisThrAlaValAspArgThrAlaGlyTrpAsnIleProMetGlyLeuLeu 474

                  1510                1530                1550                1570
cDNA     TCCAAAAATACAGACTCCTGCAGATTTGATGAATTCCTCAGTCAAAGCTGTGCCCCTGGGTCTGACCCAAGATCC
Protein  SerLysAsnThrAspSerCysArgPheAspGluPheLeuSerGlnSerCysAlaProGlySerAspProArgSer 499

                       1590                1610                1630                1650
cDNA     AAGCTCTGTGCTCTGTGTGCAGGCAACGAGGAGGGCCAGAACAAGTGTGTGCCCAACAGCAGCGAGAGATACTAT
Protein  LysLeuCysAlaLeuCysAlaGlyAsnGluGluGlyGlnAsnLysCysValProAsnSerSerGluArgTyrTyr 524

                       1670                1690                1710
cDNA     GGCTACACTGGGGCTTTCAGGTGCCTGGCTGAGAATGTTGGGGATGTTGCGTTTGTGAAAGATGTCACCGTCTTA
Protein  GlyTyrThrGlyAlaPheArgCysLeuAlaGluAsnValGlyAspValAlaPheValLysAspValThrValLeu 549

          1730                1750                1770                1790
cDNA     GACAACACTGATGGAAAGAACACTGAGCAGTGGGCTAAGGATTTGAAGCTGGGAGACTTTGAGCTGCTGTGCCTC
Protein  AspAsnThrAspGlyLysAsnThrGluGlnTrpAlaLysAspLeuLysLeuGlyAspPheGluLeuLeuCysLeu 574

                  1810                1830                1850                1870
cDNA     AATGGCACCAGGAAGCCTGTGACTGAGGCTGAGAGCTGCCACCTGGCCGTGGCCCCAAATCATGCTGTGGTATCT
Protein  AsnGlyThrArgLysProValThrGluAlaGluSerCysHisLeuAlaValAlaProAsnHisAlaValValSer 599

                       1890                1910                1930                1950
cDNA     CGGATTGATAAGGTAGCACACCTGGAACAGGTGCTGCTCCGCCAACAGGCTCATTTTGGAAGAAATGGACGAGAC
Protein  ArgIleAspLysValAlaHisLeuGluGlnValLeuLeuArgGlnGlnAlaHisPheGlyArgAsnGlyArgAsp 624

                       1970                1990                2010
cDNA     TGCCCAGGCAAGTTTTGCTTGTTCCAGTCCAAAACCAAAAACCTCCTGTTCAATGACAACACTGAGTGTCTGGCC
Protein  CysProGlyLysPheCysLeuPheGlnSerLysThrLysAsnLeuLeuPheAsnAspAsnThrGluCysLeuAla 649

          2030                2050                2070                2090
cDNA     AAACTCCAAGGCAAAACAACATATGAAGAGTATTTGGGACCACAGTATGTCACGGCCATTGCTAAGCTGAGACGA
Protein  LysLeuGlnGlyLysThrThrTyrGluGluTyrLeuGlyProGlnTyrValThrAlaIleAlaLysLeuArgArg 674

                  2110                2130                2150                2170
cDNA     TGCTCCACCTCCCCGCTTCTGGAAGCCTGCGCCTTCCTGATGAGGTAAAACTCGAAAAGCCGCCCCGCCTCCCCA
Protein  CysSerThrSerProLeuLeuGluAlaCysAlaPheLeuMetArgEnd                            689

                       2190                2210                2230                2250
cDNA     GAAGCCTCAGCCCCTGGCTGCTCGCAACCCTGATCCCAGGTGTGCTGCACCTTCCTCTCCCTTCCTGAGGGCGGA
                       2270                2290                2310
cDNA     GTTCGCCAAGCTCATCAGTTTTCACAATTCCCTGCTGTCAACTTAGCAAGAAATAAAATTAGAAATGCTGTTGGT
          2330
cDNA     TTTCATTCCCT
```

Fig. 4.28. cDNA sequence of camel milk lactoferrin and corresponding protein, with mature protein in bold. The open reading frame of the cDNA sequence is from A^{22} to G^{2145} and the polyadenylation signal in bold from A^{2302} to A^{2307}. Numbering of the amino acid chain starts from the first residue of the mature protein. ***Gly***: residue with glycosylation potential.

Lactoferrin belongs to the family of transferrins, together with blood serotransferrin (siderophilin), egg white ovotransferrin (conalbumin), melanotransferrin of malignant melanoms, the porcine inhibitor of carbonic anhydrase, and other proteins. The common property of this protein family is the binding of two metal cations, preferably Fe^{3+}, at structurally closely related binding sites. Most proteins of the transferrin type are needed for storage or transport of iron. Lactoferrin was discussed to serve for iron scavenging in body secretions (Brock, 1997). It is found in milk and different other body secretions, and in neutrophil leukocytes (Masson, 1970).

Primary Structure

PCR amplification products of a full length cDNA clone of camel lactoferrin were sequenced (Fig. 4.28; EMBL/GenBank™ accession number AJ131674). The clone was 2336 bp long, and contained a 5'-untranslated region of 21 bp and a 3'-untranslated region of 191 bp. The 5'-untranslated region contained a partial Kozak-box (Kozak, 1989) in front of the translational start site $A^{22}TG$, with a purine at –3 bp, and cytosines at –1 bp, -5 bp and –8 bp. The 3'-untranslated region contained a polyadenylation signal $A^{2302}ATAAA$. The open reading frame ranged from A^{22} to G^{2145}, and coded for a polypeptide of 707 aa residues. The start site of the mature protein was determined by similarity as Ala^{1}. The 19 aa signal peptide had 94.7% sequence similarity to the signal sequence of bovine lactoferrin, and 84.2% to the signal sequence of human lactoferrin. Mature lactoferrin was 689 aa residues long and the unmodified peptide had a molecular weight of 75.250 kDa (Table 4.3). The isoelectric point of the unmodified peptide was at pH 8.14. The protein shared 91.6% sequence similarity with bovine and with human lactoferrin, and 91.3% with porcine lactoferrin. High similarity in primary structures indicated, that there were only small variations in functional aspects.

Glycosylation

N-linked glycans contribute about 4% to 11% (3 to 9 kDa) to the total mass of bovine lactoferrin, which is about 80 kDa (Spik *et al.* 1994). Glycosylation enhances the solubility of the secreted protein and may help to bind at specific cell types, such as liver parenchymal cells (Ziere *et al.* 1993). Camel milk lactoferrin was found to contain 6.2% carbohydrates in colostral milk and 5.6% in milk collected 15 to 30 days after parturition (Mahfouz *et al.* 1997). The content of N-acetyl-glucosamine in camel milk lactoferrin was markedly higher than in ruminants' milk lactoferrins (3.35% in colostral camel milk compared to about 1.75% in colostral ruminants' milk). The

carbohydrate content of lactoferrin from end-lactational milk was 6.2%-6.8% of total protein mass, calculated as a difference between the protein mass measured by MALDI-MS and the protein mass of the amino acid sequence (Table 4.3). Possible glycosylation sites, based on pattern analysis (Gavel & Von Heijne, 1990), are Asn^{233}, Asn^{366}, Asn^{518} and Asn^{575}. In bovine lactoferrin, three of five sites with N-glycosylation potential, Asn^{368}, Asn^{476}, and Asn^{545}, are glycosylated, and contribute to an overall carbohydrate content of 11.2%. Glycosylation of bovine lactoferrin was only found at the C-lobe. Degree of glycosylation in human lactoferrin is about 6.40% (Spik *et al.* 1994), and thus similar to camel lactoferrin. Human lactoferrin contains 2 glycosylated sites, Asn^{138} and Asn^{479}, with glycans of the N-acetyllactosaminic type, which were also found in camel lactoferrin. By comparison with bovine and human lactoferrin, glycosylation of Asn^{366}, and Asn^{518} or Asn^{575}, in camel lactoferrin is proposed (Fig. 4.28).

Concentration in Camel Milk

Colostral camel milk was reported to have an extremely high lactoferrin content of 5.10 g l^{-1} on the second day after parturition, compared to about 0.50 g l^{-1} in bovine colostral milk. After 30 days of milking, the lactoferrin level in camel milk went down to 0.34 g l^{-1}, whereas in bovine milk, only about 0.06 g l^{-1} were found (Abd El-Gawad *et al.* 1996). In our studies, we used an extinction coefficient of 84540 M^{-1} cm^{-1} at 280 nm to calculate a lactoferrin concentration of 0.22 g l^{-1} in a milk sample, which was taken at the end of the lactation period, 360 days after parturition. In a sample of pooled cow milk, lactoferrin concentration was 0.14 g l^{-1}. If it is assumed, that the main function of lactoferrin in milk is inhibition of bacterial growth, a differently composed microflora in the gut of the new-born could be a reason for the apparently higher lactoferrin concentration in camel milk.

Iron Content

Lactoferrin of colostral camel milk was reported to have a low iron saturation of 9%, similar to lactoferrin of bovine colostral milk. In milk taken 15 to 30 days after parturition, camel lactoferrin was nearly completely iron saturated. Similar results were found for bovine lactoferrin from milk of the same lactational stage (Mahfouz *et al.* 1997).

Tertiary Structure and Ligand Binding

Transferrins are monomeric, structurally highly conserved proteins, nearly identical in secondary and tertiary structure, and with high homology of disulphide bonding (Schanbacher *et al.* 1993). Camel and bovine lactoferrin

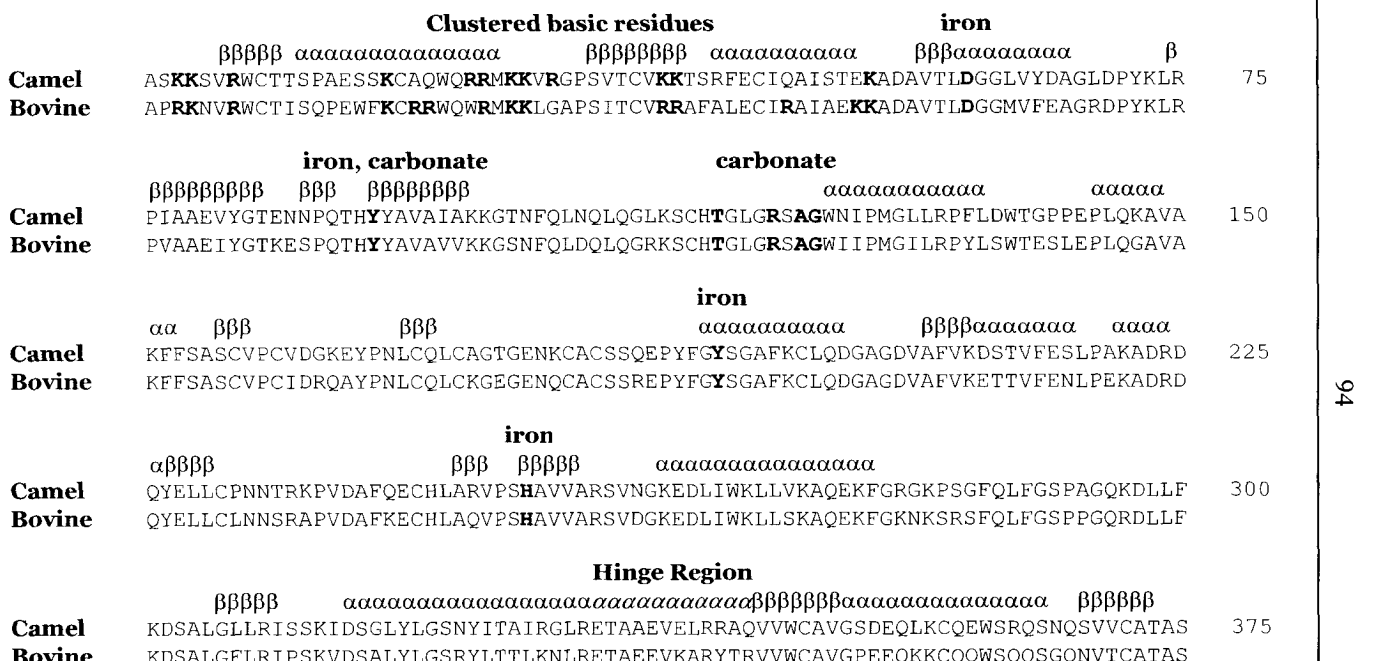
Clustered basic residues
iron
βββββ ααααααααααααα βββββββββ ααααααααααα βββααααααααα β
Camel ASKKSVRWCTTSPAESSKCAQWQRRMKKVRGPSVTCVKKTSRFECIQAISTEKADAVTLDGGLVYDAGLDPYKLR 75
Bovine APRKNVRWCTISQPEWFKCRRWQWRMKKLGAPSITCVRRAFALECIRAIAEKKADAVTLDGGMVFEAGRDPYKLR
iron, carbonate
carbonate
βββββββββ βββ βββββββββ ααααααααααα ααααα
Camel PIAAEVYGTENNPQTHYYAVAIAKKGTNFQLNQLQGLKSCHTGLGRSAGWNIPMGLLRPFLDWTGPPEPLQKAVA 150
Bovine PVAAEIYGTKESPQTHYYAVAVVKKGSNFQLDQLQGRKSCHTGLGRSAGWIIPMGILRPYLSWTESLEPLQGAVA
iron
αα βββ βββ ααααααααααα βββββααααααα αααα
Camel KFFSASCVPCVDGKEYPNLCQLCAGTGENKCACSSQEPYFGYSGAFKCLQDGAGDVAFVKDSTVFESLPAKADRD 225
Bovine KFFSASCVPCIDRQAYPNLCQLCKGEGENQCACSSREPYFGYSGAFKCLQDGAGDVAFVKETTVFENLPEKADRD
iron
αβββββ βββ βββββ ααααααααααααααα
Camel QYELLCPNNTRKPVDAFQECHLARVPSHAVVARSVNGKEDLIWKLLVKAQEKFGRGKPSGFQLFGSPAGQKDLLF 300
Bovine QYELLCLNNSRAPVDAFKECHLAQVPSHAVVARSVDGKEDLIWKLLSKAQEKFGKNKSRSFQLFGSPPGQRDLLF
Hinge Region
βββββ ααααααααααααααααααααααααααββββββββααααααααααααααα βββββββ
Camel KDSALGLLRISSKIDSGLYLGSNYITAIRGLRETAAEVELRRAQVVWCAVGSDEQLKCQEWSRQSNQSVVCATAS 375
Bovine KDSALGFLRIPSKVDSALYLGSRYLTTLKNLRETAEEVKARYTRVVWCAVGPEEQKKCQQWSQQSGQNVTCATAS

Fig. 4.29. Schematic drawing of the relationship between structure and function in camel and bovine lactoferrin. Functional residues in bold. α-helical regions designated as "α", β-pleated regions as "β".

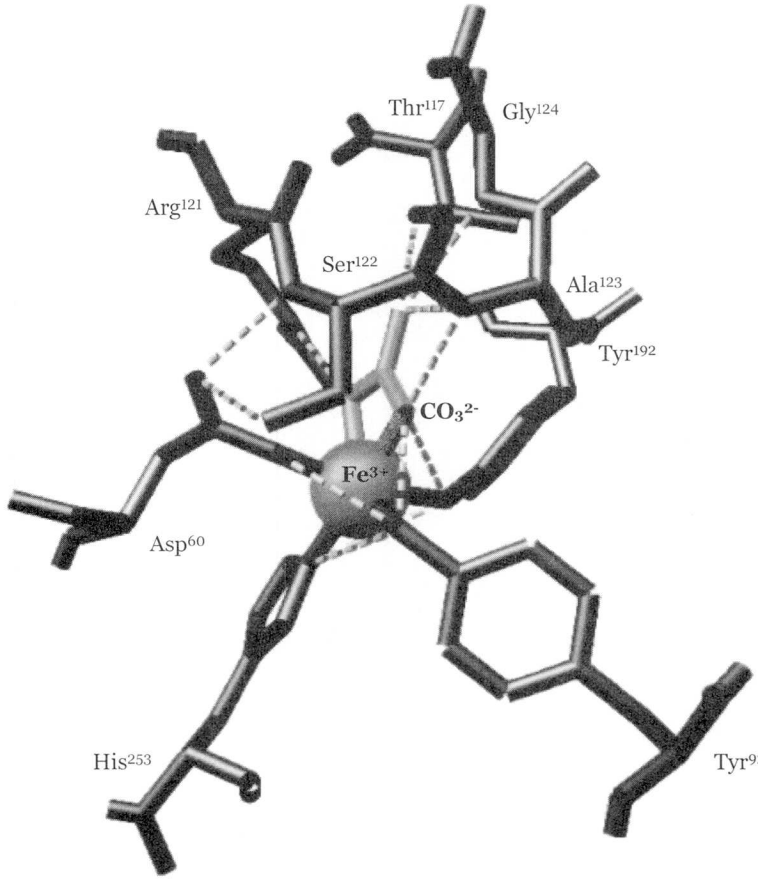

Fig. 4.30. Ligand-binding centre of bovine lactoferrin. Fe^{3+} in space filling view, CO_3^{2-} and coordinated amion acid residues as wire frame. Hydrogen bonds as dashed lines. Data from Moore *et al.* (1997).

shared 85.5% and 83.9% sequence similarity, respectively, with bovine serotransferrin. All transferrins have a polypeptide chain of about 700 amino acids, which is folded into two, tandemly arranged, asymmetrical metal binding sites, designated as N- and C-lobes. These lobes are connected by a helical hinge, which, in camel lactoferrin, extends from

Glu^{333} to Gln^{344} (Fig. 4.29). The sequence of the N-lobe, which extends from Val^{6} to Arg^{332}, shares 75.7% sequence similarity with the sequence of the C-lobe, which ranges from Val^{345} to Arg^{673}, probably as a result of gene duplication. The two lobes were reported to bind iron synergistically, but the C-lobe was found to have a lower dissociation constant than the N-lobe (Baker *et al.* 1994). Both lobes are folded into two domains, which form a cleft, where the metal cation is bound. Under physiological conditions, transferrins bind two Fe^{3+} cations with low dissociation constants of about 10^{-20} (Brock, 1997). Cation binding requires synergistic binding of a bicarbonate anion, probably for charge compensation. In bovine and camel lactoferrin, the side chains of Asp^{60}, Tyr^{92}, Tyr^{192} and His^{253} were found to be involved in binding of the cation in the N-lobe (Fig 4.30). Two oxygens from the bidentate CO_3^{2-} anion are suggested to complete a distorted octahedral geometry (Anderson *et al.* 1989). In the N-lobe, the side chains of Tyr^{92}, Thr^{117}, Arg^{121}, and Tyr^{192}, and two backbone hydrogens of Ala^{123} and Gly^{124}, were found to be involved in binding of the bicarbonate anion. The binding sites appeared to be optimised for the binding of Fe^{3+} and CO_3^{2-}, with respect to size, charge and stereochemistry. The iron affinity of lactoferrins is about 300 times higher than the affinity of serotransferrins. Lactoferrin retains its iron binding potential at pH values below pH 5.5, and even in the presence of citrate, in contrast to the other known transferrins (Brock, 1997). The binding sites of bovine and camel lactoferrin were found to be highly similar. Considering the nearly identical structural geometry, we assume that cations are bound by both, N- and C-lobe of the camel protein, with similar affinities as in bovine lactoferrin.

Bacteriostatic Activity of the N-terminal End

A high amount of Arg and Lys are clustered at the N-terminal end of lactoferrin, near and between a loop, which is formed by disulphide bonding of Cys^{19} and Cys^{36} (Fig. 4.31). The N-terminus of human and bovine lactoferrin was found to have strong bacteriostatic activity on gram-negative bacteria, as a result of non-specific binding to the negatively charged outer bacterial membrane, and subsequent release of lipopolysaccharides, thereby altering the permeability properties. The N-terminal end of human lactoferrin was shown to be the binding site of heparin, lipid A moiety of bacterial lipopolysaccharides, human lysozyme and DNA, the latter with distinct sequence preference (Van Berkel et al. 1997; Furmanski et al. 1997). These binding affinities were equally high for diferric lactoferrin and iron depleted apolactoferrin. The N-terminal part of camel lactoferrin contained 13 basic residues (Fig. 4.29), at sites more similar to bovine lactoferrin than to human lactoferrin. The isoelectric point of the N-terminal fragment from

Ala[1] to Ala[54] was at pH 10.98, compared to pH 11.57 for the bovine fragment at the same position, which had one more basic residue. As in bovine lactoferrin, most residues were found in or near the loop. Based on the high homology between camel and bovine lactoferrin in general, and particularly on the similar grouping of the N-terminal basic residues, we suggest a similar effect of camel lactoferrin as observed of bovine lactoferrin. The bacteriostatic activity of camel lactoferrin on different bacterial strains was found to have equal strength as the activity of bovine lactoferrin (Elagamy *et al.* 1992).

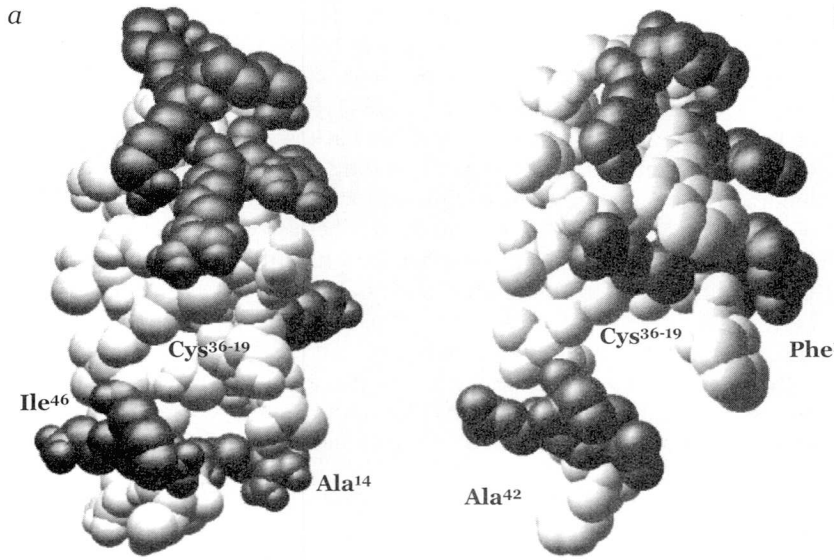

Fig. 4.31. Space filling view of the loop formed by cystin near the N-terminal end of lactoferrin. Basic residues dark shaded. (a) Model of the loop region from camel lactoferrin. (b) Loop region of bovine lactoferrin, reconstructed from X-ray crystallographic data (Moore *et al.* 1997).

Bacteriostatic Breakdown Products

Even stronger antibacterial activity was shown for peptides of lactoferrin, which were produced by aspartic protease digestion or heat treatment at low pH, imitating the conditions in the stomach. The antimicrobial peptides were found to correspond to the N-terminal end of lactoferrin (Tomita *et al.* 1994, Saito *et al.* 1994). Different N-terminal antimicrobial peptides were

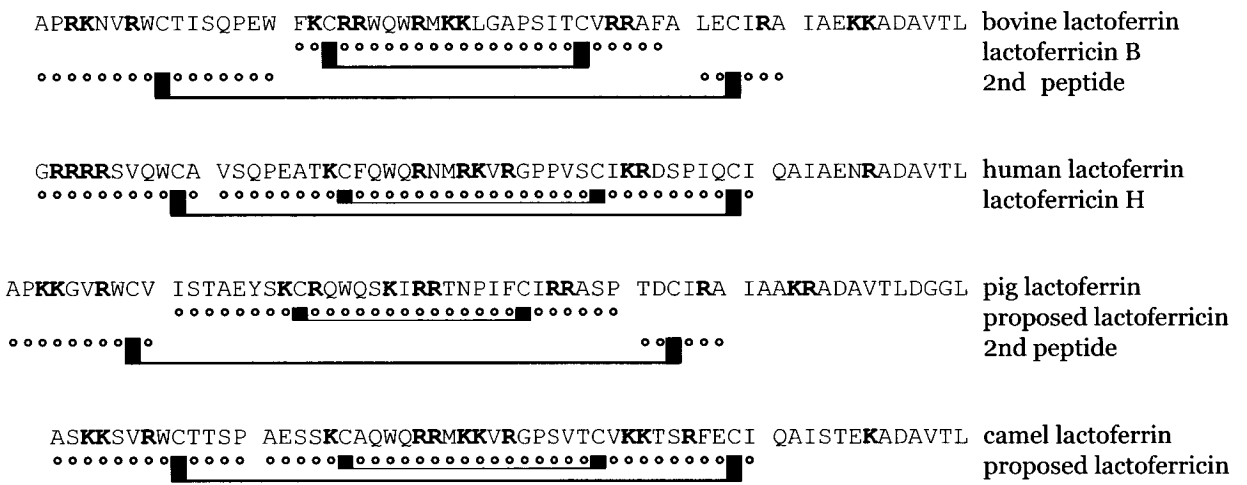

Fig. 4.32. Primary structures of lactoferrin from different species. Beneath the amino acid sequence of the N-terminal end, there are schematic drawings of bovine and human lactoferricin, and proposed structures of porcine and camel lactoferricin. Antimicrobial peptides formed upon gastric digestion designated by °. Basic residues in bold. Disulphide bonds indicated by connecting lines. 2nd peptides, which are formed upon digestion, are also shown, although these peptides exhibit weaker antimicrobial activities than lactoferricins.

obtained by digestion of human and bovine lactoferrin, since human lactoferrin does not contain a hydrophobic dipeptide suited for digestion by aspartic proteases between Cys^{37} and Cys^{46}. Antimicrobial peptides were structurally similar to well characterised, antimicrobial peptides, such as magainins from frog skin or cecropins from the hemolymph of insects. The bovine peptide, designated as lactoferricin B (Fig. 4.32), was twelve times more effective in the inhibition of gram-positive and gram-negative bacterial growth than undigested bovine apolactoferrin. Furthermore, bifidobacteria, which dominate the neonatal intestinal microflora, were less inhibited than many opportunistic pathogens. Lactoferrin may therefore help to promote the establishment of bifidobacteria in the neonatal microflora. A camel lactoferricin is proposed in analogy to bovine and human lactoferricin (Fig 4.32), based on probable pepsin cleavage sites. This lactoferricin would have more similarity to human lactoferricin, where the N-terminus is not cleaved off, since probability of cleavage between Cys^{36} and Cys^{45} of camel lactoferrin is low. Growth inhibition of this peptide was assumed to be in the range of human lactoferricin, where 100 mg l^{-1} of peptide is needed to inhibit growth of *E. coli* completely, rather than to be in the range of bovine lactoferricin, where mere 6 mg l^{-1} are sufficient for complete inhibition of bacterial growth (Tomita *et al.* 1994).

Functional Aspects
Camel and bovine lactoferrin were highly similar in most structural aspects. Compared sequences did not reveal sequence insertions or deletions. Amino acid residues surrounding the ligand binding centres were highly conserved and clustering of the basic N-terminal residues was largely identical. The only differences were found in glycosylation potential and sites of glycosylation, and in the proposed structure of lactoferricin produced upon gastric digestion. The most prominent difference between the proteins was the concentration in milk.

It can be assumed, that lactoferrin in colostral milk acts as an iron scavenger, which depletes the milk from free iron and thereby slows down microbial growth. Brock (1997) proposed, that the in vivo function of apolactoferrin is the prevention of iron-mediated lipid peroxidation, a property, which was already demonstrated with monocytes. This function is based on the ability of lactoferrin, to bind to cell membranes. The higher affinity for iron, as compared to other transferrins, would enable it to function at the reduced pH found in the stomach and upper intestine. The high resistance of apolactoferrin to proteolysis, compared with other apotransferrins, would enable it to maintain its iron-binding potential in

the face of proteolytic activity in the gut. Since diferric lactoferrin was reported to be even more resistant to proteolysis, it was supposed, that the iron-lactoferrin complex would resist degradation, and was sequestered by hepatocytes, or was excreted from the gut. A higher lactoferrin concentration also could help to prevent lipid peroxidation by free radicals in an infected udder, which has an elevated iron content.

Iron-saturated lactoferrin, which is found in milk from the second week to the end of the lactational period, may primarily prevent microbial growth in the gut. This would help the new-born, which is easily infected, to survive the first weeks, until its own immune system becomes developed, and the gut becomes adapted to food digestion. Iron saturated lactoferrin could also be a source of iron for the suckling, once the protein is degraded in the gut. The much higher concentration in camel colostrum and milk gives indication, that the suckling camel is under greater danger of getting an infection than the suckling calf. A reason for this could be the harsh environment, to which these animals are adapted, and differences in physiological aspects and metabolism.

Food Preservation
Lactoferrin was discussed to be a promising choice for preservation in food and cosmetics, since it is highly stable towards heat treatment and at low pH conditions (Saito *et al.* 1994). Since the protein helps to establish a favourable microflora, and promotes growth of bifidobacteria, it is interesting for use in functional food products. The antimicrobial peptides formed upon gastric digestion of lactoferrin are also an interesting choice for natural food preservation.

Primary structures of peptides formed from camel lactoferrin should be studied and activity of such peptides on inhibition of bacterial growth tested, to get better understanding of the action of lactoferrin in camel milk. The higher amounts of lactoferrin in camel milk are an advantage for natural preservation of the milk in arid regions, where technology for milk preservation is often not available.

4.4.3 Lactoperoxidase

Literature
Lactoperoxidase (EC 1.11.1.7) is found in milk, tears and saliva. It contributes to the non-immune host defence system, exerting bactericidal

activity, mainly on gram-negative bacteria. It is supposed that the main function in milk is the protection of the udder from microbial infections (Ueda *et al.* 1996). The finding, that lactoperoxidase remained active in the gastric juice from a new-born, being fairly resistant towards proteolytic digestion and acidic pH, led to the assumption, that the enzyme may also be a key player in the defence of the suckling animal's intestinal system from bacterial infection in the initial stages of life (Paul & Ohlsson, 1985). Lactoperoxidase was also reported to have a general growth-promotion and anti-tumour activity (Ueda *et al.* 1997). Yet another functional role for lactoperoxidase may be in the degradation of catecholamines, such as norepinephrine (Andersson *et al.* 1996). In contrast to human lactoperoxidase, which is only present in the colostrum, and becomes almost undetectable within one week after parturition, bovine lactoperoxidase activity is maintained at a high level throughout lactation (Ueda *et al.* 1997). Lactoperoxidase was only detected in the cDNA library created from mammary gland tissue taken in the second month of lactation, but could not be isolated, by cation exchange chromatography, from the camel milk sample studied, which was from the end of the lactation period, whereas the greenish stain of bovine lactoperoxidase was detected in a pooled cow milk sample.

Camel lactoperoxidase is a monomeric protein, which has 79.3% sequence similarity to human myeloperoxidase, and 79.2% sequence similarity to human eosinophil peroxidase. Myeloperoxidase is a dimeric protein, expressed in primary granules of neutrophilic granulocytes. Eosinophil peroxidase is also dimeric, and expressed in cytoplasmic granules of eosinophils. Infectious micro-organisms, which are carried into phagosomes by endocytosis, are inactivated by oxidative action of hypochloride on free sulfhydryl groups of bacterial enzymes, by myelo- or eosinophil peroxidase, prior to degradation. The genes of all three proteins were found to be located on the same gene locus in the human genome, and probably have evolved from a common ancestor by gene duplications (Ueda *et al.* 1997). Close relation (71.7%) was also found to human thyroid peroxidase, which is involved in iodination and coupling of the hormonogenic tyrosines in thyroglobulin, for production of thyroid hormones.

Lactoperoxidase is an antimicrobial enzyme of commercial interest. Industrial scale isolation from bovine whey is feasible, and use in cosmetics, dental and wound treatments is under investigation (De Wit & Van Hooydonk, 1996). In this study, we show that lactoperoxidase is expressed

in alveolar camel tissue, and exhibits high sequence similarity to bovine lactoperoxidase. Upon comparison of structural data, we can expect, that the way of action in activation of the lactoperoxidase system and in inhibition of several, mainly pathogenic, bacteria, will be nearly identical to the bovine enzyme.

Primary Structure

PCR amplification products of a camel lactoperoxidase cDNA clone were sequenced (Fig. 4.33; EMBL/GenBank™ accession number AJ131675). The clone was 2636 bp long, and contained a 3'-untranslated region of 497 bp, and a 5'-untranslated region of 4 readable bp. The truncation at the 5'-end was probably a result of incomplete reverse transcriptase action, maybe due to secondary structures in the corresponding mRNA. The 3'-untranslated region contained a polyadenylation signal $A^{2606}ATAAA$. The open reading frame started at A^{7} and extended to T^{2139}. In analogy to the bovine protein, and calculated upon the method of Nielsen *et al.* (1997), cleavage of the signal peptide probably occurs at the N-terminal side of Gln^{73}. The 19 aa signal peptide had 94.7% sequence similarity to the signal sequence of bovine lactoperoxidase, and 84.2% to the signal sequence of human lactoperoxidase. We suppose, by comparison to bovine lactoperoxidase, that the secreted protein is subsequently cleaved, probably by autocatalytic hydrolysis, either between Gln^{-1} and Lys^{1}, or between Ser^{28} and Val^{29} (Fig. 4.34). Bovine lactoperoxidase was reported to be heterogeneous in molecular weight. Two major forms were described, consisting of 76.5 kDa and 78.5 kDa, respectively (Dull *et al.* 1990). Mature lactoperoxidase contains 15 cysteines, if the N-terminal end of lactoperoxidase is Lys^{1}, and 14 cysteines, if the N-terminal end is Val^{29}. The second possible cleavage site is therefore more probable. The molecular weight of non-glycosylated mature camel lactoperoxidase was 69.460 kDa, compared to 69.569 kDa of bovine lactoperoxidase. The isoelectric point was higher than in bovine lactoperoxidase, at pH 8.63, whereas it was at pH 7.90 in the bovine protein. Mature lactoperoxidase shared 94.9% sequence similarity with bovine lactoperoxidase, and 94.1% with human salivary peroxidase, which is probably identical to human lactoperoxidase. High similarity in primary structure is necessary, since the catalytic activity of the protein depends on several geometrical constraints, which have implications on the tertiary structure.

Posttranslational Modifications and Ligand Binding

The quaternary structure of human myeloperoxidase, a protein with high sequence similarity to lactoperoxidase, was dissolved at 2.3 Å (Davey &

```
                10                  30                  50                  70
cDNA    NNAGTGATGTGGGTCCTTCTCCATCTCCCAGTCCTTTTGGCTTCCCTGACCTTGTTCCAGGCTGCAGCATCTGAC
Protein       MetTrpValLeuLeuHisLeuProValLeuLeuAlaSerLeuThrLeuPheGlnAlaAlaAlaSerAsp -77

                    90                  110                 130                 150
cDNA    ACCAATGCGCAGACCACTGCTGCCGCCATGTCTGAAGCCGTGCGTCAGGTCAAGGTGCACGTCAACAAGGCTTTC
Protein ThrAsnAlaGlnThrThrAlaAlaAlaMetSerGluAlaValArgGlnValLysValHisValAsnLysAlaPhe -52

                   170                 190                 210
cDNA    CTGGATTCCCGGACCAGGCTGAAGGCTGCCATGAGCTCTGAGGTGCCCACCACTCGACAGCTCTCAGAGTACCTC
Protein LeuAspSerArgThrArgLeuLysAlaAlaMetSerSerGluValProThrThrArgGlnLeuSerGluTyrLeu -27

          230                 250                 270                 290
cDNA    AAGCATGCCAAGGGCCGGACGCGCACAGCCATCCGCAACGGGCAGGTGTGGGAGGAGTCCTTAAAGAGACTGTGG
Protein LysHisAlaLysGlyArgThrArgThrAlaIleArgAsnGlyGlnValTrpGluGluSerLeuLysArgLeuTrp - 2

                310                 330                 350                 370
cDNA    CAGAAAGTGACCCAGACCAACATCACAGACCCCAGCCTGGACTTGACTGCACTCTCTTGGGAGGTGGGCTGTGAC
Protein GlnLysValThrGlnThrAsnIleThrAspProSerLeuAspLeuThrAlaLeuSerTrpGluValGlyCysAsp  24
                          Gly
                   390                 410                 430                 450
cDNA    GTCCCAGTTTCCGTGGTGAAATGTGACAAGAACAGCCCTTACCGCACCATCACAGGAGACTGTAATAACGGGAGG
Protein ValProValSerValValLysCysAspLysAsnSerProTyrArgThrIleThrGlyAspCysAsnAsnGlyArg  49

                        470                 490                 510
cDNA    CACCCCGCCCTGGGAGCCGCCAACCAGGCGTTGGCGCGCTGGCTGCCGGCGGAGTACGAGGACGGGCTGTCCCTG
Protein HisProAlaLeuGlyAlaAlaAsnGlnAlaLeuAlaArgTrpLeuProAlaGluTyrGluAspGlyLeuSerLeu  74

          530                 550                 570                 590
cDNA    CCCTTTGGGTGGACGCGGGGGAAAAAGCGGAATGGCTTCCCTCTCCCGCTGGCCCGTGAGGTATCCAACAAGATT
Protein ProPheGlyTrpThrArgGlyLysLysArgAsnGlyPheProLeuProLeuAlaArgGluValSerAsnLysIle  99

                610                 630                 650                 670
cDNA    GTTGGCTACCTGAATGAAGAGGGTGTTCTGGACCAAAACAGGTCCCTGCTCTTCATGCAGTGGGGTCAGATTGTG
Protein ValGlyTyrLeuAsnGluGluGlyValLeuAspGlnAsnArgSerLeuLeuPheMetGlnTrpGlyGlnIleVal 124
                                Gly
                   690                 710                 730                 750
cDNA    GACCACGACCTGGACTTTCCCCGGGACACGGAGCTGGGGAGCAGCGAGTACTCCAAAGCGCAGTGTGATGAGCAC
Protein AspHisAspLeuAspPheProArgAspThrGluLeuGlySerSerGluTyrSerLysAlaGlnCysAspGluHis 149

                        770                 790                 810
cDNA    TGTATCCGGGGAGACAACTGCTTCCCCATCATGTTCCCGCGCAACGACCGCAAGGTGATGACTCAAGGGAAATGC
Protein CysIleArgGlyAspAsnCysPheProIleMetPheProArgAsnAspArgLysValMetThrGlnGlyLysCys 174

          830                 850                 870                 890
cDNA    ATGCCTTTCTTCCGAGCTGGGTTTGTCTGCCCCAATCCACCTTACCAGTCTTTGGCCCGAGAGCAGATCAACGCC
Protein MetProPhePheArgAlaGlyPheValCysProAsnProProTyrGlnSerLeuAlaArgGluGlnIleAsnAla 199

                910                 930                 950                 970
cDNA    CTGACCTCCTTCCTGGACGCCAGCTTAGTGTACGGCTCTGAGCCCAGCCTGGCCAGCAGTCTCCGTGACCTCAGT
Protein LeuThrSerPheLeuAspAlaSerLeuValTyrGlySerGluProSerLeuAlaSerSerLeuArgAspLeuSer 224

                   990                1010                1030                1050
cDNA    AGTCCGCTGGGCCTCATGGCCGTCAACCAGGAGTTCTGGGACCACGGGGCTGGCCTACCCACCCTTTGTCAACAAG
Protein SerProLeuGlyLeuMetAlaValAsnGlnGluPheTrpAspHisGlyLeuAlaTyrProProPheValAsnLys 249

                        1070                1090                1110
cDNA    AAGCCGAGCCCCTGTGAGGTCATCAACACCACTGCCCAAGTGCCTTGCTTCCTGGCGGGAGATTCCCGAGCCTCA
Protein LysProSerProCysGluValIleAsnThrThrAlaGlnValProCysPheLeuAlaGlyAspSerArgAlaSer 274
                                Gly
          1130                1150                1170                1190
cDNA    GAGCAGATCCTGCTGGCCACTTCCCACACCCTGCTTCTCCGAGAGCACAACCGTCTGGCCAGAGAACTAAAGAAA
Protein GluGlnIleLeuLeuAlaThrSerHisThrLeuLeuLeuArgGluHisAsnArgLeuAlaArgGluLeuLysLys 299

                1210                1230                1250                1270
cDNA    CTCAACCCTCACTGGGATGGAGAGAAGCTCTACCAGGAAGCCCGGAAAATCCTGGGAGCCTTCATGCAGATTATC
Protein LeuAsnProHisTrpAspGlyGluLysLeuTyrGlnGluAlaArgLysIleLeuGlyAlaPheMetGlnIleIle 324

                   1290                1310                1330                1350
cDNA    ACCTTTAGGGACTACCTACCCATTGTGCTAGGTGATGAGATGCAGAAGTGGATCCCTCCATACCGAGGCTATAAC
Protein ThrPheArgAspTyrLeuProIleValLeuGlyAspGluMetGlnLysTrpIleProProTyrArgGlyTyrAsn 349
                                                                          Gly
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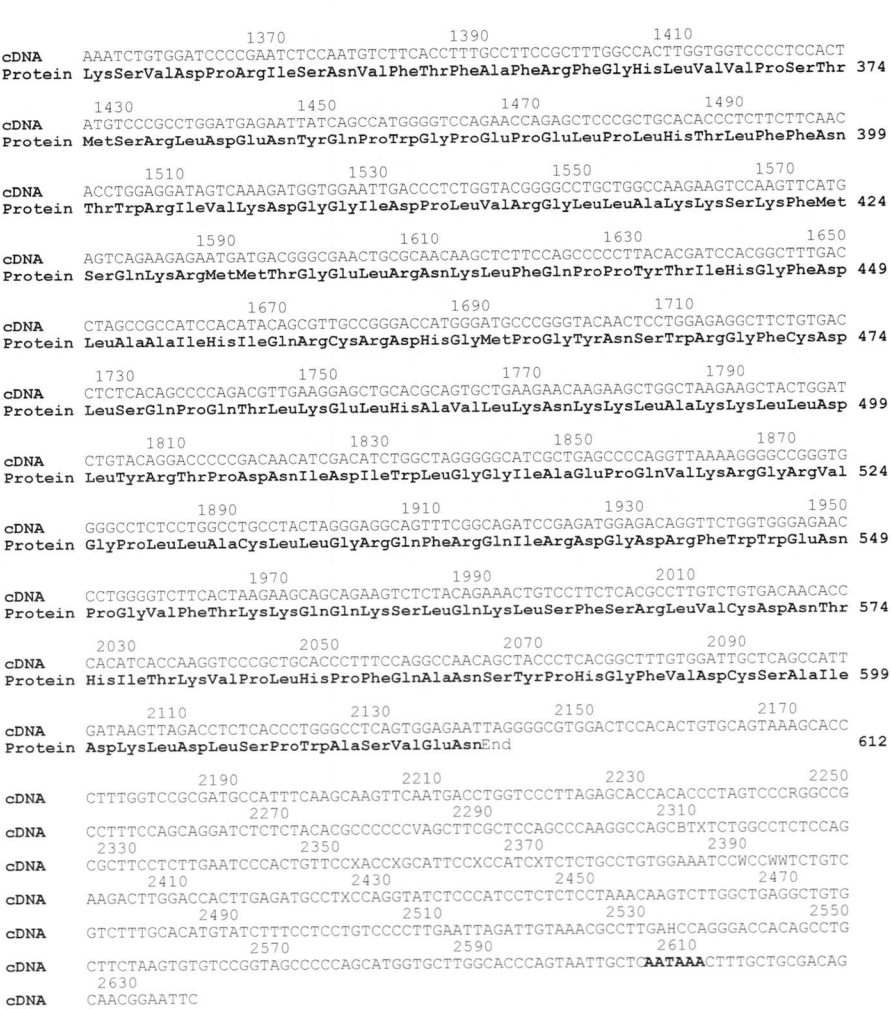

Fig. 4.33. cDNA sequence of camel milk lactoperoxidase and corresponding protein, with mature protein in bold. The open reading frame of the cDNA sequence is from A^{7} to T^{2139} and the polyadenylation signal in bold from A^{2606} to A^{2611}. Numbering of the amino acid chain starts from the first residue of the mature protein. ***Gly***, potentially glycosylated arginines.

Fenna, 1996). This protein is posttranslationally processed into a heavy and a light chain, in contrast to lactoperoxidase, which remains as a single chain peptide. A myeloperoxidase molecule consists of two heavy and two light chains with near-exact two-fold symmetry, covalently linked by a single disulfide at Cys^{155}. Residues Ala^{107} to Gly^{114} are excised from the mature chain by posttranslational processing (Fig. 4.34). Although there are significant modifications in the overall structure of the proteins, the architecture of the catalytic centre is almost the same. Upon the data from human myeloperoxidase, assumptions were made, concerning the ligand binding residues of lactoperoxidase and the catalytic centre. Cys^{184}, which corresponds to Cys^{155} in human myeloperoxidase, would be free in lactoperoxidase, since the molecule is monomeric, and may be disulphide linked to Cys^{458}, which corresponds to Ser^{427} in human myeloperoxidase. Nevertheless, the distance between the two residues is large, more than 16 Å, and covalent linkage would result in a major alteration in tertiary structure compared to myeloperoxidase. Furthermore, Cys^{152}, which is free in myeloperoxidase, and exchanged to the corresponding Gly^{181} in lactoperoxidase, is within 13 Å distance to Ser^{427} and may have a similar function in myeloperoxidase, as a free Cys^{458} in lactoperoxidase.

Both proteins contain a heme b (protoporphyrin IX) in the catalytic centre. In contrast to other known peroxidases from bacterial, fungal or plant origins, mammalian peroxidases bind the heme prosthetic group covalently, which helps to maintain a more rigid tertiary structure (Andersson *et al.* 1996). The mammalian enzymes work optimally at a broad range in pH and ionic strength. They are also able to oxidise high potential substrates, such as pseudohalides, e.g. thiocyanate, in contrast to other peroxidases (DePillis *et al.* 1997). In myeloperoxidase, the heme pyrrole ring C methyl group is covalently attached to Asp^{96} by ester linkage, the methyl group of the A ring to Glu^{244} by ester linkage and the vinyl group of the A ring to Met^{245} by sulfonium ion linkage (Fig. 4.34). Whereas aspartic and glutamic acids are found at the corresponding positions in lactoperoxidase, methionine is replaced by glutamine. DePillis *et al.* (1997) showed, that a heme b group is covalently bound to lactoperoxidase by autocatalytic processing. Speed of the reaction and amount of produced twofold ester linked heme were dependent of the peroxide concentration. Sulfonium ion linkage, as found in myeloperoxidase, was not observed in lactoperoxidase. Considering the high conservation of aspartic and glutamic acids in the active centre of the different enzymes, we suggest, that the prosthetic heme is covalently attached to camel lactoperoxidase by the corresponding residues Asp^{125} and Glu^{275} (Fig 4.34), a hypothesis supported by the study of Andersson *et al.*

(1996). Asp^{125} is adjacent to His^{126} and Glu^{275} is near Arg^{272}. These two residues are involved in the catalytic process, on the distal pocket of the active site (Fenna *et al.* 1995). In the resting enzyme, the ferric heme has high-spin pentacoordinate spectral characteristics. The fifth ligand is His^{338} in myeloperoxidase, which corresponds to His^{368} in lactoperoxidase. A sixth ligand was excluded by X-ray crystal structure analysis (Davey & Fenna, 1996).

Enzymes of the mammalian peroxidase family bind one calcium ion in distal vicinity to the C ring of the heme. In myeloperoxidase, the pentagonal bipyramidal coordinated ion is bound by backbone carbonyl oxygens of Asp^{98}, Thr^{170} and Phe^{172}, and side chain oxygens of Asp^{98}, Thr^{170}, Asp^{174} and Ser^{176}. The ion is thought to stabilise the overall structure of the protein and maintain the heme bound ferric ion in the high spin state. There was an equal ratio of calcium to iron in dialysed lactoperoxidase. Removal of calcium from bovine lactoperoxidase by dialysis against a buffer with 6 M guanidium hydrochloride and 10 mM EGTA resulted in partial precipitation of the protein (Booth *et al.* 1989).

Camel lactoperoxidase revealed four possible N-glycosylation sites at Asn^{6}, Asn^{112}, Asn^{258}, and Asn^{349}. Possible N-glycosylation sites of bovine lactoperoxidase are at Asn^{6}, Asn^{112}, Asn^{222}, Asn^{258}, and Asn^{349}. The carbohydrate content of bovine lactoperoxidase was reported to be about 10% (De Wit & Van Hooydonk, 1996), which corresponds to about 9.0 kDa (Table 4.3). This could account for four to five glycosylated sites. The structurally related human myeloperoxidase is glycosylated at Asn^{191} and at Asn^{227} by single N-acetylglucosamines, and at Asn^{319} by a fucosylated N-acetyllactosaminic type glycan (Fenna *et al.* 1995). Based on these data, glycosylation of Asn^{258} and Asn^{349} in camel lactoperoxidase is proposed. Nevertheless, glycosylation of Asn^{6} and Asn^{112} may also occur.

Tertiary Structure and Catalytic Activity

Antimicrobial activity of lactoperoxidase is performed by a so-called lactoperoxidase system (LP-system), in which hydrogen peroxide (H_2O_2) is reduced and a halide, e.g. iodide (I^-) or bromide (Br^-), or a pseudohalide, e.g. thiocyanate (SCN^-) is subsequently oxidised (Ferrari *et al.* 1997). Complex formation of lactoperoxidase with SCN^-, a molecule with low charge density, was reported to be favoured both, thermodynamically and kinetically. Change in free enthalpy was 0.592 kJ mol^{-1} for the reaction with Cl^-, which is a good substrate for myeloperoxidase. The value for the reaction with SCN^- was -7.46 kJ mol^{-1} (Ferrari *et al.* 1997). The kinetic

```
                                          Prosequence                                    ◇
           αααααααααααα         αααααααα       ααααααααα            ααααααααααα
Camel     Q TTAAAMSEAVRQVKVHVNKAFLDSRTRLKAAMSSEVPTTRQLSEYLKHAKGRTRTAIRNGQVWEESLKRLWQKV      2
Bovine    QAASTTTISDAVSKVKIQVNKAFLDSRTRLKTTLSSEAPTTQQLSEYFKHAKGRTRTAIRNGQVWEESLKRLRRDT      2

                                  ◇
           αα                    βββ                                  ααααααααααααα
Camel      TQTNITDPSLDLTALSWEVGCDVPVSVVKCDKNSPYRTITGDCNNGRHPALGAANQALARWLPAEYEDGLSLPFG     77
Bovine     TLTNVTDPSLDLTALSWEVGCGAPVPLVKCDENSPYRTITGDCNNRRSPALGAANRALARWLPAEYEDGLALPFG     77
Human                                 VTCPEQDKYRTITGMCNNRRSPTLGASNRAFVRWLPAEYEDGFSLPYG     48

                                                Hem linked Asp, distal His, Ca²⁺ binding Asp
                      αααααααααααα                  ααααααααααααα
Camel      WTRGKKRNGFPLPLAREVSNKIVGYLNEEGVLDQNRSLLFMQWGQIVDHDLDFPRDTELGSSEYSKAQCDEHCIR    152
Bovine     WTQRKTRNGFRVPLAREVSNKIVGYLDEEGVLDQNRSLLFMQWGQIVDHDLDFAPETELGSNEHSKTQCEEYCIQ    152
Human      WTPGVKRNGFPVALARAVSNEIVRFPTDQLTPDQERSLMFMQWGQLLDHDLDFTPEPA        VNCETSCVQ    123

                                                              Ca²⁺ binding region
              ββ              ββ                                      αααα   αααααα
Camel      GDNCFPIMFPRNDRKVMTQGKCMPFFRAGFVCPNPPYQSLAREQINALTSFLDASLVYGSEPSLASSLRDLSSPL    227
Bovine     GDNCFPIMFPKNDPKLKTQGKCMPFFRAGFVCPTPPYQSLAREQINAVTSFLDASLVYGSEPSLASRLRNLSSPL    227
Human      QPPCFPLKIPPNDPRIKNQADCIPFFRSCPACPGS  NITIRNQINALTSFVDASMVYGSEEPLARNLRNMSNQL    196

                                                        Distal Arg,  Hem binding Glu
                                αααα                         αααααααααααααααααααα
Camel      GLMAVNQEFWDHGLAYPPFVNKKPSPCEVINTTAQVPCFLAGDSRASEQILLATSHTLLLREHNRLARELKKLNP    302
Bovine     GLMAVNQEAWDHGLAYLPFNNKKPSPCEFINTTARVPCFLAGDFRASEQILLATAHTLLLREHNRLARELKKLNP    302
Human      GLLAVNQRFQDNGRALLPFDNLHDDPCLLTNRSARIPCFLAGDTRSSEMPELTSMHTLLLREHNRLATELKSLNP    271
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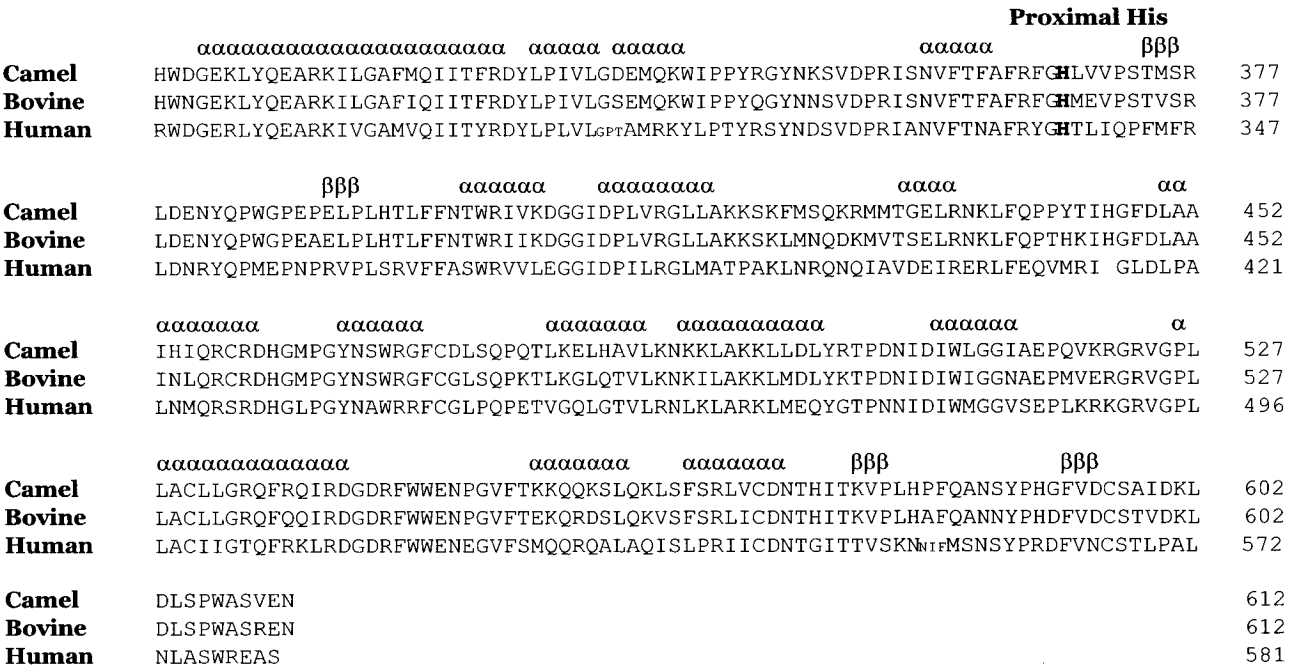

Fig. 4.34. Schematic drawing of the relationship between structure and function in camel and bovine lactoperoxidase. Numbering starts from the first residue of the presumed long variant of lactoperoxidase. Heavy and light chain of human myeloperoxidase underneath, for better comparison of functional residues. Light chain of myeloperoxidase from Val^{1} to Ala^{106}, heavy chain from Val^{15} to Ser^{581} Functional residues in bold. α-helical regions designated as "α", β-pleated regions as "β". ◇ Possible cleavage sites of the propeptide.

preference for SCN^- was discussed to be a consequence of shape and amino acid composition at the distal side of lactoperoxidase heme (De Wit & Van Hooydonk, 1996). A very low K_M value of 170 μM was reported for SCN^-. Reaction kinetics are not limited by H_2O_2, since the K_M for this co-substrate was lower than for SCN^- and for halides (Ferrari *et al.* 1997).

The architecture of the heme pocket of peroxidases is highly conserved. In mammalian peroxidases, the heme is distorted, as a result from covalent binding. At the proximal side of the pocket, the ferric ion is coordinated by His^{368} in lactoperoxidase, and His^{338} in myeloperoxidase, which function as an axial, fifth ligand. The distal side of myeloperoxidase, which provides a catalytic surface for the reaction cascade, is shown in Fig. 4.35. The channel of myeloperoxidase is about 12 Å in diameter, leading to a much narrower entrance to the distal pocket near the heme pyrrole ring D. The upper surface of the distal pocket is composed of Arg^{241} at the entrance, followed by His^{97} and Gln^{93} at the back. High occupancy binding was found at the entrance to the distal pocket between pyrrole ring D and Arg^{241} (Hori *et al.* 1994). This interaction was presumed to be electrostatic in nature, since there is an accumulation of four positive charges from the ferric iron atom of the heme and from Arg^{241}. It is supposed, that Arg^{241} is involved in initial binding of negatively charged substrates. Peroxidases undergo a two-electron oxidation by H_2O_2 to form an enzyme intermediate named compound I, which contains an oxoferryl porphyrin π-cation radical, which is readily converted into an oxoferryl protein radical. His^{97} is thought to donate a proton in its function as an acid-base catalyst, which promotes heterolytic cleavage of peroxide, while Arg^{241} plays a secondary role in helping to stabilise the resultant oxoferryl center of compound I (Davey & Fenna, 1996). A nucleophilic substrate, such as SCN^-, donates an electron to compound I, which yields the corresponding substrate free radical and an oxoferryl heme intermediate, $Fe^{4+}O$, or $Fe^{3+}R^{\cdot}$, which is named compound II. A subsequent reduction by a second nucleophilic substrate molecule leads to ferric peroxidase. Peroxidases also react with the superoxide radical anion to form compund III, a resonance hybrid between $Fe^{3+}O_2^-$ and $Fe^{2+}(O)_2$.

Natural substrates of lactoperoxidase in milk are thiocyanate and iodide, of which milk contains trace amounts. SCN^- is provided mainly by consumption of plants of the family *Cruciferae*. Kale, a cabbage, contains up to 5 g kg^{-1} thioglucosides, which are readily converted into SCN^- by enzymatic hydrolysis (Bibi, 1989). Cow milk content of SCN^- was reported

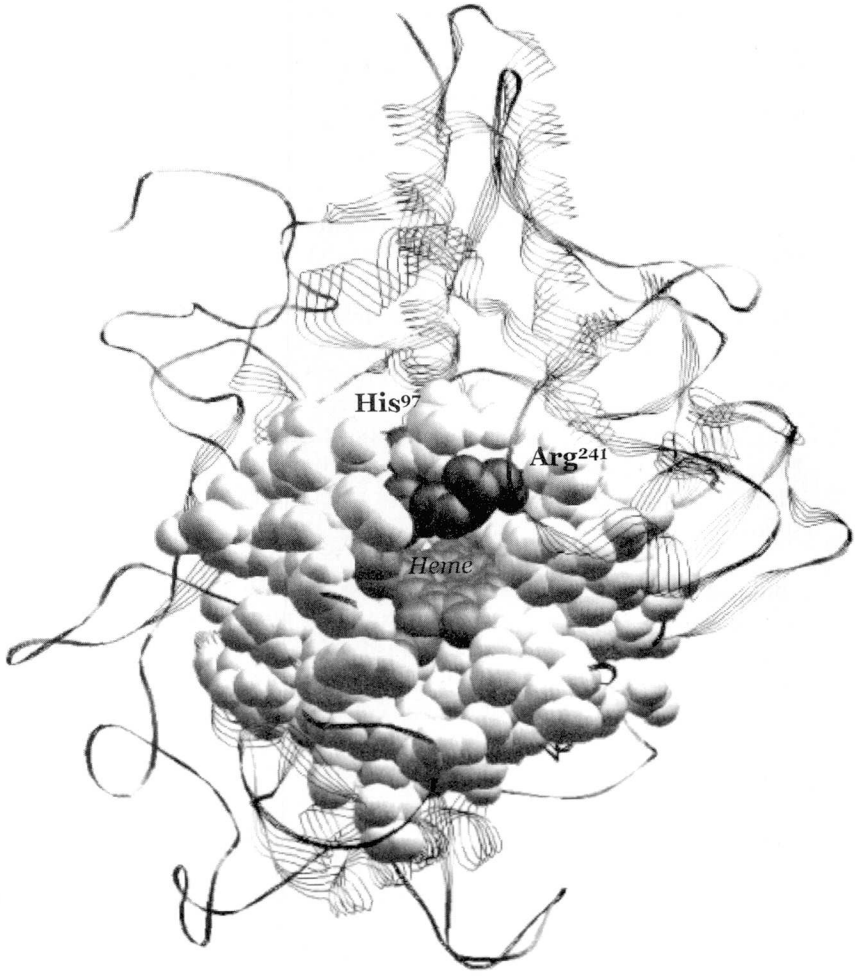

Fig. 4.35. Myeloperoxidase, a close homologue of lactoperoxidase, shown as a monomer with one heavy and one light chain. Distal side of the heme pocket, which contains the catalytic centre, in direction of vision. Distal His^{97} and Arg^{241} dark shaded. Heme medium grey shaded. Residues within 8 Å distance of the heme are drawn as Van-der-Waals surface, other residues are drafted in ribbon view. X-ray crystallographic data from Fenna *et al.* (1995).

to vary between 1 mg l^{-1} and 15 mg l^{-1}, with much lower concentrations in winter time, depending on feeding (De Wit & Van Hooydonk, 1996). Concentration of hydrogen peroxide is low in mastitis free milk. It can be generated by oxidation of xanthine by Xanthine oxidase, or supplied by catalase-negative bacteria, such as strains of lactobacilli, lactococci or streptococci, which naturally occur in milk (Bibi, 1989).

The reaction products of thiocyanate oxidation, $OSCN^-$ and HOSCN, are in chemical equilibrium at pH 5.3, and are able to oxidise free sulphhydride groups of the cytoplasmic membrane of gram-negative bacteria, similarly to hypoiodide (OI^-). The structural damage on bacterial cell membranes results in diffusion of potassium ions, amino acids and polypeptides out of the cell, whilst uptake of glucose and other metabolic substrates is inhibited. Gram-positive bacteria, such as streptococci, are probably better protected from the action of lactoperoxidase by their rigid cell wall. Nevertheless, a bacteriostatic effect on many gram-positive bacteria was also reported, which was observed in retarded acid production (De Wit & Van Hooydonk, 1996).

The advantage of $OSCN^-$ and HOSCN, compared to other hyperoxidised substrates, is, that they are inert towards mammalian cells and milk components, due to low charge density. Lactoperoxidase is not able to oxidise the chloride anion Cl^-, which is present at high concentrations in milk and digestive secretions, in contrast to the other mammalian peroxidases. It was reported, that the lactoperoxidase-chloride complex has a high dissociation constant of 1270 mM, whereas complexes with the preferred substrates thiocyanate and iodide have low K_D-values of 20.3 mM and 205 mM, respectively (Ferrari *et al.* 1997). The different substrate specificity of lactoperoxidase, compared with myeloperoxidase, as well as an anomalous electronic absorption spectrum relative to those of other heme b-containing proteins, is thought to be the result of an unusually constraint heme pocket in lactoperoxidase (Hu *et al.* 1993). The reason for the low affinity of lactoperoxidase towards Cl^- is supposed to be in the protection of mammalian organs and milk components. Nevertheless it was shown, that aromatic molecules are able to bind at the distal heme pocket of myeloperoxidase (Hori *et al.* 1994). It was suggested, that the heme pyrrole ring D, and the side chains of the distal Phe^{101}, Arg^{241}, Phe^{368}, and Phe^{409}, form a hydrophobic surface at the entrance to the distal cavity, which can bind aromatic substrate molecules. In this way, mono- or dichlorinated biphenyls (PCBs), which are accumulated in the body by digestion of animal fats, are metabolised to dihydroxy compounds and further oxidised to

reactive metabolites, which can react with cell DNA (Oakley *et al.* 1996). Lactoperoxidase and myeloperoxidase may therefore play a crucial role in the chemical induction of breast cancer (Josephy, 1996).

Lactoperoxidase is easily inhibited by irreversible binding of nitrite, azide, cyanide and carbon monoxide (Hu *et al.* 1993, Ferrari *et al.* 1997). Riboflavin (vitamin B2), found in camel milk at low concentrations of 0.4 mg l^{-1} to 0.8 mg l^{-1}, was shown to promote light induced inactivation of lactoperoxidase (Herández *et al.* 1990). Photochemical inactivation of bovine lactoperoxidase in milk was 55% after 4 h irradiation with 6,000 lux, and was inhibited by cysteine, indicating, that riboflavin damaged lactoperoxidase by oxidative action.

Potential for Milk Preservation

The LP-system is active against mainly gram-negative, psychrotrophic and mesophilic bacteria, but also against a broad range of pathogens, including viruses and moulds, organisms found in milk of poor hygienic quality (De Wit & Van Hooydonk, 1996). It was observed, that the keeping quality of raw cow milk, stored at 4 °C and pasteurised after three to four days, was much better than that of the same milk pasteurised on the first, or on the seventh day, when also stored at 4 °C, before and after pasteurisation (Ravanis & Lewis, 1995). From these results, it was concluded, that the natural LP-system in raw cow milk is most effective up to four days. Thereafter, H_2O_2 is supposed to be the limiting factor. Maximal performance of the LP-system is achieved by addition of equimolar concentrations of hydrogen peroxide and a halide, usually 0.25 mM of each (Bibi, 1989). The optimal pH range of the LP-system is at pH 5.5 to 6.8. Gram-negative, catalase-positive bacteria, such as pseudomonads, coliforms, salmonellae, and shigellae, are killed by the LP-system, provided, H_2O_2 is provided in sufficient concentrations. An activated LP-system was also shown to be effective against different *Listeria* strains (Bibi, 1989). Activation of the LP-system extends the storage time of raw cow milk at 10 °C for at least three days. Moderate cooling, e.g. using a leather bag or clay pot for storage, which allows slow water sublimation, could be a useful alternative to extend the keeping quality of the milk. A problem could be the reported light inactivation of lactoperoxidase. Light-sealed containers, such as clay pots, should be preferred to transparent plastic canisters.

The lactoperoxidase system is of special interest as an alternative method for the preservation of camel milk, to guarantee a hygienic product under difficult conditions, such as handling at high ambient temperature, long

distance transports and inadequate cleaning of milk handling equipment, e.g. due to water shortage (Bibi, 1989). Mainly the collection of evening milk was reported to be problematic, since cooling is often not feasible. To estimate the potential of the LP-system for camel milk, it would be necessary, to measure the activity of lactoperoxidase in pooled camel milk. The SCN^- concentration in different feeding areas should also be studied. Further on, bacterial strains should be found, which promote the formation of H_2O_2 in camel milk. Elagamy et al (1992) reported, that the LP-system of camel milk was bactericidal against *Escherichia coli* and *Salmonella typhimurium*, but only bacteriostatic towards *Lactococcus lactis* and *Staphylococcus aureus*.

The limited interest in the LP-system for the preservation of milk may be explained by problems with inhibition of starter cultures and by danger of milk poisoning, if the concentrations of the added chemicals are surpassed.

5 CONCLUSIONS AND OUTLOOK

In the course of the study, quantitative and structural aspects of camel milk proteins were studied. Major variations were found, when the proteins were compared to well characterised homologues of cow milk, in both, concentration, and structure of the proteins. These variations were suggested to have an impact on the technological quality of camel milk, and on the performance as a food product with antimicrobial activity.

The differences were supposed to originate from the different habitats, to which the two species are adapted, the distant evolutionary relationship, the forced breeding selection in cattle, the different lactating and suckling regime of heifer and calf, and a possible difference in natural pressure for survival of the infant.

A first aim of the study was to find an explanation for the poor renneting capability, and the low heat stability of camel milk. Research done with focus on the casein fraction and the renneting enzymes gave indication, that some problems in fermentation, pasteurisation and cheese production resulted from the low κ-CN content of casein micelles, combined with a high β-CN content, and with structural divergences between camel and bovine κ-CN. Use of recombinant camel chymosin was suggested to be a promising alternative in camel milk rennet coagulation, with focus on improvement of curd firmness. The high proportion of β-CN may also give rise to the low heat stability of camel milk. Nevertheless, it has to be considered, that other factors, which were not examined in the course of this study, may additionally influence technological properties, such as the concentration of free and bound calcium phosphate, size of casein micelles and fat globules, and concentration of total protein, fat and lactose. An analysis of protein and calcium levels in camel milk, under consideration of age, stage of lactation, feeding and stress factors, such as dehydration, would be highly desirable, to evaluate the impact of these factors on the processing quality of the milk.

Camel milk is a rich source of proteins with potential antimicrobial and protective activity. All proteins isolated from the camel whey fraction in the course of this study were found to be expressed in the lactating mammary gland, although homologous proteins of other species were reported to participate in the innate immune system, being secreted e.g. from

leukocytes or from the gastric mucosa. Some of these proteins were not found in cow milk, or only in minor amounts, such as the novel peptidoglycan recognition protein, the whey acidic protein, or lactophorin. Peptidoglycan recognition protein was easily isolated from whey and was presumed to exhibit high capacity in bacterial growth inhibition. It would be of interest to know the stability of this protein against heat and acid denaturation, and the potential of synergistic activity in combination with other milk proteins reported to exhibit antimicrobial activity, such as lactoferrin, lactoperoxidase, lysozyme, whey acidic protein, lactophorin and the different immunoglobulin types found in milk. Camel milk is used in the traditional medicine of camel keeping societies for treatment of wounds and gastric problems. It would be of interest to study the antimicrobial activity of milk from different breeds, stages of lactation, feeding and husbandry conditions, on their selective inhibition of bacterial and fungal growth, and of rotaviral spread. It should be studied, if there is a possible application of camel whey as a prebiotic additive in food and cosmetics.

The potential of the lactoperoxidase system for extension of the keeping quality of fresh camel milk should be examined by microbial assays and ABTS measurement of lactoperoxidase activity in milk from different stages of lactation. There would also be interest in selection of peroxide producing lactobacilli, lactococci and streptococci strains, which could be used for activation of the LP-s in camel milk. Another interest could be the selection of lactic acid bacterial strains, which better tolerate the natural protective system of camel milk, and which could help in production of a fermented product with constant quality.

Due to the lower protein content, and a larger contribution of whey proteins, camel milk could be an interesting alternative in infant milk formula. Absence of β-lactoglobulin, which may result in intolerance of an infant towards cow milk (Hambræus, 1992), a low amount of lactoperoxidase, which seems to be down-regulated in human milk early in lactation, a high amount of α-lactalbumin, which has a high nutritional value, of lactoferrin, which is also found at a high level in human milk, and of non-protein nitrogen, similarly to human milk, could be of advantage in utilisation of camel milk in dairy products for infants and people with allergy against cow milk products.

As a long term goal in camel research, breed classification, and determination of genetic and nutritional factors, which increase milk yield and improve consistency of milk quality should be envisaged. Genetic

characterisation in terms of large-scale cDNA sequence analysis, chromosomal mapping of genes and microsatellite sequences, study of regulation of gene expression, and detection of markers for breed classification, could help to find suitable animals for milk and meat production, as well as for transport, agricultural and racing purposes, and could be a first step in understanding the genetic and physiological reservoir of the camel as the livestock animal, which is best adapted to arid regions.

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7 CURRICULUM VITAE

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1975 – 1980	Primary School, Baden, Switzerland.
1980 – 1984	Secondary School, Baden, Switzerland.
1984 – 1988	High School, Baden, Switzerland. Completed with a Matura A.
Autumn 1988	Military Education at the Division for Artillery, Frauenfeld, Switzerland.
Spring 1989	Study of English Language at International House, Hastings, UK. Completed with Cambridge First Certificate in English, Arels Higher Certificate in Spoken English and Comprehension, and Oxford Examination, Higher Level.
1989 – 1994	Study of General Biology at the Swiss Federal Institute of Technology, Zurich, Switzerland. Completed with a Diploma Thesis in Plant Genetics, titled "Circadian Oscillations of *At*grp and *At*glp transcripts in *Arabidopsis thaliana* and Immunocytochemical Localisation of the Corresponding Proteins in *Sinapis alba*", and with a Diploma in Natural Sciences (Cell Biology, Molecular Biology, Microbiology, Plant Physiology, Biochemistry, and Biotechnology).
1995 – 1998	Assistant Research Work at the Institute of Food Technology, Swiss Federal Institute of Technology, Zurich, Switzerland.