Production and characterization of recombinant human lactoferrin

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Chapter 7

The role of N-linked glycosylation in the protection of human and bovine lactoferrin against tryptic proteolysis

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Lactoferrin (LF) is an iron-binding glycoprotein of the innate host defence system. To elucidate the role of N-linked glycosylation in protection of LF against proteolysis, we compared the tryptic susceptibility of human LF (hLF) variants from human milk, expressed in human 293(S) cells or in the milk of transgenic mice and cows. The analysis revealed that recombinant hLF (rhLF) with mutations Ile130→Thr and Gly404→Cys was about twofold more susceptible than glycosylated and unglycosylated variants with the naturally occurring Ile130 and Gly404. Hence, N-linked glycosylation is not involved in protection of hLF against tryptic proteolysis. Apparently, the previously reported protection by N-linked glycosylation of hLF [van Berkel, P.H.C., Geerts, M.E.J., van Veen, H.A., Kooiman, P.M., Pieper, F., de Boer, H.A. & Nuijens, J.H. (1995) *Biochem. J.* **312**, 107–114] is restricted to rhLF containing the Thr130 and Cys404. Comparison of the tryptic proteolysis of hLF and bovine LF (bLF) revealed that hLF is about 100-fold more resistant than bLF. Glycosylation variants A and B of bLF differed by about 10-fold in susceptibility to trypsin. This difference is due to glycosylation at Asn281 in bLF-A. Hence, glycosylation at Asn281 protects bLF against cleavage by trypsin at Lys282.

Keywords: lactoferrin; tryptic susceptibility; N-linked glycosylation; transgenic; gastrointestinal.

Lactoferrin (LF) is a metal-binding glycoprotein of M_r 77 000 that belongs to the transferrin family [1]. The molecule is found in secretions such as milk, tears and saliva, but also in the secondary granules of neutrophils (reviewed in [2]). LF is involved in nonspecific host defence against infection and severe inflammation, most notably at mucosal surfaces such as those of the gastrointestinal tract [2]. Antimicrobial activities of LF include bacteriostasis by the sequestration of free iron [3] and bactericidal activity by destabilization of the cell wall [4,5]. Anti-inflammatory actions of LF include inhibition of hydroxyl-radical formation [6], of complement activation [7] and of cytokine production [8] as well as binding and neutralization of lipopolysaccharide (LPS) [9,10].

LF consists of a single polypeptide chain that is folded in two highly homologous lobes, designated the N- and C-lobe, each of which can bind a single ferric ion concomitantly with one bicarbonate anion [11]. The amino acid sequence of human LF (hLF) shows 69% homology with bovine LF (bLF) [12]. Three and five possible N-linked glycosylation sites are present in hLF [13] and bLF [12], respectively, and differential utilization of these sites results in distinct glycosylation variants. In hLF, N-linked glycosylation occurs at one (Asn479), two (Asn138 and 479) or three sites (Asn138, 479 and 624) in about 5%, 85% and 9% of the molecules, respectively [14]. In bLF, four sites (Asn233, 368, 476 and 545) are always utilized [15] while the fifth (Asn281), located in the N-lobe, is glycosylated in about 30% of the molecules in bovine colostrum, but only in about 15% in mature milk [16–18]. The significance of glycosylation for lactoferrin is not completely understood, although protection against proteases such as the pancreatic enzyme trypsin has been suggested [19,20].

The experiments described herein further elucidate the role of N-linked glycosylation in the protection of lactoferrin against tryptic proteolysis. It appeared that glycosylation at Asn281 protects bLF against trypsin. On the contrary, N-linked glycosylation is not involved in the protection of hLF, even though hLF is much more resistant against the protease than bLF.

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Abbreviations: bLF, bovine LF; LF, lactoferrin; hLF, human LF; natural hLF, hLF purified from human milk; iron-saturated hLF, natural hLF that has completely been saturated with iron *in vitro*; rhLF, recombinant hLF; $rhLF^{gen}$, rhLF derived from an hLF-genomic sequence; $rhLF^{cDNA}$, rhLF derived from the Rey hLF^{cDNA} sequence; rhLF-Gln138/479, $rhLF^{cDNA}$ with Thr130→Ile, Cys404→Gly, Asn138→Gln and Asn479→Gln mutations.

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Materials and methods

Reagents

Bovine pancreatic trypsin (type III-S) and soybean trypsin inhibitor (SBTI, type I-S) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). *N*-glycosidase F was obtained from Roche (Mannheim, Germany) and S Sepharose fast flow was obtained from Amersham Biosciences (Uppsala, Sweden).

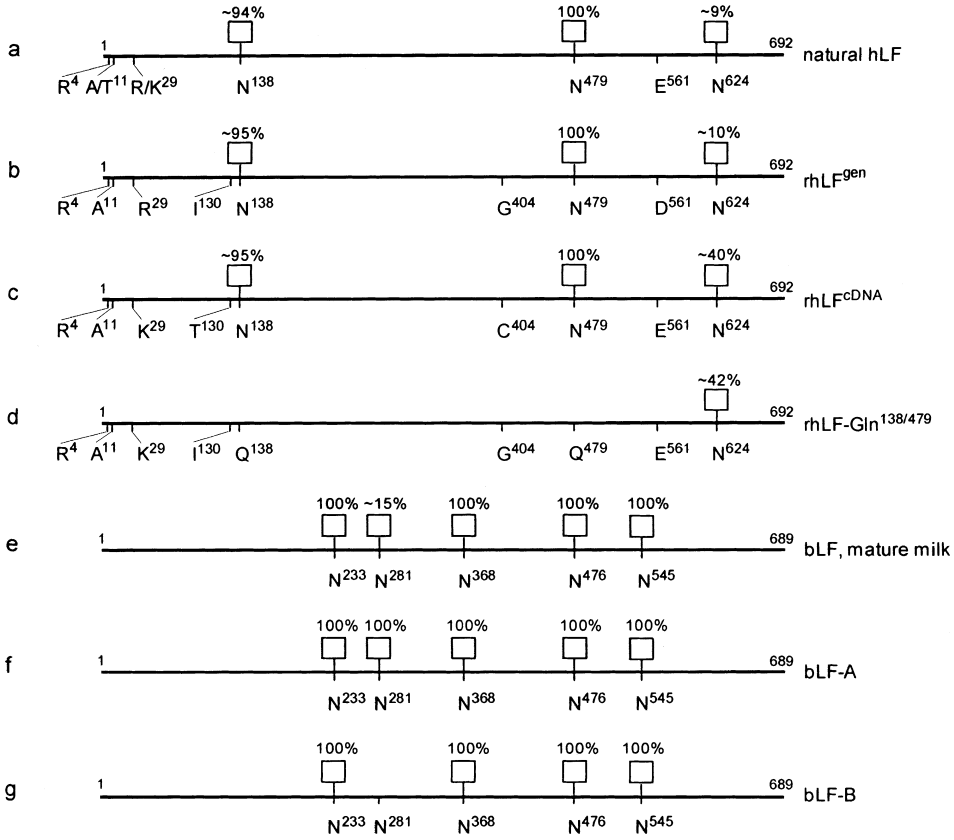

Fig. 1. Lactoferrin variants. The horizontal lines (a–g) represent the hLF and bLF variants used in this study. Short vertical lines together with the amino acids, presented by the standard one-letter code, mark the positions of polymorphic, mutation or N-glycosylation sites. Percentages (above boxes) indicate the proportion of molecules in which the glycosylation sites are actually used. Natural hLF (a) was isolated from a donor heterozygous at positions 11 and 29.

Human lactoferrin variants

Extensive analysis of hLF sequences revealed polymorphic sites in the coding sequence at amino acid position 4 (deletion of Arg), position 11 (Ala or Thr), position 29 (Arg or Lys) and position 561 (Asp or Glu) [18]. The Arg4 deletion in hLF in the Dutch population is rare, i.e. $< 5\%$, while the other polymorphic variants are more evenly distributed. The donor who supplied milk to purify natural hLF for this study was heterozygous at position 11 and 29 (Fig. 1). Natural hLF was purified from human milk as described [19] and was saturated with iron at 3%; complete saturation of hLF with iron was performed as described [21].

Production, purification and characterization of recombinant hLF (rhLF) from milk of transgenic mice and cows was described previously [21,22]. Briefly, mammary gland-specific expression vectors based on the regulatory elements from the bovine αS_1 *casein* gene and either the hLF-cDNA coding sequence published by Rey *et al.* [13], designated $rhLF^{cDNA}$, or genomic hLF sequences, designated $rhLF^{gen}$, were introduced into the murine or bovine germ line. Purified rhLF from transgenic murine and bovine milk appeared to be saturated with iron for about 90% [21] and 8% [22], respectively. Enhanced N-linked glycosylation at Asn624 was observed in $rhLF^{cDNA}$ but not in $rhLF^{gen}$. This is probably caused by a unique cysteine at amino acid position 404 in the Rey cDNA sequence ([13], Fig. 1).

A stable human kidney 293(S) based cell-line expressing rhLF-Gln138/479, a glycosylation site mutant that was derived from $rhLF^{cDNA}$, in which the unique Thr130 and Cys404 were replaced by the naturally occurring Ile130 and Gly404 and Asn138 and Asn479 were mutated in Gln, has been described previously [14]. About 57% of purified rhLF-Gln138/479 is unglycosylated, whereas about 42% of the molecules are glycosylated at Asn624 [14]. In addition, rhLF-Gln138/479 appeared to be completely saturated with iron [14]. An overview of all LF variants is provided in Fig. 1.

Purification of bovine lactoferrin and separation in its variants

Bovine LF was purified from colostrum and mature milk of Frisian Holstein cows using S Sepharose essentially as described for hLF [19]. Colostrum derived bLF was diluted in 20 mM sodium phosphate, pH 7.5 and separated subsequently into bLF-A and bLF-B variants [16] by Mono S chromatography [18]. Mono S elution fractions containing the bLF variants were diluted again and subjected to rechromatography to obtain homogeneous bLF-A and bLF-B preparations.

Analytical Mono S chromatography

Analytical Mono S cation-exchange chromatography was performed as described [18]. Briefly, purified LF was diluted in 20 mM sodium phosphate, pH 7.5 (buffer A) and applied to a Mono S HR 5/5 column (Amersham Biosciences, Uppsala, Sweden) in buffer A. The column was washed subsequently and bound proteins were eluted with a linear salt gradient from 0 to 1 M NaCl in 30 mL of buffer A at a flow rate of 1.0 $mL \cdot min^{-1}$. Eluted protein was detected by absorbance measurement at 280 nm.

Tryptic proteolysis of lactoferrin variants

Lactoferrin variants (0.4 $mg \cdot mL^{-1}$, final concentration; except where indicated otherwise) were incubated with trypsin (0.4 $mg \cdot mL^{-1}$, final concentration) at 37 °C in 50 mM Tris, pH 8.0, 0.14 M NaCl, 2 mM $CaCl_2$. At various timepoints the trypsin activity was stopped by the addition of a threefold excess of SBTI and the mixtures were subjected to nonreduced, boiled SDS/PAGE (12.5%) analysis [19]. Proteins were visualized by staining with Coomassie Brilliant Blue. Densitometry was performed using the Fluor-S Multi-Imager and QUANTITY ONE software from Biorad Laboratories, CA, USA. The tryptic susceptibility of distinct LF species was evaluated by focusing on the degradation of LF and/or by comparing the times required to degrade 50% of LF of M_r 80 000.

Results

Tryptic susceptibility of transgenic rhLF variants

Comparison of the tryptic susceptibility of $rhLF^{cDNA}$ and $rhLF^{gen}$ from transgenic mice with natural hLF and iron-saturated hLF revealed that the tryptic susceptibility of $rhLF^{gen}$, natural hLF and iron-saturated hLF was similar, whereas $rhLF^{cDNA}$ was about twofold more susceptible (Fig. 2, lanes 5–12). The C-lobe derived tryptic fragments, designated hC_1-tryp and hC_2-tryp, migrated as a doublet of protein bands in $rhLF^{gen}$, whereas single bands were observed in natural and iron-saturated hLF (Fig. 2, compare lanes 5 and 6 with 8). This difference results from glycosylation heterogeneity at glycosylation site Asn479 in $rhLF^{gen}$ [14,21]. No predominant C-terminal tryptic bands were observed for $rhLF^{cDNA}$ (Fig. 2, lanes 7 and 11), whereas similar amounts of clear-cut N-lobe fragments, designated hN_1-tryp, were observed for all iron-saturated LF species analysed.

Recombinant hLF^{gen} isolated from transgenic cow milk [22] displayed similar tryptic degradation kinetics compared to natural hLF (Fig. 3). The slightly faster migration of hC_1-tryp and hC_2-tryp of $rhLF^{gen}$ from transgenic cattle compared to natural hLF (Fig. 3, lanes 3–6) resides in differential N-linked glycosylation of the two hLF variants [22]. Similar kinetics of tryptic degradation were also found for iron-saturated $rhLF^{gen}$ from transgenic cattle and iron-saturated hLF (result not shown). The degradation kinetics of $rhLF^{cDNA}$ from transgenic cow milk revealed this variant to be more susceptible towards trypsin than natural hLF and iron-saturated hLF, i.e. similar to $rhLF^{cDNA}$ from transgenic mice (result not shown).

Taken together, these results suggest that $rhLF^{cDNA}$ with the Gly404→Cys mutation shows increased susceptibility towards trypsin, when compared to $rhLF^{gen}$ and natural hLF. Based on experiments with $rhLF^{cDNA}$, we concluded previously that N-linked glycosylation protects hLF against tryptic proteolysis [19]. As the tryptic susceptibility of $rhLF^{cDNA}$ differs from natural hLF and $rhLF^{gen}$ (Fig. 2), we decided to study the role of N-linked glycosylation in the protection of hLF in more detail, and also in LF variants with a glycine at position 404.

Susceptibility to tryptic proteolysis of unglycosylated rhLF

Similar kinetics of tryptic degradation were found for rhLF-Gln138/479 and iron-saturated hLF indicating that

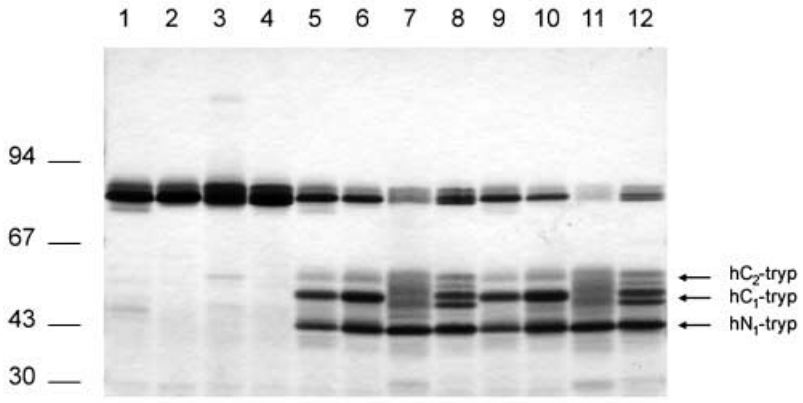

Fig. 2. Susceptibility to tryptic proteolysis of $rhLF^{cDNA}$ and $rhLF^{gen}$ from transgenic mice. Purified hLF variants (0.4 $mg \cdot mL^{-1}$) were incubated with trypsin (0.4 $mg \cdot mL^{-1}$) and subjected to nonreduced, boiled SDS/PAGE (12.5%) analysis as described in the Materials and methods. Natural hLF (lanes 1, 5 and 9), iron-saturated hLF (lanes 2, 6 and 10), $rhLF^{cDNA}$ from transgenic mice (lanes 3, 7 and 11) and $rhLF^{gen}$ from transgenic mice (lanes 4, 8 and 12); after 0, 120 and 240 min of digestion, respectively. Proteins were visualized by staining with Coomassie Brilliant Blue. Left-hand numbers ($10^{-3} \times M_r$) indicate the migration of the protein standards. hC_2-tryp, hC_1-tryp and hN_1-tryp represent the tryptic C- and N-lobe fragments of hLF bearing either 2 or 1 N-linked glycans.

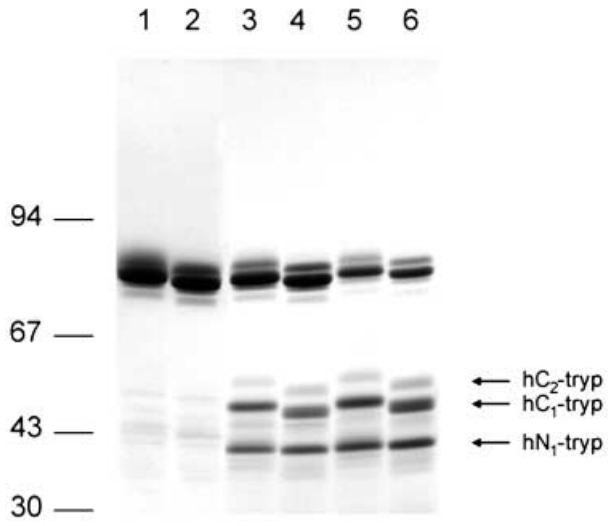

Fig. 3. Susceptibility to tryptic proteolysis of bovine transgenic rhLF^{gen}. SDS/PAGE (12.5%) analysis of tryptic digests obtained as described in the Materials and methods. Natural hLF (lanes 1, 3 and 5) and rhLF^{gen} from transgenic cow milk (lanes 2, 4 and 6); after 0, 60 and 240 min of digestion, respectively.

glycosylation at Asn138 and Asn479 is not involved in the protection of hLF against tryptic proteolysis (Fig. 4). The susceptibility to trypsin of unglycosylated- and Asn624-glycosylated rhLF in rhLF-Gln138/479 was very similar indicating that glycosylation at Asn624 is not essential to protect the molecule against trypsin (Fig. 4, lanes 1–3). These results contrast with the previous reported role of N-linked glycosylation in the protection of hLF against trypsin [19]. This observation appears to be valid only for rhLF^{cDNA} with the Gly404→Cys mutation.

Comparison of kinetics of tryptic degradation between hLF and bLF variants

When the tryptic susceptibility of hLF and bLF from mature milk was compared, hLF appeared to be about 100-fold less susceptible to trypsin than bLF (Fig. 5A). This difference confirms the observations of others [23]. It should be noted that this experiment provides no information on limited N-terminal degradation of hLF. We reported previously that the arginine-rich N-terminus of hLF is very susceptible towards tryptic proteolysis [24]. The bLF preparation used in this experiment consisted of two isoforms on Mono S chromatography [22] and SDS/PAGE (result not shown), which were previously identified as bLF-A and bLF-B [16]. Bovine LF-A and bLF-B differ in N-linked glycosylation at Asn281, which site is utilized in bLF-A, but not in bLF-B [17]. Analytical Mono S chromatography followed by peak surface integration indicated that bLF-A represents about 30% and 15% of total bLF in bovine colostrum and mature whey, respectively [18]. The two bLF variants were isolated as described in the Methods and analysed by Mono S chromatography which revealed symmetric peaks eluting at 0.76 and 0.80 M NaCl for bLF-A and bLF-B, respectively (Fig. 6A,B). The N-terminus of both variants was intact, indicating that the differential elution pattern on Mono S was not caused by limited proteolyses of the bLF N-terminus. SDS/PAGE analyses revealed homogeneous protein bands migrating at M_r 84 000 and 82 000 for bLF-A and bLF-B, respectively (Fig. 7, lanes 1–2). After deglycosylation with N-glycosidase F, both variants migrated with a M_r of 73 000 (Fig. 7, lanes 3–4), confirming that the difference in M_r between both bLF variants was caused by differences in N-linked glycosylation. Comparison of the degradation kinetics of bLF-A and bLF-B in a suboptimal buffer for trypsin activity, i.e. 0.9% NaCl, revealed that bLF-A was about 10-fold more resistant towards trypsin than bLF-B (Fig. 5B). This

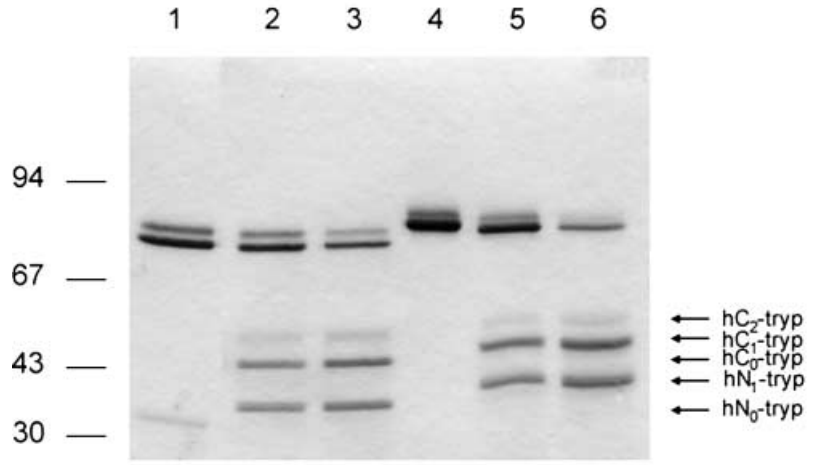

Fig. 4. Susceptibility to tryptic proteolysis of the rhLF-Gln138/479 glycosylation-site mutant. Lactoferrin (80 μg·mL^{-1}) was incubated with trypsin (80 μg·mL^{-1}) and subjected to SDS/PAGE (12.5%) analysis. rhLF-Gln138/479 (lanes 1–3) and iron-saturated hLF (lanes 4–6); after 0, 4 and 24 h of digestion, respectively. hC_2-tryp, hC_1-tryp, hC_0-tryp, hN_1-tryp and hN_0-tryp represent tryptic C- and N-lobe fragments of hLF bearing either 2, 1 or 0 N-linked glycans.

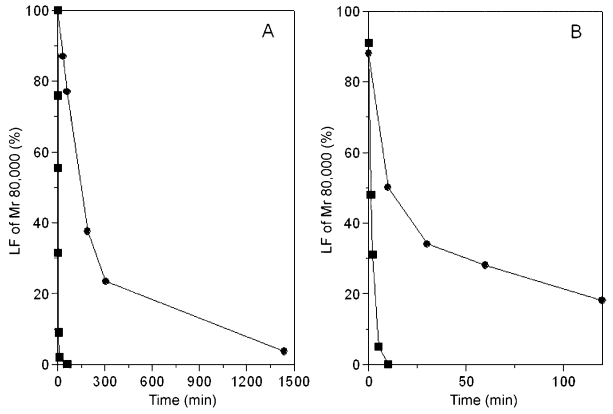

Fig. 5. Kinetics of trypsin degradation of hLF and bLF variants. (A) hLF (●) and bLF (■) from mature milk were incubated with trypsin in 50 mM Tris, pH 8.0, 0.14 M NaCl, 2 mM $CaCl_2$ and subjected to SDS/PAGE analysis as described in Materials and methods. Proteins were visualized by staining with Coomassie Brilliant Blue and residual LF migrating at $M_r \approx 80\,000$ was quantified using densitometry by reference to untreated LF, which was arbitrarily set at 100%. (B) Kinetics of tryptic proteolysis of bLF-A (●) and bLF-B (■) in 0.9% (w/v) NaCl.

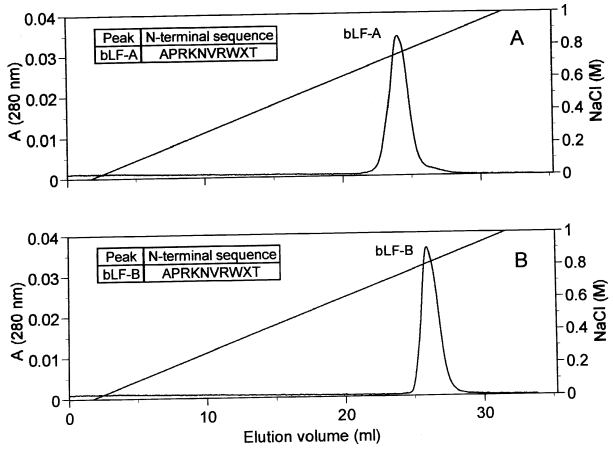

Fig. 6. Mono S chromatography and N-terminal protein sequencing of bLF variants. Forty micrograms of bovine colostrum purified bLF-A (A) and bLF-B (B) were subjected to analytical Mono S chromatography as described in the Materials and methods. The left and right abscissas indicate the absorption at 280 nm and NaCl concentration (M), respectively. The inserts provide the N-terminal protein sequencing results obtained as described [18].

suggests that glycosylation at Asn281 protects bLF against proteolysis at Lys282, the major tryptic cleavage site reported for bLF [25,26]. To further investigate this, the tryptic digests of bLF-A and bLF-B were compared on SDS/PAGE (Fig. 8), which revealed that the tryptic fragments of bLF-B (Fig. 8, lanes 4 and 6) were similar to the protein band pattern reported previously for trypsinized bLF [23,26]. Tryptic fragments, designated as bC_3-tryp, bC_3 and bN_1-tryp, with M_r values of 55 000, 46 000 and 36 000, respectively, were also present in the digest of bLF-A but it also contained an additional

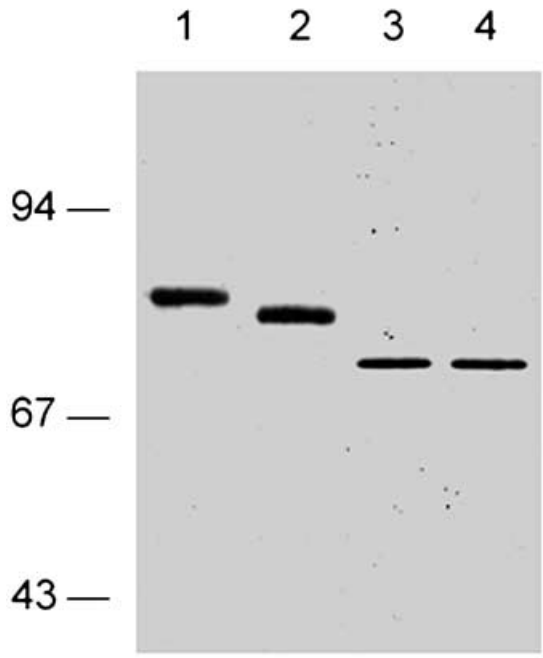

Fig. 7. SDS/PAGE analysis of deglycosylated bLF-A and bLF-B. Purified bLF-A and bLF-B were deglycosylated with N-glycosidase F [19] and subjected to nonreduced, boiled SDS/PAGE (7.5%) analysis. Lane 1, untreated bLF-A; lane 2, untreated bLF-B; lane 3, deglycosylated bLF-A; lane 4, deglycosylated bLF-B. Proteins, 300 ng per lane, were visualized by staining with silver.

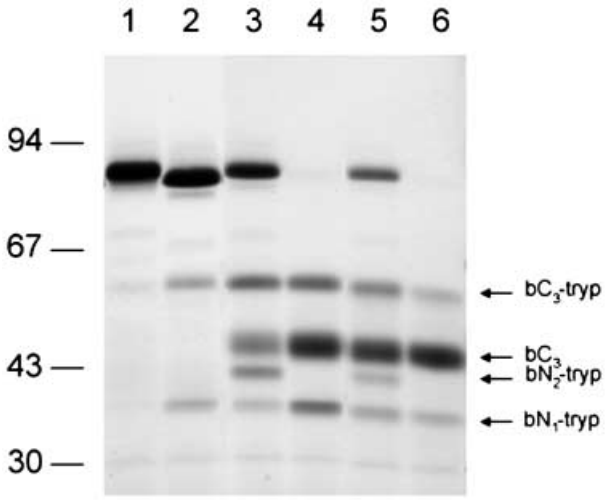

Fig. 8. SDS/PAGE analysis of tryptic digests of bLF-A and bLF-B. Tryptic digests of 10 μg of bLF variants were applied to SDS/PAGE (12.5%). bLF-A (lanes 1, 3 and 5) and bLF-B (lanes 2, 4 and 6); after 0, 30 and 240 min of digestion, respectively. bC_3-tryp, bC_3, bN_2-tryp and bN_1-tryp indicates the tryptic C- and N-lobe fragments derived from bLF bearing either 3, 2 or 1 N-linked glycans. Left-hand numbers ($10^{-3} \times M_r$) indicate the migration of the protein standards.

protein band of M_r 41 000 (Fig. 8, lanes 3 and 5). We speculated that this fragment of bLF-A represents the N-terminal tryptic fragment with two N-linked glycans attached (confirmed by deglycosylation experiments;

results not shown) and it was therefore designated bN_2-tryp. Furthermore, the change in ratio between bN_2-tryp and bN_1-tryp bands in time (Fig. 8, compare lanes 3 to 5) suggests that bN_2-tryp is generated first and subsequently degraded into a protein band of M_r 36 000.

Taken together, these results suggest that the first cleavage of bLF by trypsin is after Lys282 and that glycosylation at Asn281 in bLF-A protects the molecule against proteolysis.

Discussion

Previously, we reported differences in tryptic susceptibility between N-linked glycosylated and unglycosylated rhLF [19]. The rhLF variants used in that study were derived from the Rey sequence [13], i.e. $rhLF^{cDNA}$, and comparison of glycosylated and unglycosylated $rhLF^{cDNA}$ with natural hLF revealed that, although $rhLF^{cDNA}$ was slightly more susceptible to tryptic proteolysis, the susceptibility was enhanced strongly in unglycosylated $rhLF^{cDNA}$ [19]. Thus, we concluded that N-linked glycosylation protects hLF against tryptic proteolysis. However, here we show that, in case of naturally occurring hLF variants, N-linked glycosylation is not involved in protection of the molecule against trypsin.

First, we confirmed that $rhLF^{cDNA}$ is more susceptible to trypsin than natural hLF, iron-saturated hLF or rhLF produced from a genomic sequence ($rhLF^{gen}$). The enhanced susceptibility, about twofold, of $rhLF^{cDNA}$ is most pronounced in its C-terminus (Fig. 2). The $rhLF^{cDNA}$ sequence contains two unique mutations, i.e. Ile130→Thr in the N-lobe and Gly404→Cys in the C-lobe, when compared to other published hLF sequences [18]. The Cys404 residue may cause alternative disulphide bonding in the C-lobe, which might explain an increased tryptic susceptibility. It is to be noted that Cys404 is located near Cys406, which may explain why a putative structural difference is rather subtle and did not appear from comparative studies of natural hLF and $rhLF^{cDNA}$ by *in vitro* and *in vivo* antigenicity, iron-binding and release and binding to several ligands [21]. The only indication for a difference in conformation between $rhLF^{cDNA}$ and natural hLF was the increased glycosylation at Asn624 in $rhLF^{cDNA}$ ([21], Fig. 1) which is in line with the hypothesis that glycosylation at Asn624 in natural hLF is limited due to conformational and/or primary sequence constraints [14].

Secondly, the unglycosylated- and Asn624-glycosylated rhLF-Gln138/479 variants appeared equally resistant to trypsin when compared to iron-saturated hLF (Fig. 4). This result indicates that the absence of glycosylation in rhLF-Gln138/479, which has the naturally occurring Gly404, does not lead to increased tryptic susceptibility of the rhLF-Gln138/479 molecules.

Taken together, the results suggest that the Gly404→Cys mutation in $rhLF^{cDNA}$ results in a slightly altered conformation, when compared to natural hLF, which accounts for the increased tryptic susceptibility. Evidently, the tryptic proteolysis assay is able to reveal subtle, previously unnoticed, differences between $rhLF^{cDNA}$ and natural hLF.

Recombinant $rhLF^{gen}$ from transgenic cows and natural hLF (Fig. 3) as well as their iron-saturated counterparts (result not shown) showed similar tryptic degradation kinetics. Apparently, the polymorphic amino acid at position 561 i.e. Glu or Asp in natural hLF and $rhLF^{gen}$, respectively (Fig. 1), did not alter the tryptic degradation kinetics (Figs 2,3).

Similar to hLF, bLF occurs as a mixture of glycosylation variants, designated as bLF-A and bLF-B [16,17]. We obtained homogeneous preparations of bLF-A and bLF-B as shown by analytical Mono S chromatography (Fig. 6A,B) and SDS/PAGE (Fig. 7, lanes 1–2) and confirmed that glycosylation at Asn281 in the bLF N-lobe [17] explains for the larger molecular weight of bLF-A compared to bLF-B (Fig. 7). The major tryptic cleavage site reported for bLF is after Lys282 [25,26], which is located within the N-linked glycosylation sequon Asn281-Lys282-Ser283 [12]. N-linked glycosylation at Asn281 in bLF-A, but not in bLF-B, therefore most likely explains for the differential tryptic susceptibility (Figs 5B and 8).

The concentrations of bLF-A in colostrum (about 30% of total bLF) are higher than that in mature milk (about 15%). Recently, it was shown that bLF-A displays a higher bacteriostatic activity against *E. coli* than bLF-B [16]. As bLF-A is more resistant to proteolytic degradation than bLF-B, the first may also be superior in protection of the mammary gland and the intestinal tract of the newborn because it is more resistant to proteolytic degradation. However, even though bLF-A was about 10 times more resistant to trypsin than bLF-B, it was still much more sensitive to trypsin than hLF, i.e. hLF was found to be about 100-fold more resistant to trypsin than bLF (Fig. 5A). This is particularly interesting given the fact that Lys282 is the major trypsin cleavage site for both hLF and bLF [25]. Apparently, the conformation of bLF and hLF differs, with major cleavage sites being less accessible to trypsin in case of hLF, despite the 69% amino acid homology between the two proteins [12].

Experiments with pepsin also revealed differences between hLF and bLF i.e. hLF is less susceptible to digestion by pepsin than bLF (result not shown). The increased susceptibility of bLF, compared to hLF, to digestive proteases such as trypsin and pepsin are relevant when considering oral application of lactoferrin where the protein has to survive the harsh environment of the gastrointestinal tract. Thus, on the basis of this study, rhLF may be preferred over bLF in oral applications of lactoferrins in human healthcare.

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