Production and characterization of recombinant human lactoferrin

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Chapter 2

Analytical cation-exchange chromatography to assess the identity, purity, and N-terminal integrity of human lactoferrin

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Abstract

Human lactoferrin (hLF) is an iron-binding glycoprotein involved in the innate host defense. The positively charged N-terminal domain of hLF mediates several of its activities by interacting with ligands such as bacterial lipopolysaccharide (LPS), specific receptors, and other proteins. This cationic domain is highly susceptible to limited proteolysis, which impacts on the affinity of hLF for the ligand. An analytical method, employing cation-exchange chromatography on Mono S, was developed to assess the N-terminal integrity of hLF preparations. The method, which separates N-terminally intact hLF from hLF species lacking two (Gly^1–Arg^2) or three (Gly^1–Arg^2–Arg^3) residues, showed that 5–58% of total hLF in commercially obtained preparations was N-terminally degraded. The elution profile of hLF on Mono S unequivocally differed from lactoferrins from other species as well as homologous and other whey proteins. Analysis of fresh human whey samples revealed two variants of N-terminally intact hLF, but not limitedly proteolyzed hLF. Mono S chromatography of 2 out of 26 individual human whey samples showed a rare polymorphic hLF variant with three N-terminal arginines (Gly^1–Arg^2–Arg^3–Arg^4–Ser^5–) instead of the usual variant with four N-terminal arginines (Gly^1–Arg^2–Arg^3–Arg^4–Arg^5–Ser^6–). In conclusion, Mono S cation-exchange chromatography appeared a robust method to assess the identity, purity, N-terminal integrity, and the presence of polymorphic and intact hLF variants.

Keywords: Lactoferrin; N-terminal integrity; Mono S

Human lactoferrin (hLF)[1] is a metal-binding glycoprotein of M_r 77,000 that belongs to the transferrin family [1]. The molecule is found in milk, tears, saliva, and other secretions [2]. It is also present in the secondary granules of neutrophils [2]. Lactoferrin (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 [3]. The amino acid sequence of hLF has been determined and showed 69, 71, and 70% homology with bovine, porcine, and murine lactoferrin, respectively [4–6]. Extensive in vitro and some in vivo studies revealed that hLF is involved in the host defense against infection and severe inflammation, most notably at the mucosal surface [2]. Antimicrobial activities of hLF include bacteriostasis by sequestration of free iron [7] and bactericidal activity by destabilization of cell-wall components [8,9]. Anti-inflammatory actions of hLF include inhibition of complement activation [10] and cytokine production [11] as well as binding to lipopolysaccharide (LPS) [12]. Many of the hLF activities are mediated by its positively charged N-terminus in which two basic clusters, i.e., Arg^2–Arg^3–Arg^4–Arg^5, and Arg^{28}–Lys^{29}–Val^{30}–Arg^{31}, are juxtaposed to form a cationic cradle [13–15]. This domain binds to negatively charged ligands like LPS, DNA, and heparin [13–15], and other proteins such as lysozyme [13] as well as to specific receptors [16,17]. Several reports have indicated that the affinity of these interactions changes when consecutive arginines of the first basic cluster are removed by limited proteolysis or

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[1] *Abbreviations used:* LF, lactoferrin; hLF, human LF; milk-purified hLF, S Sepharose human milk-purified LF; LPS, lipopolysaccharide; bLF, bovine LF; RT-PCR, reverse transcriptase-polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

site-directed mutagenesis [13,16–18]. Although gel electrophoretic studies indicated that the hLF molecule is highly resistant to degradation by trypsin and chymotrypsin [19], its first basic cluster is highly susceptible to tryptic proteolysis [16]. Therefore, a simple and robust analytical method to assess the N-terminal integrity of hLF from human or recombinant sources would be of great value in structure–function relationship studies. Here we report that analytical cation-exchange chromatography on Mono S [20], a mono-dispersed 10 μm bead packed in a HR 5/5 column meets these criteria. In addition, Mono S chromatography appeared valuable for determining the identity and purity of hLF due to its unique elution profile.

Materials and methods

Reagents

Human lactoferrin and transferrin were obtained from Sigma Chemical (St. Louis, MO), Calbiochem (La Jolla, CA) and Serva (Heidelberg, Germany) or purified as described below. Neuraminidase was obtained from Behringwerke AG (Marburg, Germany). S Sepharose fast flow was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Chemicals were of pro analysis grade or higher and buffers were filtered over 0.22-μm filter (Millipore, Bedford, MA) prior to use.

Analytical cation-exchange chromatography of purified proteins

A Mono S HR 5/5 column (Amersham Pharmacia Biotech) was equilibrated in 20 mM sodium phosphate, pH 7.5 (buffer A), using an Äkta Explorer 10 equipped with a 2-mm flowcell (Amersham Pharmacia Biotech). Purified proteins were diluted in buffer A, centrifuged for 5 min at 23,000*g*, and applied to the column. The column was subsequently washed with 5 ml of buffer A and bound proteins were eluted with a linear salt gradient from 0 to 1 M sodium chloride (NaCl) in 30 ml buffer A after which 5 ml of the 1 M NaCl buffer was applied. The flow rate was at 1.0 ml/min and absorbance was measured simultaneously at 214, 280, and 465 nm. The elution position of proteins was determined after alignment of the theoretical salt gradient with the UV signal. Integration of eluting peaks was done with the Unicorn software (Amersham Pharmacia Biotech).

Purification of polymorphic variants of hLF from human milk

Sequence analysis of cDNA available from GenBanks [21–27] as well as several individuals revealed that the hLF gene is polymorphic at three sites, i.e., at position 11 (alanine or threonine), position 29 (arginine or lysine) or position 561 (aspartic acid or glutamic acid) in hLF. RT-PCR and restriction enzyme analysis on genomic DNA from individual milk donors was performed to identify their polymorphism at positions 11, 29, and 561.[2] Human LF was purified from the milk of selected individual donors and saturated with iron as described [20]. This reference also describes the purification of human lysozyme.

Purification of lactoferrins from milk of various species

Human, bovine, murine, and porcine milk to which NaCl had been added to 0.4 M final concentration was centrifuged at 10 °C for 60 min at 23,000*g* to separate fat and casein fractions from the whey. The whey was 5-fold diluted in buffer A, passed through a 0.22-μm filter, and applied to analytical chromatography on Mono S as described above. Eluted fractions were subjected to SDS–PAGE and human, murine, and bovine LF were applied to N-terminal protein sequencing.

SDS–PAGE analysis

Reduced SDS–PAGE (4–20%) analysis was performed using Novex precast gels and buffers from Invitrogen (Paisly, UK). Prior to analysis samples were boiled for 2 min to achieve denaturation and concomitant iron release [20]. The electrophoresis conditions were as recommended by the manufacturer.

Results

Analytical Mono S analysis of human milk-purified LF

Human lactoferrin is highly cationic which allows binding of this protein to strong cation-exchange media such as S Sepharose even in the presence of 0.4 M NaCl at pH 7.5. At this ionic strength and pH, other human milk proteins and LPS do not bind to hLF or the cation-exchange matrix. Reduced SDS–PAGE analysis of S Sepharose human milk-purified LF (milk-purified hLF) revealed a homogenous preparation with an identical migration pattern as the preparation obtained from Sigma (Fig. 1, lanes 1 and 2). Analytical cation-exchange chromatography of milk-purified hLF on Mono S showed that 99% of the protein eluted at 0.68 M NaCl (Fig. 2) and that the elution profile obtained at 214 nm was similar to that at 280 nm. N-terminal protein sequencing of this preparation revealed a single sequence with an intact N-terminus. The hLF recovery from

[2] P.H. Nibbering, R. de Winter, L.A. van Berkel, E. Ravensbergen, M.M. Welling, J.T. van Dissel, J.H. Nuijens, and P.H.C. van Berkel (2001) Human lactoferrin: Polymorphisms and antibacterial activity, ICAAC, Chicago, September 22–25, 2001.

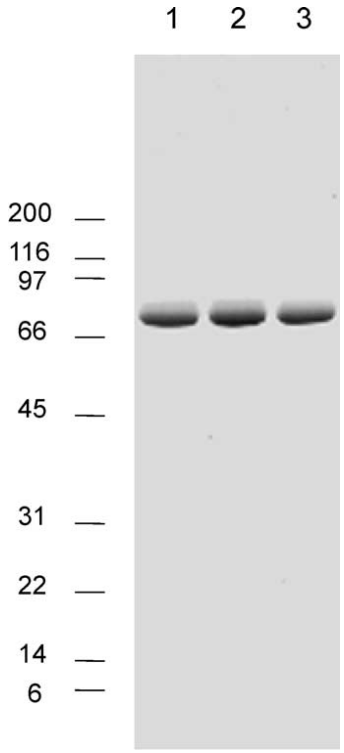

Fig. 1. SDS–PAGE analysis of purified hLF preparations. Commercially obtained hLF from Sigma (lane 1), S Sepharose-purified hLF from fresh human milk (lane 2), and hLF-fl (lane 3) were subjected to reduced SDS–PAGE (4–20%) analysis. Proteins, 2.5 μg/lane, were visualized with Coomassie brilliant blue. The migration of the standard protein markers is indicated on the left ($10^{-3} \times M_r$).

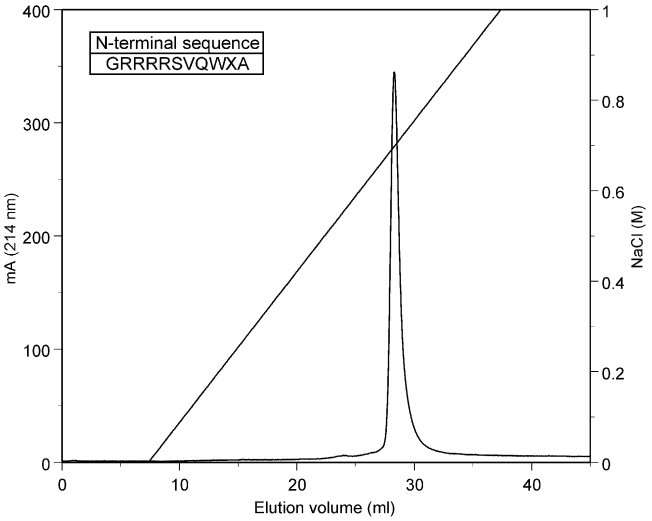

Fig. 2. Mono S chromatography and N-terminal protein sequencing of milk-purified hLF. One hundred micrograms of S Sepharose milk-purified hLF was applied to Mono S as described. The left and right abscissas give the absorption at 214 nm and NaCl concentration (M), respectively. The N-terminal protein sequencing result was obtained by the automatic Edman degradation procedure using an Applied Biosystems gas-phase sequencer, Model 473A. Sequencing results are presented by the standard one-letter code for amino acids.

Mono S was 101 ± 2% on five separate experiments with 500 μg. Follow-up experiments with varying hLF quantities revealed linearity in recovery between 2.5 and 750 μg. Mono S analysis of fully iron-saturated hLF showed no significant change in the elution profile when compared with milk-purified hLF, indicating that iron-saturation does not affect the binding to and elution of hLF from Mono S (Table 1). This result is in line with earlier observations of milk-purified hLF and iron-saturated hLF binding equally well to anionic ligands [13], but conflicts with the results of Makino and Nishimura who reported different profiles of apo- and iron-saturated hLF on Mono S [28]. Absorption measurement at 465 nm, which allows specific detection of the iron-saturated conformation of lactoferrin [29], revealed that release of iron from hLF did not occur during Mono S analysis (H.A. van Veen, unpublished data). Analysis of desialylated and fully deglycosylated hLF showed no significant differences in retention on Mono S, indicating that the N-linked glycans of hLF are not involved in the binding to and elution from Mono S (Table 1, [20]).

LF species-specific elution profiles on Mono S

Analysis of the binding and elution from Mono S of molecules closely related in size and structure to hLF revealed that human transferrin did not bind to the column, whereas heterologous LF species do, but elute at species-specific positions (Table 1). Bovine LF (bLF) eluted as a major peak at 0.80 M NaCl with a small shoulder at 0.76 M NaCl. Analysis of the latter, minor bLF variant revealed that it contains an N-linked glycan at Asn^{281}, whereas the major bLF variant eluting at 0.80 M NaCl is not glycosylated at this position.[3] Hence, in contrast to hLF, N-linked glycosylation of bLF affects its elution from Mono S. The last peaks eluting from human and bovine whey (at 0.26 and 0.27 M NaCl) before the LF peaks represent human lysozyme and bovine lactoperoxidase, respectively.

Analysis of N-terminal integrity and purity of purified hLF preparations

Previously, we reported that consecutive removal of arginines from the first basic cluster of hLF, i.e., Arg^2–Arg^3–Arg^4–Arg^5, by limited proteolysis or site-directed mutagenesis affects the elution from Mono S [13,16,20]. Human LF in commercially purchased preparations may be limitedly proteolyzed. For example, Fig. 3 shows the Mono S analysis of the hLF preparation from Sigma, which appeared homogenous

[3] H.A. van Veen, M.E.J. Geerts, J.P.J. Brakenhoff, P.H.C. van Berkel, and J.H. Nuijens (1997) *N*-glycosylation at Asn^{281} in bovine lactoferrin protects the molecule against tryptic proteolysis. Third International Conference on Lactoferrin, Le Touquet, France, May 5–9, 1997.

Table 1
Species-specific elution of LFs and other (milk) proteins from Mono S

Protein[a]	N-terminal sequence	Elution[b] (M NaCl)
Milk-purified human LF	GRRRRSVQWXA	0.68
Iron-saturated human LF	—	0.67
Desialylated-human LF[c]	—	0.68
Human LF-Arg^{29}	—	0.69
Human LF-Lys^{29}	—	0.68
Bovine LF, major variant	APRKNVRWXT	0.80
Bovine LF, minor variant	APRKNVRWXT	0.76
Murine LF	KATTVRWXAV	0.26
Murine LF, polymorphic variant	KATTVQWXAV	0.22
Porcine LF	—	0.54
Human transferrin	—	No binding
Human lysozyme	—	0.26
Bovine lactoperoxidase	—	0.27

[a] Purified proteins and whey from different species were subjected to analytical Mono S chromatography as described under Materials and methods.
[b] The molarity of NaCl required to elute each protein.
[c] Desialylated-human LF was obtained by incubation of 1 mU neuraminidase/μg hLF for 19 h at 37 °C in 50 mM sodium acetate, pH 5.5, 1 mM calcium chloride.

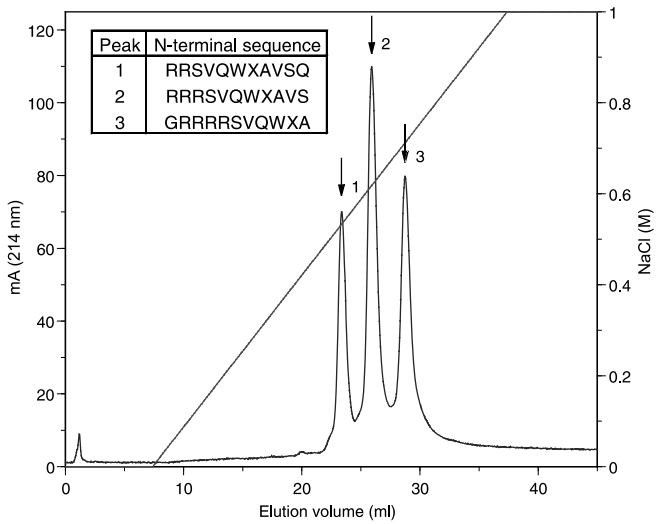

Fig. 3. Mono S chromatography and N-terminal protein sequencing of commercially obtained hLF. One hundred micrograms of Sigma hLF was subjected to Mono S analysis. Peak fractions (1–3) were applied to N-terminal protein sequencing.

on SDS–PAGE (Fig. 1, lane 1). It, nonetheless separated as three distinct peaks at 0.52, 0.61, and 0.69 M NaCl from Mono S. N-terminal protein sequencing revealed the subsequent peaks to represent hLF from which three or two N-terminal amino acids were lacking, and N-terminally intact hLF (Fig. 3). N-terminally intact hLF (peak 3) of the Sigma preparation eluted at the same position as hLF isolated from fresh human milk (Fig. 2). Analysis of six distinct hLF preparations from various commercial suppliers showed that each preparation contained N-terminally degraded hLF. The quantities of peak 1 and peak 2 material ranged from 1 to 19% and from 4 to 39%, respectively, whereas that of intact hLF ranged from 44 to 95% of total hLF. In one preparation the quantity of intact hLF, decreased in favor of peak 2 (from 39 to 49%) and peak 1 (from 18 to 30%) after repeated freezing and thawing. In addition, Mono S analysis of one commercially obtained preparation, revealed a small amount of human lysozyme (see also Table 1). These results demonstrate the analytical power of analytical Mono S chromatography to assess the quality and purity of hLF preparations.

Analysis of hLF in human whey

The N-terminal integrity of hLF in the whey of 26 individual milk donors was determined by Mono S analysis. The whey samples were obtained after addition of 0.4 M NaCl to the milk to disrupt electrostatic interactions of hLF with casein micelles, and their removal by centrifugation. Fig. 4A shows the Mono S elution profile of whey from donor D with peaks eluting at 0.54 and 0.67 M NaCl. SDS–PAGE analysis of the peak at 0.54 M NaCl revealed an identical migration pattern as hLF from Sigma and milk-purified hLF (Fig. 1, lanes 1–3). N-terminal protein sequencing showed that the protein eluting at 0.54 M NaCl, designated hLF-fl, represented N-terminally intact hLF. Unexpectedly, when hLF-fl was subjected to rechromatography on Mono S, 65% of total hLF eluted at 0.69 M NaCl (Fig. 4B). As yet we do not understand the nature of this phenomenon. Control experiments with S Sepharose-purified hLF never did yield a hLF-fl peak on Mono S. We therefore speculate that a small whey component interacts with hLF to cause a decrease in the positive charge of the N-terminus. Apparently, this interaction is lost on rechromatography. Mono S analysis of whey samples from the panel of individual milk donors revealed the presence of hLF-fl in the whey of each donor at levels ranging from 4 to 31% of total hLF. In none of the

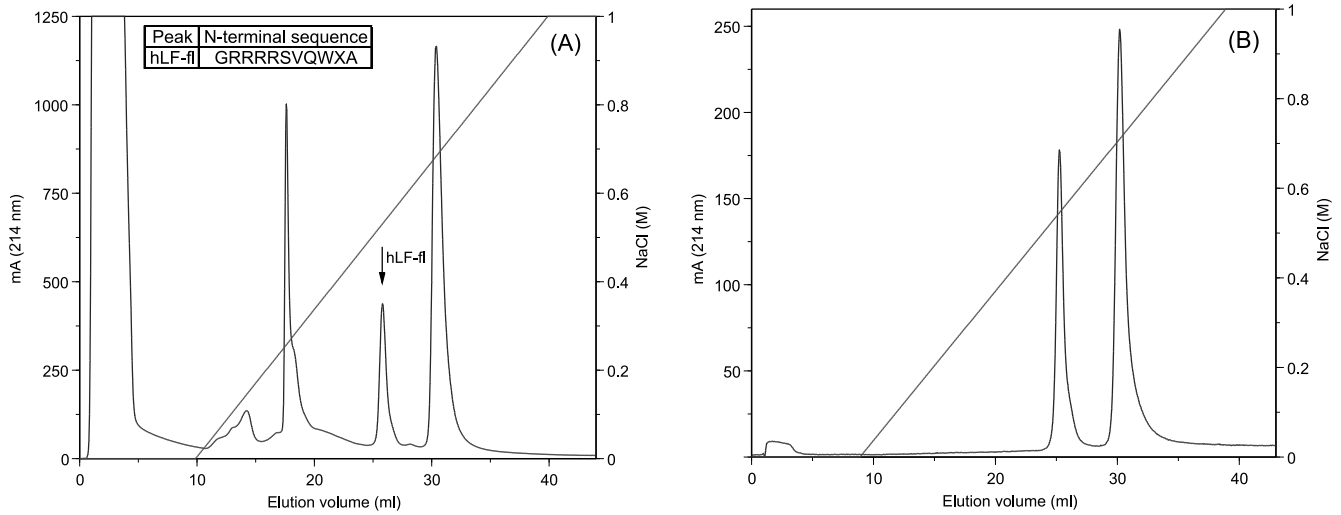

Fig. 4. Mono S analysis of human whey from donor D. Sodium chloride was added to milk from donor D to a final concentration of 0.4 M. The milk was subsequently centrifuged to separate whey from caseins and fat. Five-hundred microliters of whey was 5-fold diluted in buffer A and applied to Mono S. Nonbound proteins were removed by washing the column with 5 ml of buffer A and bound proteins were eluted with the linear salt gradient (A). The insert of panel A shows the result of N-terminal sequence analysis of hLF-fl. Panel B shows the rechromatography on Mono S of hLF-fl fractions that had been 2-fold diluted in buffer A and incubated for 7 h at 37 °C.

wheys, N-terminal degradation to an extent exceeding of 1% of total hLF was observed.

Analysis of polymorphic hLF variants

Whey samples from 24 out of the 26 donors showed elution profiles very similar to that in Fig. 4A. Two whey samples displayed a different profile on Mono S (Fig. 5, donor V), with additional peaks eluting at 0.46 and 0.60 M NaCl, designated hLF-p. N-terminal sequencing of these peaks revealed an intact N-terminus with only three arginines, i.e., Gly^1–Arg^2–Arg^3–Arg^4–Ser^5–. The peak eluting at 0.46 M NaCl apparently represented the hLF-fl variant of the peak eluting at 0.60 M NaCl. The peak eluting at 0.67 M NaCl had the expected four N-terminal arginines. Thus these two donors express two polymorphic hLF variants, one with three and one with four N-terminal arginines. Peak integration revealed that the surface area of both peaks was similar, indicating that the two allelic variants of hLF were expressed at equal ratios.

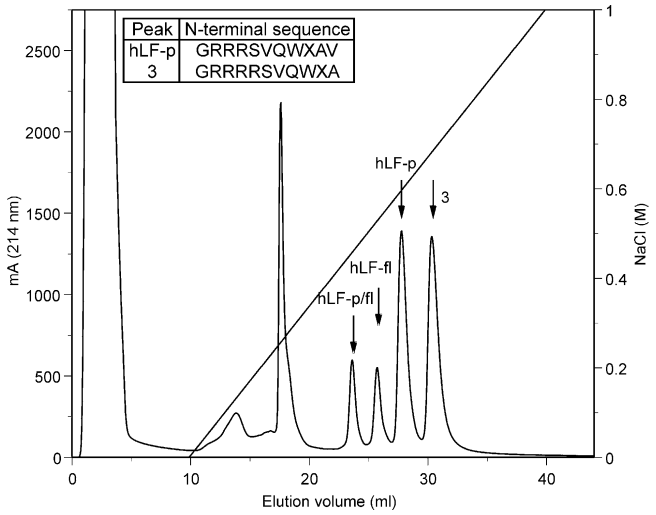

Fig. 5. Mono S analysis of human whey from donor V. Whey from donor V was subjected to Mono S analysis as described under Fig. 4. The insert shows the result of the N-terminal protein sequence analysis of hLF-p and peak 3 fractions.

Mono S analysis of hLF variants polymorfic at position 11 (alanine or threonine), position 29 (arginine or lysine; Table 1) or position 561 (aspartic acid or glutamic acid) did not reveal significant differences in the retention on Mono S. Hence, Mono S chromatography can only be used to detect polymorphism in the first basic cluster.

Reproducibility of hLF elution with different Mono S columns

Analysis of the ionic strength required to elute intact hLF from four different Mono S columns yielded highly reproducible results: the mean elution position of intact hLF from the various columns ranged between 0.67 and 0.70 M NaCl, with coefficients of variation ranging between 0.6 and 1.4% (at least 10 runs per column were performed over a period of at least 6 months).

We observed, however, that columns can retain small amounts of hLF that might elute in subsequent runs and that residual fat and casein micelles in whey preparations occasionally caused an increment in back pressure. Therefore, a protocol consisting of 25 ml of 1 M sodium hydroxide followed by 25 ml of 1% Tween 80 in 1 M acetic acid at a reversed flow rate of 0.5 ml/min was used on a regular basis to clean the column.

Discussion

This paper demonstrates that analytical cation-exchange chromatography on Mono S is a useful and reliable tool to study the identity, purity, and N-terminal integrity of hLF. Mono S fractionates hLF from other human (milk) proteins, between lactoferrins from distinct species, as well as between proteolytic, polymorphic, and milk-purified hLF variants. Mono S chromatography of hLF purified with S Sepharose from human milk to which 0.4 M NaCl had been added revealed a purity level exceeding 99% (Fig. 2). Similar purity levels were obtained on Mono S chromatography of recombinant hLF purified from the milk of transgenic cows. In addition, this analysis allows the quantification of bLF contaminating recombinant hLF, due to the distinct differences in species-specific elution profiles on this column (Table 1). We recommend the extraction of hLF from milk at high ionic strength as this procedure prevents the contamination of preparations with antibiotic proteins human lysozyme and bovine lactoperoxidase (Table 1) as well as with LPS. The high ionic strength disrupts the interaction of LF with these molecules and prevents their binding to the cation-exchange media. Contamination of (commercially obtained) LF preparations with these compounds may have blurred the interpretation of antibiotic and anti-inflammatory properties of lactoferrin in the literature. The N-terminus of hLF appeared to be highly susceptible to limited tryptic proteolysis resulting in the subsequent removal of two or three N-terminal amino acid residues [16]. We have shown previously that limited proteolysis of the N-terminus of hLF impacts on the antibiotic and anti-inflammatory activities of the protein [30]. It is shown in Fig. 3 that Mono S chromatography can be used to assess the extent of N-terminal proteolysis in hLF preparations. Limitedly proteolyzed hLF from which two or three N-terminal residues were lacking appeared to elute from Mono S at 0.52 and 0.61 M NaCl, respectively, whereas intact hLF eluted at 0.69 M NaCl. N-terminal proteolysis was present to a variable extent in all purchased hLF preparations, although most of the preparations appeared homogeneous and migrated as a doublet of protein bands of M_r 77,000 on non-reduced and reduced SDS–PAGE (Fig. 1, lane 1). One lot of hLF from commercial origin likely still contained the protease responsible for the degradation since the amount of peak 3, eluting at 0.69 M NaCl, decreased in time in favor of peak 2 and peak 1. We speculate that this protease is plasmin because of its presence in milk and its ability to bind to cation-exchange media [31]. Given the importance of the first basic cluster in hLF function [30] we advocate that the N-terminal integrity of hLF preparations is routinely assessed before and during in vitro or in vivo studies.

The Mono S profile of fresh whey samples of 24 individual human milk donors displayed a major peak representing N-terminally intact hLF eluting at 0.67 M NaCl, and a variable amount of a minor peak, designated hLF-fl, eluting at 0.54 M NaCl (Fig. 4A). N-terminal protein sequencing revealed both these peaks to represent N-terminally intact hLF. In none of the whey samples, limited proteolysis of hLF was detected by chromatographic and protein sequence analysis. Rerunning on Mono S of hLF-fl resulted in a minor hLF-fl peak and a major peak eluting at 0.69 M NaCl. Preliminary experiments have indicated that the ratio of hLF-fl versus intact hLF in whey and upon rechromatography is determined by the ionic strength and/or composition of the buffer, the temperature, and the incubation time. Thus, hLF-fl appears to represent a labile variant of intact hLF in whey, which on fractionation and rerunning on Mono S transforms into the more cationic major variant of intact hLF (Fig. 4B). In contrast, we have never observed the appearance of hLF-fl on rerunning of the more cationic variant of intact hLF. The presence of hLF-fl in human whey may relate to a specific conformation of the hLF N-terminus or to a reversible electrostatic and/or hydrophobic interaction with a small molecule present in the whey slightly decreasing the (exposed) positive charge. Phosphorylation at Ser^5 in hLF-fl may offer an alternative explanation because the sequence $–Arg^2–Arg^3–Arg^4–Ser^5–Val^6–$ is recognized by a cA(G)MP-dependent protein kinase [32]. The mechanism of putative phosphate release upon rechromatography of hLF remains unclear. Large-scale purification of hLF using S Sepharose at high ionic strength yields N-terminally intact hLF preparations containing less than 1% of hLF-fl.

During the screening of human whey samples on Mono S, we found that 2 out of 26 individual donors appeared hetrozygotes with one allele expressing N-terminal intact hLF with three N-terminal arginines next to the common hLF variant with four arginines (Fig. 5). The rare frequency of the polymorphic variant with three arginines in the Dutch population likely accounts for not having observed any individual being homozygous for this variant. The hLF polymorphism with three N-terminal arginines has been reported before [3,26] but it was debated whether it represented a true hLF polymorphism or an artifact related to cDNA production or sequencing error. The Mono S analysis and protein sequencing result prove that this polymorphic variant indeed exists. Obviously, Mono S chromatography can be used to further assess the occurrence and functional consequences of this hLF polymorphism. In contrast Mono S analysis cannot be used to discriminate between hLF polymorphic variants with either a lysine or an arginine at position 29 in the second basic cluster (Table 1).

Taken together, we show that Mono S analysis is a reliable tool to determine the purity, identity, N-terminal integrity, and presence of intact hLF variants in purified

hLF preparations. Hence, this robust method will be very useful in structure–function studies of hLF as well as a method to control and assure the quality of batches of recombinant hLF for applications in human health care.

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