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Production and characterization of recombinant human lactoferrin

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Chapter 8

Characterization of Bovine Neutrophil Gelatinase-Associated Lipocalin

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ABSTRACT

A protein of relative molecular mass of approximately 25,000 was purified from bovine colostrum by cation-exchange and size-exclusion chromatography. The N-terminus of the protein matched the sequence predicted by the National Center for Biotechnology Information for the bovine homolog of human neutrophil gelatinase-associated lipocalin, a glycoprotein of relative molecular mass 25,000 belonging to the family of lipocalins. The protein was further designated as bovine neutrophil gelatinase-associated lipocalin (bNGAL). Sodium dodecyl sulfate-PAGE of enzymically deglycosylated bNGAL indicated that the intact protein bears one N-linked glycan. Monosaccharide and mass spectrometric analyses of released N-linked carbohydrates revealed the presences of complex- and hybrid-type glycans, with galactose substituted with *N*-acetylgalactosamine. This substitution is typical for glycoproteins expressed in the bovine mammary gland. A specific ELISA revealed bNGAL concentrations in plasma and mature milk of about 0.05 and 1 $\mu\text{g/mL}$, respectively, whereas values as high as 51 $\mu\text{g/mL}$ were measured in colostrum. Thus, we have isolated and characterized a novel bovine (milk) protein that is a new member of the lipocalin family.

Key words: neutrophil gelatinase-associated lipocalin, lactoferrin, colostrum

INTRODUCTION

The lipocalins are a functionally diverse family of small extracellular proteins that belong to the superfamily of calycins (Flower, 1996). Despite their overall limited sequence homology, the lipocalins share a characteristic calyx- or cup-shaped structure that can bind to a variety of small hydrophobic ligands and macromolecules such as specific receptors (Flower, 1996). Members of the kernel subfamily of lipocalins include the species homologous proteins human neutrophil gela-

tinase-associated lipocalin (hNGAL, also named human neutrophil lipocalin), mouse 24p3/uterocalin (24p3), and rat α_2 -microglobulin-related protein (A2UMRP; Kjeldsen et al., 2000). These lipocalins are found in the specific granules of neutrophils and are secreted by the liver, kidney, bronchial, and gastrointestinal mucosa and by the mammary gland and uterus, most notably during involution (Kjeldsen et al., 2000).

A variety of functions have been attributed to hNGAL, 24p3, and A2UMRP. These include involvement in the transport of iron (Yang et al., 2002), fatty acids, and retinol (Chu et al., 1998); host defense against bacterial infection by sequestration of siderophore-bound iron (Goetz et al., 2002); and the induction of neutrophil apoptosis (Devireddy et al., 2001).

The AA sequence of hNGAL, a single-chain glycoprotein of relative molecular mass (M_r) 25,000, shows 62 and 63.5% identity with 24p3 and A2UMRP, respectively (Kjeldsen et al., 2000). X-ray crystallography (Goetz et al., 2000) and nuclear magnetic resonance spectroscopy of hNGAL (Coles et al., 1999) revealed the typical 8-stranded antiparallel β -barrel protein structure. However, the calyx appears to be unusually large and lined with positively charged AA.

Here we describe the purification and characterization of the bovine homolog of hNGAL, further referred to as bovine neutrophil gelatinase-associated lipocalin (bNGAL).

MATERIALS AND METHODS

Reagents

N-Glycosidase F and neuraminidase were obtained from Roche (Mannheim, Germany). Bovine lactoferrin (bLF) from mature milk was obtained from Sigma Chemical Co. (St. Louis, MO) or DMV (Veghel, The Netherlands) or was purified as described previously (van Veen et al., 2004). Bovine lactoferrin free of bNGAL was obtained by size-exclusion chromatography of purified bLF on a Superose 12 HR 10/30 column (Amersham Biosciences, Uppsala, Sweden). RapiGest was from Waters (Milford, MA), and the gel-filtration protein markers were from BioRad (Hercules, CA). Polyclonal rabbit anti-bNGAL antiserum was obtained after repeated intramuscular injection with purified

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bNGAL (see ensuing discussion) in Freund's adjuvant; specific antibody was purified by affinity purification on bNGAL-Sepharose and conjugated with horseradish peroxidase as described previously for human lactoferrin antibodies (van Berkel et al., 1995). Bovine leukocytes were isolated from EDTA-anticoagulated blood by successive centrifugations to remove plasma and red blood cell supernatants after lysis with ammonium chloride. The leukocyte pellet was suspended in 1% Triton X-100 and 10 mM EDTA and was frozen at -70°C until use.

Purification of bNGAL from Bovine Colostrum

Bovine NGAL and bLF were simultaneously extracted from colostrum of Friesian Holstein cows using S Sepharose (Amersham Biosciences). Bovine colostrum (3 L) to which sodium phosphate (20 mM final concentration) and NaCl (0.4 M) had been added was defatted by centrifugation ($1,600 \times g$, 15 min, 4°C) and subsequently incubated batchwise with S Sepharose (0.5 L). After incubation, the S Sepharose was washed with 20 mM sodium phosphate (pH 7.5) and 0.4 M NaCl and packed in a column (5×30 cm), and bound proteins were eluted with 20 mM sodium phosphate (pH 7.5) and 1 M NaCl at a flow rate of 10 mL/min. To separate bLF from bNGAL, the S Sepharose eluate was filtered over a Sephacryl S-200 HR column (2.6×70 cm; Amersham Biosciences) in 10 mM sodium phosphate (pH 7.4) and 1 M NaCl at a flow rate of 1.0 mL/min. The Sephacryl elution fractions containing bNGAL were diluted in 20 mM sodium phosphate (pH 7.5) and subjected to Mono S HR 5/5 chromatography (van Veen et al., 2002) to obtain a homogeneous bNGAL preparation. The recovery of bNGAL by using this procedure was about 40%.

Analytical Mono S Chromatography

Analytical cation-exchange chromatography using Mono S was performed as described previously (van Veen et al., 2002). Briefly, bovine colostrum to which NaCl was added (0.4 M final concentration) was centrifuged ($23,000 \times g$, 10°C , 150 min) to separate fat and casein fractions from the whey. The whey was filtered through a 0.22- μm filter and 500 μL was applied to a Mono S HR 5/5 column (Amersham Biosciences) equilibrated in 20 mM sodium phosphate (pH 7.5; buffer A) containing 0.4 M NaCl. The column was subsequently washed, and bound proteins were eluted with a linear salt gradient from 0.4 to 1 M NaCl in 18 mL of buffer A at a flow rate of 1.0 mL/min. Purified bNGAL (10 μg) was applied to the Mono S column in buffer A and eluted with a linear salt gradient from 0 to 1 M NaCl in 30 mL of buffer A at 1.0 mL/min.

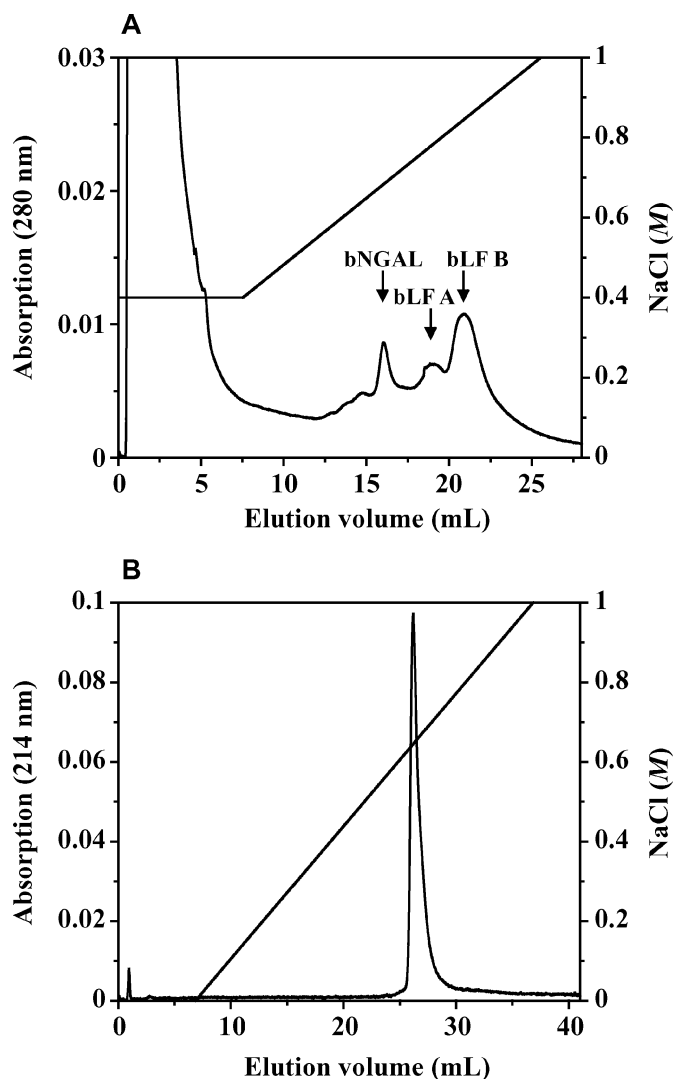


Figure 1. Analytical Mono S analysis of bovine whey and purified bovine neutrophil gelatinase-associated lipocalin (bNGAL). Whey obtained from bovine colostrum (panel A) or purified bNGAL (panel B) was analyzed on a Mono S column (Amersham Biosciences, Uppsala, Sweden). Left and right abscissas indicate absorption [at 280 nm (panel A) or 214 nm (panel B)] and NaCl concentration (M), respectively. Bovine lactoferrin (bLF) A and bLF B (panel A) represent the bLF A and B glycosylation variants (van Veen et al., 2004).

Mass Spectrometric and Monosaccharide Analysis of the bNGAL Glycans

Bovine neutrophil gelatinase-associated lipocalin (0.6 mg/mL) was incubated with 50 U/mL of N-glycosidase F and 0.5 U/mL neuraminidase in 20 mM sodium phosphate (pH 7.0) and 0.1% (wt/vol) RapiGest for 20 h at 37°C . Released desialylated N-glycans were analyzed by matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry in the reflector mode on an Applied Biosystems Voyager DE Pro mass spectrometer (Applied Biosystems, Foster City, CA).

The matrix was 2,5-dihydroxybenzoic acid (10 mg/mL) in 50:50:0.1% acetonitrile–water–trifluoroacetic acid. Spectra were obtained in the positive ion mode and glycans were detected as sodium adducts, $[M + Na]^+$. The monosaccharide analysis was performed as described previously (van Berkel et al., 2002).

Quantitative ELISA for bNGAL

Phosphate-buffered saline containing 1 μ g/mL of affinity-purified rabbit anti-bNGAL was incubated for 16 h at 20°C in microtiter plates (Polysorp; Nunc, Roskilde, Denmark). Plates were then washed with PBS and 0.02% (vol/vol) Tween-20 and incubated with serial dilutions in PTG buffer [PBS, 0.2% (wt/vol) porcine gelatin, and 0.02% (vol/vol) Tween-20] of purified bNGAL standard and test samples, which were pretreated by adding NaCl to 0.4 M. After 2 h, the plates were washed and PTG buffer containing 1% (vol/vol) rabbit serum and 0.3 μ g/mL of peroxidase-conjugated rabbit anti-bNGAL was added to each well. Plates were subsequently incubated for 1 h, washed, and tetramethylbenzidine substrate solution (Pierce, Rockford, IL) was added. Substrate conversion was stopped by the addition of 2 M H_2SO_4 , and the absorption at 450 nm was measured using a Power Wave microtiter plate reader (BioTek Instruments, Winooski, VT). All incubations were performed with 100- μ L volumes.

RESULTS

Purification of bNGAL from Bovine Colostrum

Analytical Mono S chromatography of bovine colostrum whey revealed a protein peak eluting at 0.67 M NaCl (Figure 1A, bNGAL), that is, before the lactoferrin glycosylation variants bLF A and bLF B (van Veen et al., 2004). On a preparative scale, the protein was purified using cation-exchange and size-exclusion chromatography as described in the Materials and Methods section. Analytical Mono S chromatography of the purified protein confirmed it to elute at 0.67 M NaCl and to be free of bLF (Figure 1B). Analytical size-exclusion chromatography of the purified protein on Superose 12 revealed a single peak eluting at 13.1 mL, which corresponds to a M_r of 25,000 by reference to the elution pattern of the protein standards (Figure 2).

N-Terminal Protein Sequencing of Purified bNGAL

N-Terminal protein sequencing revealed a single N-terminal sequence (Figure 3, bNGAL) indicating that the preparation was pure and devoid of proteolysis. The N-terminal sequence was determined for 41 AA. The observed N-terminus completely matched the sequence

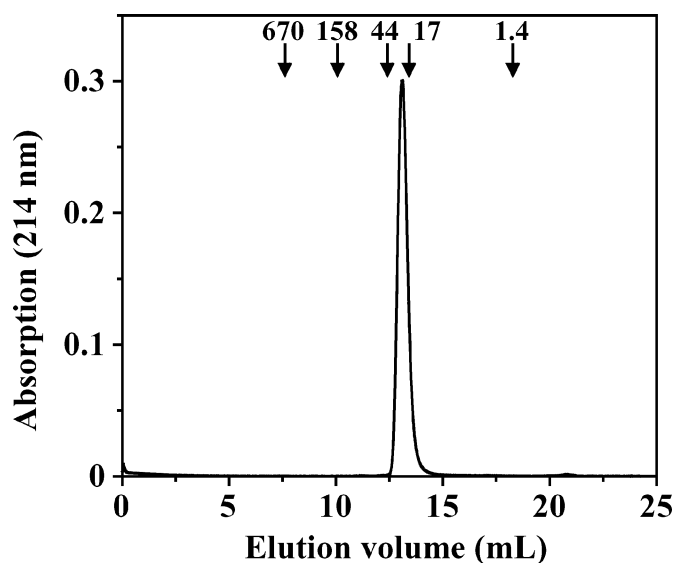


Figure 2. Superose 12 analysis of purified bovine neutrophil gelatinase-associated lipocalin (bNGAL). Bovine neutrophil gelatinase-associated lipocalin (10 μ g) was filtered over a Superose 12 HR 10/30 column (Amersham Biosciences, Uppsala, Sweden) in 20 mM sodium phosphate (pH 7.5) containing 0.75 M NaCl at a flow rate of 0.25 mL/min. The left abscissa indicates the absorption at 214 nm. The arrows indicate the elution of the protein standards ($10^{-3} \times M_r$).

predicted by the National Center for Biotechnology Information (annotation process of the bovine genome, XP_605012) for the bovine homolog of hNGAL. Identities of the observed N-termini with hNGAL, 24p3, and A2UMRP were 68, 54, and 49%, respectively. Taken together, the results indicated that the purified whey protein was a lipocalin and the bovine homolog of hNGAL, 24p3, and A2UMRP. The protein was further referred to as bNGAL.

SDS-PAGE Analysis of bNGAL

Nonreduced SDS-PAGE of purified bNGAL revealed 2 protein bands of M_r 43,000 and 25,000 (Figure 4, lane 1) that, upon reduction, shifted to M_r 45,000 and 26,000, respectively (Figure 4, lane 5). The M_r of 25,000 corresponded to the M_r of bNGAL found by size-exclusion chromatography, but a peak corresponding to a molecular mass of 43,000 was not observed on Superose 12 (Figure 2). Moreover, SDS-PAGE analysis of peak fractions taken from the Superose 12 chromatographic analysis revealed no separation between the 2 proteins (results not shown), indicating that the protein of M_r 43,000 likely represented a dimer formed upon denaturation of the bNGAL in SDS.

Sodium dodecyl sulfate-PAGE analysis of bNGAL treated with N-glycosidase F revealed protein bands of M_r 38,000 and 23,000 (Figure 4, lane 2), indicating that

Sequence	AA														
	1	5				10					15				
bNGAL	R	S	S	S	S	R	L	L	R	A	P	P	L	S	R
NCBI: XP_605012	R	S	S	S	S	R	L	L	R	A	P	P	L	S	R
hNGAL	Q	D	S	T	S	D	L	I	P	A	P	P	L	S	K
24p3	Q	D	S	T	Q	N	L	I	P	A	P	S	L	L	T
A2UMRP	Q	D	S	T	Q	N	L	I	P	A	P	P	L	I	S
	16	20				25					30				
bNGAL	I	P	L	Q	P	N	F	Q	A	D	Q	F	Q	G	K
NCBI: XP_605012	I	P	L	Q	P	N	F	Q	A	D	Q	F	Q	G	K
hNGAL	V	P	L	Q	Q	N	F	Q	D	N	Q	F	Q	G	K
24p3	V	P	L	Q	P	D	F	R	S	D	Q	F	R	G	R
A2UMRP	V	P	L	Q	P	G	F	W	T	E	R	F	Q	G	R
	31	35				40									
bNGAL	W	Y	T	V	G	V	A	G	N	A	I				
NCBI: XP_605012	W	Y	T	V	G	V	A	G	N	A	I				
hNGAL	W	Y	V	V	G	L	A	G	N	A	I				
24p3	W	Y	V	V	G	L	A	G	N	A	V				
A2UMRP	W	F	V	V	G	L	A	A	N	A	V				

Figure 3. N-Terminal protein sequencing of bovine neutrophil gelatinase-associated lipocalin (bNGAL). N-Terminal protein sequencing of bNGAL (20 μ g) was performed by the automatic Edman degradation procedure using an Applied Biosystems gas-phase sequencer, model 473A (Applied Biosystems, Foster City, CA). The sequencing result of bNGAL, presented by the standard one-letter code for AA, is aligned to the predicted sequence of bNGAL (National Center for Biotechnology Information: XP_605012) and published sequences of human neutrophil gelatinase-associated lipocalin (hNGAL), mouse 24p3/uterocalin (24p3), and rat α_2 -microglobulin-related protein (A2UMRP; Kjeldsen et al., 2000). The shaded box represents motif 1, a highly conserved sequence among lipocalins (Flower et al., 1991).

bNGAL was N-linked glycosylated. Based on the average M_r of 2,200 for a complex glycan (Spik et al., 1988), the difference in M_r of 2,000 observed on SDS-PAGE before and after deglycosylation suggests that monomeric bNGAL (M_r 25,000) bears one N-linked glycan.

Glycan Structures Present on bNGAL

Monosaccharide analysis of bNGAL showed the presence of mannose, *N*-acetylgalactosamine, *N*-acetylglucosamine, and *N*-acetylneuraminic acid (Table 1). Fucose and galactose were hardly detected, which suggested that, in the event of N-linked glycosylation of bNGAL, galactose was substituted with *N*-acetylgalactosamine. This has previously been observed for other N-linked glycoproteins produced in bovine milk and has been ascribed to the presence of an *N*-acetylgalactosaminyl transferase in mammary gland epithelium (Van den Nieuwenhof et al., 1999). The MALDI-TOF analysis of desialylated bNGAL glycans showed 7 significant peaks (Figure 5, peaks A–G). The proposed glycan compositions and structures, deduced from the mass-to-charge (m/z) values of 6 of these peaks, are shown in

Table 2 and Figure 6, respectively. The observed m/z value of each proposed structure differs maximally by 0.42 Da from the theoretical value of the glycan (Table 2). The majority of the masses could be assigned to complex- or hybrid-type glycans with only *N*-acetylgalactosamine in their antennae (Table 2, Figure 6). Glycan structures B and G, which are minor peaks in the MALDI-TOF spectrum (Figure 5), likely represent fucosylated variants of structures A and E, respectively. Major peaks C and D may, in theory, also represent complex-type glycans bearing galactose in their antennae. However, this is considered less likely given the absence of galactose in the monosaccharide analysis, although it cannot be excluded that minor amounts of glycans bearing galactose are present. Structure F could not be assigned on the basis of available data, but might represent a sulfated variant of glycan E or a hybrid-type glycan D with an additional mannose. High-performance anion-exchange chromatography—pulse amperometric detection analysis (Barroso et al., 2002) of the N-linked glycans of bNGAL before and after desialylation showed that the majority of the glycan structures contained one *N*-acetylneuraminic acid (results not shown).

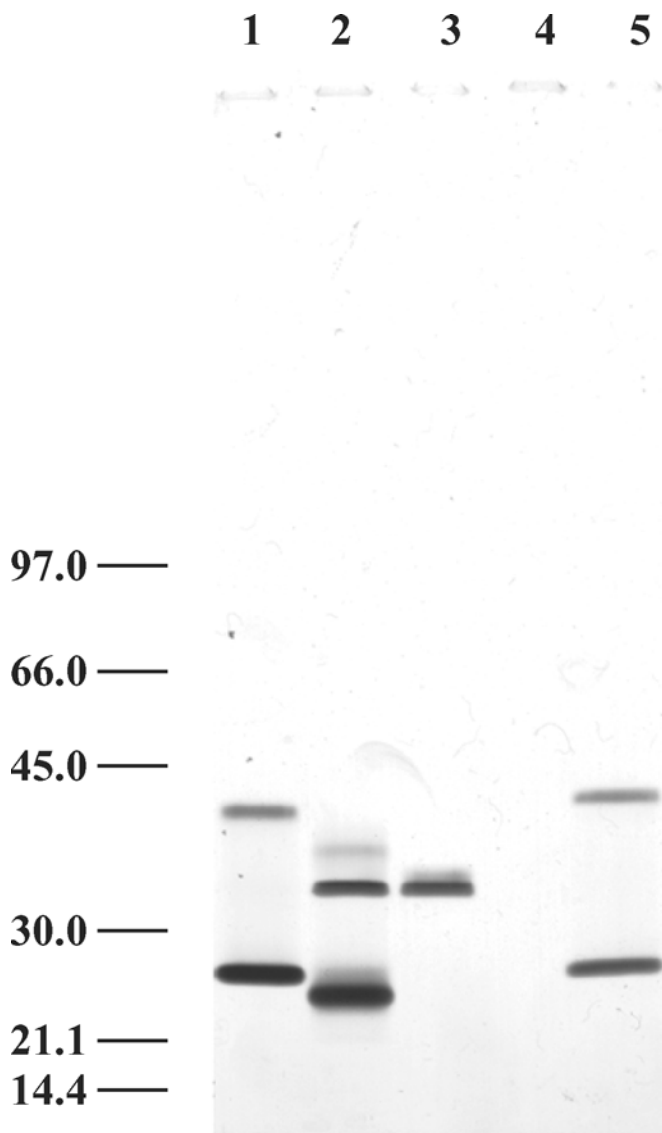


Figure 4. Sodium dodecyl sulfate-PAGE analysis of bovine neutrophil gelatinase-associated lipocalin (bNGAL). Nonreduced (lanes 1–4) and reduced (lane 5) SDS-PAGE (8–16%) analysis of bNGAL was performed as described (van Veen et al., 2002). Lanes 1 and 5: bNGAL (400 ng); lane 2: bNGAL treated with N-glycosidase F; lane 3: N-glycosidase F; lane 4: SDS sample buffer. Proteins were visualized with silver. The migration of the protein markers is indicated on the left ($10^{-3} \times M_r$).

Identification and Concentration of bNGAL in Bovine Samples

A bNGAL-specific ELISA was developed using affinity-purified rabbit anti-bNGAL antibodies. The detection limit of the ELISA was 0.5 ng/mL of bNGAL and the intra and interassay coefficients of variations were 6 and 8%, respectively. Furthermore, the ELISA did not detect bLF (Figure 7) and showed complete recovery

Table 1. Monosaccharide composition of bovine neutrophil gelatinase-associated lipocalin (bNGAL)

Monosaccharide	bNGAL
Fucose	<0.1
Mannose	3.0 ¹
Galactose	<0.1
Glucose	<0.1
N-Acetylgalactosamine	1.0
N-Acetylglucosamine	3.1
N-Acetylneuraminic acid	0.8

¹Results are expressed relative to the amount of mannose, which was arbitrarily set at 3.0.

of bNGAL when added to the biological matrices tested (results not shown).

Parallel curves were obtained for colostrum-purified bNGAL and a lysate from leukocytes (Figure 7), indicating the presence of bNGAL in leukocytes. The concentration of bNGAL in bovine plasma, as determined by reference to a purified bNGAL standard, was approximately 0.05 $\mu\text{g/mL}$. Parallel curves between purified bNGAL and bNGAL in milk were obtained after preincubation of the milk in 0.4 M NaCl. Apparently, the salt was necessary to disrupt electrostatic interactions between bNGAL and the casein micelles, which is similar to previous observations for the detection of human lactoferrin in murine milk (Nuijens et al., 1997). The bNGAL concentration in mature milk was about 1 $\mu\text{g/mL}$, whereas levels as high as 51 $\mu\text{g/mL}$ were measured in colostrum samples. Analysis of various bLF preparations in the ELISA revealed bNGAL levels of 0.7% (wt/wt) in 2 preparations purified as described previously (van Veen et al., 2004). In commercial bLF, bNGAL levels were measured to 2.2% (wt/wt). Taking into account the 3-fold difference in M_r between bNGAL (M_r 25,000) and bLF (M_r 77,000; Nuijens et al., 1996), the bNGAL contamination in bLF preparations can be substantial (i.e., about 7% on a molar ratio).

DISCUSSION

This study details the purification and characterization of the bovine homolog of hNGAL, mouse 24p3, and rat A2UMRP. The homology of the bovine protein, designated bNGAL, was based on N-terminal sequence identity with the predicted sequence and said lipocalins (Figure 3).

Bovine neutrophil gelatinase-associated lipocalin was extracted from colostrum using cation-exchange chromatography at 0.4 M NaCl (Figure 1A), which indicates that bNGAL is strongly positively charged. Further purification to homogeneity (Figures 1B, 2, and 4) involved chromatography on Sephacryl S-200 and Mono S.

Table 2. Proposed N-linked glycan compositions of desialylated bovine neutrophil gelatinase-associated lipocalin (bNGAL)

Peak	Observed ion, ¹ <i>m/z</i>	Proposed glycan composition	Theoretical ion, <i>m/z</i>
A	1,339.79	(Hex) ₃ (HexNAc) ₄ ²	1,339.48
B	1,485.87	(Hex) ₃ (HexNAc) ₄ (Deoxyhex) ₁	1,485.53
C	1,501.87	(Hex) ₄ (HexNAc) ₄	1,501.53
D	1,663.94	(Hex) ₅ (HexNAc) ₄	1,663.58
E	1,745.99	(Hex) ₃ (HexNAc) ₆	1,745.64
F	1,825.99	No assignment possible	—
G	1,892.11	(Hex) ₃ (HexNAc) ₆ (Deoxyhex) ₁	1,891.69

¹Mass-to-charge values ([M + Na]⁺) of desialylated released glycans of bNGAL (Figure 5).

²Hex = hexose; HexNAc = *N*-acetylhexosamine; Deoxyhex = deoxyhexose.

Analytical gel-filtration chromatography of bNGAL revealed an M_r of approximately 25,000 (Figure 2). Sodium dodecyl sulfate-PAGE analysis of untreated and deglycosylated bNGAL confirmed the M_r of 25,000 and showed that bNGAL bore one N-linked glycan (Figure 4). These results are highly similar to the M_r and N-linked glycosylation reported for hNGAL (Kjeldsen et al., 1993).

On SDS-PAGE, a minor portion of purified bNGAL migrated, with an M_r of 43,000. This band did not disappear upon reduction, which excludes the possibility that dimers were formed through disulfide bonding upon heating and denaturation and that bNGAL, like 24p3 and A2UMRP, does not have a free sulfhydryl group

as in hNGAL (Kjeldsen et al., 2000). Sodium dodecyl sulfate-PAGE analysis of Superose 12 fractions showed no separation of the 2 proteins by size-exclusion chromatography. Furthermore, SDS-PAGE experiments have indicated that the putative bNGAL dimer-to-monomer ratio increases with higher ionic strength prior to dilution in SDS sample buffer (result not shown). Taken together, the protein of M_r 43,000 most likely occurs as a laboratory artifact; however, it cannot be excluded that dimerization of bNGAL occurs in vivo, because this is a characteristic trait among lipocalins (Kjeldsen et al., 2000).

A bNGAL-specific ELISA detected the molecule in lysed leukocytes (Figure 7). The plasma concentration of bNGAL, likely released from the specific granules of activated neutrophils, is very similar to that of hNGAL in the plasma of healthy volunteers, that is, approximately 0.05 $\mu\text{g/mL}$ (Xu et al., 1994). Very similar to the changes in bLF concentration during lactation (Schanbacher et al., 1993), the bNGAL concentration in colostrum is much higher than in mature milk. Given the similarity in changes of expression with lactation phases, even higher bNGAL concentrations may be found at involution of the bovine mammary gland, which has been reported for bLF (Schanbacher et al., 1993), 24p3 (Ryon et al., 2002), and A2UMRP (Stoesz and Gould, 1995).

With the ELISA, a significant contamination (as high as 7% on a molar ratio) of bLF preparations with bNGAL could be detected. Although the exact physiological role of bNGAL is unknown, the molecule might have antibacterial activities similar to those ascribed to hNGAL (Goetz et al., 2002). Consequently, contamination of (commercially obtained) bLF preparations with bNGAL may have complicated experiments on the antibiotic properties of bLF. Therefore, we recommend removing all bNGAL from bLF prior to in vitro or in vivo studies. Separation of bLF from bNGAL can be achieved by cation-exchange, gel-filtration, or lectin chromatography on concanavalin A (results not shown).

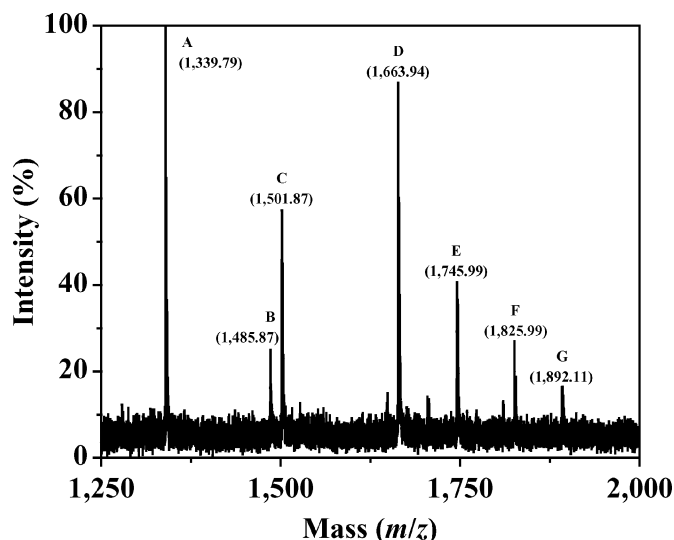


Figure 5. Matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) analysis of bovine neutrophil gelatinase-associated lipocalin (bNGAL) glycans. Bovine neutrophil gelatinase-associated lipocalin released and desialylated N-linked glycans were subjected to MALDI-TOF analysis as described in the Materials and Methods section. The results are provided by the relative intensity (%) as a function of the mass-to-charge ratio (m/z). Values in parentheses indicate the m/z value of the associated peak.

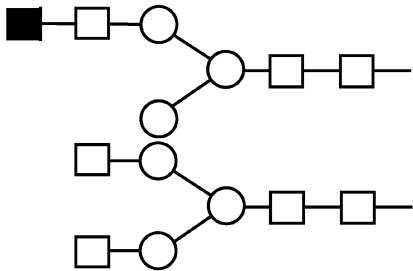
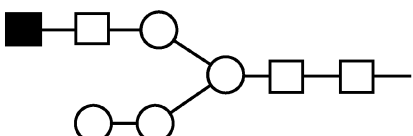
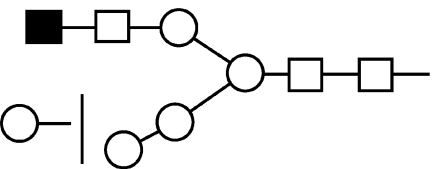
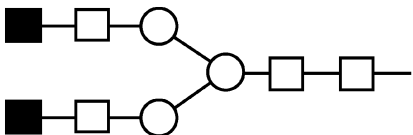
Peak	Proposed glycan structures
A	
B	Fucosylated variants of structures A
C	
D	
E	
F	No assignment possible
G	Fucosylated variants of structure E

Figure 6. Proposed N-linked glycan structures of desialylated bovine neutrophil gelatinase-associated lipocalin (bNGAL). The proposed N-linked glycan structures were deduced from the glycan compositions of the indicated peaks (Table 2). N-Acetylglucosamine: □; mannose: ○; N-acetylgalactosamine: ■.

The separation on concanavalin A underlines the differences in the N-linked glycan structures present on bNGAL and bLF. Bovine neutrophil gelatinase-associated lipocalin harbors mainly complex- and hybrid-type glycans (Table 2, Figure 6), whereas bLF also bears high-mannose type glycans (Coddeville et al., 1992), which have a higher affinity for concanavalin A.

Recently, the expression of mouse 24p3 by the mammary gland epithelium was reported (Ryon et al., 2002).

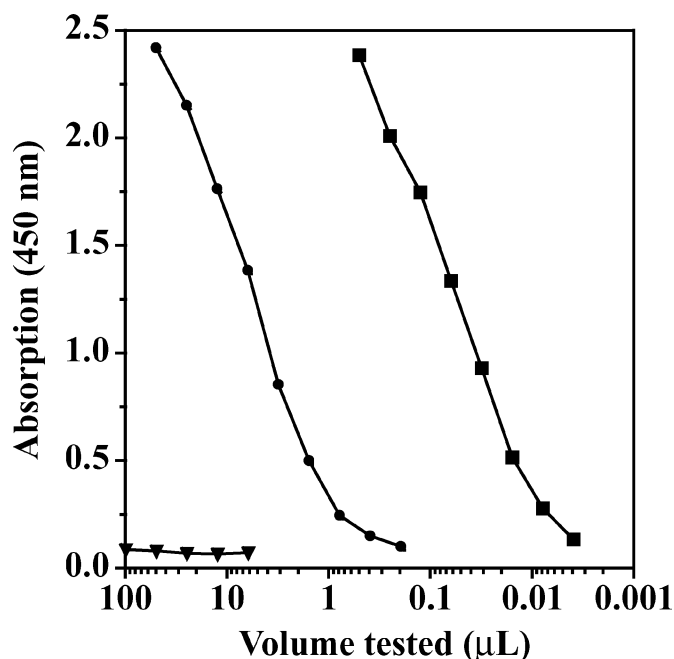


Figure 7. Dose-response curves of purified bovine neutrophil gelatinase-associated lipocalin (bNGAL) and a lysate from leukocytes in the bNGAL ELISA. Serial dilutions of purified bNGAL (●, 50 ng/mL), bovine lactoferrin (▼, 2 μg/mL), and a lysate from bovine leukocytes (■) were incubated with affinity-purified rabbit anti-bNGAL coated on microtiter plates as described in the Materials and Methods section. Bound bNGAL was detected by subsequent incubation with peroxidase-conjugated anti-bNGAL. The A_{450} values, measured as described, are plotted as a function of the experimental volume (microliters) tested.

Bovine neutrophil gelatinase-associated lipocalin might be released in milk from activated neutrophils or mammary gland epithelial cells or both. The analysis of purified colostrum bNGAL suggests that it is produced by the epithelium because substitution of galactoses with N-acetylgalactosamine is a typical feature of glycoproteins produced in the bovine mammary gland (Van den Nieuwenhof et al., 1999). In conclusion, we have isolated, identified, and characterized a novel bovine (milk) protein that is a new member of the lipocalin family and the homolog of hNGAL, 24p3, and A2UMRP.

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REFERENCES

- Barroso, B., R. Dijkstra, M. Geerts, F. Lagerwerf, P. van Veelen, and A. de Ru. 2002. On-line high-performance liquid chromatography/mass spectrometric characterization of native oligosaccharides from glycopeptides. *Rapid Commun. Mass Spectrom.* 16:1320–1329.
- Chu, S. T., H. J. Lin, H. L. Huang, and Y. H. Chen. 1998. The hydrophobic pocket of 24p3 protein from mouse uterine luminal fluid: Fatty acid and retinol binding activity and predicted structural similarity to lipocalins. *J. Pept. Res.* 52:390–397.
- Coddeville, B., G. Strecker, J. M. Wieruszski, J. F. G. Vliegenthart, H. van Halbeek, J. Peter-Katalinic, H. Egge, and G. Spik. 1992. Heterogeneity of bovine lactotransferrin glycans. *Carbohydr. Res.* 236:145–164.
- Coles, M., T. Diercks, B. Muehlenweg, S. Bartsch, V. Zolzer, H. Tschesche, and H. Kessler. 1999. The solution structure and dynamics of human neutrophil gelatinase-associated lipocalin. *J. Mol. Biol.* 289:139–157.
- Devireddy, L. R., J. G. Teodoro, F. A. Richard, and M. R. Green. 2001. Induction of apoptosis by a secreted lipocalin that is transcriptionally regulated by IL-3 deprivation. *Science* 293:829–834.
- Flower, D. R. 1996. The lipocalin protein family: Structure and function. *Biochem. J.* 318:1–14.
- Flower, D. R., C. North, and T. K. Attwood. 1991. Mouse oncogene protein 24p3 is a member of the lipocalin protein family. *Biochem. Biophys. Res. Commun.* 180:69–74.
- Goetz, D. H., M. A. Holmes, N. Borregaard, M. E. Bluhm, K. N. Raymond, and R. K. Strong. 2002. The neutrophil lipocalin NGAL is a bacteriostatic agent that interferes with siderophore-mediated iron acquisition. *Mol. Cell* 10:1033–1043.
- Goetz, D. H., S. T. Willie, R. S. Armen, T. Bratt, N. Borregaard, and R. K. Strong. 2000. Ligand preference inferred from the structure of neutrophil gelatinase associated lipocalin. *Biochemistry* 39:1935–1941.
- Kjeldsen, L., J. B. Cowland, and N. Borregaard. 2000. Human neutrophil gelatinase-associated lipocalin and homologous proteins in rat and mouse. *Biochim. Biophys. Acta* 1482:272–283.
- Kjeldsen, L., A. H. Johnsen, H. Sengelov, and N. Borregaard. 1993. Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. *J. Biol. Chem.* 268:10425–10432.
- Nuijens, J. H., P. H. van Berkel, M. E. Geerts, P. P. Hartevelt, H. A. de Boer, H. A. van Veen, and F. R. Pieper. 1997. Characterization of recombinant human lactoferrin secreted in milk of transgenic mice. *J. Biol. Chem.* 272:8802–8807.
- Nuijens, J. H., P. H. C. van Berkel, and F. L. Schanbacher. 1996. Structure and biological actions of lactoferrin. *J. Mammary Gland Biol. Neoplasia* 1:285–295.
- Ryon, J., L. Bendickson, and M. Nilsen-Hamilton. 2002. High expression in involuting reproductive tissues of uterocalin/24p3, a lipocalin and acute phase protein. *Biochem. J.* 367:271–277.
- Schanbacher, F. L., R. E. Goodman, and R. S. Talhouk. 1993. Bovine mammary lactoferrin: Implications from messenger ribonucleic acid (mRNA) sequence and regulation contrary to other milk proteins. *J. Dairy Sci.* 76:3812–3831.
- Spik, G., B. Coddeville, and J. Montreuil. 1988. Comparative study of the primary structures of sero-, lacto- and ovotransferrin glycans from different species. *Biochimie* 70:1459–1469.
- Stoesz, S. P., and M. N. Gould. 1995. Overexpression of neu-related lipocalin (NRL) in neu-initiated but not ras- or chemically initiated rat mammary carcinomas. *Oncogene* 11:2233–2241.
- van Berkel, P. H. C., M. E. J. Geerts, H. A. van Veen, P. M. Kooiman, F. Pieper, H. A. de Boer, and J. H. Nuijens. 1995. Glycosylated and unglycosylated human lactoferrins can both bind iron and have identical affinities towards human lysozyme and bacterial lipopolysaccharide, but differ in their susceptibility towards tryptic proteolysis. *Biochem. J.* 312:107–114.
- van Berkel, P. H. C., M. M. Welling, M. Geerts, H. A. van Veen, B. Ravensbergen, M. Salaheddine, E. K. J. Pauwels, F. Pieper, J. H. Nuijens, and P. H. Nibbering. 2002. Large scale production of recombinant human lactoferrin in the milk of transgenic cows. *Nat. Biotechnol.* 20:484–487.
- Van den Nieuwenhof, I. M., W. E. Schiphorst, I. Van Die, and D. H. Van den Eijnden. 1999. Bovine mammary gland UDP-GalNAc:GlcNAc- β -R β 1 \rightarrow 4-N- acetylgalactosaminyltransferase is glycoprotein hormone nonspecific and shows interaction with α -lactalbumin. *Glycobiology* 9:115–123.
- van Veen, H. A., M. E. J. Geerts, P. H. C. van Berkel, and J. H. Nuijens. 2002. Analytical cation-exchange chromatography to assess the identity, purity and N-terminal integrity of human lactoferrin. *Anal. Biochem.* 309:60–66.
- van Veen, H. A., M. E. J. Geerts, P. H. C. van Berkel, and J. H. Nuijens. 2004. The role of N-linked glycosylation in the protection of human and bovine lactoferrin against tryptic proteolysis. *Eur. J. Biochem.* 271:678–684.
- Xu, S. Y., C. G. B. Petersson, M. Carlson, and P. Venge. 1994. The development of an assay for human neutrophil lipocalin (HNL)—To be used as a specific marker of neutrophil activity in vivo and vitro. *J. Immunol. Meth.* 171:245–252.
- Yang, J., D. Goetz, J.-Y. Li, W. Wang, K. Mori, D. Setlik, T. Du, H. Erdjument-Bromage, P. Tempst, R. Strong, and J. Barasch. 2002. An iron delivery pathway mediated by a lipocalin. *Mol. Cell* 10:1045–1056.