Quantification of lactoferrin in milk from New Zealand dairy goats

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ABSTRACT

Lactoferrin (Lf), an iron-binding protein, plays a key part in host defence with diverse protective roles in infection and inflammation, including allergy. It is present in most mucosal secretions, including colostrum and milk. To quantify the levels of Lf in milk from New Zealand dairy goats, a sandwich enzyme-linked immunosorbent assay (ELISA) was established. Purified Lf, extracted from goat colostrum, was used to raise antibodies in rabbits and for ELISA standards. The ELISA method involved coating goat Lf antibody onto microtitre plates then sequential addition of standard or test samples, biotinylated-goat Lf antibody and streptavidin-biotin conjugate, with a colorimetric endpoint employing 3,3',5,5'-tetramethylbenzidine. The intra and inter assay coefficient of variation was 4.1% and 7.8%, respectively. Average recovery of added Lf was 104.5%. Cross reactivity with bovine Lf was 40% and negligible for other milk proteins tested. In goat milk samples collected over the lactation season, the average Lf concentration was 31.8 ± 7.3 (standard error of the mean); 33.4 ± 5.2 and 821.8 ± 269.4 mg/L, in early, mid- and late season, respectively. An ELISA method has been developed that accurately and reproducibly measures Lf in goat milk samples. Lf levels in New Zealand goat milk are similar to those reported for the cow.

Keywords: lactoferrin; ELISA, milk; New Zealand dairy goats.

INTRODUCTION

Lactoferrin (Lf) is an iron-binding protein with antimicrobial properties that has been shown to inhibit the growth of bacteria, viruses and fungi (Farnaud & Evans, 2003; Vorland, 1999). Lactoferrin is thought to play a key part in host defence with diverse protective roles in infection and inflammation, including allergy (Kruzel *et al.*, 2006). Early reports attributed the anti-bacterial activity of Lf to its iron-binding properties, however, it is now recognised that Lf also has anti-microbial properties that are independent of iron-binding (Farnaud & Evans, 2003).

Lactoferrin is present on mucosal surfaces, within the specific granules of polymorphonuclear leukocytes and in most mucosal secretions, including colostrum, milk, saliva and tears. It is most abundant in mammary secretions. In mammary secretions, the levels of this protein vary with the stage of lactation, with the highest concentrations occurring in the first milk, colostrum. Human breast milk has the highest reported Lf values, ranging from 5,000 to 7,000 mg/L in colostrum (Hambraeus *et al.*, 1978) and 1,000 to 2,000 mg/L in mature milk (Goldman *et al.*, 1982). In ruminants and pigs, the level of Lf ranges from 20 to 200 mg/L (Masson & Heremans, 1971). There are a few reports in the literature of Lf concentration in goat milk (Chen & Mao, 2004; Park *et al.*, 2007), with no data for levels of Lf in New Zealand dairy goat milk.

Different methodologies reported for measuring Lf in bovine milk include radial immunodiffusion (Hagiwara *et al.*, 2003) and enzyme-linked immunosorbent assays (ELISA) (Chen & Mao, 2004). Commercially available ELISA kits for the measurement of bovine Lf do not work for goat Lf. Therefore, in our study we have developed and validated a sandwich format ELISA for the measurement of Lf levels in New Zealand dairy goat milk, using reagents prepared in the laboratory.

MATERIALS AND METHODS

Purification of Lf by affinity chromatography

Lactoferrin was purified from freeze-dried skimmed goat colostrum powder based on reported methods, with some modifications (Chen & Mao, 2004; Nam *et al.*, 1999). Reconstituted colostrum powder (150 mL, 10% w/v) was fully defatted by centrifugation at 1,650 g for 30 minutes at 4°C. The skimmed solution was warmed to 37°C, the pH adjusted to 4.6 with 1 N HCl and then centrifuged at 10,000 *g* for 60 minutes at 10°C. The resultant whey was filtered through a 0.45 µm membrane (Millipore, MA, USA), the pH adjusted to 7.5 with 1 N NaOH and the immunoglobulins depleted by precipitation at 17% (w/v) Na_2SO_4 and centrifugation at 1,200 g for 20 minutes at 22°C. The supernatant was dialysed overnight at 4°C in 0.01 M sodium phosphate buffer, pH 7.4, then filtered through a 0.45 µm membrane prior to loading (150 mL; 120 mL/h) onto a heparin affinity column (16 x 125 mm; Heparin Sepharose 6 Fast Flow, Amersham Biosciences (NZ), Auckland, NZ) and eluted (120 mL/h) with NaCl in the same buffer using a combination of step and gradient increments. The Lf peak eluted during the gradient was then dialysed using 0.05 M ammonium acetate, pH 6.2, prior to freeze-drying.

Assessment of Lf purity by SDS-PAGE and Western blotting

The protein in the fractions eluted from the column and the purity of the isolated Lf was assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), based on the method of Laemmli (1970), with some modifications, and Western blotting techniques. Sample protein concentrations were estimated using the Lowry method (DC^{TM} Protein Assay; BioRad, Auckland, New Zealand). Samples were prepared in Laemmli's sample buffer containing β-mercaptoethanol (5%) and boiled for five to six minutes. SDS-PAGE was performed using a 4% stacking gel with a 12% separating gel. Samples were loaded at 10 μg protein per well on gels and run for 120 minutes at constant voltage of 150 V, then stained by the colloidal Coomassie Blue G 250 method (Neuhoff *et al.*, 1988). The Western blot procedure involved the transfer of separated proteins from the SDS-PAGE gel onto a nitrocellulose membrane (Pall Life Sciences, Florida, USA) then incubation with goat anti-bovine Lf- horse-radish peroxidase (HRP; 1:100,000; Bethyl Laboratories, Texas, USA) and the detection of bound antibody with enhanced chemiluminescence. Goat Lf was compared with bovine Lf from two sources (bovine Lf standard, Bethyl Laboratories and bovine Lf from milk, Sigma-Aldrich, Auckland, New Zealand), loaded at 0.5 μg per well.

Goat Lf ELISA

A sandwich enzyme-linked immunosorbent assay (ELISA) was established using reagents prepared "in-house". Antibodies were produced by immunizing two rabbits with purified goat Lf, according to Harlow and Lane (1988). One rabbit antibody was used for the primary coating antibody. The second rabbit antibody was biotinylated, prepared using EZ-Link No Weigh Biotin (Pierce, Rockford, Illinois, USA) following the manufacturer's recommended protocol. A checkerboard approach was used to determine the optimum concentration of primary antibody, secondary antibody and tertiary conjugate (streptavidin-HRP; Amersham Biosciences (NZ)). For the optimised ELISA protocol, plates were washed three times by an automated plate washer using 0.01 M phosphate buffer, pH 7.4 containing 0.15 M NaCl and 0.05% (v/v) Tween 20 (PBS-T). All samples and reagents were diluted with PBS-T containing 1% w/v bovine serum albumin (BSA; A-7906, Sigma) and all reagent addition volumes were 100 μL, unless otherwise stated. Lf standards were: 1000, 333.3, 111.1, 37.0, 12.4, 4.1 and 1.4 μg/L. Samples were pre-diluted 1:1,000, 1:5,000, 1:10,000 and 1:20,000. A Quality Control sample (QC) was run with each ELISA plate. Standards, QC and sample dilutions were assayed in duplicate.

ELISA plates (Maxisorp F-96 immunoplates; Nalge Nunc International, Rochester, NY, USA) were coated with rabbit anti-goat Lf (1:1,000,000 diluted in 0.05M bicarbonate buffer, pH 9.8) and incubated overnight at 4°C. Plates were washed then remaining activated sites on immunoplates were blocked by adding 250 μL PBS-T containing 1% w/v BSA to each well. Plates were incubated for 1 hour at room-temperature, washed, and then Lf standards, the QC and pre-diluted samples were added to designated wells. After 2 hours incubation at room-temperature the plates were washed and biotinylated rabbit anti-goat Lf (1:4,000) was added. Following overnight incubation at 4°C and washing, streptavidin-HRP (1:12,000) was added. Plates were incubated for 30 minutes at room-temperature, and washed prior to addition of the enzyme substrate solution (Product No TMBW-1000-01, BioFx, Maryland, USA). The reaction was stopped after 30 minutes with the addition of 50 μL of 2 M sulphuric acid. Optical density in wells was measured at 450 nm using an automated plate reader (Versa$_{max}$; Molecular Devices, CA, USA). A standard curve was constructed by plotting Lf concentration versus optical density using a four parameter curve fit, and then used for determining the unknown Lf concentrations of samples.

The intra and inter assay precision of the ELISA was determined by the repeat analysis of the QC sample. The accuracy of milk Lf measurement was determined by spiking several samples with purified Lf and measuring the Lf concentration in the ELISA. The potential interference due to components in the milk was assessed by comparing the parallelism of the Lf standards prepared in buffer with serial dilutions of milk samples. To assess the specificity of the assay, a range of commercially available bovine milk compounds were tested for cross-reactivity in the ELISA.

Milk samples

Milk samples were collected from healthy individual goats during early (n = 12), mid- (n = 40) and late (n = 12) lactation. The milk was defatted by centrifugation at 1,650 g for 30 minutes at 4°C. The skimmed milk was further processed by centrifugation at 11,800 g for 60 minutes at 4°C. Sample supernatants were stored at -20°C until required. Colostrum was collected on two separate dairy farms from a group of goats that had kidded one to three days previously, pooled within farms and then processed as described above for the milk samples.

RESULTS

Purification of Lf by affinity chromatography

Goat colostrum was first depleted of the major proteins, the caseins and immunoglobulins, by sequential precipitation steps. Following this, only one chromatography step was required, using a combination of stepwise and gradient salt increments to elute the bound proteins, to achieve a highly enriched Lf isolate. Figure 1 illustrates a typical chromatogram for the affinity purification. Lf was eluted as a single peak at a salt concentration of 0.7 to 0.9 M NaCl. The recovery of Lf was about 8 mg from 150 mL goat colostrum. Figure 2a shows a comparison of the column feedstock and the isolated Lf peak by SDS-PAGE. The Lf peak is a single band of protein with an estimated molecular weight of 80 kDa. Figure 2b shows a comparison of the purified goat Lf and two different bovine Lf (bovine Lf kit standard, Bethyl Laboratories and bovine Lf from milk, Sigma-Aldrich) detected by anti-bovine Lf antibody. The goat Lf band was visualised at a similar molecular weight to the bovine Lfs.

Goat Lf ELISA

Using reagents prepared "in-house", a Lf ELISA was developed and validated. The precision of the ELISA was determined with an intra and inter assay coefficient of variation of 4.1% and 7.8%, respectively. The accuracy of measuring Lf in goat milk samples was determined by spiking different concentrations of purified Lf into samples pre-diluted to 1/1,000 then measuring the total Lf levels in the ELISA. The average percentage recovery of Lf was 104.5%, over the range of 10 to 350 mg/L. The parallelism of serial diluted samples was compared with the Lf standards prepared in buffer. Figure 3 shows a typical Lf standard curve and three serially diluted samples. The milk samples diluted parallel to the buffer standard curve, suggesting that there were no matrix effects of milk in the ELISA. The specificity of the assay was tested with a range of commercially available bovine whey proteins including: Lf, lactoperoxidase, IgG, IgA and IgM. Cross reactivity with bovine Lf was 40% and negligible for other milk components tested.

FIGURE 1: Lactoferrin (Lf) was purified from goat colostrum. Firstly, colostrum was depleted of caseins and immunoglobulins, and then applied to a heparin affinity column in 0.01 M sodium phosphate buffer (pH 7.4). The column was washed with the same buffer prior to elution of bound proteins with step and gradient increments of NaCl. Lf eluted over the range of 0.7 – 0.9 M NaCl. (**-**) = absorbance (o) = NaCl.

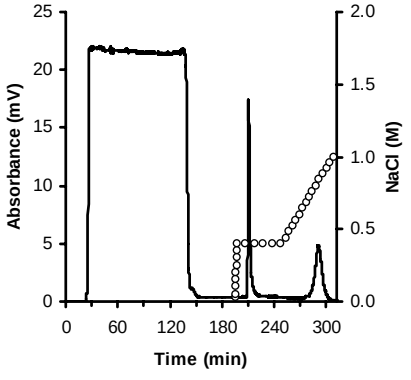


FIGURE 2: Purified lactoferrin (Lf) was evaluated by (a) SDS-PAGE analysis**:** Column feed (Lane 1), isolated goat Lf (Lane 2) and molecular weight markers (Lane 3) were run on a 12% gel and stained with Coomassie Blue. (b) Western blot analysis: Isolated goat Lf (Lane 1, 10µg/well), Bethyl bovine Lf (Lane 3; 0.5 µg/well) and Sigma bovine Lf (Lane 4; 0.5 µg/well) were detected with anti-bovine Lf antibody.

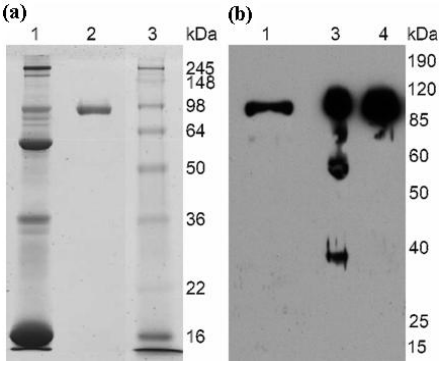
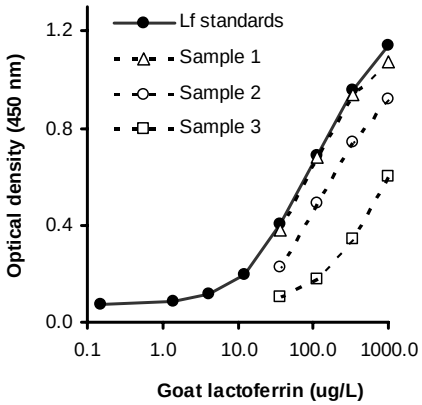


FIGURE 3: A comparison of the parallelism between lactoferrin (Lf) standards prepared in buffer and 3-fold serial dilutions of three different milk samples. The sample dilutions plotted on the X axis are expressed as an inverse of the dilution.



Milk samples

Fresh milk samples from healthy dairy goats collected at various times over the lactation season were analysed in the goat Lf ELISA. The average Lf level was 31.8 ± 7.3 (mean ± standard error of the mean; n = 12); 33.4 ± 5.2 (n = 40) and 821.8 ± 269.4 mg/L (n = 12), in the early, mid- and late season, respectively. Values in the early lactation season ranged from 4.0 to 99.8 mg/L, in the mid-lactation season from 2.0 to 120.3 mg/L and in the late lactation season from 9.4 to 2,941.8 mg/L.

Samples of the two pools of colostrum were also analysed in the ELISA. The average Lf level was 142.6 ± 22.9 mg/L.

DISCUSSION

This report describes the establishment of a sandwich format ELISA for the quantification of Lf in New Zealand dairy goats. All essential reagents were produced "in-house" because no commercial reagents or assay kits were available for the measurement of goat Lf. Although researchers have reported that anti-bovine Lf antibodies cross-react with goat Lf (Nam *et al.*, 1999), others have found that goat Lf could not be detected using a bovine Lf ELISA (Chen & Mao, 2004). This was also our experience. Using a commercially available bovine Lf ELISA kit (Bethyl Laboratories, Texas, USA), we were unable to detect levels of Lf in New Zealand dairy goat milk. Interestingly, antibody from the kit did detect goat Lf when employed in a Western blot. This may suggest that Lf from the cow and goat may be more similar in a reduced form than when the proteins are in their native form. However, the bovine antibody did show a greater affinity for the bovine Lf compared with the goat Lf, as illustrated in the Western blot where the goat protein was loaded at a 20-fold higher concentration than the bovine protein.

The accuracy of ELISA test results are dependent on the quality of the reagents, the monospecificity of the antibodies being of prime importance (Clark & Engvall, 1980). Cross-reactivity studies confirmed the specificity of the antibodies used in this Lf ELISA; bovine Lf was the only milk protein that was detected at a significant level in the assay. In addition, other studies investigating the precision and accuracy of test results and the parallelism of serially diluted milk samples with the standard curve established that the assay was suitable for the detection of Lf in milk.

Lactoferrin concentrations in colostrum and milk samples collected at various times during the lactation period suggest that the level of Lf in New Zealand dairy goat milk is similar to that reported by others for goats (Chen & Mao, 2004; Park *et al.*, 2007) and also similar to the cow (Farr *et al.*, 2002; Hagiwara *et al.*, 2003). Higher Lf concentrations observed in some samples from the late season may reflect changes in the gland due to partial drying off. The Lf content of mammary secretions has been reported to markedly increase during mammary involution when expression of the Lf gene is initiated in milk-engorged, secretory alveoli (Molenaar *et al.*, 1996).

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