Journal of Food and Drug Analysis, Vol. 12, No. 2, 2004, Pages 133-139 藥物食品分析 第十二卷 第二期

Detection of Lactoferrin in Bovine and Goat Milk by Enzyme-linked Immunosorbent Assay

PO-WEN CHEN AND FRANK CHIAHUNG MAO*

Department of Veterinary Medicine, National Chung-Hsing University, Taiwan, R.O.C.

(Received: July 16, 2003; Accepted: January 5, 2004)

ABSTRACT

Lactoferrin (LF) has been proposed as a multifaceted functional ingredient of food. A competitive enzyme-linked immunosorbent assay (ELISA) was established to determine the LF in milk by using bovine LF antiserum and bovine LF-Biotin conjugate. The LF concentration in cow milk samples could be determined by this ELISA. However, the LF concentration in goat milk could not be measured using this method. Thus, the goat LF ELISA was established using goat LF-Biotin conjugate to measure the LF concentration in goat milk. Milk samples were collected for measurement of their LF concentrations. The mean LF level for the individual cow milk samples with the somatic cell count (SCC) below 1×10^5, 1 to 2.5×10^5 or 2.5×10^5 to 5×10^5 cells/mL was found to be about 176, 466 or 742 μg/mL, respectively. On the other hand, the LF level was about 200 μg/mL in goat bulk milks with the methylene blue reduction test (MBRT) time more than 5 hr. In conclusion, both LF ELISA methods developed in this study could be successfully used to determine the LF concentration in goat and cow milk.

Key words: lactoferrin, milk, ELISA

INTRODUCTION

Lactoferrin (LF) was first discovered as an iron-binding protein in bovine milk[1]. It is produced by epithelial cells and neutrophils, and is found in most of the external secretion of mammals such as milk, reproductive tract secretion, synovial fluid, lachrymal and salivary secretion[2-4]. LF and its N-terminal region has been reported to possess antimicrobial activity that could inhibit the growth of bacteria, viruses and fungus[5,6]. LF may also modulate the immune systems through cytokine expression and has an antitumour activity both *in vitro* and in animal models[7-8]. Moreover, enzymatic activity, protease activity, transcriptional regulation and autoantibodies have also been ascribed to LF[5].

Since LF is a multi-functional protein, it has been proposed as a multifaceted ingredient for functional foods. This protein has been added in infant formulas to enhance iron absorption[4,9] and possibly inhibit the oxidation of infant formulas[10-11]. Thus, understanding the quantity of LF in formula milk or especially in normal consuming milk, would be helpful in the evaluation of the nourishing component of milk. Previous studies had shown that the LF in cow milk could be measured using the indirect enzyme-linked immunosorbent assay (ELISA) and immunodiffusion method[12-15]. However, a large quantity of antibody (1.5 to 2% anti-bovine LF antiserum) is generally needed for the immunodiffusion method. Since less antiserum is needed for ELISA, a competitive ELISA was established in our study to detect the milk LF. This competitive ELISA was found to be better than the indirect ELISA in that it could determine the LF in milk samples both from goats and cows by using only different LF-Biotin conjugates. In addition, competitive ELISA is generally more sensitive than the indirect one.

* Author for correspondence. Tel: 886-4-22861053; Fax: 886-4-22861053; E-mail: fcmao@dragon.nchu.edu.tw

MATERIALS AND METHODS

I. *Bovine LF ELISA*

Competitive ELISA was carried out with reference to the previously published β-lactoglobulin ELISA[16]. Bovine LF antiserum was obtained by immunizing three rabbits with bovine LF (Sigma, St. Louis, MO, U.S.A.) according to the report by Harlow and Lane[17]. The bovine LF was conjugated to the NHS-LC-Biotin (1:5) (Pierce, Rockford, Illinois, U.S.A.) as described in Mao and Bremel's previous report[18]. In preliminary tests, the antiserum was diluted 50,000, 100,000 and 200,000 folds in the coating buffer (0.05 M sodium carbonate-bicarbonate; pH 9.6) and coated onto the well of a microplate (Nunc, Rochester, NY, U.S.A.). The LF-LC-Biotin conjugate was also diluted 100,000, 200,000 and 400,000 folds. Subsequently, various combinations using the prepared antiserum, LF-LC-Biotin and bovine LF standard (10 and 0.001 μg/mL) were tested in an ELISA assay for the most optimum titer for antiserum and LF-LC-Biotin. The light absorbance of the above ELISA ranged from 0.1 to 1.5. Finally, the antiserum and LF-LC-Biotin were found to be the most optimum when diluted 200,000 and 400,000 folds, respectively. Thus, the antiserum was diluted 200,000 fold in coating buffer and dispensed onto an ELISA microplate at 100 μL per well. The microplate was incubated at 4˚C overnight and washed

four times with the washing buffer (0.02 M sodium phosphate, 0.12 M NaCl and 0.025% Tween 20; pH 7.2). Then, 50 µL of assay buffer (0.04 M 3-(N-Morpholino) propanesulfonic acid [MOPS], 0.12 M NaCl, 0.01 M ethylenediamine tetra-acetic acid [EDTA], 0.5 µg/mL leupeptin, 0.1% gelatin, 0.05% Tween 20 and 0.005% chlorhexidine digluconate; pH 7.2) was added to each well of the plate. The bovine LF standard was prepared with the assay buffer of 10, 1, 0.3, 0.1, 0.03, 0.01, 0.001 and 0 µg/mL and the milk samples were diluted with the same buffer at 1000 to 3000-fold. Then, the LF standards or samples in 50-µL assay buffer were added in triplicate into nonadjacent wells. The lactoferrin-LC-Biotin was diluted 400,000 fold in the assay buffer and 100 µL added into each well. The plate was then sealed and incubated at room temperature for 2 hr. After incubation, the plate was washed four times with the washing buffer, and blotted on a paper towel. ExtrAvidin-peroxidase (Sigma, St. Louis, MO, U.S.A.) was diluted 10,000 fold in the assay buffer, and 100 µL was added into each well. The plate was incubated for 2 hr at room temperature. After incubation, the plate was washed eight times with the washing buffer, and blotted on a paper towel. The substrate, prepared by mixing 19 mL of sodium acetate, 64 µL of hydrogen peroxide (Yakuri Pure Chemicals, Osaka, Japan) and 200 µL of tetra-methylbenzidine (TMB; Sigma, St. Louis, MO, U.S.A.), was added at 125 µL into each well. After incubating for 12 min, the reaction was stopped by adding 50 µL of 0.5 M H_2SO_4 into each well. The absorbance of each well was read at 450 nm (minus light absorbance at 600 nm as background value) with an auto spectrophotometer (Labsystems Multiskan, UK). In addition, the intraassay and interassay coefficients of variances for this ELISA were maintained at below 10%.

II. *Isolation of Goat Lactoferrin*

Goat LF was purified from colostrum with modification of a previous study[19]. In brief, 60 mL of colostrum (Alpine goat) was defatted by centrifugation at 2000 ×g for 30 min at 4˚C. The pH of the skim milk were adjusted to 4.6 with 5 N HCl and then centrifuged at 10,000 ×g for 1 hr to remove the casein precipitate. The whey was passed through a 0.45 mm filter (Millipore, Ireland) to completely remove the casein precipitate and its pH was readjusted to 6.0 with 1N NaOH. The immunoglobulin in the whey was removed by ammonium sulfate precipitation (48%). After passing through 0.45 mm filter, the whey was concentrated and desalted using a desalting kit (Vivascience, UK). The solution in the whey was then replaced with 0.005 M sodium phosphate buffer (pH 6.0) followed by loading into a heparin affinity column (Amersham Pharmacia Biotech, Uppsala, Sweden) and eluted by step elution with 0.005 M sodium phosphate buffer (pH 6.0) containing 0.1, 0.3 or 0.5 M NaCl. The LF was collected at the 0.5 M NaCl eluting solution and its purity was assessed by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and Western blotting as described below.

III. *Electrophoresis and Western-blotting*

SDS-PAGE and Western-blotting were carried out as described in the previous reports[20-21]. The 4% stacking and 15% separating gel was used. In addition, a molecular weight marker 97.4, 66.2, 57.5, 45, 36, 24 and 20 kDa was used. The primary antibody for Western-blotting was rabbit anti-bovine LF and the secondary antibody was horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Sigma, St Louis, Mo, U.S.A.).

IV. *Caprine LF ELISA*

Caprine LF ELISA was established as well as bovine LF ELISA (bLF ELISA) as mentioned above. However, purified goat LF and goat lactoferrin-biotin conjugate were used instead of bovine LF and bovine LF-biotin conjugate, respectively. The purified goat LF was conjugated to NHS-LC-Biotin (1:5) (Pierce, U.S.A.) according to a previous study by Mao and Bremel[18]. In preliminary tests, the antiserum and goat LF-LC-Biotin were first diluted to their optimal concentration as the bLF ELISA. Finally, the bovine LF antiserum was diluted 30,000 fold with coating buffer and then coated onto an ELISA microplate (Nunc, U.S.A.). The prepared LF-LC-biotin was diluted 250,000 fold in assay buffer to be used for the competition reaction. Moreover, the LF standard was prepared as 10, 3, 1, 0.3, 0.1, 0.03, 0.01 and 0.003 µg/mL; the milk samples were diluted by the assay buffer at 1000 to 2000-fold. ExtrAvidin-peroxidase (Sigma, U.S.A.) was diluted 10,000 fold in assay buffer to give an optimal color development. The absorbance of each well was read at 450 nm (minus light absorbance at 600 nm as background value) with an auto spectrophotometer (Labsystems Multiskan, U.K.). The intraassay and interassay coefficients of variances for this ELISA were below 10%.

Three milk samples were diluted serially 2 fold between 400 to 12800 dilution folds. The diluted samples were determined for their light absorbance using Caprine LF ELISA. The parallelism test was performed by comparing the light absorbance of standard solution and milk samples.

V. *Milk Samples*

Fifty individual milk samples were randomly selected from the dairy cows during normal lactational period in a commercial dairy farm. The cows were determined healthy by clinical inspection and the routine monitoring of their somatic cell counts (SCC) in milk (Bentley SCC 300, U.S.A.). In addition, one hundred goat milk samples were randomly collected from bulk milk in different commercial goat farms. The bulk milk, which do not contain any colostrum, was composed of milk from individual goats at different lactational periods. The LF concentration in the collected cow and goat milk samples were measured using the aforementioned LF ELISA. Furthermore, the cow milk

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samples were also measured for their milk SCC (Bentley SCC 300, U.S.A.) and the goat milk samples for their methylene blue reduction time (MBRT)[22], respectively.

RESULTS AND DISCUSSION

I. *Establishment of Bovine LF ELISA*

The bLF ELISA was easily established in this study because purified bovine LF could be purchased commercially. Thus, the bovine LF standard and the biotin-conjugate were prepared without difficulty. The parallelism test showed that this ELISA is suitable for detecting LF in milk between 1000 to 10000 dilution points. In addition, the specificity of this ELISA was confirmed by Western blotting analysis using anti-bovine LF antibody as the primary antibody to detect the LF in milk samples (data not shown). The standard curve was fitted by the four parameter logistic method as shown in Figure 1. The detection limit of this ELISA was found to be about 0.002 μg/mL (n = 6) as calculated by subtracting the highest light absorption value from twice its standard deviation and extrapolating it accordingly to the LF concentration. Suitability of the constructed standard curve for detecting the milk LF was also evaluated by the parallelism test.

Nam *et al*. (1999) found that the anti-bovine LF antibody could cross-react with the Korean goat and Saanen goat LF. They suggested that the Korean goat LF share similar epitope with that of bovine LF[19]. However, our

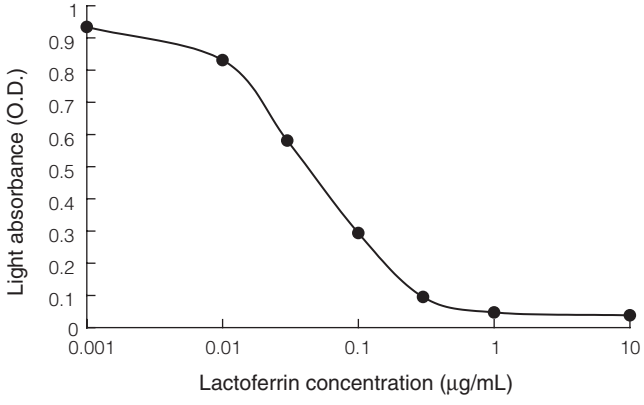

Figure 1. Standard curve for the competitive bovine lactoferrin ELISA. Bovine lactoferricin at concentrations of 10, 1, 0.3, 0.1, 0.03, 0.01, 0.001 and 0 μL/mL were used as standards.

bLF ELISA was tried out to detect the LF in goat milk samples without success. The LF level in goat milk determined by the bLF ELISA was found to be very low due to the higher light absorbance by each of the milk samples. This higher light absorbance in bLf ELISA indicates that most of the coated antibody on the microplate was bound to the bovine LF-conjugate. In other words, goat LF as well as the other proteins in goat milk could not bind to the coated antibody in the presence of the bovine LF-conjugate. Thus, we suggest that the LF in the goat milk could not compete with bovine LF-biotin in the binding to bovine LF antibody. Therefore, we purified the goat LF to establish the goat LF ELISA (gLF ELISA).

II. *Purification of LF from Goat Colostrum*

The goat LF was purified from the colostrum with modification of a previous study[19]. To save the purification time, one affinity column wad used in our studey instead of the two column systems used by Nam *et al*. (1999)[19]. Figure 2 shows the results of the SDS-PAGE analysis of the fractions collected from the elution profile of the LF passing through theaffinity column. It was observed that most milk protein was eluted by the phosphate buffer containing 0.1 M and 0.3 M NaCl. A single band of KF (80 kDa) was eluted by the phosphate buffer containing 0.5 M NaCl. Figure 3 shows the Western blot analysis for the cross-reactivity between bovine and goat LF. The result indicates that the anti-bovine LF could

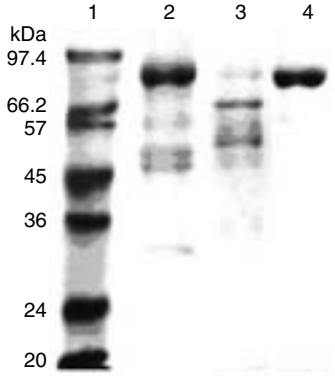

Figure 2. Profiles of SDS-PAGE (15%) after affinity-heparin column chromatography for purifying goat lactoferrin from colostrum. 1 = Protein marker; 2 = fraction eluted by the phosphate buffer (contained 0.1 M NaCl); 3 = fraction eluted by the phosphate buffer (contained 0.3 M NaCl); 4 = fraction eluted by the phosphate buffer (contained 0.5 M NaCl).

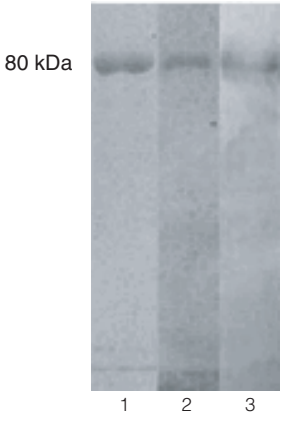

Figure 3. Western blotting analysis for the cross-reactivity between bovine and goat lactoferrin. Anti-bovine lactoferrin antibody was used to detect bovine lactoferrin (lane 2) and goat lactoferrin (lane 3). Lane 1, bovine lactoferrin (80 kDa).

cross-react with goat LF (Saanen goat LF). This observation was in accordance with the previous report[19]. On the other hand, although the anti-bovine LF antibody could bind to both bovine and goat LF, this antibody showed greater affinity to bovine LF. This is because the amount for antibody used in the bovine LF ELISA (antiserum was diluted 200,000 folds) is 6 fold less than that used in the goat LF ELISA (antiserum was diluted 30,000 folds). This finding supports the non-feasibility of using bovine LF ELISA (competitive ELISA) to detect the goat LF in goat milk.

The recovery of LF was about 3 mg from 60 mL of colostrum (50 mg/L) in our study. This observation is similar to that using the two-column system to purify LF from Korean goat colostrum (about 66 mg/L; 200 mg from 3 L of colostrum)[19] as reported previously. Although the purification method used in our study could produce a large quantity of LF from the goat colostrum, the binding capacity of the heparin column is limited to about 60 mL of colostrum. Nevertheless, the 3 mg of LF purified from 60 mL of colostrum is enough for about 800 microplates (about 19,200 milk samples).

III. *Establishment of Goat LF ELISA*

The gLF ELISA was successfully established by the use of the purified goat LF. The standard curve for this ELISA was fitted by the 4 parameter logistic method as shown in Figure 4. The detection limit of this ELISA was 0.012 μg/mL (n = 6) as calculated by subtracting the highest light absorption value from twice its standard deviation value and extrapolated it accordingly to the LF concentration. The light absorptions values for the serially diluted milk samples were found to be parallel to that of the standard curve between points at 800 to 3200 dilutions (Figure 5). This finding indicates that the ELISA established here is suitable for detecting the LF in milk samples in this range. The specificity of this ELISA was also confirmed by using the anti-bovine LF antibody as the primary antibody in the Western blotting analysis for the goat milk samples. The lactoferrin in goat milk could be detected specifically as a major band, despite the abundance of other proteins in the goat milk samples.

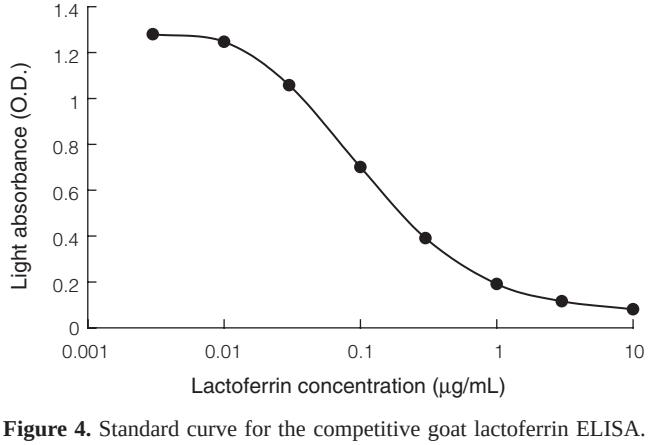

Figure 4. Standard curve for the competitive goat lactoferrin ELISA. Goat lactoferrin at concentration of 10, 3, 1, 0.3, 0.1, 0.03, 0.01, and 0.003 μg/mL were used as standards.

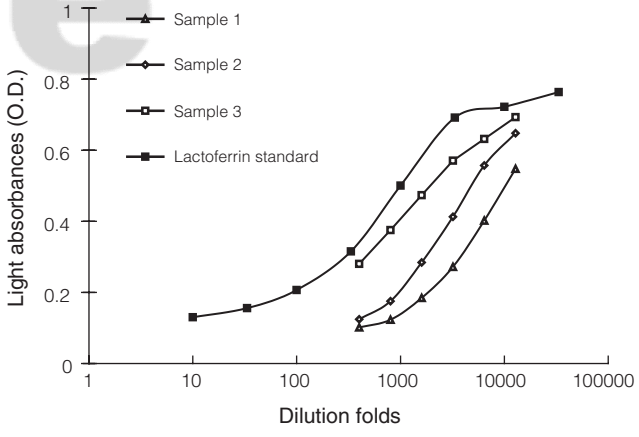

Figure 5. The ELISA parallelism test for goat lactoferrin standard and serially diluted milk samples. Dilution folds are expressed as log10 scale. Milk samples were serially 2 fold diluted between 400 to 12,800 dilution folds.

IV. *LF Level in Cow and Goat Milk for Consumption*

The SCC for the milk from cows with subclinical mastitis is often greater than 2.5×10^5 cells/mL[23]. The International Dairy Federation (IDF) suggested that the SCC threshold for differentiation between the normal and subclinical mastitis milk is 5×10^5 cells/mL[24]. This SCC threshold is accepted in Taiwan for the diagnosis of subclinical mastitis for dairy cows. The diagnosis of subclinical mastitis or clinical mastitis for dairy cows is important because the milk quality would be compromised by the internal halves inflammation[25-26] and thus would not be suitable for human consumption. The milk SCC is being used in Taiwan to monitor milk quality and subclinical mastitis. In our study, the collected cow milk samples were divided into three categories according to their SCC values. The milk SCC in group 1 was below 1×10^5 cells/mL. In group 2, the milk SCC ranged from 1 to 2.5×10^5 and in group 3 from 2.5 to 5×10^5 cells/mL. The milk LF concentration in the three groups above is shown in Table 1. The mean LF level in milk group 1, 2 and 3 was 176, 466 and 742 μg/mL, respectively. Moreover, the LF level in the three milk groups was found to be significantly ($P < 0.05$) different from each other (one-way ANOVA). Furthermore, the milk LF level in the three groups seems to correlate negatively with the respective milk SCC. Indeed, statistical analysis of the relationship between milk LF and milk SCC showed that the LF concentration negatively correlates ($r = 0.437$, $P < 0.05$) with the milk SCC that is below 1×10^5 cells/mL ($P < 0.01$) (Pearson correlation test). However, no significant relationship between milk LF and milk SCC in the other two groups (Pearson correlation test) was observed. This finding needs further investigation because the milk samples in our study were randomly collected

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Table 1. The lactoferrin concentration in cow and goat milks

Milk	Number	LFC (μg/mL)		SCC	MBRT (hr)
		Mean	Standard deviation	($\times 10^5$ cells/mL)	
Bovine	50	176.8	120.3[a]	≦1	ND
	15	466	508.5[b]	1-2.5	ND
	10	742.1	374.2[c]	2.5-5	ND
Goat	50	166.4	53.8[d]	ND	≧8
	50	217.0	75.4[e]	ND	5-8

[a,b,c]Significant difference in bovine group ($P < 0.05$).
[d,e]Significant difference in goat group ($P < 0.05$).
ND: not determined.

from cows with different lactational periods. On the other hand, Harmon *et al*. (1975) reported that the LF in milk from cows in normal lactational period ranged from 20 to 200 μg/mL[27]. Thus, the LF level seen in our study in the higher SCC milk (SCC between 1-5 $\times$ 10^5 cells/mL) was slightly higher than the previously reported value. This discrepancy may be due to the different methodology used and the time when milk samples were collected. In addition, previous reports showed that the LF the subclinical or clinical mastitis milk was higher than that of normal milk[12-13]. Milk SCC at 2.5 to 5 $\times$ 10^5 cells/mL has been regarded as an indication of infection in one of the cow's teats[23]. Thus, the higher mean LF level for the milk group with higher SCC value might be attributed to the collection of subclinical mastitis milk.

The goat LF in normal milk for consumption is shown in Table 1. The goat milk samples were divided into two groups based on their MBRT time. The MBRT time for goat milk is one of the criteria for price setting of the goat milk in Taiwan. The time needed for the reducion reaction to be completed in the MBRT has been demonstrated to be inversely related to the number of bacteria in milk[28]. Thus, the MBRT that is more than 8 hr would be considered a good quality of milk, whereas that between 5 to 8 hr would be viewed as normal quality and those below 5 hr would not be accepted for sale. We found that the LF level in milk with MBRT of more than 8 hr was about 166 μg/mL, and those between 5 to 8 hr was 217 μg/mL, respectively. The LF concentration in normal goat milk observed in our study is similar to the previously reported 20 to 200 μg/mL as detected by the radial immunodiffusion method (pooled milk)[14]. Otherwise, the LF concentration in the two milk groups would be significantly different from each other (student's t test; $P < 0.001$).

An advantage of ELISA in detecting the milk lactoferrin is that less amount of antiserum is needed. In our study, the antiserum used in the bovine and goat LF ELISA are diluted 200,000 folds (equal to 0.0005%) and 30,000 folds (equal to 0.003%), respectively. The antiserum used in our study is considerably less than that used in the immunodiffusion method (1.5 to 2% antiserum is need)[14]. In addition, our competitive ELISA was found to be more sensitive than the indirect one previously reported[15]. The detection limit for LF in the bovine LF ELISA and goat LF ELISA were 2 ng/mL and 12 ng/mL, respectively. In contrast, bovine LF level ranging from 50 to 500 ng/mL was used in the indirect ELISA[15].

V. *Possible Role of LF in Milk for Consumption*

In vitro study showed that the minimum inhibitory concentration (MIC) of bovine LF for *E. coli* was 2000 μg/mL[29]. The mean LF level in cow milk in our study was observed to be below 1000 μg/mL. Paulsson *et al*. (1993) studied the thermal stability of bovine LF (apolactoferrin) and found that UHT (ultra high temperature pasteurization) treatment of LF (135˚C, 4 sec) abolished the bacteriostatic activity of apolactoferrin[30]. Based on these finding, cow milk for consumption processed by the UHT might not possess the antibacterial activity. However, Oria *et al*. (1993) reported that the heat treatment had only little effect on the interaction of LF with monocytes after a treatment 137˚C for 8 sec, as assessed by the displacement of labeled LF or cell prolification[31]. Therefore, the bovine LF processed by the UHT might still retain its immunomodulatory effect. On the other hand, the LF in cow milk was reported to be 22% iron-saturated[32]. The bovine LF was also shown to have antioxidant effect which was related to its iron-chelating ability[33-34]. Abe *et al*. (1991) demonstrated the heat stability of bovine LF by showing that preheating at 70˚C for 3 min followed by UHT at 130˚C for 2 sec resulted in only 3% loss in residual iron-binding capacity[35]. Thus, the LF in milk for consumption not only retain its immunomodulatory ability but also its antioxidant activity. However, study on heat stability of goat LF is rare. Since the LF concentration in goat milk determined here was low, the LF in goat milk for consumption might not be responsible for the antibacterial activity but for other physiological function. Nevertheless, further experiment is needed to test the antioxidant or immunomodulatory effect of cow or goat milk based on the LF concentrations.

In conclusion, two ELISA methods for measuring the LF level in cow and goat milk had been successfully established and may be applied to detect the LF level in milk products or other biological fluids from bovine and caprine.

ACKNOWLEDGMENTS

This work was supported in part by a grant 92AS-3.1.3-AD-U1 from the Council of Agriculture, Executive

Yuan, Taiwan.

REFERENCES

1. Sorensen, M. and Sorensen, M. P. L. 1939. The proteins in whey. C.R. Trav. Lab. Carlsberg 23: 55-59.
2. Masson, P. L., Heremans, J. F. and Dive, C. 1966. An iron-binding protein common to many external secretions. Clin. Chem. Acta. 14: 735-739.
3. Masson, P. L., Heremans, J. F. and Schonne, E. 1969. Lactoferrin, an iron binding protein in neutrophilic leukocytes. J. Exp. Med. 130: 643-658.
4. Levay, P. F. and Viljoen, M. 1995. Lactoferrin: a general review. Haematologica 80: 252-267.
5. Brock, J. H. 2002. The physiology of lactoferrin. Biochem. Cell Biol. 80: 1-6.
6. Chen, P. W., Shyu, C. L. and Mao, F. C. 2003. Antibacterial activity of short hydrophobic and basic-rich peptides. Am. J. Vet. Res. 64: 1088-1092.
7. Ward, P. P., Uribe-Luna, S. and Conneely, O. M. 2002. Lactoferrin and host defense. Biochem. Cell Biol. 80: 95-102.
8. Tsuda, H., Sekine, K., Fujita, K. I. and Iigo, M. 2002. Cancer prevention by bovine lactoferrin and underlying mechanisms- a review of experimental and clinical studies. Biochem. Cell Biol. 80: 131-136.
9. Chierici, R., Sawatzki, G., Tamisari, L., Volpato, S. and Vigi, V. 1992. Supplementation of an adapted formula with bovine lactoferrin. 2. effects on serum iron, ferritin and zinc levels. Acta. Paediatr. 81: 475-479.
10. Raghuveer, T. S., McGuire E. M., Martin, S. M., Wagner, B. A., Rebouche C. J., Buettner G. R. and Widness J. A. 2002. Lactoferrin in the preterm infant's diet attenuates iron-induced oxidation products. Pediatr. Res. 52: 964-972.
11. Satue-Gracia, M. T., Frankel, E. N., Rangavajhyala, N. and German, J. B. 2000. Lactoferrin in infant formulas: effect on oxidation. J. Agric. Food Chem. 48: 4984-4990.
12. Hagiwara, S., Kawai, K., Anri, A. and Nagahata, H. 2003. Lactoferrin concentrations in milk from normal and subclinical mastitic cows. J. Vet. Med. Sci. 65: 319-323.
13. Kawai, K., Hagiwara, S., Anri, A. and Nagahata, H. 1999. Lactoferrin concentration in milk of bovine clinical mastitis. Vet. Res. Commun. 23: 391-398.
14. Masson, P. L. and Heremans, J. F. 1971. Lactoferrin in milk from different species. Comp. Biochem. Physiol. B 39: 119-129.
15. Tu, Y. Y., Chen, C. C., Chang, J. H. and Chang, H. M. 2002. Characterization of lactoferrin (LF) from colostral whey using anti-LF antibody immunoaffinity chromatography. J. Food Sci. 67: 996-1001.
16. Mao, F. C. and Bremel, R. D. 1995. Prediction of milk yields from serum β-lactoglobulin concentrations in pregnant heifers. J. Dairy Sci. 78: 291-295.
17. Harlow E, Lane D. ed. 1988. Antibodies: A Laboratory Manual. pp. 92-199. Cold Spring Harbor. New York. U. S. A.
18. Mao, F. C. and Bremel, R. D. 1990. Enzyme-linked immunosorbent assays for bovine β-lactalbumin and α-lactoglobulin in serum and tissue culture media. J. Dairy Sci 74: 2946-2951.
19. Nam, M. S., Shimazaki, K. I., Kumura, H., Lee, K. K. and Yu, D. Y. 1999. Characterization of Korean native goat lactoferrin. Comp. Biochem. Physiol. B 123: 201-208.
20. Leonard, G. D., Michael, K. and James, F. B. 1994. Electrophoresis of proteins on sodium dodecyl sulfate polyacrylamide gels. In "Basic Methods in Molecular Biology". 2nd ed. pp. 661-668. Appleton and Lange. Connecticut, U. S. A.
21. Leonard, G. D., Michael K. and James, F. B. 1994. Western blotting of proteins. In "Basic Methods in Molecular Biology". 2nd ed. pp. 680-690. Appleton and Lange. Connecticut, U. S. A.
22. Richardson, G. H. ed. 1985. Standard Methods for the Examination of Dairy Products. 15th ed. pp. 261-262. Am. Publ. Health Assoc. Inc. Washington, DC, U. S. A.
23. O'Rourke, D. J. and Blowey, R. W. 1992. Cell counts and mastitis monitoring. In "Bovine Medicine. Diseases and Husbandry of Cattle". pp. 305-312. Andrews, A. H., Blowey, R. W., Boyd, H. and Eddy, R. G. ed. Blackwell Science publication. London, U. K.
24. International Dairy Federation. 1981. Recommended methods for somatic cell counting in milk. Document 132 pp. 5-16.
25. Bramley. 1992. Mastitis. In "Bovine Medicine. Diseases and Husbandry of Cattle". pp. 289-290. Andrews, A. H., Blowey, R. W., Boyd, H. and Eddy, R. G. ed. Blackwell Science publication. London, U. K.
26. Rajala-Schultz, P. J., Grohn, Y. T., McCulloch, C. E. and Guard, C. L. Effects of clinical mastitis on milk yield in dairy cows. J. Dairy Sci. 82: 1213-1220.
27. Harmon, R. J., Schanbacher, F. L., Ferguson, L. C. and Smith, K. L. 1975. Concentration of lactoferrin in milk of normal lactating cows and changes occurring during mastitis. Am. J. Vet. Res. 36: 1001-1007.
28. Dabbah, R., Tatini, S. R. and Olson, J. C. 1967. Comparison of methods for grading milk intended for manufacturing purposes. J. Milk Food Techol. 30: 71-76.
29. Tomita, M., Takase, M., Wakabayashi, H. and Bellamy, W. 1994. Antimicrobial peptides of lactoferrin. Adv. Exp. Med.Biol. 357: 209-218.
30. Paulsson, M. A., Svensson, U., Kishore, A. R. and Naidu, A. S. 1993. Thermal behaviour of bovine lactoferrin in water and its relation to bacterial interaction and antibacterial activity. J. Diary Sci. 76: 3711-3720.
31. Oria, R., Ismael, M., Sanchez, L., Calvo, M. and Brock, J. 1993. Effect of heat treatment and other milk proteins on the interaction of lactoferrin with monocytes. J. Dairy Res. 60: 363-369.

32. Lönnerdal, B., Forsum, E. and Hambraeus, L. 1976. A longitudinal study of the protein, nitrogen and lactose of human milk from Swidish well-nourished mothers. Am. J. Clin. Nutr. 29: 1127-1133.
33. Gutteridge, J. M. C., Paterson, S. K., Segal, A. W. and Halliwell, B. 1981. Inhibition of lipid peroxidation by the iron-binding protein lactoferrin. J. Biochem. 199: 259-261.
34. Huang, S. W., Satué-Gracia, M. T., Frankel, E. N. and German, J. B. 1999. Effect of lactoferrin on oxidative stability of corn-oil emulsions and liposomes. J. Agric. Food Chem. 47: 1356-1361.
35. Abe, H., Saito, H., Miyakawa, H., Tamura, Y., Shimamura, S., Nagao, E. and Tomita, M. 1991. Heat stability of bovine lactoferrin at acidic pH. J. Dairy Sci. 74: 65-71.