Existence of functional lingual antimicrobial peptide in bovine milk

N. Isobe,¹ J. Nakamura, H. Nakano, and Y. Yoshimura

Graduate School of Biosphere Science, Hiroshima University, Higashi-Hiroshima, 739-8528 Japan

ABSTRACT

The lingual antimicrobial peptide (LAP) belongs to the β -defensin family in cattle and is localized in epithelial cells of alveoli in mammary glands. The aim was to investigate whether LAP is secreted into milk and whether the secreted LAP has antimicrobial activity. Decaseinated bovine skim milk was applied to sample extraction cartridges, and the eluate was used for competitive enzyme immunoassay and Western blotting to test for the presence of LAP in milk. After tricine-SDS PAGE, the gel was stained using the periodic acid-Schiff reaction to examine the possibility of glycosylation of LAP. The eluate obtained from the sample extraction cartridges was subjected to a LAP antibody-coupled affinity column, after which the antimicrobial activity of its eluate against *Escherichia coli* was investigated with radial diffusion plate assay and colony-forming unit enumeration following the culture of bacteria with the sample. The immunoreactive LAP was detected in the eluate by competitive enzyme immunoassay (optical density = 0.437 ± 0.012 vs. 0.468 ± 0.016). In the Western blotting analysis, immunoreactive bands were seen around 8, 14, and 17 kDa. The bands at 14 and 17 kDa, but not 8 kDa, were periodic acid-Schiff reaction-positive. The eluate of LAP antibody-coupled affinity column had antimicrobial activity against *E. coli* (cfu/control = 0.17 ± 0.18). These results suggest that bovine milk contains functional LAP-like substances that exert antimicrobial activity.

Key words: lingual antimicrobial peptide, milk, bovine, defensin

INTRODUCTION

Mastitis is a serious disease of dairy cows, caused by bacterial infection of the mammary gland, that has detrimental effects on the quantity and quality of milk. To prevent infections, mammary glands prepare sequentially orchestrated mechanisms of immunity. Innate immunity plays an important role in the first defense system against infection in the mammary gland as well as the entire body. This type of immunity consists of various antimicrobial proteins such as lactoperoxidase,

lysozyme, and lactoferrin (Polis and Shmukler, 1953; Chandan et al., 1964; Bellamy et al., 1993). These proteins kill bacteria in different ways; for example, lactoferrin chelates iron ions essential for bacterial growth. The antimicrobial peptide defensin is found in the innate immunity systems of mammals (Ganz et al., 1985; Selsted and Ouellette, 2005). Defensin causes permeabilization of target bacteria membranes, resulting in cell lysis and eventual bacterial death (Ganz, 2003). Both gram-negative and gram-positive bacteria can be the target of defensin (Schonwetter et al., 1995).

The bovine β -defensin includes lingual antimicrobial peptide (**LAP**; Schonwetter et al., 1995), bovine neutrophil β -defensin (Selsted et al., 1993; Goldammer et al., 2004), tracheal antimicrobial peptide (Diamond et al., 1991), and enteric β -defensin (Tarver et al., 1998). Lingual antimicrobial peptide was first isolated from inflamed bovine tongue epithelium (Schonwetter et al., 1995). Stolzenberg et al. (1997) found widely spread LAP mRNA expression in infected bovine intestinal and respiratory tissues. The expression of β -defensin mRNA was reported in the mammary glands of cows (Swanson et al., 2004). A positive relationship between SCC in milk and LAP mRNA expression and localization of mRNA in epithelial cells of mastitic tissue by in situ hybridization was described. Singh et al. (2008) reported an increase in LAP mRNA expression in the bovine alveolar tissue at 192 h postmilking upon involution. Immunohistochemical studies demonstrated the expression of LAP peptide in healthy (Isobe et al., 2009) and *Escherichia coli*-inoculated mammary tissues (Petzl et al., 2008).

Jia et al. (2001) and Armogida et al. (2004) reported abundant human β -defensin-1 and 2 in human milk. If the milk of healthy cows contains LAP, it may play roles not only in the defense of mammary tissues, but also in neonatal immunity. Nevertheless, it is not clear whether the LAP peptide is secreted from mammary tissue into the milk. Therefore, the present study was undertaken to identify the presence of LAP peptide in bovine milk.

MATERIALS AND METHODS

Milk Sample Preparation

Milk was collected twice by machine from a Holstein-Friesian dairy cow ($n = 1$; 3 yr old) at the Hiroshima

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¹Corresponding author: niso@hiroshima-u.ac.jp

University Farm. The cow was fed in accordance with the regulations of Hiroshima University for animal experiments. The cow was multiparous and several months postpartum (early stage of lactation). The composite milk with low SCC ($<100,000$ cells/mL) was used. Removal of fat and casein was performed as described previously (Bushe and Oliver, 1987). Fresh raw milk was heated at 100°C for 5 min and skimmed by centrifugation at $1,700 \times g$ for 30 min at 4°C . The casein was removed by addition of acetic acid until pH = 4.5 and centrifugation. The supernatant was loaded into C18 Sep-Pak cartridges (Waters Corp., Milford, MA). Elution was achieved by flow of 80% acetonitrile in 0.1% trifluoroacetic acid followed by evaporation.

Competitive Enzyme Immunoassay

An enzyme immunoassay procedure was performed as described previously (Isobe et al., 2009) with minor modifications. A 96-well microtiter plate was coated with $2\text{ }\mu\text{g/mL}$ anti-LAP antibody in carbonate buffer (pH 9.7) at 4°C overnight, followed by blocking with 0.05 M borate buffer supplemented with 0.2% BSA at pH 7.8. Evaporated samples were resuspended in the borate buffer and 0.05 mL was pipetted into wells with incubation for 2 h at room temperature. After washing 4 times with PBS, the wells were incubated with 0.05 mL of horseradish peroxidase-labeled LAP for 1 h. The wells were washed with PBS and incubated with 0.15 mL of tetramethyl benzidine solution for 30 min. The optical density was measured at 655 nm. The assay was repeated 3 times.

Western Blotting

Evaporated eluate of the Sep-Pak cartridges was mixed with sample buffer followed by immersion into boiling water as described previously (Yoshimura and Bahr, 1991). The sample (0.01 mg) was electrophoresed on tricine-SDS PAGE gels containing 15% acrylamide (Schagger and von Jagow, 1987), and electroblotted onto nitrocellulose membrane (GE Healthcare, Uppsala, Sweden). For immunodetection, the membrane was blocked with Tris-buffered saline supplemented with 0.5% Triton X-100 and 1% goat serum for 1 h, and cultured with anti-LAP antibody at $1\text{ }\mu\text{g/mL}$ overnight at 4°C . The membrane was cultured with $5\text{ }\mu\text{L/mL}$ anti-rabbit IgG antibody labeled with biotin (Vector Laboratories Inc., Burlingame, CA). The avidin-biotin-complex reaction was then performed using a Vectastain ABC kit (Vector Laboratories Inc.) for visualization of immunoreacted bands using diaminobenzidine solution consisting of 0.02% (wt/vol) 3, 3'-diaminobenzidine tetrahydrochloride and 0.005% (vol/vol) H_2O_2 in 0.05 M Tris-HCl buffer, pH 7.6. Blotting was repeated twice.

Carbohydrate Staining of Milk Peptides

Carbohydrate staining was performed as reported (Cagatay and Hickford, 2008). Immediately following electrophoresis, the gels were washed in 50% (vol/vol) ethanol for 30 min and fixed for 1 to 2 h using a fixation solution (10% acetic acid and 35% methanol). The gels were placed in periodate solution [7 g/L periodic acid (H_5IO_6) in 5% (vol/vol) acetic acid] for 30 min before washing 6 times in distilled water for 5 min per wash. A sodium metabisulfite solution [2 g/L $\text{Na}_2\text{S}_2\text{O}_5$ in 5% acetic acid] was added to the gels and allowed to react for 5 to 10 min. Fresh sodium metabisulfite solution was then added and left for another 5 to 10 min. The gels were placed in Schiff's reagent and incubated in the dark at room temperature until red or magenta bands appeared (2 h). The gels were then washed with 0.1% (wt/vol) $\text{Na}_2\text{S}_2\text{O}_5$ in 10 mM HCl for 6 h in the dark.

LAP Peptide Purification by Anti-LAP Antibody-Coupled Affinity Column

Anti-LAP antibody was purified by the HiTrap *N*-hydroxysuccinimide-activated high-performance affinity column (GE Healthcare) coupled with synthetic peptide (RNSQSCRRNKG; 11 amino acids out of 40 amino acids of mature peptide; Schonwetter et al., 1995) according to the manufacturer's directions. This affinity-purified LAP antibody was further coupled to the HiTrap *N*-hydroxysuccinimide-activated HP affinity column. Decaseinated skim milk was subjected to this affinity column to purify LAP peptide in the milk. Eluted LAP peptide was tested for antimicrobial activity.

Antimicrobial Activity

Antimicrobial activity was examined by 2 methods using *E. coli* (IFO3301); the first was a radial diffusion plate assay as described by Zasloff (1987), and the second was colony-forming unit enumeration after the culture of bacteria with the sample. In the former method, cultured bacteria were added to 10 mL of 0.7% agar in nutrient broth and poured over a 90-mm Petri dish containing 10 mL of nutrient agar (pH 7, 1.5% agar, Eiken Chemical Co., Tokyo, Japan). Antibacterial activity was assayed by suppression of bacterial growth dependent on application of fractions ($2\text{ }\mu\text{L}$) to the top agar surface.

The quantitative antimicrobial activity of the sample was determined as follows. Cultured bacteria were washed with 0.01 M phosphate buffer thrice and resuspended in the same buffer. The cell concentrations were

Figure 1. Competitive enzyme immunoassay of bovine Sep-Pak eluted milk proteins. The control is the buffer without milk. ^{a,b}Different letters indicate significant difference in optical densities between groups ($P < 0.05$).

estimated by measuring the optical density at 600 nm. The suspensions were diluted to 1×10^7 cfu/mL and mixed with the sample, followed by incubation at 37°C for 3 h. Serial 10-fold dilutions of the suspension were performed using phosphate buffer and surface-plated onto nutrient agar in a 90-mm Petri dish. After a 24-h incubation, emerging colonies were counted.

Statistical Analysis

Differences among optical densities in the enzyme immunoassay and ratio of colony-forming units in the treatment to control were analyzed by one-way ANOVA followed by Tukey's multiple range test. A probability of $P < 0.05$ was considered significant.

RESULTS

The eluate from the Sep-Pak cartridges was examined with competitive enzyme immunoassay to confirm the presence of LAP peptide in the milk. Optical density in the eluate was less ($P < 0.05$) compared with the buffer without milk components (Figure 1),

Figure 2. Western blotting with lingual antimicrobial peptide antibody (WB) and periodic acid-Schiff staining (PAS) of bovine milk. Three bands (8, 14, and 17 kDa: arrows) can be seen in the immunoblotting. Two bands (14 and 17 kDa) were positive for periodic acid-Schiff staining.

indicating that immunoreactive peptide was present in the milk. Further, Western blotting analysis showed 3 immunoreactive bands of approximately 8, 14, and 17 kDa (Figure 2). These molecular weights were heavier than the predicted size of mature LAP peptide (4 kDa). Therefore, carbohydrate staining was conducted following PAGE to examine the possibility of glycosylation of the LAP peptide. Two bands of approximately 14 and 17 kDa were found periodic acid-Schiff reaction positive (Figure 2).

The eluate of the Sep-Pak cartridges was applied to an LAP antibody-coupled affinity column to isolate the LAP peptide. Processing 60 mL of milk resulted in the isolation of 62 µg of LAP peptide after affinity purification. Therefore, the average concentration of LAP peptide in the milk sample was 1 µg/mL. The affinity column eluate was examined with competitive enzyme immunoassay. As the eluate was diluted up to 64 times, optical density increased ($P < 0.05$, Figure 3), which meant concentration decreased with greater dilution.

The antimicrobial activity of the affinity eluate was investigated. In the radial diffusion plate assay, the eluate inhibited *E. coli* proliferation (Figure 4A). The quantitative antimicrobial activity method found the colony-forming unit count of eluate without dilution was low, although it increased ($P < 0.05$) by diluting it 2 or 4 times (Figure 4B).

DISCUSSION

After removal of fat and casein, the milk sample was subjected to a competitive enzyme immunoassay to confirm the presence of LAP peptide. The immunoreactive

Figure 3. The competitive enzyme immunoassay of milk affinity purified with a lingual antimicrobial peptide antibody-coupled column. Affinity eluate was diluted 2 to 128 times and assayed ($n = 3$). Bars represent mean $\pm$ SD. ^{a-f}Different letters indicate significant difference in optical densities between groups ($P < 0.05$).

component of LAP antibody was found, suggesting that milk contained LAP peptide. The eluate of the LAP antibody-coupled affinity column showed competitive immunoreaction in the enzyme immunoassay. Further, the antimicrobial activity against *E. coli* was found in the affinity column eluate. These results indicate that functional LAP peptide was found in the bovine milk. Jia et al. (2001) and Armogida et al. (2004) reported that abundant human β -defensin-1 and 2, respectively, were found in human breast milk at concentrations of 1 to 10 $\mu\text{g/mL}$. In the present study, the concentration of LAP peptide (1 $\mu\text{g/mL}$ or more) was similar to that of human β -defensin-1. Human β -defensin-1 and 2 in breast milk and breast tissue have broad-spectrum protective functions for the mother against bacteria (Jia et al., 2001; Armogida et al., 2004). The β -defensin had an impact on neonatal immunity through immunomodulatory effects. It remains to be elucidated whether or not LAP peptide in bovine milk has a similar role.

Immunoreaction in the electroblotting membrane showed 3 bands at 8, 14, and 17 kDa, which were much heavier than that of the predicted size of mature LAP peptide (4 kDa, Schonwetter et al., 1995). One of the possibilities was that the propeptide form of LAP is secreted into the milk rather than the mature counterpart. Canty et al. (2006) found that some proteins such as procollagen may be secreted in the propeptide form. Extracellular secretion occurs when the propeptide is released before the detachment of the signal peptide

Figure 4. Antimicrobial activity of milk protein that has been affinity purified with lingual antimicrobial peptide (LAP) antibody-coupled column. A) Radial diffusion plate assay. Milk LAP: milk contents after affinity purification. Control: phosphate buffer without LAP. B) Examination of colony-forming units. Affinity purified LAP was diluted 1 to 8 times followed by assessment of antimicrobial activity ($n = 3$). Vertical axis expresses ratio of colony-forming units of sample to that of phosphate buffer without LAP. Bars represent mean $\pm$ SD. ^{a,b}Different letters indicate a significant difference in ratios between dilutions ($P < 0.05$).

from the functioning protein (Dommett et al., 2005). The propeptide form of LAP had a molecular weight of 7 to 8 kDa (Schonwetter et al., 1995) that was almost identical to the lowermost band detected in the present study. Alternatively, it was assumed that the LAP was glycosylated because the macaque β -defensin, DEFB126, was reported to include carbohydrate moiety (Yudin et al., 2005). To investigate this possibility, the gel was stained using the periodic acid-Schiff reaction. Positive carbohydrate staining resulted in the 14 and 17 kDa bands, suggesting that 2 kinds of glycosylated

LAP with different molecular weights of 14 and 17 kDa exist in bovine milk.

Mastitis is one of most serious and common diseases in dairy cows. When bacteria invade the mammary glands, many kinds of immune cells and components gather to defend the host organism. The LAP is one of these immune components and exhibited direct action against bacteria. Swanson et al. (2004) found a positive relationship between SCC in milk and LAP mRNA expression and localization of mRNA in epithelial cells of mastitic tissue by in situ hybridization. Expression of LAP peptide in the mammary tissue increased after *E. coli* inoculation (Petzl et al., 2008). The present study found that LAP was present in the milk and has antimicrobial activity. Therefore, it would be interesting to examine whether IMI stimulates the secretion of LAP into milk and what causes its increased concentration in milk. Bovine milk containing functional LAP-like substances that exert antimicrobial activity for human health and the dairy industry has important nutritional and hygienic implications.

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