Identification of milk proteins enhancing the antimicrobial activity of lactoferrin and lactoferricin

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ABSTRACT

Lactoferrin (LF) is known as an iron-binding antimicrobial protein present in exocrine secretions such as milk and releases the potent antimicrobial peptide lactoferricin (LFcin) by hydrolysis with pepsin. The antimicrobial activity of LF and LFcin has been studied well; however, their cooperative action with other milk proteins remains to be elucidated. In this study, we identified milk proteins enhancing the antimicrobial activity of bovine LF and LFcin against gram-negative bacteria, gram-positive bacteria, and fungi. As the target fraction, we isolated a minor milk protein fraction around 15 kDa, which was identified as bovine RNase 5 (angiogenin-1), RNase 4, and angiogenin-2 by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry. As these proteins are collectively known as the RNase A family, we referred to the target protein fraction as milk RNase of 15 kDa (MR15). The number of colony-forming units of *Escherichia coli* and other pathogenic microorganisms with the addition of MR15 to LF (MR15:LF ratio = 16:1,000) was dramatically lowered than that with LF alone. On the other hand, MR15 itself did not show any reductions in the number of colony-forming units at the concentrations tested. Similarly, the antimicrobial activities of LFcin against various microorganisms were significantly enhanced by the addition of MR15. These results suggest that LF and MR15 may be concomitantly acting antimicrobial agents in milk.

Key words: lactoferrin, lactoferricin, milk-derived RNase, antimicrobial activity

INTRODUCTION

Lactoferrin (LF), an iron-binding glycoprotein of the transferrin family, is present in milk, tears, saliva and most other exocrine body fluids. Lactoferrin has been considered to play a host defensive role at mucosal surfaces. Lactoferrin exhibits antimicrobial activity

against *Escherichia coli*, *Streptococcus mutans*, *Candida albicans*, and a variety of other pathogenic microorganisms (Arnold et al., 1980). Lactoferrin has iron-binding capacity and also binds to the outer membrane of gram-negative bacteria, resulting in the release of LPS (Ellison et al., 1988). It has been shown that pepsin-hydrolyzed LF (LF-hyd) has stronger antimicrobial activity than the undigested protein (Bellamy et al., 1992b). A potent antimicrobial peptide named lactoferricin (LFcin) was isolated from LF-hyd (Bellamy et al., 1992a).

Lactoferrin is an innate-defense protein and a component of milk. In previous studies, lysozyme and IgA, components in milk, have been reported to have synergistic effects with LF for growth inhibition of *E. coli* (Rogers and Synge, 1978; Ellison and Giehl, 1991). It is also known that combinations of various antimicrobial drugs (Wakabayashi et al., 1996; Wakabayashi et al., 2002) and antimicrobial peptides, including nisin (Murdock et al., 2007) and β -defensin 2 (Bals et al., 1998), enhance the antimicrobial activity of LF in a synergistic manner. To date, a large number of minor proteins in bovine milk have been identified by proteomic methods (Smolenski et al., 2007); however, the interactions among such minor milk proteins and their roles in the host-defensive effect have not been elucidated in detail. As previous studies suggested that some agents can act as enhancers of antimicrobial activity of LF, we investigated whether minor components of bovine milk have any influence on the antimicrobial activity of LF.

MATERIALS AND METHODS

Sample Preparation

Preparation of LF, LF Pepsin Hydrolysate, and LFcin. Bovine LF was purified from fresh skim milk by cation-exchange chromatography. The hydrolysate of bovine LF (LF-hyd) and the antimicrobial peptide LFcin B were produced by methods reported previously (Tomita et al., 2002). Briefly, LF-hyd was produced by digestion of LF with porcine pepsin. The LFcin B was isolated from LF-hyd and validated by reverse-phase HPLC.

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Isolation of Milk Protein Fractions Using Cation-Exchange Chromatography. Fresh skim milk was stored at 4°C until use. The target protein fraction was prepared by cation-exchange chromatography by the following procedure: skim milk was incubated in a rotating shaker with CM Sephadex C-50 resin (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) at 4°C. The CM Sephadex C-50 resin was washed with deionized water, which was followed by elution with 0.27 M NaCl and 1.7 M NaCl in series, to obtain protein fractions.

Membrane Separation of Target Protein Fractions. The eluate from the cation-exchange resin was adjusted with 0.5 M NaCl solution and permeated by centrifugal ultrafiltration of molecular weight cut-off (MWCO) 30,000 (Amicon Ultra 30K; Millipore, Merck, Darmstadt, Germany). Then, the permeate from ultrafiltration of MWCO 30,000 was desalted and concentrated using centrifugal ultrafiltration of MWCO 10,000 (Amicon Ultra 10K; Millipore, Merck) with the addition of deionized water. Concentrations of protein fractions were determined by measuring the absorbance at 280 nm with a spectrophotometer. Fractions were collected and were characterized by SDS-PAGE and mass spectrometry, and tested for antimicrobial activity.

Gel Filtration Chromatography. The eluate of the cation-exchange resin was dissolved in 20 mM citrate buffer (0.5 M NaCl, pH 5.2) at 5 mg/mL. This solution was applied to the TSKgel filtration column (TSKgel 2000SW; Tosoh Corp., Tokyo, Japan) connected to an HPLC system (Hitachi, Ibaraki, Japan), including an L-5200 Intelligent Pump, L-4000 UV detector, and D-2500 Chromato-Integrator. HPLC separation was performed using 20 mM citrate buffer (0.5 M NaCl, pH 5.2) at a flow rate of 3.0 mL/min.

Analytical Methods of the Protein Fraction

SDS-PAGE. The membrane-separated protein fraction was analyzed by SDS-PAGE. The protein fraction from ultrafiltration between MWCO 30,000 and 10,000 was diluted with NuPAGE lithium dodecyl sulfate (LDS) sample buffer (Invitrogen, Life Technologies Corp., Carlsbad, CA) containing β -mercaptoethanol and was heated in a block incubator (BI-525A; Astec Co. Ltd., Fukuoka, Japan) at 70°C for 10 min; NuPAGE 4 to 12% Bis-Tris gel (Invitrogen) and 3-(N-morpholino)propanesulfonic acid (MOPS) or 2-(N-morpholino)ethanesulfonic acid (MES) running buffer (Invitrogen) were used. Electrophoresis was performed at 200 V for 45 min. Imaging of protein bands on gels stained with SimplyBlue SafeStain (Invitrogen) was captured by a Densitograph AE-6920 image sensor (Atto Corp., To-

kyo, Japan) and analyzed by a Lane and Spot Analyzer version 6.0 (Atto Corp.).

Two-Dimensional SDS-PAGE. The membrane-separated protein fraction was analyzed by 2-dimensional SDS-PAGE (2-dimensional gel electrophoresis, **2D-GE**). For 2D-GE, a ZOOM IPGRunner system (Invitrogen) was used. The target fraction sample was separated based on the intrinsic charge as the first dimension by isoelectric focusing with rehydrated immobilized pH gradient ZOOM strips pH 3 to 10. Then, proteins were separated based on mass as the second dimension using NuPAGE Novex 4 to 12% Bis-Tris ZOOM gels and MOPS running buffer. The 2D-GE was performed at 200 V for 35 to 50 min. Protein bands on gels were stained with SimplyBlue SafeStain (Invitrogen) and images were captured in the same manner as SDS-PAGE.

Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight Mass Spectrometry. Mass spectrometric analysis of target proteins was performed using a matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (**MALDI-TOF MS**) autoflex II instrument (Bruker Daltonics Inc., Billerica, MA). Visible protein spots were excised from SDS-PAGE or 2-dimensional SDS-PAGE gels and destained. Destained gel pieces were processed to in-gel protein digestion using trypsin. Peptides were extracted from gel pieces and desalted by ZipTip C18 pipette tips (Millipore, Merck). The purified peptide solution was spotted onto a MALDI sample plate. Then, MALDI-TOF MS was performed and the peaks of the peptides were detected using the MALDI-TOF instrument.

Protein identification was carried out using the MALDI-TOF mass spectral data matching search engine Mascot (Matrix Science Ltd., London, UK). The MS data were searched against the National Center for Biotechnology Information (NCBI, Bethesda, MD) database. Protein matches were considered to be valid if there were at least 6 matched peptides with Mascot scores summing greater than 67 ($P < 0.05$).

Antimicrobial Testing

Bacteria Strains and Culture. For gram-negative bacteria, the following 6 strains were used: *E. coli* K-12 [NITE Biological Resource Center (NBRC) 3301], *E. coli* O111, *Cronobacter sakazakii* American Type Culture Collection (ATCC) 12868, *Salmonella enteritidis* IID 604, *Klebsiella pneumoniae* JCM 1662T, and *Pseudomonas aeruginosa* MMI 603. For gram-positive bacteria, the following 2 strains were used: *Staphylococcus aureus* JCM 2151, and *Strep. mutans* JCM 5705T. For fungi, *C. albicans* TIMM 1768 was used. The following bacteria were incubated in 1% Bacto Peptone

broth (Becton, Dickinson and Co., Franklin Lakes, NJ): *E. coli* K-12, *E. coli* O111, *Cronobacter sakazakii* ATCC 12868, *Salmonella enteritidis* IID604, *Klebsiella pneumoniae* JCM 1662T, and *Pseudomonas aeruginosa* MMI 603. *Strep. mutans* JCM 5705T was incubated in PYG broth containing 1% Bacto Peptone, 1% glucose (Wako Pure Chemical Industries Ltd., Osaka, Japan), and 0.05% yeast extract (Becton, Dickinson and Co.). *Candida albicans* TIMM 1768 was incubated in Sabouraud broth containing 1% Bacto Peptone, and 2% glucose (Wako Pure Chemical Industries Ltd.).

Effect of the Target Protein Fraction on Antimicrobial Activity of LF, LF-hyd, and LFcIn. To determine the antimicrobial activity, we conducted 2 types of tests. For the colony-forming units assay, about 10^6 viable cells/mL of *E. coli* K-12 preincubated for 6 h at 37°C were used as an inoculum. Test bacteria were incubated in 1% Bacto Peptone broth for indicated time at 37°C in the presence of LF (2,000 µg/mL), LF-hyd (400 µg/mL), or LFcIn B (8 µg/mL) with target protein fractions. The control culture contained all components except for test proteins or peptides. To determine the number of colony-forming units, serial 10-fold dilutions were prepared in 0.85% NaCl and plated onto Rapid Media RM-SPC agar plates (Morinaga Milk Industry Co. Ltd., Tokyo, Japan). After incubation of the plates for 16 h, the number of colony-forming units was determined. The detection limit was set to 250 cfu/mL. Each sample was tested in triplicate. To determine the MIC, 2-fold serial dilutions of LF and LFcIn B were tested. About 10^6 viable cells/mL of test bacteria were used as an inoculum. The test bacteria, except for *Strep. mutans* and *C. albicans*, were incubated in 1% Bacto Peptone broth at 37°C for the indicated time in the presence of LF, LF-hyd, or LFcIn B, with target protein fractions in a 96-well microtiter plate (Becton, Dickinson and Co.). For *Strep. mutans*, PYG broth was used as the test medium. For *C. albicans*, Sabouraud broth was used as the test medium. The control culture contained all components except for test proteins or peptides. After incubation for the indicated time, the MIC was determined by measuring the absorbance at 630 nm by a microplate reader (Corona Electric Co. Ltd., Ibaraki, Japan). The MIC was determined as the lowest concentration of target protein and LF or LFcIn B to inhibit the growth of the test bacteria to 50% of control.

Statistical Analysis

The colony-forming unit data were expressed as mean $\pm$ standard deviation. The Student's *t*-test was used for intergroup comparisons of antimicrobial activity and *P* < 0.05 was regarded to be significantly different.

RESULTS

Isolation of the Target Protein Fraction

Our preliminary experimental results indicated that the crude milk-protein fraction from cation-exchange chromatography, containing basic proteins such as LF and others along with a minute amount of proteins around 15 kDa (Figure 1, lane 1), exhibited a markedly higher antimicrobial activity against *E. coli* than that with highly purified LF. Therefore, we noted this protein fraction around 15 kDa as the target. Using membrane separation, we separated and concentrated the target fraction with a molecular size between 10 and 30 kDa from the crude cation chromatography eluate. The purity of the target protein fraction was determined by SDS-PAGE. After the purification procedure, the target protein around 15 kDa (Figure 1, lane 3) was separated from the higher molecular protein around 80 kDa, which was mainly LF (Figure 1, lane 2). The purity of the target protein fraction around 15 kDa was >98%.

Effects of the Target Protein Fraction on the Antimicrobial Activity of LF-Related Compounds Against *E. coli* K-12

First, the antimicrobial activity of LF against *E. coli* K-12 was evaluated in the presence or absence of the target protein fraction around 15 kDa by colony-forming unit tests (Figure 2 a). After incubation at 37°C for 17 h, *E. coli* K-12 was grown from 10^6 to 10^8 cfu/mL in the control culture. The addition of LF (2,000 µg/mL) alone decreased the number of colony-forming units of *E. coli* K-12 to about 10^6 cfu/mL, which is the bacteriostatic effect reported in previous studies. Furthermore, the addition of the target protein fraction around 15 kDa (32 µg/mL) to LF (2,000 µg/mL) dramatically decreased the number of colony-forming units of *E. coli* K-12 to less than 250 cfu/mL (detection limit; *P* < 0.05 for target protein addition to LF vs. LF alone). On the other hand, the addition of the target protein fraction (32 µg/mL) alone did not cause any reductions in the number of colony-forming units after 17 h of incubation. Similarly, antimicrobial activity tests using LF-hyd and LFcIn B were performed. In the LF-hyd test, the addition of LF-hyd (400 µg/mL) alone decreased the number of colony-forming units of *E. coli* K-12 to 10^5 cfu/mL after 17 h of incubation (Figure 2b). The addition of the target protein fraction around 15 kDa (32 µg/mL) to LF-hyd (400 µg/mL) clearly decreased the number of colony-forming units of *E. coli* K-12 to less than 250 cfu/mL (detection limit; *P* < 0.05 for target protein addition to LF-hyd vs. LF-hyd alone). The

Figure 1. Sodium dodecyl sulfate PAGE analysis of fractions during different stages of purification of the target protein fraction around 15 kDa from bovine milk. Lane 1 = the initial eluate fraction including the target protein around 15 kDa after cation chromatography; lane 2 = retentate of UF molecular weight cutoff (MWCO) 30,000; lane 3 = the target protein-enriched fraction (permeate of UF MWCO 30,000); M = molecular weight markers.

target protein fraction also showed an enhancing effect on the antimicrobial activity of LFCin B ($P < 0.05$ for target protein addition to LFCin B vs. LFCin B alone), the same as for LF and LF-hyd (Figure 2c).

Identification of Proteins Enhancing the Antimicrobial Activity of LF-Related Compounds

To analyze the composition of the target protein fraction, the eluate fraction including the target protein of 15 kDa was separated by 2D-GE and proteins were identified using MALDI-TOF MS. A proteomic map of the eluate fraction is shown in Figure 3, with accompanying data on protein identification given in Table 1. The stained 2D-GE gel gave a pattern of spots with clearly dominant proteins/peptides in relatively lower mass and high isoelectric point (pI) regions of the gel. The molecular weight distribution at pI 9 (vertical line) was accounted for by densitometric analysis of these spots. The minute amount of the 40-kDa spot (1.4%), 30-kDa spot (12%), 17-kDa spot (8.4%), and an abundance of 14- to 15-kDa spots (78%) were also observed (data not shown).

As shown in Table 1, MALDI-TOF MS spectra of these spots revealed that proteins of 15 and 30 kDa were bovine RNase 5, the protein of 14 kDa was bovine

RNase 4, and the protein of 17 kDa was bovine angiogenin (ANG)-2. Several other lower-molecular weight forms of RNase-5 were also identified in the basic pI range, although at very low levels (Figure 3). A trace amount of an 80-kDa spot (1.4% of total protein) was identified as bovine LF. As these proteins, including RNase 5, RNase 4, and ANG-2, are known as milk-RNases belonging to RNase A family, we referred to the target protein fraction as milk RNase of 15 kDa (MR15).

The Effects of Further Purified Milk RNases on the Antimicrobial Activity of LF

To validate that each milk RNase identified from the target fraction has an antimicrobial activity-enhancing effect, we conducted isolation of RNase 5, RNase 4, and ANG-2. Using gel filtration chromatography, 3 subfractions, including F1 (RNase 5 + ANG-2), F2 (RNase 5), and F3 (RNase 4) to LF were separated and confirmed by SDS-PAGE and MALDI-TOF MS analysis (Figure 4).

The antimicrobial activity-enhancing effect of each of RNase 5, RNase 4, and RNase 5 + ANG-2 was confirmed by combining with LF, using the same method as for the whole fraction described above. As shown in Figure 5, the 3 isolated subfractions [F1 (RNase 5 + ANG-2), F2 (RNase 5), and F3 (RNase 4)] themselves did not show any reductions in the number of colony-forming units after 17 h of incubation. Addition of each isolated subfraction [F1 (RNase 5 + ANG-2), F2 (RNase 5), F3 (RNase 4)] provided significant reductions in the number of colony-forming units relative to LF alone ($P < 0.05$). However, the reduction rates of the number of colony-forming units by LF with these subfractions (F1, F2, and F3) and the mixed fractions (F1 + F3 and F2 + F3) were relatively weaker (3.5 to 4.5 log₁₀ cfu/mL reduction) than that by LF with the whole MR15 fraction (shown in Figure 2a; 5.4 log₁₀ cfu/mL reduction).

Antimicrobial Activity of LF and LFCin B with MR15 Against Various Pathogens

Because the antimicrobial activity of LF-related compounds (LF, LF-hyd, and LFCin B) against *E. coli* K-12 was potently enhanced, we performed MIC tests against other various microorganisms. As shown in Tables 2 and 3, the growth of all microorganisms tested was not inhibited by the addition of MR15 (32 µg/mL) alone. With the addition of MR15 against all microorganisms tested, MIC of LF were reduced 2-fold to 16-fold (Table 2). In the same manner as LF, the MIC of LFCin B were also reduced 2-fold to 4-fold when

Figure 2. Effect of the addition of the target protein around 15 kDa on the antimicrobial activity of lactoferrin (LF; a), pepsin-hydrolyzed LF (LF-hyd; b), or lactoferricin B (LFcin B; c) against *Escherichia coli* K-12. The antimicrobial activity of LF (2,000 μg/mL), LF-hyd (400 μg/mL), and LFcin B (8 μg/mL) were determined by plating cells after incubation for 17 h and counting colonies on the agar plate. Results are means of 3 tests per condition, with standard deviations denoted by error bars. * $P < 0.05$ (significantly different for the results for control, LF, LF-hyd, or LFcin B). Dotted lines indicate the detection limit (250 cfu/mL).

used together with MR15 (32 μg/mL) against all species tested (Table 3).

DISCUSSION

In previous animal and human studies, oral administration of LF alone was shown to improve intestinal bacterial flora (Roberts et al., 1992; Teraguchi et al., 1995a,b). Recently, it has become evident that oral administration of LF exerts various beneficial health effects such as antiinfective activities not only in infants,

but also in adult animals and humans (Wakabayashi et al., 2006; Tomita et al., 2009).

In this study, we isolated a protein fraction around 15 kDa, which enhanced the antimicrobial activity of LF, from bovine milk by cation-exchange chromatography and ultrafiltration. Proteins around 15 kDa in this fraction were identified as bovine RNases, including RNase 4, RNase 5 (ANG-1), and ANG-2, reported as the minor components of bovine milk. These proteins belong to the RNase A family and we referred to the protein fraction as milk RNase of 15 kDa (MR15). Further-

Table 1. Results of the identification of the protein fraction by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) analysis

Source of organism	Molecular weight (kDa)	Protein	Mascot protein score ¹	Number of mass values matched	pI ²
<i>Bos taurus</i>	40	Bovine lactoferrin	96	11	8.52
<i>Bos taurus</i>	17	Bovine angiogenin-2 (ANG-2)	73	6	9.97
<i>Bos taurus</i>	15	Bovine angiogenin-1 (RNase 5)	140	15	9.10
<i>Bos taurus</i>	14	Bovine RNase 4	102	9	9.18

¹Protein scores greater than 67 are significant ($P < 0.05$). Scores were determined using the MALDI-TOF mass spectral data matching search engine Mascot (Matrix Science Ltd., London, UK).
²Isoelectric point.

more, our results indicated that MR15 could enhance the antimicrobial activity of LF-hyd and LFcin B in a manner similar to LF.

As shown in Figure 5, the 3 subfractions of MR15 isolated by gel filtration chromatography, including F1 (RNase 5 + ANG-2), F2 (RNase 5), and F3 (RNase 4), enhanced the antimicrobial activity of LF. However, the reduction rate of the number of colony-forming units by LF with these subfractions was relatively weaker than that by LF with the whole MR15 fraction before gel filtration (Figure 2a). Moreover, when the subfractions of MR15 were remixed again (F1 + F3 and F2 + F3), the antimicrobial activity enhancing effect did not recover to the level before isolation. A similar result was also obtained using F1 + F2 + F3 in another test (data not shown), which means that the antimicrobial activity-enhancing effect of MR15 is fully exerted when applied

as a whole MR15 fraction, but not as a subfraction. For this reason, it is possible that the irreversible complex structure of these components is important in the action of MR15. Analysis of physicochemical features, for example protein-protein interaction among MR15 subfraction proteins or between MR15 and LF, may offer some insights to understanding the mechanisms of action.

Recently, the biological role of RNases, some of them described as ANG, has been attracting attention (Ganz, 2003; Rosenberg, 2008). Many RNase A family proteins, such as eosinophil cationic protein (Rosenberg and Domachowske, 2001) and RNase 7 (Harder and Schröder, 2002; Huang et al., 2006), are highly cationic and are considered to have antiinfective properties. Hooper et al. (2003) found that mouse and human ANG (RNase 5), but not bovine RNase 5, displayed antimicrobial activity, especially against *C. albicans*.

Ribonuclease 5 was originally isolated from the human colon adenocarcinoma cell line HT-29 for its angiogenic activity, and so has been termed ANG (Fett et al., 1985). The presence of proteins with RNase activity in bovine milk was first reported in 1988 (Maes et al., 1988), hereafter referred to as RNase 5. Bovine ANG-2, whose RNase activity, amino acid sequence, and glycosylation profile differ from those of bovine

Figure 3. Two-dimensional gel electrophoresis (2D-GE) analysis of the target protein fraction around 15 kDa prepared by ultrafiltration of a cation-exchange eluate fraction of bovine milk. Arrows of each spot indicate proteins identified by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) and the National Centre for Biotechnology Information (NCBI, Bethesda, MD) protein database using Mascot search software (Matrix Science Ltd., London, UK). RNase 5 = angiogenin-1; ANG-2 = angiogenin-2; M = molecular weight markers.

Figure 4. Sodium dodecyl sulfate PAGE analysis of milk RNase of 15 kDa (MR15) subfractions separated by gel filtration chromatography. M = molecular weight markers; lane 1 = F1 [angiogenin-2 (ANG-2) + RNase 5]; lane 2 = F2 (RNase 5); lane 3 = F3 (RNase 4).

Table 2. Minimum inhibitory concentrations of lactoferrin (LF) and milk RNase of 15 kDa (MR15) alone or in combination against various microorganisms

Test microorganisms	MIC ¹ of LF (μg/mL)		
	LF alone	With MR15 (32 μg/mL)	MIC ¹ of MR15 (μg/mL)
Gram-negative bacteria			
<i>Escherichia coli</i> O111	2,000	1,000	>32
<i>Cronobacter sakazakii</i> ATCC 12868	8,000	1,000	>32
<i>Salmonella enteritidis</i> IID 604	8,000	1,000	>32
<i>Klebsiella pneumoniae</i> JCM 1662T	8,000	1,000	>32
<i>Pseudomonas aeruginosa</i> MMI 603	8,000	1,000	>32
Gram-positive bacteria			
<i>Staphylococcus aureus</i> JCM 2151	500	125	>32
<i>Streptococcus mutans</i> JCM 5705T	8,000	500	>32
Fungi			
<i>Candida albicans</i> TIMM 1768	1,000	125	>32

¹The MIC resulting in a 50% suppression of growth, as measured by absorbance at 630 nm. Results were confirmed in 2 tests.

RNase 5, was also isolated from serum and milk in 1997 (Strydom et al., 1997). However, the inherent roles of these RNases in milk have not been clearly understood for a long time. More recently, Harris and colleagues have reported that RNase 5 isolated from bovine milk suppressed the growth of *C. albicans*, but not any other microorganisms tested, including *E. coli*, *Enterococcus faecalis*, *Streptococcus pneumoniae*, and *Pseudomonas aeruginosa* (Harris et al., 2010). Harris et al (2010) concluded that the concentrations of RNase 5 required to suppress the growth of *C. albicans* (MIC: 55 μM = 825 μg/mL) was higher than that naturally found in milk (3–19 μg/mL). In the same study, the authors demonstrated for the first time the presence of RNase 4 in bovine milk and concluded that RNase 4 did not suppress the growth of any of the microorganisms tested.

Interestingly, a substantial number of antimicrobial proteins are known to contain basic amino acid-rich sequences. As referred to above, all components of MR15 belong to the RNase A superfamily of proteins, recognized for their RNase activity and highly basic properties (pI >9.0). The mechanism of action of MR15 has still been unknown; however, it is possible that the electrostatic interaction between MR15 and the bacterial membrane may contribute to strengthen the antimicrobial activity of LF. Further study is needed to elucidate whether RNase activity or any other biochemical activity is essential for the antimicrobial activity-enhancing effect of MR15 toward LF.

CONCLUSIONS

This is the first report to provide new insight concerning the cooperative action of LF and milk-derived RNases, and the biological importance of their coexistence in milk. The killing activity against a wide range

of pathogens of both intact LF and some digested LF (LF-hyd) may be enhanced by their administration with MR15. We suggest that MR15 may have an important role in host defense by acting together with LF, especially in the case of newborn infants. In the future, we would like to study the synergistic effects of ingestion containing MR15 and LF or LF-hyd on

Figure 5. Effect of the addition of separated milk RNase of 15 kDa (MR15) subfractions on the antimicrobial activity of lactoferrin (LF) against *Escherichia coli* K-12. The antimicrobial activity of LF, F1 (angiogenin-2 + RNase 5), F2 (RNase 5), F3 (RNase 4), and their combinations was determined by plating cells after incubation for 17 h and counting colonies on the agar plate. Results are means of 3 tests, with standard deviations denoted by error bars. * $P < 0.05$ (vs. LF).

Table 3. Minimum inhibitory concentrations of lactoferricin B (LFcin B) and milk RNase of 15 kDa (MR15) alone or in combination against various microorganisms

Test microorganisms	MIC ¹ of LFcin B (µg/mL)		
	LFcin B alone	With MR15 (32 µg/mL)	MIC ¹ of MR15 (µg/mL)
Gram-negative bacteria			
<i>Escherichia coli</i> O111	8	4	>32
<i>Cronobacter sakazakii</i> ATCC 12868	32	16	>32
<i>Klebsiella pneumoniae</i> JCM 1662T	32	8	>32
<i>Pseudomonas aeruginosa</i> MMI 603	32	16	>32
Gram-positive bacteria			
<i>Staphylococcus aureus</i> JCM 2151	8	4	>32
<i>Streptococcus mutans</i> JCM 5705T	32	8	>32
Fungi			
<i>Candida albicans</i> TIMM 1768	>32	32	>32

¹The MIC resulting in a 50% suppression of growth, as measured by absorbance at 630 nm. Results were confirmed in 2 tests.

protection against infectious diseases and improvement of intestinal flora.

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