Proteomic and peptidomic study of proteolysis in quarter milk after infusion with lipoteichoic acid from *Staphylococcus aureus*

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ABSTRACT

Mastitic milk is associated with increased bovine protease activity, such as that from plasmin and somatic cell enzymes, which cause proteolysis of the caseins and may reduce cheese yield and quality. The aim of this work was to characterize the peptide profile resulting from proteolysis in a model mastitis system and to identify the proteases responsible. One quarter of each of 2 cows (A and B) was infused with lipoteichoic acid from *Staphylococcus aureus*. The somatic cell counts of the infused quarters reached a peak 6 h after infusion, whereas plasmin activity of those quarters also increased, reaching a peak after 48 and 12 h for cow A and B, respectively. Urea-polyacrylamide gel electrophoretograms of milk samples of cow A and B obtained at different time points after infusion and incubated for up to 7 d showed almost full hydrolysis of β - and α_{S1} -casein during incubation of milk samples at peak somatic cell counts, with that of β -casein being faster than that of α_{S1} -casein. Two-dimensional gel electrophoretograms of milk 6 h after infusion with the toxin confirmed hydrolysis of β - and α_{S1} -casein and the appearance of lower-molecular-weight products. Peptides were subsequently separated by reversed-phase HPLC and handmade nanoscale C_{18} columns, and identified by matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry. Twenty different peptides were identified and shown to originate from α_{S1} - and β -casein. Plasmin, cathepsin B and D, elastase, and amino- and carboxypeptidases were suggested as possible responsible proteases based on the peptide cleavage sites. The presumptive activity of amino- and carboxypeptidases is surprising and may indicate the activity of cathepsin H, which has not been reported in milk previously.

Key words: bovine milk, peptidomics, proteolysis, somatic cell count

INTRODUCTION

Mastitis is a major disease in dairy cattle that causes significant economical losses due to decreased milk production, increased veterinary costs, and discarded milk (Everson, 1984; Windig et al., 2005). Mastitis involves inflammation of the mammary gland resulting from the introduction and multiplication of pathogenic microorganisms in the mammary gland. Causative pathogens include *Escherichia coli* and *Staphylococcus aureus* (Heringstad et al., 2000; Bradley, 2002). The invasion of bacteria into the mammary gland and the bacteria's multiplication induce the action of the innate mammary gland immune defense system. Somatic cells such as polymorphonuclear leucocytes (PMN) are one of the major cellular immune components that respond to infection; these cells migrate to the site of infection to kill bacteria, resulting in a rapid increase in milk SCC. Milk SCC is thus used to assess inflammation, the health status of the mammary gland, and milk quality.

Polymorphonuclear leucocytes and macrophages, another somatic cell type, respond to bacteria or bacterial products by secreting various substances that mediate the response to the infection (Pugin, 1994; Le Roux et al., 2003; Haddadi et al., 2006). For example, cytokines disrupt the tight junctions between the secretory epithelial cells, which results in increased levels of the principal proteolytic enzyme in milk, plasmin, and its zymogen, plasminogen, in the infected glands. The plasmin system has been widely investigated in normal and mastitic milk (Andrews and Alichanidis, 1983; Pugin, 1994).

Proteases in milk may potentially arise from different sources, for example, secretion into milk from the mammary epithelium, transfer from blood to milk due to a compromised barrier between milk and blood at mastitis, or secretion or leakage from invading microorganisms or from bovine somatic cells (Politis et al., 1989a,b; Haddadi et al., 2006; Larsen et al., 2006). Furthermore, some bacteria secrete activators of protease zymogens, which, in synergy with bovine proenzymes, can increase the proteolytic potential of fresh and stored milk (Jackson et al., 1981; Leigh, 1994; Larson et

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al., 2006). Plasmin is transported into milk from blood as plasminogen and then activated, whereas other proteases in milk are thought to be released from somatic cells. Both neutrophils and macrophages contain active lysosomal acid and neutral proteases, such as cathepsins, procathepsins, and elastase (and protease zymogens; Kirschke and Barrett, 1987; Travis, 1988, Sordillo et al., 1997; Kelly et al., 2006).

Proteolysis of casein thus increases in bovine milk during elevated SCC and mastitis (de Rahm and Andrews, 1982; Andrews, 1983), due both to a direct increase in the level of proteases present (Larsen et al., 2006) and, in some instances, to increased activation of zymogens of proteases, leading to increased enzymatic activities (e.g., of plasminogen; Heegaard et al., 1994). Increased proteolytic activity of milk then decreases levels of casein (Le Roux et al., 1995). Whereas plasmin is the most important protease in milk from healthy cows, nonplasmin proteases become more important with the increasing severity of udder inflammation. Proteolysis of the CN leads to decreases in CN proportions, especially β -CN and α_{S1} -CN, with simultaneous clear increases in γ -CN and proteose peptone levels (Fox and Kelly, 2006). The dairy industry has particular challenges in processing high SCC milk as the proteolysis of casein results, for example, in decreased cheese yields, off-flavor development, and decreased shelf life of dairy products (Ali et al., 1980; de Rahm and Andrews, 1982). Knowledge of the proteases responsible for proteolysis in milk is important as it enables the identification of the enzyme systems that are most detrimental to milk protein quality.

In addition to the characterization of proteases and measurement of enzyme activities, new peptidomic methods using mass spectrometry are now available, by which the actual result of these proteolytic activities in specific milk samples can be studied and characterized. In an earlier study, the peptide profile of 3 different milk types, a normal milk sample, milk with medium SCC (about 500,000 cells/mL), and milk obtained during clinical mastitis after infection with *Streptococcus uberis*, was studied using liquid chromatography–matrix-assisted laser desorption/ionization (LC-MALDI) spotting and MS detection of peptides (Wedholm et al., 2008). By these methods, a range of peptides corresponding to CN fragments were identified and, by consulting the AA sequences of CN, cleavage sites were identified and on this basis some potentially responsible proteases were suggested based on earlier reported specificities.

In the absence of bacteria, model infections can be induced by using bacterial antigens to bring about experimental mastitis. In previous studies, cows were infused with gram-negative LPS of *E. coli* (Andrews,

1983; Moussaoui et al., 2002, 2004; Sládek et al., 2002; Sohn et al., 2007). Another model system uses the bacterial lipoteichoic acid (LTA) of gram-positive *Staph. aureus* to initiate mastitis (Strandberg et al., 2005). In this study, the inflammatory response of mid-lactation cows to *Staph. aureus* LTA was investigated. The present study is a further development of this approach, reporting a proteomic and peptidomic study of bovine milk from cows during early immunological response to infusion with bacterial LTA, thereby eliminating the contribution from mastitis bacteria themselves to the proteolytic cascade.

MATERIALS AND METHODS

Materials

Acetic acid was purchased from J. T. Baker (Deventer, Holland), acetonitrile from Rathburn Chemicals (Walkerburn, UK), and trifluoroacetic acid (TFA) from Merck (Darmstadt, Germany). Water was purified using a Milli-Q Gradient system (Millipore, Bedford, MA). Buffer solutions for HPLC were filtered through a 0.45- μ m Magna nylon filter from Frisette (Ebeltoft, Denmark) before use. Fluorescamine (Fluram) was from Fluka (Sigma-Aldrich, Copenhagen, Denmark). Leucine standard and the matrix α -cyano-4-hydroxycinnamic acid were from Sigma-Aldrich. Column material (C18) for batch elution of peptides was from PerSeptive Biosystems (Framingham, MA). The peptide calibration standard, 1,000 to 4,000 Da (PepMix), used for calibration of the MALDI-time of flight (TOF) MS/MS and the MALDI AnchorChip target plate was from Bruker Daltonics GmbH (Bremen, Germany).

Sample Preparation

Immediately after the morning milking, 2 dairy cows were infused in 1 quarter each with 10 μ g of LTA from *Staph. aureus* that caused mastitis (Yang et al. 2008), diluted in 10 mL of saline (9 g/L). One control quarter received only saline. Milk samples (300 mL) were collected from the gland to be infused and from the control gland before infusion ($t = 0$), and after 6, 12, 48, and 144 h; samples were obtained by hand milking during routine milking procedures. Somatic cell count measurements were performed with a DeLaval cell counter (Tumba, Sweden). Whole milk samples used for subsequent studies were frozen (-18°C) until analysis. Before analysis, milk samples were thawed and skim milk was prepared by centrifugation ($3,000 \times g$, 15 min, 4°C) with a Heraeus multifuge 3 S-R (Kendro Laboratory, Albertslund, Denmark). After removal of the fat layer, the skim milk was centrifuged again to remove fat remnants.

Determination of Plasmin Activity

Milk samples were mixed 3:1 with 0.4 M trisodium citrate (Richardson and Pearce, 1981) and centrifuged at $15,800 \times g$ for 30 min before assaying the supernatant for plasmin activity. Plasmin activity in milk was determined as described by Richardson and Pearce (1981) using the substrate *N*-succinyl-L-alanyl-L-phenylalanyl-L-lysyl-7-amido-4-methyl coumarin (Sigma Chemical Co., St. Louis, MO), from which plasmin produces 7-amido-4-methylcoumarin (AMC). Plasmin activity was expressed as nanomoles of 7-amido-4-methylcoumarin released/min per milliliter of milk.

Assessment of Proteolysis During Storage

Milk samples were incubated at 37°C for 0, 1, 3, or 7 d, with sodium azide (0.5 g/L) to prevent microbial growth. Milk samples were then mixed 1:1 with sample buffer and incubated at 40°C for 5 min. Urea-PAGE of milk samples was then performed using a Protean II xi vertical slab gel unit (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) with a separating and stacking gel system according to the method of Andrews (1983). Gels were prerun at 280 V for 30 min before sample application, and samples were run at 300 V. Gels were stained using 0.1% (wt/vol) Coomassie Brilliant Blue R 250 in a 5:1:4 mixture of methanol, acetic acid, and deionized water and destained in a 7:5:88 mixture of methanol, acetic acid, and deionized water until the gels became clear. Gels were then scanned using a GS-800 calibrated densitometer (Bio-Rad Laboratories Ltd.).

Two-Dimensional Electrophoresis

Isoelectric focusing was carried out with a Protean IEF cell (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. The milk protein samples (40 μ L) were mixed with a rehydration buffer (8 M urea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 50 mM dithiothreitol, 0.2% Bio-Lyte 3/10 ampholyte, and 0.001% bromophenol blue). Mixed samples (125 μ L) were loaded onto immobilized pH gradient strips (pH 3–10, 7 cm; Bio-Rad), which were then passively rehydrated for 8 h, followed by active rehydration overnight for 8 h. The immobilized pH gradient strips were then focused at 20°C until 20,000 kV was reached. After focusing, the strips were immediately used for second-dimension SDS-PAGE. The SDS-PAGE was carried out under reducing conditions using 15% acrylamide in a Criterion Dodeca cell unit (Bio-Rad). Prior to SDS-PAGE, the focused immobilized pH gradient strips were equilibrated with 50 mM Tris HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS,

and 130 mM dithiothreitol and alkylated in the same buffer containing 130 mM iodoacetamide instead of dithiothreitol. The gels were run at 200 V for 50 min, stained using colloidal Coomassie blue (Chevalier et al., 2004), and images from stained gels were digitized at 300 dpi with a GS-800 calibrated densitometer (Bio-Rad Laboratories Ltd.).

Preparation of Peptide-Enriched Acid Whey Fractions and Fluorescamine Assay

Acid whey fractions were prepared by acidification with 30 to 35 μ L of 10% acetic acid/mL of skim milk sample to pH 4.6. An equal volume of 1 M sodium acetate was then added to maintain the pH at 4.6 and centrifugation was carried out at $1,500 \times g$ for 10 min at 5°C to separate the whey fraction from the CN. The whey fraction (500 μ L) was further ultrafiltered under centrifugation, at $10,000 \times g$ for 45 min at 10°C, in Micron YM-10 filter units (Millipore) with a molecular cut-off value of 10,000 Da. Aliquots of ultrafiltered acid whey samples were stored at -18°C until further analysis. The levels of free amino terminals in the peptide-enriched acid whey fractions were determined by the fluorescent probe, fluorescamine, using the method described by Larsen et al. (2004). The fluorescence values were expressed as millimolar leucine equivalents based on a standard curve of leucine (0.5 to 1.5 mM) dissolved in 1 mM HCl.

Separation of Peptides by Capillary RP-HPLC

An Agilent 1100 Series capillary HPLC system (Agilent Technologies 2000, Waldbronn, Germany), consisting of a capillary pump, autosampler, valve, micro-vacuum degasser, thermostatically controlled column compartment, variable wavelength detector, cooled microwell-plate autosampler/MALDI target spotter, and the Agilent Chemstation software was used. The whey filtrate (0.5 μ L) was injected onto a C18 reversed-phase (RP)-HPLC column (Agilent Zorbax 300SB, 150×0.5 mm, 300 Å, 5 μ m) and eluted using a step-based gradient from 100% buffer A (0.1% TFA in ultrapure water) to 55% buffer B (0.1% TFA in 80% acetonitrile) for 15 min, over 5 min to 60% B, and to 100% B for 10 min. Column temperature was 20°C and the flow rate was 10 μ L/min. Detection was monitored at 210 and 280 nm. The eluted peptides in each peak were spotted by peak according to absorbance at 210 nm directly onto the anchors of the MALDI target plate. A saturated solution (0.4 μ L) of the matrix α -cyano-4-hydroxycinnamic acid in 70% acetonitrile, 0.1% TFA, was added to all dried spots.

Table 1. Somatic cell count (thousands of cells/mL) of milk samples from cow A and B¹

Time (h)	Cow A control	Cow A infused	Cow B control	Cow B infused
0	11	62	135	32
6	63	2,093	296	3,794
12	26	467	106	2,780
48	15	323	53	3,185
144	8	42	70	114

¹Time indicates hours after infusion with lipoteichoic acid.

Batch Elution of Peptides from Ultrafiltered Acid Whey Using Handmade RP Columns

Handmade chromatographic columns were used as an alternative method for desalting and concentration of the peptides in the peptide-enriched acid whey preparations before mass spectrometric analysis according to the method of Gobom et al. (1999). The column was equilibrated in 0.1% TFA. Twenty microliters of the peptide-enriched acid whey fraction were loaded, the column was washed in 0.1% TFA, and the peptides were eluted in α -cyano-4-hydroxycinnamic acid (20 mg/mL) in 50% acetonitrile directly onto the MALDI target plate in volumes of 0.5 to 1 μ L.

Identification of Peptides by MALDI-TOF MS/MS

A peptide calibration standard (0.2 μ L) was manually spotted to a separate anchor of the MALDI target plate. The calibration standard contained 7 standard peptides ranging in molecular mass from 1,046.54 to 3,147.47 Da. A Bruker Ultraflex MALDI-TOF tandem mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) was used in reflectron mode for mass analysis of separated peptides. The instrument was calibrated using the monoisotopic masses of the peptides in the calibration standard. The ion accelerating voltage was 25 kV, with a delay time of 40 ns, the laser frequency was 50 Hz, and 200 laser shots were accumulated for each spectrum. Peptides were measured as $[M+H]^+$. The ion accelerating voltage in MS/MS mode was 19 kV. For MS/MS, dominant parent peaks in the MS spectrum were selected for further fragmentation in MS/MS mode followed by mass searches in the Swiss Prot database (Swiss Institute of Bioinformatics, Geneva, Switzerland) using the ion search program Mascot (Matrix Science, Boston, MA). Mascot settings included "none" for enzyme, and 1 missed cleavage was allowed. Error tolerance was 100 ppm for parent mass and 0.5 Da for the fragment ion. In Mascot, the experimental mass value obtained from MS/MS is compared with calculated masses from a database. A scoring algorithm is used to identify the closest match and significant (P

< 0.05) peptide identifications were reported. A few of the peptides were manually elucidated from the fragments from the parent ion instead of using Mascot.

RESULTS

SCC, Total Proteolysis, and Plasmin Activity After Infusion

The SCC of milk from the control and infused quarters of cow A and B are shown in Table 1. Both cows showed a very large increase in SCC, from normal initial levels expected for healthy animals (within 6 h of infusion), to a greater extent in cow B than in cow A, with SCC in both cases slowly returning to near original levels thereafter (Table 1).

Overall levels of proteolysis in acid whey samples prepared from the milk samples during the time course of infusion were estimated by determination of the level of free amino terminals in the whey samples, which clearly increased after infusion with LTA; the highest level was observed in animal A milk 6 h after infusion (Table 2). In both animals, the levels increased from 0 to 6 h after infusion but, in milk from animal A, the level decreased again from 6 h onward, whereas in animal B, essentially the same level was found 12 h after infusion.

Plasmin activity in milk samples likewise varied somewhat between the 2 animals. Activity in milk from the infused quarters reached its highest level 48 h after infusion for cow A, but 12 h after infusion for cow B, where a very high activity was observed (Figure 1), which was later than the peaks in SCC and free amino terminals.

Electrophoretic Studies of Proteolysis

The proteolysis patterns in milk samples from cows infused with LTA and incubated for up to 7 d at 37°C were first studied with urea-PAGE. The control (i.e., milk from the uninfused quarter) samples from cow A showed slow proteolysis, mainly of β -CN and to a lesser extent α _{S1}-CN, for all samples during incubation for 7 d at 37°C. However, electrophoretograms of the milk samples from the LTA-infused quarter of cow A showed a very significant increase in overall proteolysis

Table 2. Level of free amino terminals in milk from cow A and B infused glands, expressed as mM leucine equivalents (mean values of 5 replicates with SD)

Time after infusion (h)	Cow A	Cow B
0	2.53 $\pm$ 0.059	2.58 $\pm$ 0.039
6	5.85 $\pm$ 0.084	3.59 $\pm$ 0.083
12	3.66 $\pm$ 0.072	3.65 $\pm$ 0.033

Figure 1. Plasmin activity of control quarters and infused quarters of cow A control (—●—), cow A infused (·····○·····), cow B control (---●---), and cow B infused (---○---).

over incubation (Figure 2). Although the 0-h samples showed only a little breakdown of the CN over incubation, samples taken 6 h after infusion showed almost complete hydrolysis of β - and α_{S1} -CN within 1 d of incubation, with hydrolysis of β -CN being faster than that of α_{S1} -CN. Samples taken 12 and 48 h after infusion also showed rapid hydrolysis of CN, but not as fast as that after 6 h. After 144 h, the proteolysis profile returned to almost normal (e.g., similar to the 0-h sample). The urea-PAGE pattern for milk samples from control and LTA-infused quarters of cow B also showed a massive increase in hydrolysis of β - and α_{S1} -CN in the sample taken 6 h after infusion, and a subsequent gradual return to a more normal level of hydrolysis (not shown).

The protein patterns in the control and infused quarter samples from cow A (not shown) and cow B (control quarter and quarter infused after 6 h) were also analyzed by using 2-dimensional gel electrophoresis. The resulting protein maps (Figure 3) compare milk samples from a control quarter (A) and a quarter into which LTA had been infused, at the height of the response (B). Immediately after infusion, the profile showed much intact α_{S1} - and β -CN, but 6 h after infusion, the protein profile showed hydrolysis of α_{S1} - and β -CN, with the extent of breakdown being higher for β -CN than for α_{S1} -CN. In addition, higher molecular weight products appeared on the gel in the higher isoelectric point regions, as did lower molecular weight products.

Identification of Peptides

Peptides generated as a result of increased proteolysis after infusion were further characterized by peptidomic methods to identify possible proteases responsible for hydrolysis through the determination of cleavage sites. Initially, the peptides were analyzed by capillary RP-HPLC of the peptide-enriched acid whey fractions from cow A and B taken 0, 6, and 12 h after infusion (Figure 4a-c). Consistent with the level of free amino terminals (Table 2), both the number and concentration of detected peptides (i.e., the area under the curve) increased from 0 to 6 h after infusion, whereas the number and area of peaks decreased again from 6 to 12 h after infusion in the samples from animal A. By contrast, in cow B, the peptide profiles of the samples 6 and 12 h after infusion were almost identical (not shown), consistent with the results for free amino terminals (Table 2). The peptide profiles of the 6- and 12-h samples from animal

Figure 2. Urea-PAGE electrophoretograms of milk from the quarter samples of cow A infused with lipoteichoic acid and sampled after 0, 6, 12, 48, or 144 h, and then incubated for 0, 1, 3, or 7 d at 37°C. Lane 1–4: 0-h sample, incubated at 37°C for 0 (1), 1 (2), 3 (3), or 7 (4) d; lane 5–8: 6-h sample, incubated at 37°C for 0 (5), 1 (6), 3 (7), or 7 (8) d; lane 9–12: 12-h sample, incubated at 37°C for 0 (9), 1 (10), 3 (11), or 7 (12) d; lane 13–16: 48-h sample, incubated at 37°C for 0 (13), 1 (14), 3 (15), or 7 (16) d; lane 17–20: 144-h sample, incubated at 37°C for 0 (17), 1 (18), 3 (19), or 7 (20) d.

Figure 3. Two-dimensional gel electrophoretograms of milk from (A) the control quarter of cow B 0 h after infusion and (B) the infused quarter of cow B 6 h after infusion. The gels show molecular weights (M_r) and isoelectric focusing at pH 4 to 7.

B were very similar to the 12 h sample from animal A (Figure 4).

For MS characterization and identification, the LC-separated peptides in the sample with the highest level of peptides (i.e., the 6 h sample from animal A) were spotted directly onto a MALDI target plate, based on the peak profile at 210 nm. Peptides that could be identified by MS/MS eluted at between 22 and 30 min (Figure 4); MS identifications obtained by the LC-MALDI spotting procedure are shown in Table 3. The peptides obtained from this spotting procedure are designated with the prefix S. A total of 9 peptides were identified in peaks 10, 12, 13, 14, 15, and 23; peptides were not detected in other peaks in the chromatogram. Six of the identified peptides were derived from α_{S1} -CN (S1–3, S7–9), and 3 were from β -CN (S4–6).

All peptides were identified by MS/MS analysis of the parent ions; an example of MS/MS analysis of 1 of the peptides (peptide S6; Table 3) is shown in Figure 5. The most dominant ion masses were a, b, and y ions, according to the fragmentation nomenclature proposed by Roepstorff and Fohlmann (1984). Not all ions in the 3 series were detected, but the peptide sequence was assigned by the combined information from the 3 series. The measured mass of the parent peptide was 1,881.2 Da. Taken together, the combined data demonstrated the detection of the peptide S6 with the sequence YQEPVLGPVRGPFPIIV, corresponding to a peptide from the C-terminal part of β -CN (193–209). The other peptides were similarly analyzed.

Because more peptides were expected to be present, especially in the unidentified peaks of the chromatograms, but could not be identified in the spotted ma-

Figure 4. Capillary reverse-phased-HPLC profiles of peptides in peptide-enriched acid whey prepared from milk from animal A at 0, 6, and 12 h (panels a, b, and c, respectively) after infusion with LTA, respectively. Peaks were collected for tandem mass spectrometry analysis, and peptides (S1–7) were identified in peaks 10, 12, 13, 14, 15 and 23 (Table 3). Color version available in the online PDF.

Table 3. Identity of peptides in peptide-enriched acid whey samples after liquid chromatography-matrix-assisted laser desorption/ionization (LC MALDI) spotting (see Figure 4 for location of peptides)¹

Peak	Peptide	Measured mass ²	Expected mass	Sequence tag ³	Probable identity
10	S1	1,447.06	1,445.79	VAPFGVFGKEKV	α_{S1} -CN 25–37
10	S2	2,460.73	2,459.38	PKHPIKHQGLPQEVLNENLLR	α_{S1} -CN 2–22
12	S3	1,603.87	1,602.84	HQGLPQEVLNELL	α_{S1} -CN 8–21
13	S4	1,151.80	1,150.68	GPVRGPFPIIV	β -CN 199–209
14	S5	1,994.33	1,993.14	LYQEPVLGPVRGPFPIIV	β -CN 192–209
14	S6	1,881.20	1,880.06	YQEPVLGPVRGPFPIIV	β -CN 193–209
15	S7	1,200.51	1,199.62	IQKEDVPSEK	α_{S1} -CN 81–90
23	S8	1,111.67	1,110.60	YLGYLEQLL	α_{S1} -CN 91–99
23	S9	1,267.79	1,266.70	YLGYLEQLLR	α_{S1} -CN 91–100

¹The peptide masses and peptide sequences were determined by matrix-assisted laser desorption/ionization–time of flight tandem mass spectrometry analyses.

²The measured masses were detected as the mass $[M+H]^+$.

³The sequence tag used for tandem mass spectrometry identification.

terial from the LC (probably due to lower concentrations), an alternative approach to obtaining identifiable peptides was applied. By this method, the peptides in the 6-h sample from animal A were applied onto a handmade column containing RP material packed in a pipette tip, and concentrated 40 times by batch elution (i.e., 20 μ L of each whey sample was loaded onto this column instead of the 0.5 μ L loaded on the LC-system). The peptides eluted from the column were then directly and dropwise applied onto the MALDI target plate. An MS spectrum of the parent ions measured in one of the effluent drops on the AnchorChip plate (Bruker Daltonik GmbH, Bremen, Germany) is shown in Figure 6, in addition to the resulting probable identities of the peptides as subsequently determined by MS/MS. A total of 14 peptides, including 5 from α_{S1} -CN (A1–5) and 9 from β -CN (B1–9), were identified with this batch-elution method. Three of the peptides were identified by both methods (S1 = A6, S2 = A2, and S6 = B8). Some of the peptides were derived from similar regions of the protein backbones, but showed minor differences in chain lengths. From α_{S1} -CN, the α_{S1} -CN fragment 1–23 (A1) was identified, in addition to its processed variants, α_{S1} -CN 2–22 (A2) and 2–23 (A3). Furthermore, the resulting counterpart of α_{S1} -CN after the liberation of the α_{S1} -CN N-terminal fragment was represented by the peptide A4, in addition to a processed form, peptide A5. For β -CN, all peptides identified (B1–9) comprised different peptides of varying chain length derived from the region between residue 163 and the C-terminus of the molecule, at position 209.

DISCUSSION

Both LTA from gram-positive *Staph. aureus* and LPS from gram-negative *E. coli* have been used in earlier infusion studies to elicit experimental mastitis (Moussaoui et al., 2002; Strandberg et al., 2005), but the effects on the milk proteolytic systems mainly have been

studied in LPS infusion studies (Moussaoui et al., 2004; Haddadi et al., 2006). *Staphylococcus aureus* and *E. coli* account for the majority of clinical mastitis cases in cattle (Barkema et al., 1998), and it has been shown that not only infection with these live bacteria, but also infusion with LPS or LTA elicit different innate immune responses in bovine mammary cells (Bannerman et al., 2004; Strandberg et al., 2005) through differential expression of cytokines. The cytokine response to LTA was limited compared with that elicited by LPS, which suggested a reason as to why mastitis caused by gram-positive bacteria more often leads to chronic infection than that by gram-negative bacteria (Strandberg et al., 2005). The differential cytokine responses led to differences in the composition and maturation of somatic cells, and possibly affected the secretion of cellular enzymes into milk.

Mastitis induced by intramammary infusion with LTA from gram-positive *Staph. aureus* resulted in a significant increase in SCC in both cows studied, as was expected and is consistent with other studies of *E. coli* or *Staph. aureus* mastitis (Michelutti et al., 1999; Bannerman et al., 2004; Moussaoui et al., 2004; Haddadi et al., 2006). A high SCC is accompanied by an increase in proteolytic activity and subsequent negative effects on the yield and quality of dairy products such as cheese. The increased plasmin activity in the milk from infused glands is also consistent with previous results showing activation of the plasmin system during experimentally induced mastitis infections in cows by either *Staph. aureus* or *E. coli* (Grieve and Kitchen, 1985; Saeman et al., 1988; Heegaard et al., 1994; Moussaoui et al., 2002). It is interesting that the plasmin activity peaked later than did maximum SCC, which may suggest a separate mechanism for increasing activity or a time lag due to activation of the enzyme by cell-associated PA.

Increased proteolysis in milk from infused quarters was evident from multiple analytical measurements.

Figure 5. Matrix-assisted laser desorption/ionization–time of flight tandem mass spectrometry (MS/MS) of peptide S6 with parent mass of 1,881.2 Da. The a, b, and y ion series detected by MS/MS of the parent ion are indicated. The AA single-letter code is given for the sequence of the identified peptide S6, YQEPVLGPVRGPFPIIV. The sequence was obtained by combination of the masses assigned to the a, b, and y-ion series.

Urea-PAGE electrophoretograms of the milk from the control quarter of cow A sampled after 0 and 6 h showed only slow hydrolysis of α_s - and β -CN during incubation at 37°C, which is consistent with previous reports for good quality milk; this indicates that hydrolysis of β -CN is due primarily to plasmin (Kelly and McSweeney, 2003; Schroeder et al., 2008). In contrast, electrophoretograms of the milk from the infected quarter sampled after 6 h and then incubated at 37°C showed complete hydrolysis of α_s - and β -CN, with β -CN being hydrolyzed faster. This initial phase of extensive proteolysis was in agreement with the increased plasmin activity. Greatly increased proteolysis after infusion with LTA was also apparent from measurements of levels of free amino terminals.

Other studies have also shown that CN undergoes extensive hydrolysis in milk from infected glands (Schaar, 1985; Le Roux et al., 1995; Leitner et al., 2006). In many previous studies of proteolysis during mastitis, infection was caused by live bacteria (e.g., *Strep. uberis*, Larsen et al., 2004; or *E. coli*, Moussaoui et al., 2004). In such cases, the bacteria themselves may have contributed to the proteolysis occurring (e.g., as observed for *E. coli*, Haddadi et al., 2006). However, in this study, the contribution of bacterial proteases to the observed proteolytic changes in mastitic milk may be excluded as the cows were infused with LTA only.

Barry and Donnelly (1981) reported that 90% of the proteinase activity in mastitic milk could be attributed to plasmin, but in contrast, de Rahm and Andrews

Figure 6. Matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MS) profile of parent peptides obtained by batch elution of peptides. The measured masses of parent ions used for tandem MS analyses are indicated together with the identified peptides. The masses were determined as $[M + H]^+$. The peptides identified included: A1: RPKHPIKHQGLPQEVLENLLRF; A2: PKHPIKHQGLPQEVLENLLR; A3: PKHPIKHQGLPQEVLENLLRF; A4: FVAPFPEVFGKV; A5: VAPFPEVFGKEKV; B1: LLSQSKVLPVPQKAVPYQQRDMPIQAF; B2: LLSQSKVLPVPQKAVPYQQRDMPIQAF; B3: FLYQEPVLGPVRGPFPII; B4: LLYQEPVLGPVRGPFPI; B5: YQEPVLGPVRGPFPI; B6: YQEPVLGPVRGPFPI; B7: YQEPVLGPVRGPFPII; B8: YQEPVLGPVRGPFPIV; and B9: QEPVLGPVRGPFPI.

(1982) reported that only one-third of the proteinase activity in such milk was due to plasmin. Similarly, Saeman et al. (1988), Le Roux et al. (1995), and Somers et al. (2003) showed that plasmin was not the only enzyme responsible for hydrolysis of CN in milk from cows with mastitis and concluded that proteinases from somatic cells and blood may also play a significant role in the CN hydrolysis of such milk. In LPS-induced mastitis, it has been shown that neutral proteinase activity was associated with immature PMN, the major somatic cell type in early inflammation (Saad and Östensson, 1990), whereas acidic proteinase activities, which could include cathepsins, were associated with mature PMN (Moussaoui et al., 2002). Some of the proteolysis products were subsequently identified in such studies, and comprised fragments of all 4 CN produced by somatic cell proteases (Moussaoui et al., 2003). Enzyme activities associated with the PMN were later measured as elastase, collagenase, and cathepsins (Haddadi et al., 2006).

Proteases potentially responsible for the cleavages giving rise to these peptides are suggested in Table 4, based on earlier reports of enzyme specificities for CN substrates in model studies. Peptide S1 (α_{S1} -CN 25–37)

could be the result of cathepsin B, D, or elastase activity, as in the case of cathepsin B or D combined with subsequent aminopeptidase trimming. Of the α_{S1} -CN peptides, 2 were differentiated only by 1 residue (S8 and S9); it is possible that peptide S8 is the result of carboxy-terminal trimming of the C-terminal arginine of S9, originally generated by plasmin. The peptides identified from β -CN comprised 3 related peptides (S4, S5, and S6), all derived from the C-terminal part of β -CN. Cathepsin D has been shown (Hurley et al., 2000) to be able to cleave the Leu₁₉₁–Leu₁₉₂ and the Leu₁₉₂–Tyr₁₉₃ bonds of this protein, giving rise to peptides S5 and S6, respectively (Table 4).

In Table 5, the enzymes likely responsible for the cleavages generating the peptides identified by batch elution are presented, based on their known preferences for cleavages in the CN as observed in model studies. All peptides had at least 1 cleavage site (N- or C-terminal) that could be assigned to a potential responsible bovine protease, including cathepsins and elastase. Several peptides appeared in different related versions and, as for the RP-HPLC-separated peptides (Table 4), strongly indicated the occurrence of peptide trimming by aminopeptidase or carboxypeptidase activities. This

Table 4. Suggested enzymes potentially responsible for the generation of the peptides S1–9, based on earlier reports¹

Peptide	N-terminal cleavage site	Enzyme suggestion	C-terminal cleavage site	Enzyme suggestion	Reference
S1	α_{S1} -CN Phe ₂₄ -Val ₂₅	Cathepsin B or cathepsin D or elastase	Val ₃₇ -Asn ₃₈	Elastase or cathepsin B/D + AP ²	Reimerdes et al., 1979; Considine et al., 2000; Hurley et al., 2000; Considine et al., 2004
S2	α_{S1} -CN Arg ₁ -Pro ₂	AP	Arg ₂₂ -Phe ₂₃	Cathepsin D + CP ²	Hurley et al., 2000; Reimerdes et al., 1979; Considine et al., 2004
S3	α_{S1} -CN Lys ₇ -His ₈	Cathepsin B or plasmin	α_{S1} -CN Leu ₂₁ -Arg ₂₂	Not known ³	
S4	β -CN Leu ₁₉₈ -Gly ₁₉₉	Not known ³	β -CN Val ₂₀₉	No cleavage ⁴	
S5	β -CN Leu ₁₉₁ -Leu ₁₉₂	Cathepsin D	β -CN Val ₂₀₉	No cleavage ⁴	Hurley et al., 2000
S6	β -CN Leu ₁₉₂ -Tyr ₁₉₃	Cathepsin D	β -CN Val ₂₀₉	No cleavage ⁴	Hurley et al., 2000
S7	α_{S1} -CN His ₈₀ -Ile ₈₁	Not known ³	α_{S1} -CN Arg ₉₀ -Tyr ₉₁	Plasmin	Le Bars and Gripon, 1993
S8	α_{S1} -CN Arg ₉₀ -Tyr ₉₁	Plasmin	α_{S1} -CN Leu ₉₉ -Arg ₁₀₀	Plasmin + CP ²	Le Bars and Gripon, 1993; Reimerdes et al., 1979
S9	α_{S1} -CN Arg ₉₀ -Tyr ₉₁	Plasmin	α_{S1} -CN Arg ₁₀₀ -Leu ₁₀₁	Plasmin	Le Bars and Gripon, 1993

¹AP = aminopeptidase; CP = carboxypeptidase. More information on peptides S1–9 is available in Table 3.

²An AP or a CP, eventually in combination with other protease(s), may be responsible for the cleavage.

³Not known indicates that a possible responsible protease for generation of this cleavage site could not be suggested.

⁴No cleavage indicates that the residue is located at the C-terminal position of the protein from which it was derived and therefore not the result of a proteolytic cleavage at that position.

was observed, for example, for the α_{S1} -I CN fragment (A1), which also appeared in the processed forms A2 and A3. Various forms of the C-terminal fragment of β -CN also were identified, and indicated trimming, especially involving carboxy peptidase activity. In some instances, the assigned peptide appeared to be the result of combined actions of endo- and exoproteinase activities, as suggested for peptides B1, B2, and B9.

Taken together, the proteases suggested as potentially responsible for proteolysis in the present study included plasmin, cathepsin B, cathepsin D, neutrophil elastase, and one possible instance of cathepsin G, in addition to presumed AA carboxypeptidase activities. All of these enzyme activities were suggested based on the milk sample analyzed 6 h after infusion, and thus indicate that both the plasmin/plasminogen system and somatic cell proteases seem to be involved in the breakdown of protein observed in early inflammation after infusion of LTA.

Several cleavage sites appeared to be the result of the action of either amino- or carboxy-peptidases, eventually in combination with other enzymes. The many indications of amino- or carboxy peptidase activities in the analyzed samples are surprising. The presence of aminopeptidase activity in milk serum has been previously reported (Reimerdes et al., 1979), but has not been confirmed by later studies. However, a potential candidate for such aminopeptidase activity could be cathepsin H, which, apart from its endoproteinase activity, is an amino peptidase (Barrett and Kirschke, 1981). Cathepsin H is one among several lysosomal cysteine proteases with exopeptidase activity as well,

but the presence of cathepsin H in milk has not been previously demonstrated.

Of the many potential cathepsin D cleavage sites in the CN (McSweeney et al., 1995; Larsen et al., 1996), only a small number were observed in the present study, including cleavage of the Phe₂₃-Phe₂₄ bond of α_{S1} -CN, resulting in the production of the α_{S1} -I CN fragment, which is well known to be generated during cheese ripening. That more cathepsin D cleavage sites were not identified probably reflects large differences in the susceptibility of pure individual caseins compared with caseins in milk systems. This may be due to both differences in pH and ionic strength of model systems compared with milk, but also to the fact that the caseins in milk are organized in micelles, which may protect in some degree toward hydrolysis. Possible cleavage sites for cathepsin B also were identified, but none of these was assigned to only this protease. Cathepsin B is a cysteine protease that requires a reducing milieu for activity, and this fact may limit its activity in milk. Apart from its endoproteinase activity, cathepsin B also acts as a carboxy dipeptidase (i.e., it can cleave dipeptides from the C-terminus of some substrates; Barrett et al., 1988), and it has been reported that the endoproteinase activity of cathepsin B is relatively low compared with its carboxy dipeptidase activity (Barrett and Kirschke, 1981).

Nine peptides were identified by the LC-MALDI spotting method. The level of some of the further peptides detected at 210 nm was apparently too low for MS identification by the procedure used, and therefore all of the peptide material in 1 of the samples was

Table 5. Suggested enzymes potentially responsible for the generation of the identified peptides¹

Peptide	Probable identity	N-terminal cleavage site	Enzyme suggestion	C-terminal cleavage site	Enzyme suggestion	Reference
α_{SI} -CN						
A1	α_{SI} -CN 1–23	Arg ₁	No cleavage ²	Phe ₂₃ –Phe ₂₄	Cathepsin B or cathepsin D	Hurley et al., 2000; Considine et al., 2004
A2	α_{SI} -CN 2–22	Arg ₁ –Pro ₂	AP ³	Arg ₂₂ –Phe ₂₃	Cathepsin D	Hurley et al., 2000; Reimerdes et al., 1979
A3	α_{SI} -CN 2–23	Arg ₁ –Pro ₂	AP ³	Phe ₂₃ –Phe ₂₄	Cathepsin B or cathepsin D	Hurley et al., 2000; Considine et al., 2004; Reimerdes et al., 1979
A4	α_{SI} -CN 24–37	Phe ₂₃ –Phe ₂₄	Cathepsin B or cathepsin D	Val ₃₇ –Asn ₃₈	Elastase or cathepsin G	Considine et al., 2000,2002,2004; Hurley et al., 2000
A5	α_{SI} -CN 25–37	Phe ₂₄ –Val ₂₅	Cathepsin B or cathepsin D or elastase	Val ₃₇ –Asn ₃₈	Elastase	Considine et al., 2000; Hurley et al., 2000; Considine et al., 2004
β -CN						
B1	β -CN 163–190	Val ₁₆₂ –Leu ₁₆₃	Cathepsin D + AP ³	Phe ₁₉₀ –Leu ₁₉₁	Elastase	Considine et al., 1999; Hurley et al., 2000; Reimerdes et al., 1979
B2	β -CN 163–191	Val ₁₆₂ –Leu ₁₆₃	Cathepsin D + AP ³	Leu ₁₉₁ –Leu ₁₉₂	Cathepsin D	Hurley et al., 2000; Reimerdes et al., 1979
B3	β -CN 190–208	Ala ₁₈₉ –Phe ₁₉₀	Elastase	Ile ₂₀₈ –Val ₂₀₉	CP ³	Considine et al., 1999
B4	β -CN 191–207	Phe ₁₉₀ –Leu ₁₉₁	Elastase or cathepsin G	Ile ₂₀₇ –Ile ₂₀₈	CP ³	Considine et al., 1999, 2002
B5	β -CN 193–206	Leu ₁₉₂ –Tyr ₁₉₃	Cathepsin D	Pro ₂₀₆ –Ile ₂₀₇	CP ³	Hurley et al., 2000
B6	β -CN 193–207	Leu ₁₉₂ –Tyr ₁₉₃	Cathepsin D	Ile ₂₀₇ –Ile ₂₀₈	CP ³	Hurley et al., 2000
B7	β -CN 193–208	Leu ₁₉₂ –Tyr ₁₉₃	Cathepsin D	Ile ₂₀₈ –Val ₂₀₉	CP ³	Hurley et al., 2000
B8	β -CN 193–209	Leu ₁₉₂ –Tyr ₁₉₃	Cathepsin D	Val ₂₀₉	No cleavage ²	Hurley et al., 2000
B9	β -CN 194–207	Tyr ₁₉₃ –Gln ₁₉₄	Cathepsin D + AP ³	Ile ₂₀₇ –Ile ₂₀₈	CP ³	Hurley et al., 2000; Reimerdes et al., 1979

¹AP = aminopeptidase; CP = carboxypeptidase; A1 = RPKHPIKHQGLPQEVLENLLRF; A2 = PKHPIKHQGLPQEVLENLLR; A3 = PKHPIKHQGLPQEVLENLLRF; A4 = FVAPFPEVFGKV; A5 = VAPFPEVFGKEKV; B1 = LLSQSKVLPVPQKAVPYPQRDMPIQAF; B2 = LLSQSKVLPVPQKAVPYPQRDMPIQAF; B3 = FLYQEPVLGPVRGPFPII; B4 = LLYQEPVLGPVRGPFPI, B5 = YQEPVLGPVRGPFPI; B6 = YQEPVLGPVRGPFPI; B7 = YQEPVLGPVRGPFPII; B8 = YQEPVLGPVRGPFPII, and B9 = QEPVLGPVRGPFPI.

²No cleavage indicates that the residue is located at the C-terminal position of the protein from which it was derived and therefore not the result of a proteolytic cleavage at that position.

³An AP or a CP, eventually in combination with another protease(s), may be responsible for the cleavage.

concentrated by batch elution from the C18 column material, which resulted in identification of 14 peptides. It is possible that even more peptides were present, but the identification of these would require a separate study and, for example, the use of complementary MS methods to improve detection and reliability (Stoop et al., 2008). The peptides identified included peptides from α_{S1} - and β -CN; no peptides were identified from α_{S2} -CN. In an earlier study of mastitic milk, which was obtained after infection with the mastitis pathogen *Strep. uberis*, peptides from α_{S1} -, α_{S2} - and β -CN were identified (Wedholm et al., 2008). In the study of Mous-saoui et al. (2003), peptides from all 4 CN were identified, but in this study a different approach was used involving N-terminal sequencing of protein bands from SDS-PAGE, thereby favoring larger fragments that could be retained in the gel. All peptides identified as originating from α_{S1} -CN were derived from the N-terminal part of that molecule, whereas all β -CN peptides were cleaved from the C-terminal part of the molecule, except for 3 peptides (S7–9) from α_{S1} -CN, which were cleaved from the middle part. These peptides were all derived from the nonphosphorylated regions in the CN, and therefore no phosphorylated peptides were identified. This is probably because of difficulties in detecting the phosphopeptides by the MS method applied; this aspect could be further studied by applying modified methods designed for detection of phosphorylated peptides (Stensballe and Jensen, 2004).

In relation to the peptide profile identified earlier in mastitic milk using LC-MALDI spotting (Wedholm et al., 2008), the peptide S4 identified in this study is identical to the peptide P8 identified earlier, and corresponds to β -CN 199–209, whereas peptide S7 (α_{S1} -CN 81–90) from this study is almost identical to P2 (α_{S1} -CN 80–92) identified in the earlier study. This shows that some of the cleavages and peptides occurring are the same in the 2 studies, but also that many cleavages and peptides are specific to different conditions of mastitis and elevated SCC.

Among the peptides identified, several had potential bioactive properties. These included α_{S1} -CN 91–99 and 91–100, with opioid properties, and α_{S1} -CN 1–23 (Is-racidin) and β -CN 193–209, with immune-modulating and antimicrobial activities (Silva and Malcata, 2005). This indicates that some of the endogenous enzymes in milk are capable of contributing to the generation of bioactive peptides in some milk types, and raises the possibility of bovine somatic cell enzyme involvement in the generation of antimicrobial peptides as a means to fight bacterial infection.

It is possible that further peptidomic studies of samples obtained from different cows or at other time points

would identify additional peptides and suggest other enzymes to be involved. Further indications of actual enzyme activities could be obtained by characterization of specific enzyme activities, and this is currently being carried out, together with further characterization of larger CN hydrolysis products through the use of gel electrophoresis proteomic-based methods.

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