Proteolytic and proteomic changes in milk at quarter level following infusion with *Escherichia coli* lipopolysaccharide

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ABSTRACT

Mastitis is a major disease in dairy cattle, which causes significant economic losses due to decreased milk production, veterinary costs, and discarded milk. *Escherichia coli* is one of the most prevalent species of gram-negative bacteria that induce clinical mastitis. The objective of the present study was to characterize the proteolytic and proteomic changes in milk in response to infusion with lipopolysaccharide (LPS) at quarter level in a model mastitis system. One quarter of each of 2 cows was infused with 0.1 or 5 μ g of LPS. The somatic cell count of the infused quarters reached a peak 6 h after infusion to a greater extent in the cow infused with 5 μ g of LPS and changes in plasmin activity in milk differed between the 2 animals. Urea-polyacrylamide gel electrophoretograms of milk samples of the cow infused with 5 μ g of LPS 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 due to indigenous proteolytic activity. Two-dimensional gel electrophoretograms of milk at 0, 6, or 12 h after infusion with LPS showed hydrolysis of α_s -casein and β -casein as well as the appearance of lower molecular weight products. Eleven fragments from proteolysis of the caseins were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and, in addition, proteolysis patterns of casein by the indigenous bovine milk proteases plasmin and cathepsin D were studied in model studies using 2-dimensional gel electrophoretograms. Twelve hours after infusion, lower abundance markers of inflammation were identified, including serotransferrin, fibrinogen β chain, protein S100 A12, and the antimicrobial polypeptide cathelicidin.

Key words: coliform mastitis, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, 2-dimensional gel electrophoretogram, proteolysis

INTRODUCTION

It is well known and documented that proteolysis increases in bovine milk at elevated SCC (e.g., in mastitis; de Rham and Andrews, 1982; Andrews, 1983; Le Roux et al., 1995; Moussaoui et al., 2004). This increase in proteolysis is mainly due to an increase in the level of proteases present in the milk (Larsen et al., 2006), as well as increased activation of protease zymogens, leading to increased enzymatic activities (e.g., as observed for the plasminogen system in milk; Heegaard et al., 1994).

The proteases in milk may potentially arise from different sources; they can be secreted into milk from the mammary epithelium, be transferred from blood to milk, particularly in cases of a compromised barrier between milk and blood in mastitis, or be secreted or leaked from invading microorganisms or bovine somatic cells, or both (Politis et al., 1989; 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, 1999; Larsen et al., 2006). Depending on the proteolytic enzyme, different secretion pathways can be expected to be prevalent; plasmin, the principal protease in normal milk with low SCC, is transported into milk from blood in its zymogen form, plasminogen. Both neutrophils and macrophages contain active lysosomal acid and neutral proteases and protease zymogens, such as cathepsins, procathepsins, and elastase (Kirschke and Barrett, 1987; Travis, 1988; Sordillo et al., 1997; Kelly et al., 2006). 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 the protein quality in milk. Generally, hydrolysis of proteins in raw milk is undesirable (e.g., through negative effects on cheese yield; Auldist and Hubble, 1998), although proteolysis is beneficial in some dairy products, like cheese, where proteolysis can contribute to the ripening process (Wium et al., 1998; Hurley et al., 2000; Gagnaire et al., 2001).

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In addition to characterization of proteases and measurement of enzyme activities, new peptidomic methods using proteomic tools and mass spectrometry are now available, by which the actual result of these proteolytic activities in specific milk samples can be studied and characterized. In a recent study, 1 quarter of each of 2 cows was infused with lipoteichoic acid from *Staphylococcus aureus* (Larsen et al., 2010).

The objective of this study was to analyze the peptide products of proteolysis in the model mastitis system. Twenty different peptide products were obtained and characterized and the proteases responsible were identified. The peptides obtained were shown to have been derived from α_{s1} - and β -CN. On the basis of the peptide cleavage sites, it was possible to propose that the possible proteases responsible for the proteolysis were plasmin, cathepsin B and D, elastase, and amino- and carboxypeptidases.

Dairy producers are particularly concerned with mastitis infections arising from gram-negative bacteria because 1) it has been established that coliform pathogens are not successfully treated with antimicrobial drugs and other traditional remedies (Shim et al., 2004), 2) a coliform treatment vaccine has had limited effectiveness (Hogan et al., 1992), and 3) typically, a loss of up to 25% of the cows infected with gram-negative mastitis occurs as a result of culling or death because of complications due to the infection (Eberhart et al., 1987). These serious consequences of a coliform mastitis infection for dairy producers and the difficulties of counteracting it have led to much research in the field (Hirvonen et al., 1999; Bannerman et al., 2003, 2004).

Escherichia coli strains have frequently been used to experimentally induce mastitis infection in research animals because *E. coli* is the most common gram-negative bacterium to cause mastitis in cows (Andrews, 1983; Hirvonen et al., 1999; Moussaoui et al., 2002; Sládek et al., 2002; Bannerman et al., 2004; Moussaoui et al., 2004; Schmitz et al., 2004; Sohn et al., 2007). The present study is a further development of this approach and reports a proteomic and peptidomic study of bovine milk from 2 cows during early immunological response to infusion with LPS from the cell walls of the gram-negative *E. coli* (Schalm and Ziv-Silberman, 1968; Moussaoui et al. 2002; Strandberg et al. 2005; Haddadi et al., 2006), but it eliminates the contribution from mastitis bacteria themselves to the proteolytic cascade.

MATERIALS AND METHODS

Sample Preparation

Immediately after the morning milking, 2 dairy cows were infused in one quarter with 0.1 μ g or 5 μ g of LPS

from *E. coli* 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 6, 12, 48, and 144 h after infusion; samples were obtained by hand milking during routine milking procedures. Somatic cell count measurements were performed with a DeLaval cell counter (DeLaval International AB, Tumba, Sweden). Whole milk samples were skimmed as described by Larsen et al. (2010).

Determination of Plasmin Activity and Assessment of Proteolysis During Storage

Plasmin activity was performed as described by Larsen et al. (2010) and expressed in amino methyl coumarin (AMC) units (nanomoles of AMC released per minute)/mL of milk. 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 taken at each time point. Urea PAGE of stored milk samples was then performed as described by Larsen et al. (2010). Gels were then scanned using a GS-800 calibrated densitometer (Bio-Rad Laboratories Inc., Hercules, CA).

Model Studies with Indigenous Milk Enzymes

Casein (Sigma-Aldrich, St. Louis, MO) was dissolved by stirring overnight at room temperature (20°C) at 10 mg/mL in 0.05 M phosphate buffer and 0.035 M NaCl at pH 6.8. Bovine plasminogen (Sigma-Aldrich) or bovine cathepsin D (Sigma-Aldrich) were added at $t = 0$ at enzyme to substrate ratios of 1/100 wt/wt. Plasminogen was activated with urokinase (Calbiochem/VWR, Herlev, Denmark) before addition to the samples as described by Larsen et al. (2004). After addition of enzymes, the samples were incubated at 37°C, and samples were drawn after 6, 24, and 36 h. Three parallel incubations were carried out: A) control with no enzyme addition, B) with activated plasminogen, and C) with cathepsin D. All experiments were carried out in triplicate in parallel incubations of enzymes added to aliquots of the stock substrate solution.

Proteomic Analysis

Skim milk samples were analyzed by 2-dimensional gel electrophoresis (2-DE) essentially as described by Larsen et al. (2010). The first dimension of protein separation was carried out on immobilized 11-cm IPG strips (pH 4–7), and 7.5 $\times$ 11-cm 8 to 16% gradient criterion gels were used for the second dimension (Bio-Rad

Table 1. Somatic cell count (thousands of cells per milliliter) of milk samples from cows infused with a high (HLPS) or low (LLPS) level of LPS (time indicates hours after infusion with LPS)

Time after infusion (h)	LLPS control	LLPS infused	HLPS control	HLPS infused
0	36	31	90	243
6	—	1,706	—	3,814
12	—	517	—	3,135
24	102	118	56	3,821
48	—	160	—	3,944

Laboratories Inc.). A volume (185 μ L) corresponding to 300 μ g of milk protein sample was applied to each gel according to the Bradford assay. For the studies of milk samples from LPS-infused cows, the gels were stained with colloidal Coomassie Brilliant Blue (CBB) G-250 (SERVA Electrophoresis GmbH, Heidelberg, Germany) as described by Larsen et al. (2010), whereas the gels used in the model study were silver stained (Wedholm et al., 2008b). The 2-DE gels were photographed using a Vilber Lourmat digital camera (Image House A/S, Copenhagen, Denmark) equipped with Gel-Pro Analyzer software (Media Cybernetics Inc., Bethesda, MD). Protein spots of significance were subjected to in-gel digestion by addition of trypsin and identified by peptide mass fingerprinting (PMF) using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) essentially as described earlier (Larsen et al., 2010). Custom-made chromatographic columns were used for desalting and concentration of the peptide mixture before mass spectrometric analysis. The peptides were eluted in 0.5 μ L of matrix solution (15–20 g/L of α -cyano-4-hydroxycinnamic acid in 70% acetonitrile; Sigma-Aldrich) directly onto the MALDI target plate (Bruker Daltonik GmbH, Bremen, Germany). Mass spectra were obtained by PMF using an Ultraflex MALDI-TOF tandem mass spectrometer (Bruker Daltonik GmbH) in reflection mode. Proteins were identified by PMF mass searches in the database Swiss Prot (Swiss Institute of Bioinformatics, Geneva, Switzerland) followed by mass searches in the database using the ion search program Mascot (Matrix Science Inc., Boston, MA). In this program, the experimental

mass value obtained from MS is compared with calculated peptide masses from a database. A scoring algorithm is used to identify the closest match. Significant protein identifications (protein scores above 62, $P < 0.05$) were reported and manually verified.

RESULTS

SCC and Plasmin Activity After Infusion

The SCC of milk from the control and infused quarters of the cow infused with low LPS concentration (LLPS) and the cow infused with high LPS concentration (HLPS) are shown in Table 1. Both cows had normal initial levels expected for healthy animals, but showed a very large increase in SCC within 6 h of infusion, to a greater extent in cow with HLPS than in the case of LLPS. In the sample taken 48 h following infusion, the SCC for both cows was still higher than the original level, again to a greater extent in the cow with HLPS than in the cow with LLPS.

Changes in plasmin activity in milk differed between the 2 animals; the activity in milk from the infused quarter after 0 h of infusion with LPS was higher in the LLPS cow than in the HLPS cow (Table 2). However, milk from the HLPS cow showed higher plasmin activity 6 h after infusion than milk from the LLPS cow.

Electrophoretic Studies of Proteolysis

The proteolysis patterns in milk samples from 2 cows infused with HLPS or LLPS incubated for up to 7 d

Table 2. Plasmin activity [nmol of amino methyl coumarin (AMC) released per minute per milliliter of milk] of milk samples from cows infused with a high (HLPS) or low (LLPS) level of LPS (time indicates hours after infusion with LPS) at 37°C for up to 7 d

Time after infusion (h)	LLPS		HLPS	
	d 0	d 7	d 0	d 7
Control				
0	0.0144	0.0325	0.0500	0.0247
Infused				
0	0.0105	0.0055	0.0076	0.0135
6	0.0030	0.0070	0.0465	0.0301

Figure 1. Urea-PAGE electrophoretograms of milk from the control and infused quarters of a cow infused with 5 µg of LPS (high LPS concentration, HLPS) and sampled after 0 and 6 h, and then incubated for 0, 1, 3, or 7 d at 37°C. Lane 1–4: control sample, incubated at 37°C for 0 (lane 1), 1 (lane 2), 3 (lane 3), or 7 (lane 4) d; lane 5–8: 0-h infused quarter sample, incubated at 37°C for 0 (lane 5), 1 (lane 6), 3 (lane 7), or 7 (lane 8) d; lane 9–12: 6-h infused quarter sample, incubated at 37°C for 0 (lane 9), 1 (lane 10), 3 (lane 11), or 7 (lane 12) d.

at 37°C were initially studied using urea PAGE. Electrophoretograms of the milk samples from the HLPS-infused quarter of one cow after 6 h showed a very significant increase in overall proteolysis over incubation (Figure 1, lane 9–12). Urea PAGE patterns for the control milk sample (i.e., milk from the uninfused quarter) of the cow with HLPS showed little hydrolysis of the caseins over incubation (Figure 1, lane 1–4). Samples taken 0 h after infusion also showed only slow hydrolysis, mainly of β -CN over incubation. However, samples taken 6 h after infusion showed almost complete hydrolysis of β - and α_{s1} -CN within 1 d of incubation (Figure 1).

The urea PAGE patterns of the control milk sample (i.e., milk from the uninfused quarter) of the cow with LLPS also showed only little hydrolysis of β -CN and no hydrolysis of α_{s1} -CN over incubation. Samples taken immediately (0 h) after infusion also showed only little hydrolysis after 7 d of incubation at 37°C (Figure 2). However, electrophoretograms of the milk samples infused with LLPS after 6 h showed almost complete hydrolysis of β - and α_{s1} -CN after 7 d of incubation but the breakdown was not as significant as in milk from the cow treated with HLPS (Figure 1), as can be seen by the lower amount of β -CN-derived fragments appearing on the gel.

The protein patterns in the control and infused quarter samples from a cow with LLPS (not shown) and a cow with HLPS (control quarter, quarter infused

after 0, 6, or 12 h) were further analyzed by 2-DE and spots were identified by PMF using MALDI-TOF MS. Representative gels from milk samples from a control quarter and the infused quarter after 0, 6, and 12 h were chosen to illustrate the protein profiles generated in the present study (Figures 3A and 3B and Figures 4A and 4B). The resulting protein map from the control quarter 0 h after infusion and the infused quarter after 0 h (Figures 3A and 3B) exhibited an abundance of casein proteins including α_{s1} -CN, β -CN, and κ -CN variants, and the whey proteins BSA, β -LG, and α -LA.

In contrast, the protein pattern of the infused quarter after 6 h (Figure 4A) and after 12 h (Figure 4B) showed a decreased abundance of α_{s1} -CN and β -CN but a marked increase in BSA. κ -Casein spots were still evident in the infused quarters after 6 or 12 h but in decreased amounts. Marked changes in the protein profiles of milk from infused quarters included several new spots appearing in the lower isoelectric point (pI) range and with lower molecular mass than the intact casein, in addition to several additional new spots with higher pI values, with various masses, both below and above the masses of the caseins (Figure 4 A and B).

Mass spectrometry identifications of 26 spots analyzed are shown in Table 3. Of these, half of the proteins identified corresponded to α_{s1} - or α_{s2} -CN. Eight spots were identified as α_{s1} -CN isoforms with apparent molecular masses from 18.0 to 27.0 kDa and apparent pI from 4.2 to 4.3; 4 spots were identified as α_{s2} -CN

Figure 2. Urea-PAGE electrophoretograms of milk from the control and infused quarters of a cow infused with 0.1 μ g of LPS (low LPS concentration, LLPS) and sampled after 0 and 6 h, and then incubated for 0, 1, 3, or 7 d at 37°C. Lane 1–4: control sample, incubated at 37°C for 0 (lane 1), 1 (lane 2), 3 (lane 3), or 7 (lane 4) d; lane 5–8: 0-h infused quarter sample, incubated at 37°C for 0 (lane 5), 1 (lane 6), 3 (lane 7), or 7 (lane 8) d; lane 9–12: 6-h infused quarter sample, incubated at 37°C for 0 (lane 9), 1 (lane 10), 3 (lane 11), or 7 (lane 12) d.

isoforms (22.0–23.0 kDa, pI of 5.3–5.4). Most of these isoforms corresponded to breakdown products, presumably originating from the action of indigenous bovine proteolytic enzymes, producing several peptides with different molecular masses and pI values. Together with these 2 main groups of casein-derived spots, BSA (4 spots) was identified as well as some highly abundant proteins including serotransferrin (2 spots), vitamin D-binding protein, cytoplasmic actin (4 spots), and the β chain of fibrinogen (1 spot). Additional proteins were identified in the lower mass region and higher pI area, such as protein S100-A12, and 2 antimicrobial peptides (AMP) in the cathelicidin family.

To further study by 2-DE the breakdown patterns of caseins by indigenous bovine milk proteases, a model study of proteolysis by such proteases was conducted. Casein from bovine milk was dissolved at normal milk pH (6.8) and hydrolyzed with the bovine milk proteases plasmin (in the form of activated bovine plasminogen due to limited shelf life stability of the plasmin enzyme itself) or bovine cathepsin D. The progress in proteolysis after 6 and 24 h by plasmin is shown, together with the result of digestion with cathepsin D after 24 h (Figure 5A–D). In the case of hydrolysis by plasmin, breakdown of α_s - and β -CN was evident after 6 h of incubation at 37°C, with a range of new spots in both the α_{s1} -CN product area (box in Figure 5) and at higher pI values. This hydrolysis was observed to progress by

time, and extensive breakdown of α_s - and β -CN, and to some extent also κ -CN, was evident after 24 h, when new spots as a result of plasmin-induced hydrolysis were apparent just below the κ -CN train of spots in the higher pI range, in addition to a large amount of further hydrolysis products (Figures 5B and 5C). In the case of cathepsin D, extensive cleavage of α_{s1} -CN into larger, well-defined fragments (box in Figure 5D) was evident and κ -CN was also clearly hydrolyzed.

DISCUSSION

Mammary tissue is permeated with neutrophils and lymphocytes that, together with immune-cell-secreted cytokines, chemokines, and other cell defense mechanisms, can attack invading bacteria, including mastitis infections (Bannerman et al., 2003). Lipopolysaccharide from gram-negative *E. coli* has been used in earlier infusion studies to elicit experimental mastitis (Moussaoui et al., 2004; Schmitz et al., 2004; Bruckmaier and Meyer, 2005; Haddadi et al., 2006; Boehmer et al., 2008; Danielsen et al., 2010; Wellnitz et al., 2010) and different innate immune responses in bovine mammary cells through differential expression of cytokines were observed (Bannerman et al., 2004; Strandberg et al., 2005).

Mastitis induced by intramammary infusion with gram-negative LPS of *E. coli* resulted in a significant

Figure 3. Two-dimensional gel electrophoretograms of milk from A) the control quarter of cow with high LPS (HLPS) 0 h after infusion; B) the infused quarter of cow with HLPS concentration after 0 h. The gels show molecular weights (M_r) and isoelectric points (pI) in the range 4–7.

increase in SCC over time in both cows studied but to a greater extent in the cow infused with HPLS than in the cow infused with LLPS. This is in agreement with other studies of *E. coli* mastitis (Michelutti et al., 1999; Bannerman et al., 2004; Moussaoui et al., 2004; Haddadi et al., 2006; Wellnitz et al., 2010). Milk SCC is a

well-established udder indicator of udder condition and milk quality (O'Brien et al., 2001). Increases in milk SCC can cause an increase in the amount of proteolytic activity and can decrease the yield and quality of dairy products such as cheese. The increased plasmin activity in the milk from infused glands is also consistent

Figure 4. Two-dimensional gel electrophoretograms of milk from A) the infused quarter of cow with high LPS (HLPS) concentration 6 h after infusion and B) the infused quarter of cow with HLPS 12 h after infusion. The gels show molecular weights (M_r) and isoelectric points (pI) in the range 4–7. The gels were stained with Colloid Coomassie Brilliant Blue G-250 (SERVA Electrophoresis GmbH, Heidelberg, Germany). The numbered arrows indicate the 26 spots that were identified by matrix-assisted laser desorption/ionization (MALDI) in Table 3.

with previous results showing activation of the plasmin system during experimentally induced *E. coli* mastitis infections in cows (Grieve and Kitchen, 1985; Saeman et al., 1988; Moussaoui et al., 2002).

Increased proteolysis in milk from infused quarters was also studied using urea PAGE. The proteolysis pattern of the milk from the control quarter of the cow

with HLPS sampled at 0 and 6 h after infusion showed only little hydrolysis of α_{s1} - and β -CN during incubation at 37°C, which is in agreement with previous reports for good quality milk (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

Table 3. Identification of the 26 most abundant spots from the 2-dimensional (2-DE) gel of mastitis milk (Figures 4A and 4B) by peptide mass fingerprinting using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)¹

Spot no.	Identified protein	Ref. Swiss Prot	pI		MW (kDa)		Score	Sequence coverage (%)
			Theor.	Obs.	Theor.	Obs.		
1	α_{s1} -CN	P02662	4.91	4.2	22.97	24.0	65	35
2	α_{s1} -CN	P02662	4.91	4.2	22.97	23.0	57	30
3	α_{s1} -CN	P02662	4.91	4.3	22.97	23.0	60	35
4	α_{s1} -CN	P02662	4.91	4.2	22.97	18.0	46	34
5	Vitamin D-binding protein	Q3MHN5	5.19	5.2	51.53	58.0	97	41
6	Actin cytoplasmic	P60712	5.29	5.3	41.60	38.0	143	45
7	Actin cytoplasmic	P60712	5.29	5.4	41.60	38.0	186	62
8	Actin cytoplasmic	P60712	5.29	5.4	41.60	38.0	181	69
9	Actin cytoplasmic	P60712	5.29	5.4	41.60	38.0	231	72
10	BSA	P02769	5.60	5.7	66.43	53.0	95	21
11	BSA	P02769	5.60	5.7	66.43	53.0	101	22
12	BSA	P02769	5.60	5.8	66.43	53.0	113	24
13	BSA	P02769	5.60	5.9	66.43	53.0	79	20
14	Serotransferrin	Q29443	6.50	6.4	75.82	66.0	219	47
15	Serotransferrin	Q29443	6.50	6.5	75.82	66.0	170	38
16	Fibrinogen β chain	P02676	8.75	6.5	51.15	53.0	184	43
17	α_{s1} -CN	P02662	4.91	4.3	22.97	27.0	62	37
18	α_{s1} -CN	P02662	4.91	4.3	22.97	27.0	53	26
19	α_{s1} -CN	P02662	4.91	4.3	22.97	21.0	61	36
20	α_{s2} -CN	P02663	8.34	5.3	24.34	23.0	78	28
21	α_{s2} -CN	P02663	8.34	5.3	24.34	22.0	76	37
22	α_{s2} -CN	P02663	8.34	5.4	24.34	23.0	66	28
23	α_{s2} -CN	P02663	8.34	5.7	24.24	22.0	69	28
24	Cathelicidin	P22226	9.3	5.8	17.6	16.0	53	36
25	Cathelicidin	P22226	9.3	6.3	17.6	15.0	62	35
26	Protein S100 A12	P79105	5.91	6.5	10.55	12.0	70	72

¹Protein reference (Ref.) corresponds to the Swiss Prot accession number from the Swiss Prot database (Swiss Institute of Bioinformatics, Geneva, Switzerland); theoretical (Theor.) molecular mass and isoelectric point (pI) of proteins are as according to the amino acid primary sequence and without consideration of any post-translational or degradation modifications. Observed (Obs.) molecular mass and pI are as observed with the position of the corresponding spots on the 2-DE electrophoresis gel.

of α_{s1} - and β -CN. This initial phase of extensive proteolysis of β -CN was in agreement with increased plasmin activity, whereas the proteolysis of α_{s1} -CN could be due to both plasmin activity and enzymes from somatic cells. Urea PAGE electrophoretograms of the control quarter of the cow infused with LLPS showed only little hydrolysis over incubation, whereas milk from the infused quarter after 6 h and incubated at 37°C after 7 d showed hydrolysis of α_{s1} - and β -CN, resulting in new bands appearing on the gels, but to a lesser extent than in milk from the cow infused with HLPS. Other studies have also detected protein fragments resulting from hydrolysis, mainly from α_s - and β -CN (Ballou et al., 1995; Le Roux et al., 1995; Aslam and Hurley 1997; Urech et al., 1999; Moussaoui et al., 2002).

Results of the 2-DE gels demonstrate that exposure to LPS profoundly alters milk protein profiles. Protein patterns after 6 and 12 h of infusion with LPS showed an increase in hydrolysis of α_{s1} - and α_{s2} -CN. Results of other studies (Saeman et al., 1988; Le Roux et al., 1995; Somers et al., 2003; Wedholm et al., 2008a; Larsen et al., 2010) have indicated that the hydrolyses of casein in milk from cows with mastitis, was not exclusively linked to the enzyme plasmin. These studies concluded that

proteinases from somatic cells and from blood may also be responsible for some hydrolysis of casein in the milk from cows with mastitis. The main physiological role of somatic cells of the udder is to protect from infection. This is achieved by the release of active proteolytic enzymes from lysosomes of these cells (Kelly and McSweeney, 2003). A rapid increase in proteolytic activity in quarters injected with LPS after 6 and 12 h resulted in an increase in plasmin activity and was associated with the migration of polymorphonuclear neutrophils (PMN) from blood into the milk component (Mignatti and Rifkin, 1993), resulting in the release of many proteolytic enzymes (e.g., elastase and cathepsins). These proteolytic enzymes damage the blood-milk barrier. Plasmin, elastase, and cathepsins have all been shown to be able to degrade extracellular matrix glycoproteins (Bonney and Legrand, 2000). Elastase and cathepsins have also been found to exhibit proteolytic activity toward bovine casein (Considine et al., 2004). Other LPS-induced mastitis studies showed 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 be cathepsins, were associated

Figure 5. Two-dimensional gel electrophoretograms of model studies of casein digested after 0 (A), 6 (B), or 24 h (C and D) by plasmin (B, C) or cathepsin D (D). The gels were silver stained, and the isoelectric point (pI) range was 4–7. The boxes indicate regions of particulate appearance of new spots in the α_{s1} -degradation zone. The positions of the intact caseins are indicated in (A). M_r = molecular weight.

with mature PMN (Moussaoui et al., 2002). Some of the proteolysis products were subsequently identified in such studies, and comprised fragments of all 4 caseins produced by somatic cell proteases (Moussaoui et al., 2004). Enzyme activities associated with the PMN were later measured as elastase, collagenase, and cathepsins (Haddadi et al., 2006).

The model studies carried out confirmed that plasmin as expected, extensively hydrolyzed β - and α_s -CN, whereas κ -CN was more resistant, though still cleaved to some extent. The amount of plasmin added in the present model study was 0.1 mg/mL, which is approximately 50 times above the level in good-quality milk with low SCC (i.e., 1–2 μ g/mL; Benfeldt et al., 1995), although in high-SCC milk, plasmin content is elevated. Furthermore, the pH used in the model studies, corresponding to the pH of normal milk (6.7–6.8), is more in favor of plasmin activity than that of cathepsin D, which has a lower pH optimum (Hurley et al., 2000). However, cathepsin D still was able to hydrolyze the casein to a significant extent at the conditions applied, which corresponded to a significantly higher level of cathepsin D (in the range of 250 fold) than that present in normal milk with low SCC, which has been reported to be around 0.4 μ g/mL in skim milk, although it is also elevated at elevated SCC (Hurley et al., 2000).

The appearance of BSA, serotransferrin, fibrinogen β chain, vitamin D-binding protein, and cytoplasmic actin in relatively high abundance shown on the 2-DE gels of milk quarters infused with LPS supports the leakage of serum proteins into milk after cytokine production and altered vascular permeability of the mammary epithelium and these proteins are apparently related to the host response. Bovine serum albumin, serotransferrin, and fibrinogen have been identified previously in whey from mastitic milk (Hogarth et al., 2004; Smolenski et al., 2007; Boehmer et al., 2008). Bovine serum albumin was detected in both control and infused milk quarter samples, but in the current study, multiple forms BSA were detected in the milk of infused quarters after 6 and 12 h. Boehmer et al. (2008) also found multiple isoforms of BSA in mastitic whey.

The presence of bovine AMP in the milk of a cow infected with LPS from gram-negative *E. coli* is evidence for neutrophils in the mammary gland (Saad and Östensson, 1990). The cytoplasmic granules of circulating neutrophils are a well-characterized source of AMP belonging to the cathelicidin family, which are released upon neutrophil degranulation in the mammary gland (Tomasinsig and Zanetti, 2005). Studies on the cathelicidin family of peptides have suggested that these peptides can neutralize endotoxins and can suppress the proinflammatory cytokine levels (Scott et al., 2002; Wiese et al., 2003; Zanetti, 2004; Smolenski et al.,

2007; Boehmer et al., 2008; Boehmer et al., 2010).. The presence of the cathelicidin AMP family in bovine milk from cows with mastitis is evidence for the proposal that AMP acts as a defense in the host mechanism. This has previously been reported by Boehmer et al. (2008) and Danielsen et al. (2010).

As this is a model study with a small number of cows, care must be taken in extrapolating the data, but the results still represent a useful contribution to understanding in detail the response of cows to LPS. However, further peptidomic studies of samples obtained from different cows or at other time points may be required to determine, in particular, cow-cow variation in response to such stresses.

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