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Proteolysis in Milk During Experimental *Escherichia coli* Mastitis

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ABSTRACT

This work consisted of the intramammary infections (IMI) of 8 heifers by high doses of *Escherichia coli* to study both the proteolytic activity in milk and the resulting peptides. Therefore, a milking kinetic has been followed, and several parameters have been studied, such as proteose peptones (PP) fraction (quantitative and qualitative changes), plasmin activity (PA), milk somatic cell count (SCC), and bacterial count. A qualitative study of milk proteins and PP was performed by sodium dodecyl sulfate-PAGE, and the peptides recovered in PP during the acute phase of inflammation were amino-terminal micro-sequenced. A BSA increase in milk over time supported the hypothesis of an increase in the permeability of the epithelial barrier. A significant increase in PP content, considered to be an indicator of proteolysis, was observed from postinfusion hours (PIH) 12 to 48. Both the *E. coli* bacterial count and the SCC increased from PIH 3 to 216. Plasmin activity was increased noticeably from PIH 15 to 24. The respective increases in SCC, bacterial count, and PA suggest their involvement in a global mechanism responsible for the increase in proteolysis in milk after *E. coli* challenge. Somatic cell count and *E. coli* may be involved from PIH 3 to 216, and PA involvement might be highlighted during the maximum proteolysis, from PIH 15 to 24. A qualitative study of PP fraction by electrophoresis revealed the apparition of 5 peptide bands: P1 and P2 previously recovered during the lipopolysaccharide challenge, and E1 (27.0 kDa), E2 (15.5 kDa), and E3 (9.0 kDa) were specific to *E. coli* challenge; E1, E2, and E3 contained casein fragments. The roles played by leukocytes and *E. coli* are discussed.
(**Key words:** *Escherichia coli* mastitis, milk, proteolysis, polymorphonuclear neutrophil)

Abbreviation key: PA = plasmin activity, **PIH** = postinfusion hour, **PMN** = polymorphonuclear neutrophils, **PP** = proteose peptones, **t-PA** = tissue plasminogen activator, **u-PA** = urokinase-type plasminogen activator.

INTRODUCTION

Mastitis is a major problem for dairy producers, as it involves great financial losses. The effect of udder health on the yield and quality of milk and, consequently, on cheese production and quality has been established. The SCC is an indicator of the intensity of the cellular immune defense. Milk from healthy udders exhibits a physiological basal cell count, which varies between 50,000 and 200,000 cells/mL of milk, depending on the age of the cow (Harmon, 1994). Upon the onset of mastitis, the increased permeability of the blood-milk mammary epithelial barrier leads first to an influx of serum constituents, such as plasminogen (plasmin zymogen) and numerous other enzymes, and second to a massive recruitment of somatic cells with mainly polymorphonuclear neutrophils (**PMN**; Le Roux et al., 2003) containing a large panel of proteases (Owen and Campbell, 1999). In addition, bacteria exhibit their proteases in the environmental medium after phagocytosis and lysis.

Previous work has been dedicated to assessing the changes in milk composition, especially proteins, and protease activities, including plasmin and PMN enzymes (Moussaoui et al., 2002, 2003) during LPS experimental mastitis. To study the level of protein breakdown, proteose peptone (**PP**) fraction was studied both qualitatively and quantitatively, because this is considered to be an indicator of proteolysis (Le Roux et al., 1995; Moussaoui et al., 2002, 2003). The identification of some fragments from PP that were released from CN breakdown underlined the role of PMN in endogenous proteolysis (Moussaoui et al., 2003).

Lipopolysaccharide from *Escherichia coli* allows clinical mastitis to be imitated, avoiding, however, the involvement of bacteria and making it possible to study endogenous proteolysis. The presence of bacteria, in-

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cluding its endotoxin, would enable a relevant comparison with a model very much closer to reality, as far as both the direct and the nondirect roles of the bacteria are concerned. In addition, *E. coli* itself contains numerous proteases including metallo-, serine- and aspartic-proteases, capable of degrading CN and, in particular, some abnormal proteins such as oxidized proteins (Rozkov, 2001).

Mastitis is mostly induced by bacteria. The recovered pathogen and the corresponding relative frequency vary from country to country. In addition, *E. coli* is one of the most important pathogens causing mastitis in dairy cow (Bradley, 2002). The outcome of experimental *E. coli* mastitis ranges from mild to severe in individual dairy cows and can be quantified by bacterial counts in milk after infection (Lohuis et al., 1990). The clinical effects of the infection can, however, be very severe, leading to death during the periparturient period (Sordillo and Babiuk, 1991). Proteolysis in milk during *E. coli* mastitis has received very little attention. Very little information is available either on the involvement of the bacteria on CN breakdown or on the intensity of proteolytic machinery during the infection.

Therefore, the aim of this work was to explore milk proteolysis during *E. coli* experimental mastitis primarily to investigate sequentiality, to precisely define the roles of both endogenous proteases (including plasmin and leukocyte enzymes) and bacterial proteases. The PP protein fraction was used as an indicator of proteolysis. Both PP content and qualitative composition were investigated; PP composition change was compared with CN breakdown by leukocytes. In addition, plasmin activity (**PA**), SCC, and cfu were observed for several hours after the induction of *E. coli* mastitis.

MATERIALS AND METHODS

Experimental Animals and Study Facilities

All heifers (n = 8) had calved less than 1 wk before arrival at a Ghent University dairy farm (Commercial Dairy Farm Oudenaarde, Oudenaarde, Belgium). The heifers were on a system of zero grazing from arrival until the end of the inoculation trial and were fed twice daily. The ration consisted of corn silage, hay, and water ad libitum. Concentrates (Sandilac; Dumoulin Voeders Sanders, Moorslede, Belgium) were distributed according to milk production.

For inclusion in the intramammary inoculation trial, no treatment of clinical diseases (mastitis) was allowed within 10 d before the intramammary inoculation. Therefore, only clinically healthy animals, free of major mastitis pathogens on 2 consecutive bacteriological negative examinations, with an SCC <200,000/mL on quarter level, were finally included in the intramammary *E. coli* challenge.

Mechanical milking was performed twice daily using a quarter milking device (Packo & Fullwood, Zedelgem, Belgium).

Intramammary Inoculation Procedure

A stock of *E. coli* strain P4:O32 (Bramley, 1976) was maintained in lyophilization medium at –20°C. For experimental use, the bacteria were subcultured in brain-heart infusion broth (CM225; Oxoid, Nepean, ON, Canada) at 37°C for 3 consecutive days. The bacterial suspension was washed 3 times with pyrogen-free PBS, and resuspended in PBS.

Before the challenge, the teat ends were disinfected with 70% ethanol containing 0.5% chlorhexidin. Just before inoculation, the suspension was diluted in pyrogen-free PBS to a final concentration of 1×10^4 cfu/mL (n = 4) or 1×10^6 cfu/mL (n = 4). On d 0, 30 min after morning milking (1.5 h after feeding), the cows were inoculated in the left front and rear quarters with a suspension containing 1×10^4 cfu or 1×10^6 cfu *E. coli* P4:O32 in a total volume of 10 mL of pyrogen-free saline solution (0.9%) per quarter. After IMI, the bacterial suspension was thoroughly distributed into the udder cistern through a 30-s massage. All bacterial suspensions were infused into the teat cistern using a sterile, pyrogen-free teat cannula (7 cm; Me.Ve.Mat, Deinze, Belgium).

Sampling Procedure

Milk samples were collected before and at postinfusion hours (**PIH**): –24, 0, 3, 6, 9, 12, 15, 18, 21, 24, 33, 48, 57, 72, 144, and 216.

Foremilk (5 mL) was aseptically collected for bacteriologic examination for major pathogens before inoculation and for quantification of the *E. coli* population level (cfu) from PIH 3 onward. Furthermore, milk samples (100 mL) were collected for the determination of SCC and the preparation of milk whey through centrifugation. All samples were kept on melting ice (1°C) during transportation.

Clinical Examination

Standard clinical parameters were examined. Rectal temperature, heart rate, respiration rate, rumen motility, fecal appearance, appetite, general behavior, and aspects of the mammary gland (abnormal milk, swelling, teat relaxation, and milk leakage) (Massart-Leën et al., 1988) were recorded by a veterinarian each time blood and milk samples were collected.

SCC and Milk Composition

Somatic cell count was determined using a fluoro-opto electronic method (Fossomatic 400 cell counter; Foss Electrics, Hillerød, Denmark). The PP content was determined as described previously (Le Roux et al., 1995).

Quarter Milk Production

Daily quarter milk production, the yield of the evening and subsequent morning milking, was measured using a quarter milking device (Packo and Fullwood, Zedelgem, Belgium). Quarter milk production, expressed as L/d, was measured at d –4, –1, 0, 1, 2, and 3.

Colony-Forming Units in the Inoculated Quarters

The *E. coli* population level (cfu/mL) after experimental inoculation was determined by appropriate 10-fold dilutions of the sample in PBS. Ten microliters of these dilutions were plated out on Columbia Sheep Blood agar (Biokar Diagnostics, Beauvois, France). All dilutions were performed in duplicate. Colonies were counted after 24-h incubation at 37°C. The colony count was converted to cfu/mL based on the dilution factor, and the final results were expressed as $\log_{10}$(cfu)/mL.

PA

Plasmin activity was measured by a method based on the release of a yellow compound (measured at 405 nm) when a synthetic plasmin-sensitive substrate (D-Val-Leu-Lys p-nitroanilide dihydrochloride; Sigma) was hydrolyzed after the addition of a dissolving reagent (Linden et al., 1987).

Peptide Characterization

An SDS-PAGE was performed according to the method of Laemmli and Favre (1973) with a 5% (wt/vol) polyacrylamide stacking gel in 0.125 *M* Tris-HCl (pH 6.8) and with 15% (wt/vol) polyacrylamide separating gel in 0.38 *M* Tris-HCl (pH 8.8) in the presence of 0.1% (wt/vol) SDS and 5% (vol/vol) 2-mercaptoethanol. Quantities of 60 μg protein for PP and 100 μg for milk per well were loaded into the gel for each sample.

Proteins were fixed in gel using 12% (wt/vol) TCA and stained with 0.1% (wt/vol) Coomassie blue R250 in 50% (vol/vol) ethanol and 10% (vol/vol) acetic acid for at least 2 h. Destaining was performed with a solution of 30% (vol/vol) ethanol and 7.5% (vol/vol) acetic acid. Quantification of the electrophoretic bands was performed by densitometry at 633 nm (Ultrascan XL densitometer; Pharmacia Fine Chemicals, Uppsala, Sweden), and the amount of BSA in milk was deduced from the amount of protein loaded per well in the gel. Results were expressed as a ratio between the BSA amount at time t and the amount before challenge PIH 0. The ratio at PIH 0 was set at 100%.

For microsequencing analysis, proteins were electrotransferred after electrophoresis onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Milford, MA) for 5 h at 4°C in 10 m*M* 3-[cyclohexylamino]-1-propanesulfonic acid buffer (pH 11), containing 10% (vol/vol) methanol (Towbin et al., 1979). Proteins were stained with 0.2% (wt/vol) Ponceau S Red, and the membrane was washed with ultrapure water. After excision, bands of interest were amino-terminal microsequenced by Edman degradation on an automated 476 A protein microsequencer (Applied Biosystems, Foster City, CA).

Statistical Analysis

A mixed model was fitted to evaluate the effect of an IMI on PP, PA, and BSA with time, dose, and their interaction as categorical fixed effects and cow as a random effect. Because the dose and its interaction with time were not significant (see results section), a mixed model with only time as categorical fixed effect and cow as random effect was fitted.

Within this model, PP and PA at each time point were compared with the baseline value at time 0 using Dunnett's multiple comparisons adjustments technique with an overall type I error equal to 5%.

RESULTS

Clinical Response to *E. coli*

All quarters were free of major pathogens before the challenge, and no infections other than *E. coli* occurred during the entire experimental period. Quarter swelling and elevated quarter temperature occurred onward in the infected quarters from PIH 9. Teat relaxation, milk leakage, and diarrhea, considered as indicators of severe clinical illness, only appeared in a small number of animals. Changes to abnormal milk included clots, flakes, and a watery appearance. This was mostly visible in all infected animals at PIH 18. All primiparous cows had a moderate clinical outcome of *E. coli* mastitis based on their quarter milk production. Rectal temperature increased from PIH 9 onward, with peak fever reached at PIH 12. Rumen motility was depressed from PIH 9 onward, resulting in a decrease in appetite. Primiparous cows returned to normal appetite and reticulorumen motility around PIH 21. Daily milk production in infected and uninfected quarters averaged 8.43 ±

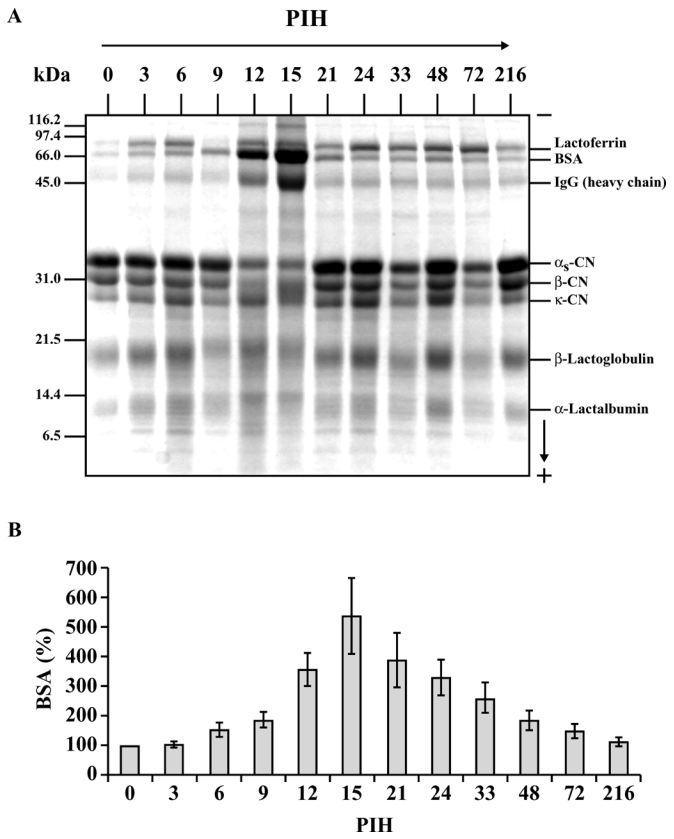

Figure 1. A) SDS-PAGE electrophoregrams of skim milk over time after *Escherichia coli* challenge. The electrophoregram presented was representative of the height gels performed (n = 8); 100 μg of protein per well was loaded, and staining was performed with Coomassie blue R 250. PIH = postinfusion hour. B) The BSA amount over time was calculated according to densitometric data based on the electrophoregram of skim milk. Results were expressed in mean values of a ratio between BSA amount at time t and BSA amount before challenge (PIH 0). Ratio at PIH 0 was fixed at 100%. Time 0 h was considered as a standard before infection. Error bars, when not visible, were included in symbols (n = 8).

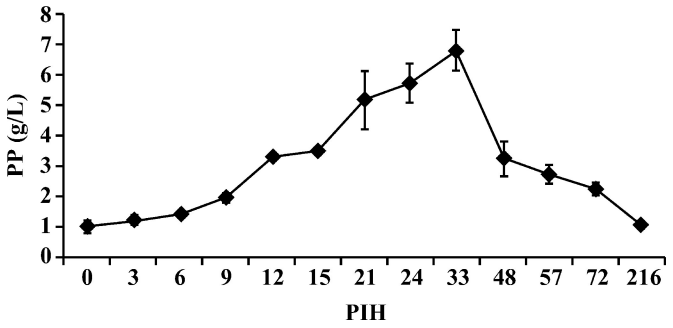

Figure 2. Mean values of proteose peptones (PP) content in skimmed milk of inflamed quarters after intramammary inoculation of *Escherichia coli*. Analyses were performed at times 0, 3, 6, 9, 12, 15, 21, 24, 33, 48, 57, 72, and 216 h. Time 0 h was considered as a standard before infection. PIH = postinfusion hour. Error bars, when not visible, were included in symbols (n = 8).

0.40 L/d preinfection. On the day of infection, milk production decreased in both infected quarters (–65%; 2.81 ± 0.46 L/d) and uninfected quarters (–35%; 5.53 ± 0.46 L/d). Two days after infection, milk production in the contralateral uninfected quarters returned to normal (±100%). As for fever, rectal temperature increased from PIH 6 and peaked significantly at PIH 9, reaching a maximum of 40.8°C (±0.5) on average with a range from 38.2 to 41.7°C. A return to initial values was observed at PIH 18. Heart rate peaked at PIH 9, reaching 101 beats/min (±7.0).

Milk Protein Compositional Change

Protein from serum, including lactoferrin, BSA, and Ig G, were slightly visible in milk before challenge (Figure 1A). The 3 proteins were easily visible from PIH 6 after challenge with a maximum at PIH 12 to 15 for BSA and Ig G on the one hand and PIH 24 to 72 for lactoferrin on the other hand. Milk proteins, including CN, β-lactoglobulin, and α-lactalbumin, were present during the complete kinetic. A decrease in CN band intensity was, however, noted and particularly at PIH 12 and 15.

The BSA increase over time was significantly higher from PIH 0 compared with PIH 6, with 1.5 times the level before inoculation (Figure 1B). It peaked at PIH 15 with 5.3 times the initial level and returned to normal level after PIH 72.

PP content. For PP, there was no significant difference between the 2 doses ($P = 0.6238$), but PP did vary over time ($P < 0.0001$). There was no interaction between the dose and the time ($P = 0.7848$) so that it can be assumed that PP evolved in a similar way for the 2 doses. The PP content before challenge averaged 0.97 g/L (±0.20), and it differed significantly from this baseline value from PIH 12 (adjusted $P = 0.027$) onward until PIH 48 (adjusted $P = 0.034$). It reached its maximum of 6.52 g/L (±0.22) at PIH 33. The average increase was 7.3× (±1.7). Return to normal values was observed at PIH 216 (PIH 0 vs. PIH 216; $P < 0.05$; Figure 2).

SCC in Milk

All cows had a SCC before challenge <200,000 cells/mL, averaging 59,000 cells/mL. Log SCC averaged 4.3 before challenge and increased significantly from PIH 3; a steady state was noted for all cows from PIH 6 to 72 with an average of 6.6 (±0.1). A slight decrease was then observed until PIH 216. No return to baseline was observed at PIH 216 (Figure 3).

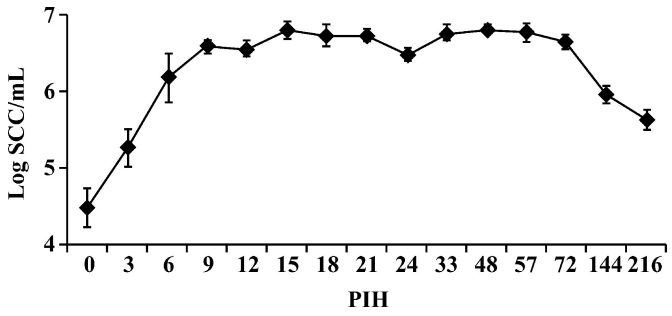

Figure 3. Mean values of SCC in milk over time after intramammary inoculation of *Escherichia coli*. Results were expressed in Log SCC/mL. Analyses were performed at times 0, 3, 6, 9, 12, 15, 18, 21, 24, 33, 48, 57, 72, 144, and 216 h. Time 0 h was considered as a standard before infection. PIH = postinfusion hour. Error bars, when not visible, were included in symbols (n = 8).

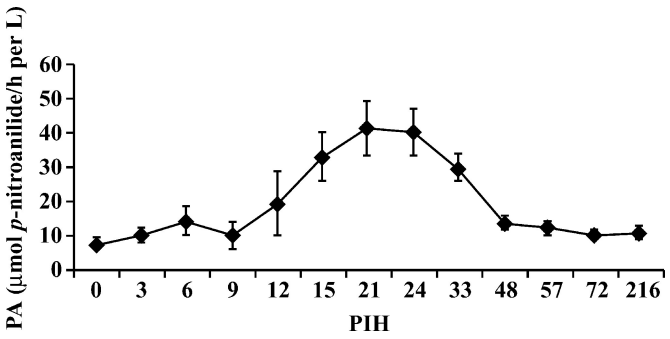

Figure 5. Mean values of plasmin activity (PA) change in whole milk over time after intramammary inoculation of *Escherichia coli*. Results were expressed in μmol *p*-nitroanilide/h per L. Analyses were performed at times 0, 3, 6, 9, 12, 15, 21, 24, 33, 48, 57, 72, and 216 h. Time 0 h was considered as a standard before infection. PIH = postinfusion hour. Error bars, when not visible, were included in symbols (n = 8).

Colony-Forming Units of *E. coli* in Milk

Milk from all cows was free of *E. coli* before inoculation. A maximum Log cfu was reached at PIH 3 with 4.3 (±0.3). No return to baseline was noted even at PIH 216 (Figure 4).

PA

For PA, there was no significant difference between the 2 doses ($P = 0.7848$), but PA did vary over time ($P < 0.0001$). There was no interaction between dose and time ($P = 0.2385$), so that it can be assumed that PA evolved in a similar way for the 2 doses. Before infusion, the average PA level was 7.7 μmol (±1.64) *p*-nitroanilide/h per L. Plasmin activity did not differ significantly from this baseline value until PIH 12, but at PIH 15, the PA level was significantly higher than baseline ($P = 0.015$) until PIH 24 ($P = 0.0001$). Maximum increase in comparison with mean values before challenge averaged 4.2× at PIH 24 (Figure 5).

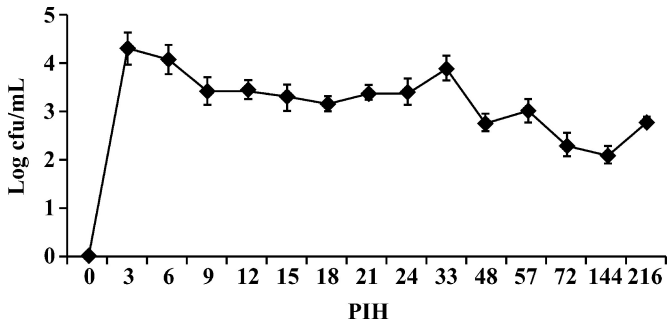

Figure 4. Mean values of cfu in milk over time after intramammary inoculation of *Escherichia coli*. Results were expressed in Log cfu/mL. Analyses were performed at times 0, 3, 6, 9, 12, 15, 18, 21, 24, 33, 48, 57, 72, 144, and 216 h. Time 0 h was considered as a standard before infection. PIH = postinfusion hour. Error bars, when not visible, were included in symbols (n = 8).

Composition Change in PP Fraction

The SDS-PAGE showed a PP fraction before challenge mainly containing 2 bands corresponding to PP3 (f1 to 135) for the upper one and β-CN (f1 to 105/107) + PP3 (f54 to 135) for the faster migrating band (Figure 6). During the challenge, 2 types of profiles appeared. The first type showed that at PIH 215 additional electrophoretic bands appeared, although not visible before the challenge. These were named E1, P1, P2, E2, and E3. The second type showed that at PIH 24 a single band appeared named P2. The respective molecular masses of the cited bands were 27.0, 17.5, 21.0, 15.5, and 9.0 kDa. Table 1 gives the corresponding sequences with CN fragments for all bands and an additional fragment of BSA for E3.

DISCUSSION

Clinical Response to *E. coli* Challenge

Mastitis caused by IMI of *E. coli* involved a local and systemic response of the immune system. Indeed, the increase in SCC at sites of inoculation (the 2 left quarters), the decrease in milk production, fever, and increased heart rate were in accordance with previous studies of *E. coli* challenges (Michelutti et al., 1999; Blum et al., 2000; Hoeben et al., 2000) and support this idea. Clinical signs and loss in milk production are typically observed in cases of clinical mastitis (Michelutti et al., 1999; Moussaoui et al., 2002). Milk secretion is known to be impaired by systemic and local effects. In addition, a recent study showed that CN fragments,

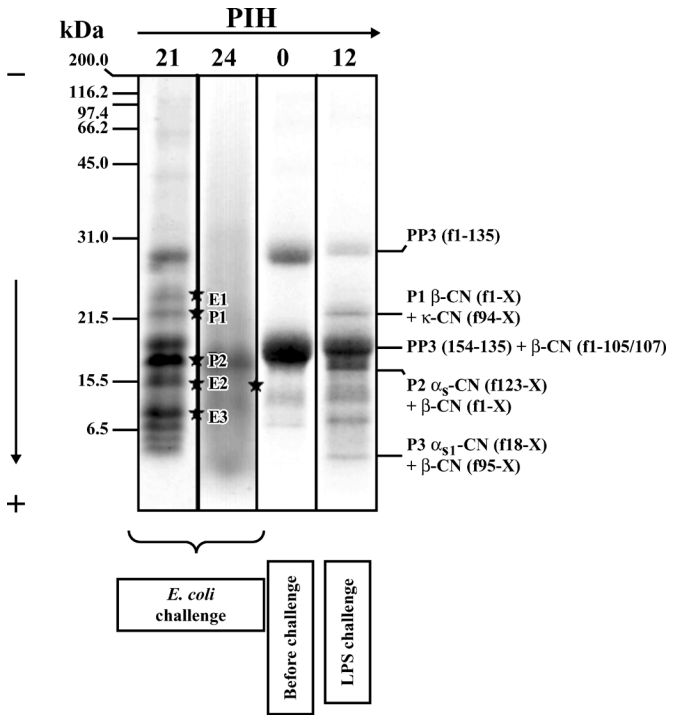

Figure 6. SDS-PAGE electrophoregrams of proteose peptone (PP) fraction at highly representative times of the kinetics following *Escherichia coli* challenge. The PP fraction of milk before challenge was held to be a standard fraction. The PP fraction obtained during lipopolysaccharide (LPS) challenge (Moussaoui et al., 2003) was representative of the phase when P1, P2, and P3 were the most visible. Sixty micrograms of protein per well were loaded. Staining was performed with Coomassie blue R 250. P1, P2, E1, E2, and E3 were the bands appearing in PP fraction during the *E. coli* experimental mastitis. PIH = postinfusion hour.

present in milk of the mammary gland cistern, were capable of disrupting the tight junctions (Shamay et al., 2003) or blocking potassium channels (Silanikove et al., 2000) and, therefore, preventing epithelial cells from synthesizing milk constituents.

The increased permeability of the mammary epithelial barrier was associated with inflammation and was supported here by the influx of serum proteins, including lactoferrin, BSA, and IgG, into milk, which is also in accordance with previous work (Hamann and Krömker, 1997).

There are several explanations that may explain the long duration and the later occurrence of the increased permeability after *E. coli* challenge in comparison with LPS challenge. In milk, *E. coli* releases very low concentrations of LPS in comparison with a direct intramammary injection of LPS, thereby explaining the later and lower response observed with the whole bacteria. Another explanation could be the engulfment of bacteria by epithelial cells (Döpfer et al., 2000, 2001) during the highest response of the immune system and the release of *E. coli* intact when the immune defense starts to decrease.

Sequentiality in Milk Proteolysis

Somatic cells. The SCC increased from PIH 3 and then reached a plateau from PIH 6 to 216. For LPS challenge, SCC decreased already from PIH 16 onward. In addition, maximum SCC was lower after *E. coli* challenge (3.98×10^6 cells/mL) than after LPS challenge (27×10^6 cells/mL; Moussaoui et al., 2002). This supported previous clinical response and PP content results, as far as the immune response is concerned. In addition, the massive influx of leukocytes was supported by the

Table 1. Partial identification of the peptides of bands P1, P2, E1, E2, and E3 characterized by SDS-PAGE and Edman amino-terminal microsequencing. Proportion (%) that corresponded to the yield of the sequencer.[1]

Peptide band	Apparent molecular mass (kDa)	Amino-terminal sequence	Proportion (%)	Identity	Amino-terminal cleavage sites	Protease likely to be involved
P1	17.5	$_{NH2}$**R-E-L-E-E-**	90	β-CN (f1-X)	—	EL[2]
		$_{NH2}$**T-M-A-R-H-**	10	κ-CN (f94-X)	Thr_{93}-Thr_{94}	ND
P2	21.0	$_{NH2}$**L-N-R-E-Q-L-S-T-**	70	α_{s2}-CN (f123-X)	Thr_{122}-Leu_{123}	ND
		$_{NH2}$**R-E-L-E-E-L-N-**	30	β-CN (f1-X)	—	ND
E1	27.0	$_{NH2}$**R-E-L-E-E-L-N-V-P-**	55	β-CN (f1-X)	—	EL[2]
		$_{NH2}$**T-M-A-R-H-P-H-P-H-**	45	κ-CN (f94-X)	Thr_{93}-Thr_{94}	ND
		$_{NH2}$**A-Q-(T)-Q-S-L-V-Y-P-F-P-**	39	β-CN (f53-X)	Phe_{52}-Ala_{53}	CD, CG, EL[3]
E2	15.5	$_{NH2}$**E-I-V-E-S-L-S-S-S-E-E-**	25	β-CN (f11-X)	Gly_{10}-Glu_{11}	ND
		$_{NH2}$**N-L-L-R-F-F-V-A-P-F-P-**	20	αs1-CN (f19-X)	Glu_{18}-Asn_{19}	ND
		$_{NH2}$**Y-Y-A-K-P-A-A-V-R-S-P-**	16	κ-CN (f60-X)	Pro_{59}-Tyr_{60}	ND
E3	9.0	$_{NH2}$**A-Q-T-Q-S-L-V-Y-P-F-P-**	50	β-CN (f53-X)	Phe_{52}-Ala_{53}	CD, CG, EL[3]
		$_{NH2}$**E-Y-G-F-Q-N-A-L-I-V-**	50	BSA (f405-X)	Ala_{404}-Leu_{405}	ND

[1]CD = cathepsin D, CG = cathepsin G, EL = elastase from leukocytes, and ND = not determined.

[2]According to Moussaoui et al. (2003).

[3]According to Larsen et al. (1996) for CD, to Considine et al. (2002) for CG, and to Considine et al. (1999) for EL.

increasing permeability of the epithelial barrier according to the BSA increase in milk over time.

E. coli. Colony-forming unit increase was effective from PIH 3, and the plateau lasted until PIH 216. The bacteriostactic effect combined with the lag time could last between 3 and 6 h after *E. coli* inoculation, which corresponded to weak bacterial growth caused by some milk proteins with an inhibitory effect including lactoferrin, lysozyme, and some CN fragments resulting from proteolysis (Lahov and Regelson, 1996; Smithers et al., 1996).

PA. Plasmin activity was significantly higher than baseline between PIH 15 and 24. The increase in PA was lower after *E. coli* challenge (maximum of 41.4 μmol of *p*-nitroanilide/h per L) than after LPS challenge (75.8 μmol of *p*-nitroanilide/h per L; Moussaoui et al., 2002). Plasmin is known to be the main protease involved in CN breakdown in milk (Kaartinen et al., 1988; Le Roux et al., 1995). The increase in its activity is linked to that of the permeability of the milk epithelial barrier during inflammation and, therefore, to the increase in BSA amount over time. This is supported by previous data related to the increase in permeability (Nickerson and Pankey, 1984) and therefore to an important influx of plasminogen, its zymogen (Kaartinen et al., 1988). In addition, numerous activators of plasmin and its zymogen come from blood stream, PMN, and bacteria. During mastitis, the serine protease activator concentration, including plasmin and plasminogen in blood and also in milk, increases sharply according to some researchers (Yamagata et al., 2001; Le Roux et al., 2003;). Polymorphonuclear neutrophils have a pool of plasminogen activators, such as urokinase type (**u-PA**) and, to a lesser extent, tissue (**t-PA**), according to Moir et al. (2001). *Escherichia coli* is reported to have both plasmin and plasminogen receptors on its surface, and these enhance enzyme activation and express plasminogen activators. An indirect plasmin activation channel is reported as being due to the secretion of t-PA by epithelial cells, thanks to cell stimulation by IL-1, in turn resulting from the release of monocytes, consequent to the contact with *E. coli* (Lähteenmäki et al., 2001).

PP. The PP fraction is widely considered as a relevant indicator of proteolysis (Le Roux et al., 1995; Urech et al., 1999; Moussaoui et al., 2002). The increase in PP content as well as the maximum content (from PIH 12 to 33) was effective later than that following LPS challenge (Moussaoui et al., 2002). The PP increase was much more marked for *E. coli* (0.97 g/L at PIH 0 vs. 6.52 g/L at PIH 33) than for LPS challenge (1.02 g/L at PIH 0 vs. 2.49 g/L at PIH 8; Moussaoui et al., 2002). In addition, the increase lasted longer with *E. coli* than after LPS challenge. Casein breakdown occurred with a lag time, but was more intense in the case of *E. coli* mastitis. One explanation of the lag time could be the longer delay required for the immune response in the presence of *E. coli*. The slight increase in caseinolysis would be linked to the first immune barrier that was non-specific and corresponded to mainly macrophages.

Sequentiality. The increases in SCC, cfu, and PA over time suggested their involvement in a global mechanism responsible for the increase of proteolysis in milk after *E. coli* challenge. Somatic cell count and *E. coli* may be involved from PIH 3 to 216, and PA involvement might be highlighted during the maximum proteolysis, from PIH 15 to 24. The maximal increase in PP content might correspond to very high protease concentrations in the milk. This very high PP content (up to 6.52 g/L) could result from an interaction between *E. coli*, the system plasmin-plasminogen, and neutrophils. Indeed, the regulatory mechanisms remain key in explaining the non-additional increase of proteolytic activities. Indeed, proteases of neutrophils as well as plasmin are known to permit the chemotaxis of cells in the site of inflammation. Furthermore, they are involved in the limitation in time of the immune response, e.g., by the cleavage of some cytokines such as IL-2, IL-6, IL-8, etc. Among these proteases, collagenase IV (also called MMP-9) activates cathepsin G as well as plasmin and inhibits serpines, the inhibitors of serine proteases. During the inflammation of the mammary gland, numerous protease activators and inhibitors come from the blood stream such as α2-macroglobulin, α1-antiplasmin, serpines, u-PA, and t-PA (Le Roux et al., 2003). The involvement of *E. coli* consists of an activator effect on pro-gelatinase A (also called pro-MMP-2) by *E. coli*-derived serine proteases (Takeda et al., 2000). The third (late) phase, from PIH 33 to 216, corresponded to the return to the initial level, with an increased level of SCC and cfu. In conclusion, sequentiality in the mechanism responsible for the increase of proteolysis in milk after *E. coli* challenge was suggested according to SCC, PA, and cfu data variation over time. Both leukocyte and *E. coli* taking parts were noted from PIH 3 to 216, but PA was significantly increased during the maximal proteolysis, from PIH 15 to 33.

CN Breakdown and the Resulting Peptides

During challenge, a compositional change in the PP fraction was noted with the apparition of 5 electrophoretic bands, including P1 and P2, previously reported during LPS challenge (Moussaoui et al., 2003) as well as E1, E2, and E3 specific to the *E. coli* challenge. The endogenous origins of CN breakdown are illustrated by previous studies. These demonstrate that concomitant increases in SCC and neutrophil enzymes play a key

role in caseinolysis and are described by a recent work dealing with some CN peptides generated during LPS mastitis (Moussaoui et al., 2003). Indeed, P1 and P2 are known to be generated after CN breakdown by PMN proteases (Moussaoui et al., unpublished data) and P1 by elastase (Moussaoui et al., 2003), which elicits the involvement of PMN during the inflammatory response to *E. coli* challenge. Polymorphonuclear neutrophils degrade CN in extracellular compartments during degranulation (Owen and Campbell, 1999) as well as in intracellular compartments such as CN fragments, which are internalized by neutrophils (Paape and Guidry, 1977). The activity of neutral proteases would be effective by degranulation; however, for acidic proteases, 2 mechanisms can be hypothesized: 1) activity at local pH levels in the close surroundings of the cell and 2) lysosomal action after the internalization of CN fragments followed by a release into the milk. As for the exogenous origins of CN breakdown, *E. coli* is known to contain several proteases likely to be involved. Nevertheless, the protease of *E. coli* best known to degrade CN is called ClpAP (caseinolytic protease with ATPase), according to Katayama et al. (1988). This protease, also called Ti, consists of a chaperone component, ClpA, and a proteolytic component, ClpP, which is a serine protease (Hwang, 1988). Numerous other proteases of *E. coli* can be cited, including serine and metallo-proteases, a majority of which are known to degrade CN (Rozkov, 2001). In addition to the large number of proteases and also that of enzyme activity during mastitis caused by bacteria, it is noteworthy that many of them degrade specifically abnormal proteins such as oxidized ones (Rozkov, 2001). Indeed, oxidized proteins forming aggregates, are selectively recognized and degrade by the intracellular proteasomal system (Merker and Grune, 2000) of activated macrophages (Gieche et al., 2001) as well as proteases from *E. coli* (Davies and Lin, 1988). This is highlighted by the production of reactive oxygen and nitrogen species during the inflammatory process, which can enhance CN breakdown. Indeed, reactive oxygen species and nitric oxide production during inflammation, and more precisely during LPS and *E. coli* mastitis, are well known (Blum et al., 2000; Sethi et al., 2001). Interestingly, this oxidative stress is strongly increased by serine proteases during inflammation (Aoshiba et al., 2001), which is highlighted by the increase in some serine protease activity during inflammation, e.g., elastase and cathepsins G during LPS mastitis (Le Roux et al., 2003). In conclusion, *E. coli* challenge induced the generation of 5 peptide fractions in PP: 2 had previously been recovered following LPS challenge and 3 were specific to *E. coli* challenge. Besides endogenous origins, proteolysis during *E. coli* challenge exhibits 2 specific origins: the large panel of bacterial proteases and the protein oxidation that increases their susceptibility to proteolysis.

CONCLUSION

The present study enabled a better understanding of proteolysis in milk during clinical *E. coli* mastitis. The *E. coli* model was also much closer to reality than the LPS model, more commonly used because of its ease of reproduction. The respective increases in SCC, cfu, and PA suggested their involvement in a global mechanism responsible for the increase of proteolysis in milk after *E. coli* challenge. Somatic cell count and *E. coli* may be involved from PIH 3 to 216, and PA involvement might be highlighted during the maximum proteolysis, from PIH 15 to 24. Casein breakdown was highlighted by the recovery, on the one hand, of the same CN fragments noted during LPS challenge (P1, P2) and previously shown to result from neutrophil proteases and, on the other hand, by 3 specific fragments resulting from *E. coli* challenge (E1, E2, E3). The latter are likely to be generated by bacterial proteases and need to be confirmed by further research.

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