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Original article

E. coli proteolytic activity in milk and casein breakdown

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Abstract – Previous studies have focused on both LPS and *E. coli* experimental mastitis and underlined the respective roles of endogenous proteolysis (including plasmin from the blood stream and other proteases from milk leukocytes), as well as the presence of *E. coli* in a more intricate system. The aim of this study was to assess the role of *E. coli* in milk proteolysis and especially that of its proteases in casein breakdown. The first part consisted in the incubation of 10^4 cfu·mL^{-1} of the *E. coli* strain in raw milk at 37 °C for 24 h; the same milk was also incubated with 0.04% sodium azide. Several parameters were evaluated: CFU, plasmin activity, gelatinase activity and pH 4.6 insoluble peptides, including the proportion of γ-CN. The profile of gelatinase activity was determined by zymography and identified by immunoblotting. In the second part of the study, we examined the profile of CN (α*s*-, β- and κ-CN) breakdown by *E. coli* lysate. The results suggest that *E. coli* proteases have a direct effect on CN, and the increase of γ-CN in inoculated milk may be generated by both plasmin and the gelatinase. Moreover, the gelatinase activity in the inoculated milk was higher after 24 h of incubation.

caseins / γ-CN / *E. coli* / gelatinase activity / milk / plasmin

Abbreviations: CFU: colony-forming units; CN: casein; IMI: intra-mammary infusion; PA: plasmin activity; PI: post inoculation; SCC: somatic cell count.

1. INTRODUCTION

Mastitis is an inflammation of the mammary gland with an increase of somatic cell count (SCC) in milk which results mainly from the presence of bacterial pathogens. Mastitic milk exhibits greatly increased proteolytic activity [1]. The proteolysis of milk has been studied both in vivo and in vitro [2–4] and can be attributed to both native proteases (endogenous) including plasmin (E.C. 3.4.21.7) and proteases from leukocytes, and non-native (exogenous) proteases from microorganisms infecting milk [5].

Lysosomes in somatic cells contain proteases such as elastase (E.C. 3.4.21.37), cathepsin G (E.C. 3.4.21.20), collagenases (E.C. 3.4.24.3) and gelatinases (E.C. 3.4.24.35),

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which are the predominant enzymes associated with polymorphonuclear neutrophils (PMN) in mastitic milk [6]. Considine et al. [7] reported the cleavage specificity of elastase and cathepsin B (E.C. 3.4.22.1) on α_{s1}- and β-CN, and showed that caseins are very suitable substrates for proteolysis by such enzymes. Plasmin is the major native milk protease [8]. This alkaline proteinase with an optimum pH of 7.5 readily hydrolyses β-CN and α_{s2}-CN and acts more slowly on α_{s1}-CN [9]. One of the indicators of proteolysis that is most commonly used is pH insoluble peptides including γ-caseins directly resulting from casein (CN) breakdown [2–4].

The involvement of bacteria during mastitis in milk proteolysis received little attention in the literature until the work of Andrews [10] about the significance of proteinases originating from milk leucocytes and a comparison of the action of leucocytes, bacterial and natural milk proteinases on casein.

Escherichia coli is one of the most important pathogens that cause mastitis in dairy cows [11], it alone contains numerous proteases including metallo-, serine- and aspartic proteases, capable of degrading CN, as well as some abnormal proteins such as oxidised proteins [12].

Matrix metalloproteinases (MMP) are a class of secreted enzymes that play a major role in the degradation and remodelling of all components of the extracellular matrix [13]. Gelatinases also degrade types V, VII and XI collagens and act synergistically with interstitial collagenases by degrading denatured collagens (gelatins) [14].

The number of bacteria in milk depends on the balance between the growth rate of bacteria and the elimination rate by host defence mechanisms. Milk from individual cows could differ in its growth medium properties for *E. coli*, resulting in different levels of bacterial load within the first hours after experimental infusion and, ultimately, in differences in severity of *E. coli* mastitis [15].

The lipopolysaccharide and *E. coli* experimental mastitis were used at the laboratory in order to study the changes in milk composition. Proteolysis was much more marked for *E. coli* than for LPS. The aim of the present work was to assess the role of *E. coli* in milk proteolysis and that of its proteases in casein breakdown. This was achieved, first, by studying bacterial growth in raw milk as well as numerous parameters linked to proteolysis, such as plasmin activity (PA), gelatinase activity and the proportion of pH 4.6 insoluble peptides, including γ-CN. Secondly, casein breakdown was investigated by incubating *E. coli* lysate with α*s*-, β- and κ-CN: the results were evaluated by SDS-PAGE. The results of our experiment were compared to naturally occurring endotoxin mastitis caused by *E. coli*.

2. MATERIALS AND METHODS

2.1. Animals and milk sample collection

Four cows were selected according to the method of Harmon et al. [16]. All quarters were free of both major and minor pathogens. Somatic cell counts (SCC) were quantified by Fossomatic 5000 (Foss Electric, Hillerød, Denmark). The SCC of each quarter was lower than 100 000 cells·mL^{-1} and had no history of clinical mastitis during the previous and current lactation. Milk samples were collected aseptically from the four quarters during the morning milking, the milks were mixed and homogenised. Before each sampling, the udders and teats were wiped clean with a dry paper towel. The teat ends were swabbed with cotton drenched in methylated spirits. Milk was collected in a sterile glass bottle for diagnostic bacteriological culture and for biochemical analyses. The samples were immediately put on ice and transported to the laboratory.

The milk was divided into two parts. The first part was mixed with 0.01% (wt·vol^{-1}) NaN_3 for the control and the second part was kept for further inoculation with *E. coli*.

2.2. Bacterial growth assay in raw milk

The *E. coli* strain P4:O32 (kindly provided by the Department of Physiology, Biochemistry and Biometrics of Professor Burvenich, Ghent, Belgium), previously isolated from a clinical case of bovine mastitis and freeze-dried, was stored at –20 °C. For the purpose of the experiment, the bacteria were subcultured in brain-heart infusion (CM225; Oxoid, Nepean, ON, Canada) and incubated at 37 °C under agitation overnight for one day. Before the start of the bacterial growth assay, the suspension of the bacteria was further diluted in 9‰ NaCl buffer, until a final concentration of 1×10^5 colony-forming units (cfu)·mL^{-1} was reached. In order to follow the bacterial count throughout time, one mL of the bacterial suspension was added to 9 mL of all of the 14 sterile tubes, until the final bacterial concentration was 10^4 cfu·mL^{-1} in all of the tubes. All samples were kept in an incubator with agitation at 37 °C. Sampling was performed at times 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 24 h post-inoculation for bacteriological counts and pH measurements in order to control milk acidity.

In order to follow milk compositional change, 340 mL of the bacterial suspension were added to 3.4 L of whole milk to obtain inoculums at 10^4 cfu·mL^{-1}. After homogenisation, 120 mL were distributed in sterile glass bottles and incubated at 37 °C under agitation. All samples were assayed in duplicate at times 0, 4, 8, 12 and 24 h post-inoculation for further biochemical analyses.

2.3. Plasmin activity

Plasmin activity was measured by a method based on the release of a yellow compound (measured at 405 nm) when a synthetic substrate (D-Val-Leu-Lys p-nitroanilide dihydrochloride; Sigma) for plasmin was hydrolysed, after addition of a dissolving reagent [17].

2.4. Zymography

Detection of gelatinase in milk was achieved by zymography, using a gelatin substrate [18]. For each sample, 15 mg of lyophilised milk per well were used, in the presence of 0.1% (wt·vol^{-1}) SDS. Zymography was performed in 10% acrylamide gel containing 1 mg of porcine skin gelatin·mL^{-1} (Sigma) as a substrate or 1 mg of casein·mL^{-1} of gel. The samples were run at 100 V for 60 min. After electrophoresis, the gels were washed in 2.5% Triton X-100 for 30 min and then incubated for 24 h at 37 °C in a gelatin essay buffer (50 mmol·L^{-1} Tris-HCl, 150 mmol·L^{-1} NaCl, 10 mmol·L^{-1} $CaCl_2$, 2.5% Tween 80, and 0.02% sodium azide, pH 7.6). After incubation, the gel was washed in distilled water and stained with 0.1% (wt·vol^{-1}) Coomassie brilliant blue R-250 in 50% ethanol (vol·vol^{-1}), 10% (vol·vol^{-1}) acetic acid, and destained in 30% (vol·vol^{-1}) ethanol and 7.5% (vol·vol^{-1}) acetic acid. The proteolytic activity appeared as clear bands on a blue background. Quantification of the clear bands indicating enzymatic activity was performed by densitometry (GS-800; Calibrated densitometer, Bio-Rad, Hercules, CA). Densitometric results were calculated in the area mode.

2.5. Immunoblotting

Before Western Immunoblotting was performed, the samples were run on 10% SDS-PAGE at 100 V for 45 min. The proteins were transferred onto polyvinyl difluor membranes (Millipore, France) by applying a constant current of 300 mA for 60 min with a semi-dry blotting (Trans-Blot, Bio-Rad, France) [18]. The membranes were used for immunodetection. The membranes were incubated with primary antibodies (rabbit polyclonal anti-human MMP-9, 1:1 000, dilution Biomol, Playmouth Meeting, PA, USA) for 12 h at 4 °C. Biotinylated anti-rabbit IgG (Biovaley, France) was subsequently incubated (1:500 dilution) for 2 h at room temperature. Antigen-antibody conjugates were visualised by their reaction to streptavidin alkaline phosphatase (1:1 000) (Biovaley, France) for 30 min. Enzymatic staining was performed with 4-nitroblue tetrazolium chloride and

5-bromo-4 chloro-3-indolylphosphate using a commercial kit (Sigma, France).

2.6. CN fraction proportions

The proportions of pH 4.6 insoluble peptides including γ-CN, was determined by fast pressure liquid chromatography (FPLC) according to the method of Collin et al. [19].

2.7. CN breakdown in solution by *E. coli* lysate

A stock of *E. coli* strain P4:O32 was cultured in four litres of broth brain-heart at 37 °C for 24 h. After centrifugation (4 000 × *g*, 30 min, 4 °C), the bacteria were washed twice in 80 mL sodium acetate buffer 2 *M* (wt·vol^{-1}), pH 7.2, containing 0.04% (wt·vol^{-1}) NaN_3. Finally, the bacteria were resuspended in 80 mL sodium acetate buffer. The cells were lysed by sonication (3 min at 100 W) and kept on crushed ice in order to prevent protein denaturation by heat. The suspension was then centrifuged (12 000 × *g*, 50 min, 4 °C) and the supernatant containing the enzymes was subsequently freeze-dried and stored at –20 °C.

Different ratio *E. coli* lysate/CN (1/1, 1/5 and 1/10) ratios were tested in order to obtain an optimal degradation of CN. The ratio of 1/1 was chosen for this experiment. The α*s*-, β-, and κ-caseins (Sigma Chemical Co, St. Louis, MO, USA) (5 mg·mL^{-1}) were dissolved separately in sodium acetate buffer 0.2 *M* (wt·vol^{-1}), pH 7.2 containing 0.04% (wt·vol^{-1}) NaN_3. The supernatant of *E. coli* lysate (5 mg·mL^{-1} final concentration of supernatant proteins) was added, and the mixture was incubated at 37 °C for up to 24 h. Aliquots (100 µL) were taken periodically at times 0, 0.5, 1, 2, 4, 6, 8, 10, 12 and 24 h for SDS-PAGE analysis. After sampling, proteases of *E. coli* lysates were inactivated in each aliquot by heating at 100 °C for 3 min.

2.8. Peptide characterisation

SDS-PAGE was performed according to the method of Laemmli and Favre [20] with a 5% (wt·vol^{-1}) polyacrylamide stacking gel in 0.125 *M* Tris-HCl (pH 6.8) and with 15% (wt·vol^{-1}) polyacrylamide separating gel in 0.38 *M* Tris-HCl (pH 8.8), in the presence of 0.1% (wt·vol^{-1}) SDS and 5% (vol·vol^{-1}) 2-mercaptoethanol. Twenty-five micrograms of casein were loaded in each well of the gel.

Proteins were fixed in the gel using 12% (wt·vol^{-1}) trichloroacetic acid (TCA) and stained with 0.1% (wt·vol^{-1}) Coomassie blue R250 in 50% (vol·vol^{-1}) ethanol and 10% (vol·vol^{-1}) acetic acid for at least 2 h. Destaining was performed with a solution of 30% (vol·vol^{-1}) ethanol and 7.5% (vol·vol^{-1}) acetic acid. Quantification of the electrophoretic bands was performed by densitometry (GS-800; Calibrated densitometer, Bio-Rad, Hercules, CA). The CN degradation rate was expressed in % per hour in comparison with the CN in milk before inoculation.

2.9. Statistical analysis

The evaluation of plasmin activity and pH 4.6 insoluble peptides including γ-CNs in milk, was determined by analysis of variance using the repeating measures option of the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC, USA). The model included a fixed factor sampling time. The covariance structure between the different sampling times was defined as being autoregressive after verification of Akaike and Schwarz-Bayesian criteria [21].

A value of $P < 0.05$ was considered to be significant, using the error of the sum of square type III. The values of the analysed variables were presented as least squares means and were analysed with the Tukey test.

3. RESULTS AND DISCUSSION

3.1. Origins of proteolysis

3.1.1. E. coli *growth in raw milk and gelatinase activity*

After inoculation of milk, the concentration of bacteria increased sharply and

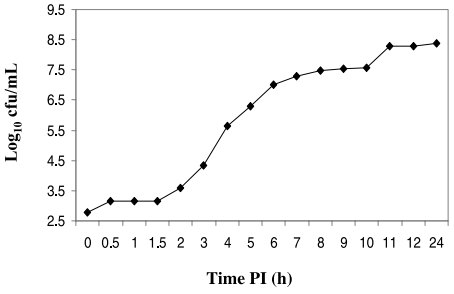

Figure 1. Mean values of bacterial counts in milk over time after 24 h of incubation at 37 °C with 10^4 cfu·mL^{-1} of *E. coli*. The results are expressed in Log cfu·mL^{-1}. The analyses were performed at times 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 24 h. PI: post inoculation.

exceeded 10^6 cfu·mL^{-1} after 5 h post inoculation (PI). The plateau lasted until 8 h PI. At 8 h PI, *E. coli* growth reached 10^8 cfu·mL^{-1} (Fig. 1). This lag time corresponded to the pre-adaptation of *E. coli* in milk and the start of proteolytic activity in milk [22]. The response profiles of *E. coli* growth in milk were in accordance with the previous findings of Kornalijnslijper et al. [23] that showed that milk from individual cows differs in its growth medium properties for *E. coli*. Variations in in vitro growth in whole milk after 6 h of incubation were more than 200-fold between individual cows. Bacterial growth in milk in vitro is different from bacterial growth in milk in an udder quarter of a cow. One of the processes occurring in the cow but not in vitro is the interaction of cells present in milk with bacteria and with mammary epithelial cells. Gelatin zymographic analysis was used to detect the presence of gelatinase in milk whose rate increased after 8 h PI (Fig. 2). This technique allows the gelatinase to be separated from potentially co-secreted inhibitors during electrophoresis. The gelatinase presence in the milk samples was also analysed by Western Blotting and detected after 8 h PI (data not shown). *E. coli* produces numerous proteinases, including collagenolytic enzymes [24]. The presence of one lysis band was detected in parallel with the basal levels in the milk control and in inoculated milk, suggesting that serum is the major source of this enzyme in milk. Our results were in accordance with Driessen et al. [25] who showed that psychrotrophic bacteria produce proteinases at the end of their growth phase and during the stationary phase.

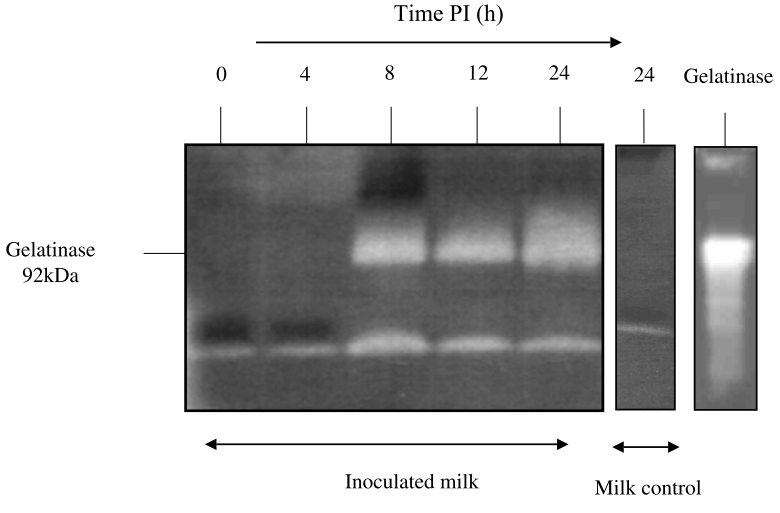

Figure 2. Gelatinolytic activity in the milk control and in milk incubated at 37 °C with 10^4 cfu·mL^{-1} of *E. coli* during 24 h. PI: post inoculation.

A comparison of our results to in vivo data revealed that the bacteriostactic effect was observed after an intra-mammary infusion (IMI) of *E. coli* in cows. The bacterial count decreased soon after the infection, thanks to the immune system [26]. The bacteriostactic effect was not observed under experimental conditions. The main increase of gelatinase levels in milk in vivo, including both intracellular and extracellular gelatinases, is associated with the increase of the neutrophil count in milk [18]. Bacterial count in milk from mastitic udders is variable and depends on the strain of bacteria and the immune defence of the udder. Mehrzad et al. [27] reports that milk PMN viability plays a critical role in the pathogenesis and outcome of coliform mastitis. The value of the bacterial count in milk did not exceed 10^5 cfu·mL^{-1} for some authors [17, 23], whereas it exceeded 10^7 cfu·mL^{-1} in previous studies by Riollet et al. [28]. Bacterial growth in milk in vitro is different from growth in the udder quarter of a cow. In vivo milk contains a variety of antibacterial factors such as phagocytic cells, immunoglobulins, lactoperoxydase and lactoferrin, suggesting that bacterial growth in milk is inhibited [29].

3.1.2. Plasmin activity

Plasmin is known to be the main protease involved in the CN breakdown in milk [2, 30]. In inoculated milk, plasmin activity before inoculation was around 15 µmol *p*-nitroanilide/h/L. It significantly decreased ($P < 0.05$) at 8 h PI and reached a minimum of 8 µmol *p*-nitroanilide/h/L after 24 h (Fig. 3). There was no supply of plasmin-plasminogen activators in vitro. In *E. coli* inoculated milk, the decrease of plasmin activity may be partially explained by the presence of serine protease inhibitors released by *E. coli* during the stationary phase of growth [31]. In contrast, in control milk containing sodium azide, plasmin activity did not significantly increase, between 8 h and 24 h PI. This result could be due to plasminogen activators such as tissue-type (t-PA) and urokinase-type (u-PA) present in milk [32]. Bacterial proteases can disrupt the casein micelle and release enzymes [33]. Although the relationship between bacterial proteases in milk and the plasmin system has been hypothesised [34], little experimental data are available on this relationship.

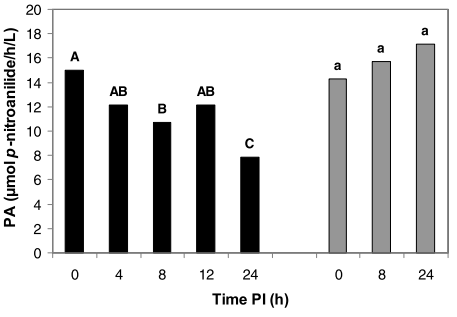

Figure 3. Plasmin activity change in milk during 24 h of incubation at 37 °C with 10^4 cfu·mL^{-1} of *E. coli* (dark histograms), and in the milk control containing 0.01% (wt·vol^{-1}) sodium azide (grey histograms). Time 0 h was considered as a standard before inoculation. For each treatment (inoculated and milk control), two points with at least one similar letter are not significantly different. ($P < 0.05$). PI: post inoculation.

A comparison of our results to in vivo data, revealed that the increase of plasmin activity is linked to the permeability of the blood-milk barrier during inflammation [35], and numerous activators of plasmin and its zymogen come from the blood stream [36]. However, in our in vitro study, a basal level of plasmin activity was considered with no turnover. This variation could therefore be primarily due to *E. coli* whose growth was effective up to 24 h PI. Working with a basal plasmin activity made it possible for us to focus on exogenous proteolysis in our study.

In conclusion, the bacterial count and gelatinase activity increased while plasmin activity decreased over time, emphasising the role of *E. coli* in plasmin activity inhibition.

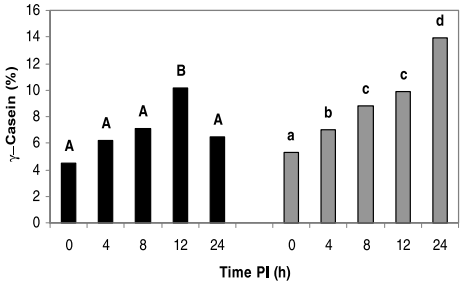

Figure 4. Evolution of pH 4.6 insoluble peptides, including -caseins. Percentage of casein after 24 h of incubation at 37 °C with 10^4 cfu·mL^{-1} of *E. coli* (dark histograms), and in a milk control containing 0.01% (wt·vol^{-1}) sodium azide (grey histograms). Time 0 h was considered as a standard before inoculation. For each treatment (inoculated and milk control), two points with at least one similar letter are not significantly different. ($P < 0.05$). PI: post inoculation.

3.2. Proteolysis and casein breakdown

3.2.1. pH 4.6 insoluble peptides including γ-caseins

In inoculated milk, the percentage of pH 4.6 insoluble peptides, including γ-CN, increased during the kinetics ($P < 0.05$) and reached a maximum of 10% at 12 h PI (Fig. 4). This increase could be related to the degradation of β-CN. Indeed Aslam and Hurley [37] reported that heterogeneous peptides (γ-CN and PP) are generated from β-CN degradation. In our case, the increase of the proportion of γ-CN between 0 h and 12 h could be explained by plasmin and gelatinase activity. The cleavage of β-CN by MMP was reported by Grieve and Kitchen [38] and showed γ-CN production after in vitro incubation of CN with psychotrophic bacteria. After 12 h PI, the rapid decrease of pH 4.6 insoluble peptides including γ-CN could be explained by the hydrolysis of γ-CN by *E. coli* proteases, particularly by a large concentration of MMP in *E. coli* [12]. Indeed, the casein hydrolysis products are nutrients for the bacteria and mastitis milk is enriched in these products [39]. However, the γ-CN for the milk control sharply increased and reached 14%, which can be mainly explained by plasmin activity, which hydrolyses β-CN and generates γ-CN.

In vivo data reveals that after *E. coli* inoculation, the percentage of pH 4.6 insoluble peptides, including γ-CN starts to increase significantly and reaches 30% [40]. The effect suggested by the above results is not supported by the intricate phenomenon of regulatory processes involved in the proteolytic machinery [41]. However, this clearly indicates, that *E. coli* is highly involved in CN breakdown and therefore in the degrading of pH 4.6 insoluble peptides including γ-CN.

3.2.2. Casein breakdown by **E. coli** *lysate*

The hydrolysis profiles of αs-, β- and κ-CN by *E. coli* proteinases were compared (Fig. 5). Their degree of susceptibility to CN proteolysis varied (Fig. 6). CN breakdown by proteinase from *E. coli* resulted in the decrease of intensity of CN bands together with the appearance of a large number of unidentified polypeptide hydrolysis products. Three phases seem to characterise the hydrolysis of the CN. During the first phase (0 to 2 h PI), the CN breakdown rate was degraded by *E. coli* lysate and appeared to be in the following order: κ- > β- >> α_s-CN (Tab. I). The degradation sensitivity of CN-κ by the *E. coli* lysate was higher than that of CN-β and CN-α_s. CN-κ was degraded by more than 45% at 2 h PI, whereas β-CN and α_s-CN were degraded by 30% and 10%, respectively. Our results were in accordance with previous observations by Grieve and Kitchen [38] with psychrotrophic bacteria. When the extracellular proteinase of *Serratia marcescens* is in contact with β-CN and κ-CN, they are degraded faster than α_s-CN. During the second phase (2 to 8 h PI), the CN breakdown rate appeared to be in the following order: α_s-CN ≥ β ≥ κ-CN. The degradation sensitivity was similar for different CN, but α_s-CN and β-CN were hydrolysed at a faster rate than κ-CN.

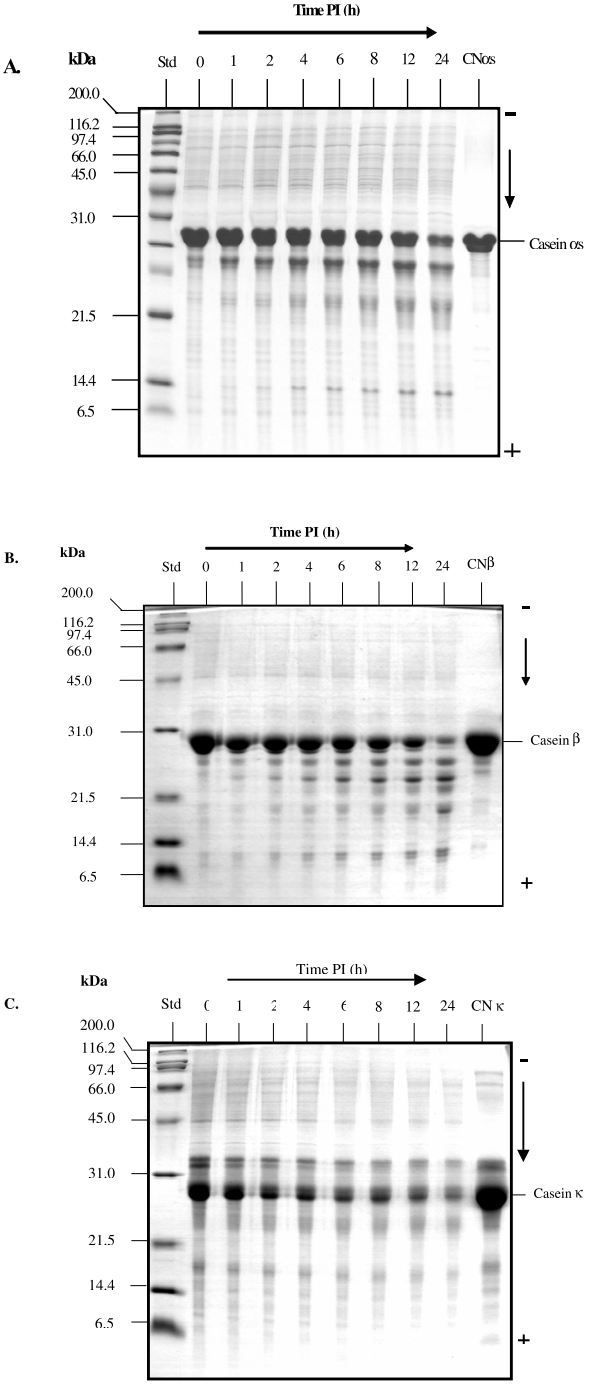

Figure 5. SDS-PAGE electrophoregrams of hydrolysis of αs- (A), β- (B) and κ-CN (C) by *E. coli* lysate. αs-, β- and κ-CN (5 mg·mL^{-1}) were incubated separately with *E. coli* lysate (5 mg protein·mL^{-1}) in sodium acetate buffer 0.2 *M* (wt·vol^{-1}), pH 7.2, NaN_3 0.04% (wt·vol^{-1}) at 37 °C up to 24 h. An amount of 25 µg of protein per well was loaded. Std = molecular mass standards. PI: post inoculation.

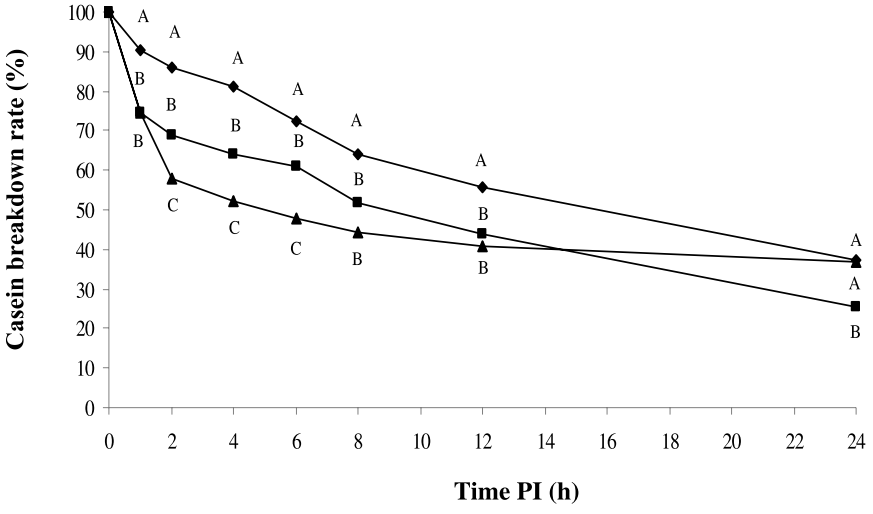

Figure 6. Total casein breakdown of αs- (♦), β- (■) and κ-caseins (▲) incubated separately with *E. coli* lysate (5 mg protein·mL^{-1}) in sodium acetate buffer 0.2 *M* (wt·vol^{-1}), pH 7.2, NaN_3 0.04% (wt·vol^{-1}) at 37 °C up to 24 h. Two time points with a least one similar letter are not significantly different. ($P < 0.05$). PI: post inoculation.

Table I. Degradation sensitivity of CN expressed in %/h by *E. coli* lysate. Two points per line with at least one similar letter are not significantly different. ($P < 0.05$). PI: post inoculation.

Period PI (h)	Degradation sensitivity	CN α	CN β	CN κ
0–2		[c] 7.02	[b] 15.52	[a] 21.04
2–8		[a] 3.68	[ab] 2.84	[b] 2.27
8–24		[a] 1.67	[a] 1.64	[b] 0.47

During the third phase (8 to 24 h PI), the CN breakdown rate appeared to be in the following order: α_s- = β- ≥ κ-CN. The degradation sensibility was similar for CN-α_s and CN-β but lower for CN-κ ($P < 0.05$). The degradation of κ-CN remained stable and weak (0.47 %·h^{-1}) between 8 and 24 h. We can therefore speculate that the peptides generated by the hydrolysis of κ-CN may have contributed to the inhibition of *E. coli* enzymes. The degradation sensitivity of CN varied with the type of individual caseins subjected to proteolysis and with the incubation time.

When the different means of casein hydrolysis by leukocyte proteases, plasmin and *E. coli* proteases were compared, their polypeptide maps differed from each other in the degree of susceptibility of proteolysis caseins and in the generated polypeptide fragments. A comparative study of the caseinolysis profiles of PMN and plasmin revealed that the rate at which CN were degraded by PMN proteases appeared to be in the following order α_s- > β- >> κ-CN. Profiles of CN hydrolysis by bovine plasmin showed that β-CN was hydrolysed at a faster rate than α_s-CN. κ-CN appeared to be

relatively resistant to proteolysis by these proteases.

The increase of the proportion of pH 4.6 insoluble peptides, including γ-CN and that of collagenase activity, occurred during optimal bacterial growth. Moreover, CN breakdown by the bacterial lysate supported the hypothesis of an involvement of *E. coli* in milk proteolysis.

The two experiments carried out in this study, including raw milk inoculated with *E. coli* and the incubation of CN with *E. coli* lysate, were complementary. On the one hand, milk proteolysis was studied with live bacteria. On the other hand, the direct effect of bacterial proteases on CN was investigated. This corresponded to the facts that there is a limited bacterial growth in vivo and that proteases are mainly released in milk as a result of lysis and phagocytosis. The combination of the two experimental approaches provided additional information as to the role of *E. coli* in milk proteolysis during *E. coli* mastitis.

4. CONCLUSIONS

In conclusion, our model of raw milk inoculated with *E. coli* successfully demonstrated the role of live *E. coli* in milk proteolysis with a weak SCC and plasmin activity. This hypothesis is supported by the following main conclusions of this study. First, the increase of pH 4.6 insoluble peptides, including γ-CN and gelatinase activity, occurred during optimum bacterial growth while plasmin activity decreased considerably. The second experiment emphasised the direct CN breakdown by bacterial proteases in solution, which is the main pathway observed in vivo during the inflammatory process. These findings may help to explain the action of *E. coli* proteases in milk. However, similar studies should be conducted in the case of the interaction of PMN proteases with *E. coli* proteases in order to better understand proteolysis mechanisms.

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