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Heterogeneity of proteolytic enzyme activities in milk samples of different somatic cell count

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Milk contains the alkaline proteinase plasmin and lysosomal proteinases; the significance of the latter is ill-defined. The objective of this study was to investigate composition and activities of several different proteolytic enzymes in milk samples of varying somatic cell count (SCC). Increasing milk SCC was correlated with increased plasmin, cathepsin D and cysteine protease activities, with concomitant increases in proteolysis in milk. Addition of plasmin inhibitors confirmed the heterogeneity of proteinase activities in milk, as urea-PAGE analysis of milk samples showed casein hydrolysis in milk after 7 d storage even in samples with inhibitors added; extent and heterogeneity of proteolysis was correlated with milk SCC. Rennet coagulation properties were not significantly correlated with SCC, or activities of measured enzymes. Milk of increasing SCC also exhibited decreased physical stability during incubation of milk at 37 °C. Pasteurized milk was more stable than raw milk, suggesting that the enzyme(s) or mechanisms leading to such instability are impaired by pasteurization. Overall, milk has a very heterogeneous proteolytic enzyme population, with a higher significance of non-plasmin enzymes, such as cathepsin D and cysteine proteinases, than perhaps previously recognised.

Keywords: Plasmin, milk, proteolysis, somatic cell count, cathepsin.

The milk alkaline proteinase, plasmin, preferentially hydrolyses β-casein to proteose peptones and γ-caseins, with optimum activity at pH 7·5 and 37 °C (Bastian & Brown, 1996). Most potential plasmin activity is in the form of the inactive zymogen, plasminogen, which is activated by plasminogen activators (PA) associated with somatic cells and casein micelles (White et al. 1995). Bovine milk also contains plasmin inhibitors and inhibitors of PA.

There are a number of other proteolytic enzymes in milk, including proteolytic enzymes from lysosomes of milk somatic cells (Andrews, 1983*b*). In milk of somatic cell count (SCC) $>2{\cdot}5 \times 10^5$ cells/ml, almost 40% of proteolytic activity can be due to non-plasmin enzymes (Le Roux et al. 1995). One of the principal lysosomal enzymes in milk is cathepsin D, an acid proteinase with optimal proteolytic activity at pH 2·8–4·0 and 37 °C. Four forms of cathepsin D have been identified in milk, of which procathepsin D is the main form (Larsen & Petersen, 1995). The presence of cysteine protease activity in milk was shown by Suzuki & Katoh (1990) and O'Driscoll et al. (1999), and Magboul et al. (2001) provided immunological evidence that cathepsin B was one of several such enzymes in milk.

The relative importance of different proteolytic enzymes in milk may be estimated by use of specific proteinase inhibitors. For example, the serine proteinase inhibitor aprotinin inhibits PA and plasmin activity in the micellar fraction of milk (de Rham & Andrews, 1982). The lysine analogue 6-aminohexanoic acid (AHA), which induces dissociation of plasmin from the casein micelles (Richardson, 1983) and inhibits PA activity in milk (Fang & Sandholm, 1995), may inhibit plasmin activity in milk without affecting somatic cell proteinases (Verdi & Barbano, 1988). Finally, milk cathepsin D activity may be inhibited by pepstatin (O'Driscoll et al. 1999).

However, understanding of the relative significance of individual proteinases in milk samples of different SCC and the relationship between the activity of such enzymes and milk quality and processability remains incomplete. The objective of this study was to investigate the relationships between the composition, rennet coagulation properties and enzyme activities of milk samples of varying SCC, in part through the use of various proteinase inhibitors.

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Materials and Methods

Gross analysis of milk samples

Milk samples from 41 individual cows of varying SCC (range 30×10^3 to 2349×10^3 cells/ml) were obtained from the Dairy Production Research Centre (Moorepark, Fermoy, Co. Cork, Ireland) and immediately preserved using sodium azide (0·5 g/l).

Milk composition (fat, protein, lactose, total solids) and pH were measured using a Milkoscan FT 120 (Foss Electric, Hillerød, Denmark), and a PHM62 Standard pH meter (Radiometer, Copenhagen, Denmark), respectively. Rennet coagulation properties of milk (rennet coagulation time (RCT, min), rate of curd aggregation ($1/K_{20}$) and curd firmness 60 min after rennet addition (A_{60}, mm of amplitude)) were measured at the natural pH of the milk using a Formagraph (Type 11700, Foss Electric), as described by O'Brien et al. (1998), after overnight storage at 4 °C.

Measurement of milk enzyme activities

Plasmin activity in milk was measured using the method of Richardson & Pearse (1981). Plasminogen-derived activity was determined after activation by urokinase (Sigma Chemical Co., Dorset, UK) for 1 h at 37 °C. Both activities were expressed in AMC units/ml, where 1 AMC unit of plasmin releases 1 nmol of 7-amido-4-methyl coumarin/min under the conditions of the assay.

Cathepsin D and cysteine protease activities in pH 4·6-soluble extracts of milk samples were assayed as described by O'Driscoll et al. (1999), using the synthetic heptapeptide substrate Pro-Thr-Glu-Phe-[NO_2–Phe]-Arg-Leu (Bachem Feinchemikalien AG, Switzerland).

Measurement of proteolysis

Each fresh (d0) milk sample was divided into five portions (A–E), which were treated as follows:

A Control (raw) milk
B Pasteurized milk (63 °C × 30 min)
C Raw milk+aprotinin (10 μg/ml, Sigma)
D Pasteurized milk+aprotinin (10 μg/ml)
E Raw milk+6-amino-hexanoic acid (100 mM).

All samples were incubated at 37 °C for up to 7 d and samples taken for plasmin and plasminogen determination (d0), measurements of cathepsin D and cysteine protease activity (d0) and urea-PAGE analysis (d0 and d7). The samples were also monitored daily for signs of gelation or other changes in physical stability. The Lowry assay (Lowry et al. 1951) was used to determine the effect of experimental treatments on levels of pH 4·6-soluble N (SN) in milk at d0 and d3 (Kelly et al. 1998).

Proteolysis in milk was examined using Urea-PAGE in polyacrylamide gels (12·5 % C, 4 % T, pH 8·9) in a Proean II xi vertical slab-gel unit (Bio-Rad Laboratories Ltd., Watford, Herts, U.K.), as described by Andrews (1983*a*). Milk samples for electrophoresis were diluted 1 : 4 with sample buffer and 12 μl loaded onto gels, which were run at 280 V until samples had passed through the stacking gel and at 300 V through the separating gel. Gels were stained overnight according to the method of Blakesley & Boezi (1977) and destained in several changes of distilled water.

Statistical analysis

Statistical analyses were performed using Minitab v.12 (Minitab Ltd., Coventry, U.K.). Two separate types of statistical analysis were performed. Firstly, to allow presentation of absolute values of measured parameters, milk samples were divided on the basis of SCC into four groups, corresponding approximately to milk of (i) low SCC (<100 000 cells/ml), (ii) typical SCC for healthy cows (101 000–400 000 cells/ml), (iii) high SCC or probable subclinical mastitis (401 000–800 000 cells/ml) and (iv) very high SCC or probable clinical mastitis (>800 000 cells/ml). The effects of experimental treatments on measured parameters was then evaluated by one-way ANOVA, comparing means within these four categories using Tukey's pairwise comparisons at the 95 % confidence level. Secondly, to examine linear (continuous) changes in milk quality with SCC or enzyme activity, relationships between certain measured parameters were also studied using regression analysis of linear correlations.

Results

Milk composition

While protein content was numerically higher in milk samples of SCC $>400 \times 10^3$ cells/ml than in samples of lower SCC, and lactose content generally decreased with increasing SCC, overall SCC had no significant effect on milk composition (Table 1), or rennet coagulation properties. There was no difference in milk pH or fat content between groups (not shown).

Milk proteolytic enzyme activity

Plasmin activity in fresh raw milk increased with increasing SCC (Table 1) and was reduced by batch pasteurization. There was no clear trend in plasminogen levels. Cysteine protease activity was higher ($P<0·05$) in milk of SCC> 801×10^3 cells/ml than in milk of lower SCC. Milk cathepsin D activity also increased in milk of increased SCC, while residual levels of the substrate used for the simultaneous analysis of these enzyme activities decreased with increasing SCC, although in both cases the effect of SCC group was not significant.

There were numerical differences in levels of pH 4·6-SN in fresh (d0) milk between the different SCC groups (Table 2). At d0 and d3 of incubation, levels of pH 4·6-SN generally increased with increasing SCC. The effect of SCC group on level of pH 4·6-SN after 3 d incubation was

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Table 1. Composition, rennet coagulation properties and enzyme activities of milk samples

	Group 1	Group 2	Group 3	Group 4	P_{ANOVA}
SCC range $\times 10^{-3}$ cells/ml	<100	101–400	401–800	>801	
SCC mean $\times 10^{-3}$ cells/ml	55	199	614	1382	
Number of cows, *n*	9	13	11	8	
Protein, g/l	33·6	33·4	34·0	35·8	NS
Lactose, g/l	45·3	45·0	44·3	43·9	NS
RCT, min	25·31	30·41	25·83	29·25	NS
$1/K_{20}$	0·086	0·082	0·098	0·100	NS
A_{60}, mm	32·02	28·85	32·31	32·05	NS
Plasmin activity, AMC units/ml					
Raw milk	0·25[a]	0·39[ab]	0·35[ab]	0·58[b]	*
Pasteurized milk	0·21	0·20	0·22	0·34	NS
Plasminogen-derived activity, AMC units/ml					
Raw milk	2·26	2·41	1·99	2·03	NS
Pasteurized milk	0·87	1·03	1·03	0·79	NS
Cysteine proteinase activity†	17·84[a]	19·15[a]	17·40[a]	31·50[b]	*
Cathepsin D activity†	12·46	15·19	16·00	20·90	NS
Residual substrate†‡	97·30	93·59	88·26	78·89	NS

NS, non-significant; * $P<0·05$
[a, b] Means within a row with a different superscript were significantly different ($P<0·05$)
† Arbitrary units derived from HPLC peak areas
‡ Amount of substrate used to measure cathepsin D and cysteine protease activities remaining after the 4 h incubation period used in the assay

Table 2. Levels of pH 4·6-soluble N in milk during incubation at 37 °C

	Group 1	Group 2	Group 3	Group 4	P_{ANOVA}
SCC range $\times 10^{-3}$ cells/ml	<100	101–400	401–800	>801	
Number of cows, *n*	9	13	11	8	
Total pH 4·6-SN, mg/ml					
Raw milk, d0	7·84	8·65	8·51	9·39	NS
Raw milk, d3	11·08	11·65	12·53	13·18	NS
Pasteurized milk, d3	8·89	10·77	11·89	11·04	NS
Raw milk+Ap.†, d3	9·20[a]	10·17[ab]	11·58[b]	11·95[b]	**
Pasteurized milk+Ap., d3	7·87[a]	9·37[ab]	9·29[ab]	10·00[b]	*
Raw milk+AHA†, d3	9·78	10·71	10·93	12·00	NS

NS, non-significant; * $P<0·05$; ** $P<0·01$
[a, b] Means within a row with a different superscript were significantly different
† Ap., aprotinin; AHA, 6-aminohexanoic acid

significant for both raw and pasteurized milk with aprotinin added milk. When proteolysis was estimated quantitatively by increases in pH 4·6-SN during 3 d storage, there was a trend towards increased proteolysis with increasing SCC, even in samples containing aprotinin, but least so in pasteurized samples (not shown).

Electrophoretic analysis of proteolysis in milk

Urea-PAGE analysis indicated significant effects of milk SCC and treatment on hydrolysis of casein in milk (electrophoretograms of two samples are shown in Fig. 1). Milk of SCC 2349×10^3 cells/ml showed greater proteolysis, for all experimental treatments, than the low SCC milk sample (101×10^3 cells/ml). For low SCC milk, proteolysis at d7 was most extensive in pasteurized milk, with almost complete hydrolysis of β-casein, while samples containing aprotinin or AHA showed little proteolysis during storage. For the high SCC sample, however, quantitatively different proteolysis patterns were evident, with a number of bands of electrophoretic mobility less than the γ-caseins appearing during incubation. Proteolysis was also evident in high SCC samples containing aprotinin and AHA, further indicating the activity of non-plasmin proteinases.

Physical stability of milk

Milk samples were also monitored daily during incubation for signs of physical instability such as gelation or sedimentation (Table 3). Pasteurized milk was most stable,

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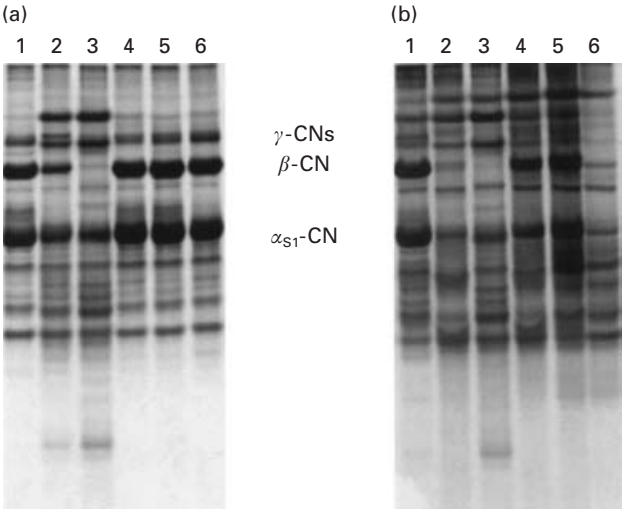

Fig. 1. Urea-PAGE electrophoretograms of milk samples of varying SCC, after various experimental treatments, during storage at 37 °C. (a) Milk of SCC 101×10^3 cells/ml (from group 2). Lane 1: raw milk, d0; Lane 2: raw milk, d7; Lane 3: pasteurized milk, d7; Lane 4: raw milk with aprotinin, d7; Lane 5: pasteurized milk with aprotinin, d7; Lane 6: raw milk with AHA, d7. (b) Lanes 1–6: as (a), but for milk of SCC 2349×10^3 cells/ml (from group 4).

while raw milk with added aprotinin was least stable. The occurrence of instability was, however, not clearly correlated with SCC.

Regression analysis of measured parameters

Finally, linear relationships between measured parameters were assessed by regression analysis (Table 4). According to the results of such analysis, protein concentration in milk was positively correlated ($P<0{\cdot}05$) with cysteine proteinase and cathepsin D activities, and with milk SCC. Milk pH was positively correlated ($P<0{\cdot}05$) with cysteine protease activity, while lactose content was negatively correlated ($P<0{\cdot}05$) with cysteine protease activity, plasmin activity and SCC. SCC was correlated with cysteine proteinase, cathepsin D and plasmin activities ($P<0{\cdot}01$). Rennet coagulation properties were not correlated with enzyme activity, plasmin activity or SCC (not shown). Levels of pH 4·6-SN in fresh milk were correlated ($P<0{\cdot}05$) with enzyme activity, plasmin activity and SCC. After 3 d incubation, there were generally weaker correlations for raw and pasteurized milk, but levels of pH 4·6-SN remained closely correlated with enzyme activities and SCC when milk was incubated with aprotinin or AHA.

Plasmin activity in fresh raw milk was positively correlated with cysteine protease activity ($P<0{\cdot}001$) but was not correlated with cathepsin D activity. SCC was correlated with plasmin activity in fresh raw milk ($P<0{\cdot}001$) but was not correlated with plasmin activity following pasteurization.

Table 3. Estimation of physical stability of milk samples during storage, expressed as % of samples exhibiting physical instability during 7 d storage at 37 °C (numbers in brackets refer to average number of days until instability occurred for those samples)

	Group 1	Group 2	Group 3	Group 4
SCC range $\times 10^{-3}$ cells/ml	<100	101–400	401–800	>801
Raw milk	55 (3)	69 (1·6)	82 (2·6)	75 (2·2)
Pasteurised milk	11 (7)	10 (5)	30 (6)	40 (1)
Raw milk+Ap.†	67 (2·5)	77 (2·5)	91 (2·1)	75 (2·3)
Pasteurised milk+Ap.	22 (3·5)	31 (2·8)	18 (5·5)	38 (3·3)
Raw milk+AHA†	33 (0·3)	77 (2)	82 (1·8)	38 (2·3)

† Ap., aprotinin; AHA, 6-aminohexanoic acid

Discussion

Many dietary, lactational and management factors influence the SCC, composition and quality of milk (Auldist et al. 1996*a*). However, in the current study, such variables were balanced, and milk samples were selected solely on the basis of SCC, which presumably reflects the current and/or historical infection status of the cow.

High SCC milk had, in general, higher protein contents and lower lactose contents than low SCC samples, as previously reported (Ng-Kwai-Hang et al. 1982; Auldist et al. 1996*a*). However, SCC had little effect on rennet coagulation properties of milk, in contrast to some previous reports (Okigbo et al. 1985; Politis & Ng-Kwai-Hang, 1988), but in agreement with the findings of Bastian et al. (1991).

Plasmin activity in fresh raw milk increased with increasing SCC, as reported by Saeman et al. (1988) and Politis et al. 1989*a*); this may be due either to plasminogen activation or increased transport of plasmin from blood (Politis et al. 1989*b*). Plasmin activity in pasteurized milk at d0 was lower than that in raw milk, as reported by Richardson (1983).

While the plasmin system in milk has been quite well studied, there have been very few studies of other proteinases in milk, although there have been preliminary reports that cysteine protease and cathepsin D activities in milk were correlated with SCC (Suzuki & Katoh, 1990; O'Driscoll et al. 1999). In this study, a synthetic substrate which is not hydrolysed by plasmin but is cleaved by both cathepsin D and cysteine proteases (O'Driscoll et al. 1999) confirmed significant activities of lysosomal proteinases in milk, increasing with SCC, with at least part of such activity being due to the activities of both cysteine proteases and cathepsin D. This strongly indicates a heterogeneous population of proteolytic activities in milk, the activity of which was also apparent from qualitative differences in electrophoretograms of casein breakdown in high and low SCC milk samples during storage. Extensive hydrolysis of α_{s1}-casein in high SCC milk, more so than in low SCC milk samples, is consistent with elevated cathepsin D activity in the former, as cathepsin D preferentially hydrolyses α_{s1}-casein (McSweeney et al. 1995).

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Table 4. Significant linear correlations between measured parameters

Values are regression coefficients, *R*

	Enzyme activity			
	Cysteine Proteinase	Cathepsin D	Plasmin, raw milk	SCC
pH	0·33*	NS	NS	NS
Protein	0·38*	0·35*	NS	0·33*
Lactose	−0·42**	NS	−0·54***	−0·41**
SCC	0·53***	0·62***	0·49***	—
Total pH 4·6-SN,				
Raw milk, d0	0·38**	0·33*	0·46***	0·40**
Raw milk, d3	NS	NS	0·56***	0·30*
Pasteurized milk, d3	NS	NS	NS	NS
Raw milk+Ap.†, d3	0·33*	0·40**	0·40**	0·48***
Pasteurized milk+Ap., d3	0·46**	0·45**	0·39**	0·47***
Raw milk+AHA†, d3	0·33*	0·39**	0·35*	0·44**
Plasmin activity				
Raw milk	0·52***	NS	—	0·49***
Pasteurized milk	NS	NS	0·54***	NS
Residual substrate‡	0·59***	0·62***	NS	0·44**

NS, non-significant; *$P<0·05$; **$P<0·01$; ***$P<0·001$

† Ap., aprotinin; AHA, 6-aminohexanoic acid

‡ Amount of substrate used to measure cathepsin D and cysteine protease activities remaining after the 4 h incubation period used in the assay

Levels of pH 4·6-SN in fresh milk were significantly correlated with SCC and all measured enzyme activities, which may reflect increases in whey protein levels and, possibly, products of proteolysis produced in milk during storage in the mammary gland pre-milking. Differences in levels of pH 4·6-SN remained proportional to SCC during incubation, which may reflect the initial state of proteins rather than changes due to subsequent proteolysis. Addition of inhibitors of plasmin or PA decreased production of pH 4·6-SN across all SCC groups, but less so in higher SCC samples, again indicating increased non-plasmin activity in such samples.

The influence of SCC on the physical stability of milk has received little attention, except in the case of UHT milk, where increasing SCC was weakly associated with accelerated age gelation (Auldist et al. 1996*b*). Physical instability of both raw and pasteurized milk during 7 d storage at 37 °C was evident in this study, particularly in the case of the former, although this did not clearly depend on milk SCC. Addition of the inhibitors AHA and aprotinin either had little effect on, or accelerated, physical changes in milk, suggesting that plasmin activity is not involved in such instability. However, tests on a small number of samples (not shown) indicated that addition of pepstatin did not prevent or delay such instability, indicating that cathepsin D is unlikely to be involved, although it can release glycomacropeptide from κ-casein (McSweeney et al. 1995), and thus could potentially contribute to coagulation of milk. Pasteurized milk was the most physically stable during storage, which implies that the enzymes(s) or factors causing instability are affected by pasteurization.

In conclusion, this study presents new information on the level and activity of cathepsin D and lysosomal cysteine proteases in milk. It appears that, in high SCC milk, the activity of such enzymes is significant, and may result in degradation of casein and hence alteration of milk or dairy product functionality. Compared with plasmin, these enzymes have received remarkably little attention, but further research into their activity and significance may be warranted, on the basis of results presented herein.

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