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Original article

Contribution of plasmin to primary proteolysis during ripening of cheese: effect of milk heat treatment and cheese cooking temperature

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Abstract – The effects of milk heat treatment and cooking conditions during cheese manufacture on plasmin activity and proteolysis during ripening were determined. Milk heat treatments studied were raw (no heating), 63 ºC for 30 min and 75 ºC for 1, 5 or 10 min. In separate experiments, cooking temperatures of 38, 43, 48 and 55 ºC were examined. Varying cooking temperature did not significantly affect cheese composition except that moisture content was significantly lower ($P < 0.05$) at higher cooking temperatures and salt content also significantly ($P < 0.001$) differed between treatments. Increasing cooking temperature increased plasmin activity and plasminogen activation during ripening, but decreased chymosin activity, as seen from urea-PAGE analysis. Increasing severity of milk heat treatment had no significant effects on cheese composition, but decreased plasmin activity in cheese and, overall, had less significant effects on proteolysis during ripening than cooking conditions. Thus, the importance of activators and inhibitors of the plasmin system, and their heat stability, in cheese ripening, and the effect of processing, conditions thereon, were apparent.

cheese / pasteurisation / cooking / plasmin / proteolysis

Résumé – **Contribution de la plasmine à la protéolyse primaire pendant l'affinage de fromage : effet du traitement thermique du lait et de la température de chauffage du caillé.** Le présent travail traite des effets du traitement thermique du lait et des conditions de chauffage du caillé lors de la fabrication du fromage sur l'activité de la plasmine et la protéolyse pendant l'affinage. Les traitements thermiques ont été étudiés sur du lait cru (sans chauffage), du lait chauffé à 63 ºC pendant 30 min et 75 ºC pendant 1, 5 et 10 min. Différentes températures de chauffage du caillé ont été examinées séparément : 38, 43, 48 et 55 ºC. Faire varier la température de chauffage du caillé n'a pas affecté significativement la composition du fromage, à l'exception de la teneur en humidité qui a significativement diminué ($P < 0,05$) aux plus hautes températures et de la teneur en sels qui était

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significativement ($P < 0,001$) différente entre les traitements. L'augmentation de la température de chauffage du caillé a entrainé l'augmentation de l'activité de la plasmine et de l'activation du plasminogène lors de l'affinage, mais a diminué l'activité de la chymosine, comme l'ont montré les déterminations enzymatiques spécifiques et l'analyse PAGE-urée. Augmenter la sévérité du traitement thermique du lait n'a eu aucun effet significatif sur la composition du fromage, mais a diminué l'activité de la plasmine, et, dans tous les cas, a eu un effet moins significatif sur la protéolyse lors de l'affinage que les conditions de chauffage du caillé. L'importance des activateurs et inhibiteurs du système de la plasmine, et de leur thermostabilité, pendant l'affinage et l'effet des conditions de fabrication a donc été mise en évidence.

fromage / pasteurisation / chauffage du caillé / plasmine / protéolyse

1. INTRODUCTION

Plasmin (E.C. 3.4.21.7), the principal technologically significant indigenous milk protease, may affect the quality of various dairy products and plays an important role in cheese ripening [3]. The plasmin system in milk is complex and is comprised of the enzyme, its zymogen, plasminogen, plasmin inhibitors, plasminogen activators (PA) and inhibitors of PA.

Plasmin is optimally active at pH 7.5 and 37 °C and is associated with the casein micelles in milk [11]. Plasmin has a relatively high heat stability, with Kaminogawa et al. [14] reporting that heating to 80 °C for 10 min was needed to inactivate the enzyme. Pasteurisation at 75 °C for 15 s significantly increases proteolytic activity in milk, while pasteurisation at 63 °C for 30 min leads to a smaller increase [24]. This is thought to be due to the inactivation of an inhibitor of plasminogen activators following pasteurisation [25]. More severe heat treatment significantly reduces plasmin activity, due to interactions of components of the plasmin system with denatured β-lactoglobulin [16].

Plasmin may significantly affect cheesemaking properties of milk, especially in terms of cheese ripening [3]. Plasmin activity in cheese depends on a number of technological parameters, including cooking temperature, with plasmin activity being highest in cheeses cooked at higher temperatures, possibly due to increased plasminogen activation resulting from inhibitors of plasminogen activators and plasmin, which are located in the serum phase of the milk, being inactivated or lost in the whey during cheesemaking [8]. It has also been reported that increased proteolysis due to increased plasmin activity in cheese results in improved flavour and overall quality [9, 10]. Strong heat treatment of cheese milk reduces plasmin activity in cheese, with concomitant decreases in breakdown of β- and α_{s2}-caseins during ripening [4].

The objective of this study was to examine and compare the relative effects of milk heat treatment and cooking conditions during cheese manufacture on the contribution of plasmin activity to proteolysis during the early stages of ripening of a miniature cheese model system.

2. MATERIALS AND METHODS

2.1. Milk heat treatment and analysis

Raw milk was obtained from CMP Dairies, Cork and batch pasteurised in a water bath under different conditions (63 °C for 30 min or 75 °C for 1, 5 or 10 min), cooled to 32 °C and divided into 200 mL portions. Compositional analysis of milk samples was carried out using a Milkoscan FT 120 (Foss Electric, Hillerød, Denmark). Plasmin activity was determined using the fluorescent assay method of Richardson and Pearce [26].

2.2. Cheese manufacture

Miniature cheeses were manufactured from milk heat treated as described above, using the method of Ur-Rehman et al. [27]. The starter culture used was *Lactococcus lactis* subsp. *cremoris* 223 (Christian Hansen, Little Island, Cork, Ireland) and the rennet used was Standard Plus 190 (Christian Hansen). To investigate the effect of cooking temperature, curds made from a number of batches of milk, pasteurised at 63 °C for 30 min, were cooked at 38 °C (control), 43 °C, 48 °C or 55 °C. All cheeses were brine salted in a 20% NaCl, 0.05% $CaCl_2$ solution for 30 min, vacuum packed, and ripened at 8 °C for up to 90 d. All experimental treatments were replicated three times, on separate days.

Plasmin activity in whey was determined using the method described for milk samples by Richardson and Pearce [26].

2.3. Analysis of cheese

Cheese was analysed at 1 d for fat, moisture, protein and salt by standard methods [2]. Plasmin activity in cheese was measured using the method of Richardson and Pearce [26].

Samples of cheese were examined using urea polyacrylamide gel electrophoresis (Urea PAGE), as described by McSweeney et al. [22]. The water-soluble nitrogen (WSN) fraction of the cheese was extracted [18], lyophilised and examined by reverse phase high performance liquid chromatography (RP-HPLC) using a Beckman HPLC system (Beckman, San Ramon, Ca., USA), consisting of a model 502 autosampler, a model 126 pump and a model 166 UV spectrophotometric detector. A nucleosil C_8 column (250 × 4.6 mm, 5 μm particle diameter, 300 Å pore size; JVA Analytical, Unit 1, Longmile Business Centre, Longmile Road, Dublin 12, Ireland) was used for all separations. The gradient was prepared from 0.1% trifluoroacetic acid (TFA) in HPLC-grade water (Solvent A) and 1.1% TFA in acetonitrile (HPLC grade, Rathburn Chemicals, Walkerburn, Scotland; Solvent B), and elution was performed using the gradient described by Kelly and O'Donnell [17].

2.4. Statistical analysis

All statistical analyses were performed using Minitab v.12 (Minitab Ltd., Coventry, UK). Effects of experimental treatments and replication on the composition, proteolysis and enzyme activities of the cheese were examined by one-way analysis of variance (ANOVA) with Tukey's pairwise comparisons at the 95% confidence level. Comparisons of milk heat treatment and cooking experiments were carried out separately, with the control in each case being cheese made from milk pasteurised at 63 °C for 30 min and cooked to 38 °C during manufacture. All results reported are the means of triplicate trials.

3. RESULTS

3.1. Milk analysis

Milk composition was reasonably uniform throughout the trial (not shown). Fat content ranged from 3.1–4.1% and protein content ranged from 3.24–3.4%. Plasmin activity in milk was significantly affected by heat treatment (Tab. I). Heating the milk to 63 °C for 30 min numerically increased plasmin activity as compared to raw milk, while more severe heat treatment significantly decreased milk plasmin activity.

3.2. Cheese composition

Cooking temperature and milk heat treatment did not significantly affect cheese composition, except in the case of moisture content and salt content (Tab. II). Increasing the cooking temperature significantly

Table I. Effect of milk heat treatment on plasmin activity in milk samples.

Milk heat treatment (° C) (min)	n	Plasmin activity (AMC units mL^{-1})
Raw	3	0.162_{A}
63 for 30 min	3	0.174_{A}
75 for 1 min	3	0.133_{AB}
75 for 5 min	3	0.094_{BC}
75 for 10 min	3	0.056_{C}
Significance[2]		***

[1] Upper-case subscripts indicate differences within heat treatment comparisons (means without common upper-case subscript are significantly different, $P < 0.05$).
[2] ***, $P < 0.001$.

T**able II.** Composition of experimental cheeses made using different milk heat treatments and cheese cooking temperatures, and plasmin activities of whey therefrom (measured at d1 of ripening).

	n	Moisture (%)	Protein (%)	Fat (%)	Salt (%)	Whey plasmin activity (AMC units mL^{-1})
Control[1]	3	42.8_{a}[2]	22.4	27.7	1.99_{ABa}	0.02_{aAC}
Cooking temperature, °C						
43	3	42.3_{a}	24.1	28.2	2.25_{b}	0.05_{b}
48	3	42.0_{ab}	23.5	29.3	2.25_{b}	0.08_{c}
55	3	38.9_{b}	25.4	29.3	1.73_{c}	0.07_{bc}
Significance[4]		*	NS	NS	***	***
Milk heat treatment, °C						
Raw	3	43.2	23.3	27.7	1.93_{A}[3]	0.04_{B}
75 for 1 min	3	44.0	21.9	29.7	2.11_{B}	0.02_{AB}
75 for 5 min	3	44.6	21.9	28.3	1.92_{A}	0.02_{AC}
75 for 10 min	3	47.4	20.9	27.3	2.04_{AB}	0.00_{C}
Significance		NS	NS	NS	*	***

[1] Control was pasteurised at 63 ºC for 30 min and cooked to 38 ºC during manufacture.
[2] Lower-case subscripts indicate differences within cooking temperature comparisons (means without common lower-case subscript are significantly different ($P < 0.05$)).
[3] Upper-case subscripts indicate differences within heat treatment comparisons (means without common upper-case subscript are significantly different ($P < 0.05$)).
[4] *, $P < 0.05$; ***, $P < 0.001$; NS, non-significant.

($P < 0.05$) decreased moisture content. Salt content was significantly affected by both cooking temperature ($P < 0.001$) and the severity of milk heat treatment ($P < 0.05$), but the patterns were not clear and the changes were of small magnitude. Increasing the severity of milk heat treatment increased the moisture content and decreased

the protein content of cheese, but not significantly.

Plasmin activity in whey generally increased with increasing cooking temperatures and decreased significantly with increasing severity of milk heat treatment (both $P < 0.001$).

3.3. Enzyme activity in cheese

Plasmin activity in cheese was significantly affected at 1 mo of ripening by cooking temperature ($P < 0.01$) and at 3 mo of ripening by both cooking temperature ($P < 0.05$) and milk heat treatment ($P < 0.05$). Cheese manufactured from milk pasteurised at 63 °C for 30 min had the highest plasmin activity throughout ripening, which was slightly higher than that in cheese made from raw milk (Fig. 1). Increasing severity of milk heat treatment decreased plasmin activity in cheese at d1 and 1 mo of ripening, but not significantly. At 3 mo of ripening, cheese made from milk heated to 75 °C for 10 min had significantly ($P < 0.05$) lower plasmin activity than that made from cheese made from milk pasteurised at 63 °C for 30 min.

Plasmin activity in cheese after 1 and 3 mo of ripening increased with increasing cooking temperature (Fig. 2). At 1 mo of ripening, activity in cheese cooked to 55 °C was significantly higher than that in cheese cooked to lower temperatures ($P < 0.05$), while at 3 mo plasmin activity in cheese cooked at this temperature was significantly higher than that in all cheeses cooked at lower temperatures ($P < 0.05$), and activity in cheese cooked to 48 °C was significantly higher than that in cheese cooked to 38 or 43 °C ($P < 0.05$). Plasmin activity increased in all cheeses over ripening, presumably due to activation of plasminogen, but the rates of increase were different. The increases were greatest in cheese cooked to 55 °C and the lowest increase was seen in the cheese manufactured from milk heated to 75 °C for 10 min.

When plasmin activities were calculated on a dry matter basis, the increase in activity with cooking temperature, the decrease in activity with increasing severity of heat treatment of milk, and the relative rates of increase during ripening, remained clear (not shown), indicating that differences in cheese dry matter content did not significantly affect the relative plasmin activity in cheese.

3.4. Urea-PAGE analysis

Both pasteurisation temperature (Fig. 4) and cooking temperature (Fig. 5) considerably affected primary proteolysis during cheese ripening. Increasing the cooking temperature had a greater effect on

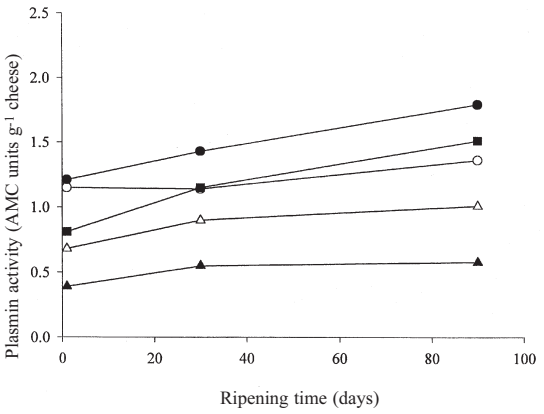

Figure 1. The effect of milk heat treatment on plasmin activity in cheese during ripening. Raw (○), 63 °C × 30 min (●), 75 °C × 1 min (■), 75 °C × 5 min (△), 75 °C × 10 min (▲).

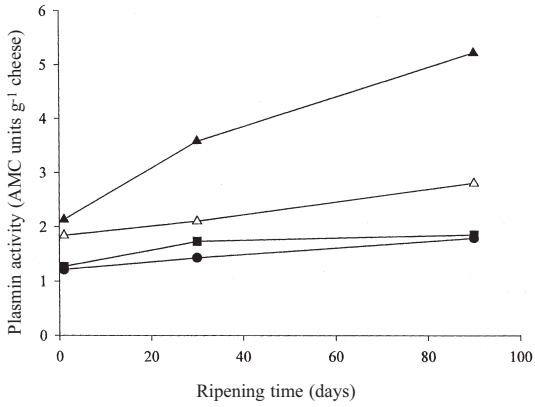

Figure 2. Effect of cheese cooking temperature on plasmin activity during ripening. Cheese was cooked at 38 °C (●), 43 °C (■), 48 °C (△) or 55 °C (▲)

plasmin-induced proteolysis of β-casein to γ-caseins than increasing the severity of the milk heat treatment, with the former treatment increasing apparent breakdown of β-casein during ripening, while the reverse was noted for the latter treatment.

Increased severity of milk heat treatment had little effect on chymosin activity, as observed by α_{s1}-casein degradation to α_{s1}-I casein, relative to control cheese (Fig. 3). However, increasing cooking temperature markedly decreased degradation of α_{s1}-casein to α_{s1}-I casein at 1 mo (Fig. 4). This is in direct contrast to the plasmin activity results where increased cooking temperature increased plasmin activity in the cheese, with a subsequent increase in β-casein breakdown.

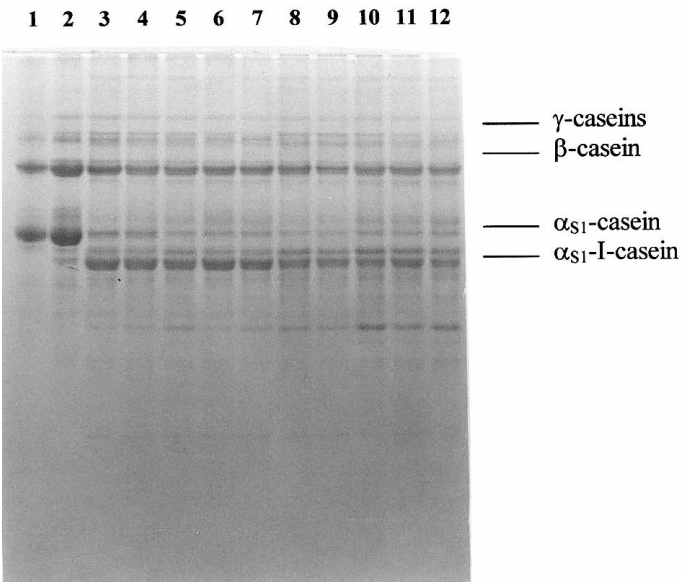

Figure 3. Urea-PAGE electrophoretograms of experimental cheese, made from milk heated under different regimes, after 1 mo and 3 mo. *Lane 1*, sodium caseinate, *lane 2*, cheese made from raw milk at d1, *lanes 3-7*, cheese made from raw milk, milk heated to 63 °C × 30 min, milk heated to 75 °C × 1 min, milk heated to 75 °C × 5 min, milk heated to 75 °C × 10 min, all after 1 mo ripening. *Lanes 8-12*, as lanes 3-7, but after 3 months ripening.

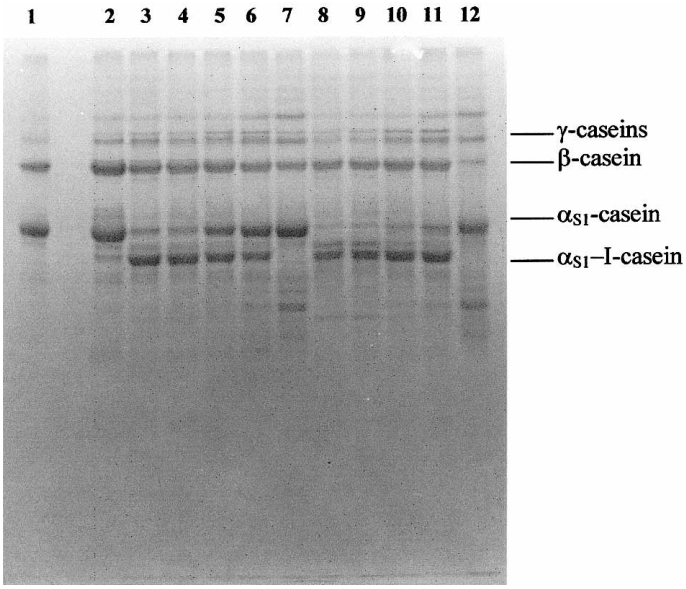

Figure 4. Urea-PAGE electrophoretograms of experimental cheeses, cooked to different temperatures, after 1 mo or 3 mo of ripening. *Lane 1*, sodium caseinate, *lane 2*, cheese made from raw milk at d1, *lane 3*, cheese made from raw milk cooked at 38 °C after 1 mo ripening, *lanes 4-7*, cheese made from milk pasteurised at 63 °C × 30 min and cooked at 38 °C, 43 °C, 48 °C, 55 °C, all after 1 mo ripening. *Lanes 8-12*, as lanes 3-7, but after 3 mo ripening.

3.5. Analysis of WSN

Analysis of the WSN fraction of cheese using RP-HPLC (a selection of representative chromatograms is presented in Fig. 5) showed differences between experimental cheeses in the region of the chromatograms of retention time 55–58 min, which has been previously recognised as being characteristic of plasmin activity [2, 16]. Higher levels of peptides in this region were seen in the cheese cooked at 55 °C at both 1 mo and 3 mo ripening than in the cheese cooked at lower cooking temperatures. Increasing the severity of milk heat treatment did not have as marked an effect on the peptide patterns seen in the chromatograms as cooking temperature.

4. DISCUSSION

Increasing the severity of heat treatment of milk increased cheese moisture content, although not significantly. This has been previously reported [1, 12], and is probably due to the adverse effects of high heat treatment of milk on the syneresis of rennet curd. Such effects may include micelle-bound β-lactoglobulin-κ-casein complexes impeding the agglomeration of renneted micelles [20, 21], or increased water binding capacity of the paracasein-whey protein complex [12].

The significant decrease in moisture content concomitant with increasing cooking temperature is in agreement with previous reports by Wilkinson et al. [28] and Yun et al. [29], who reported that increasing cooking temperature decreased the moisture content of Cheddar cheese and Mozzarella cheese, respectively.

Differing milk heat treatments and cooking temperatures had little effect on the gross composition of whey (not shown) but, however, significantly affected plasmin activity in whey. Plasmin activity in whey generally corresponded to that of cheese, with the greatest activity being found in whey from cheese cooked at higher temperatures and the lowest activity in whey from cheese made from severely heat-treated milk. The former observation may be due either to inactivation of

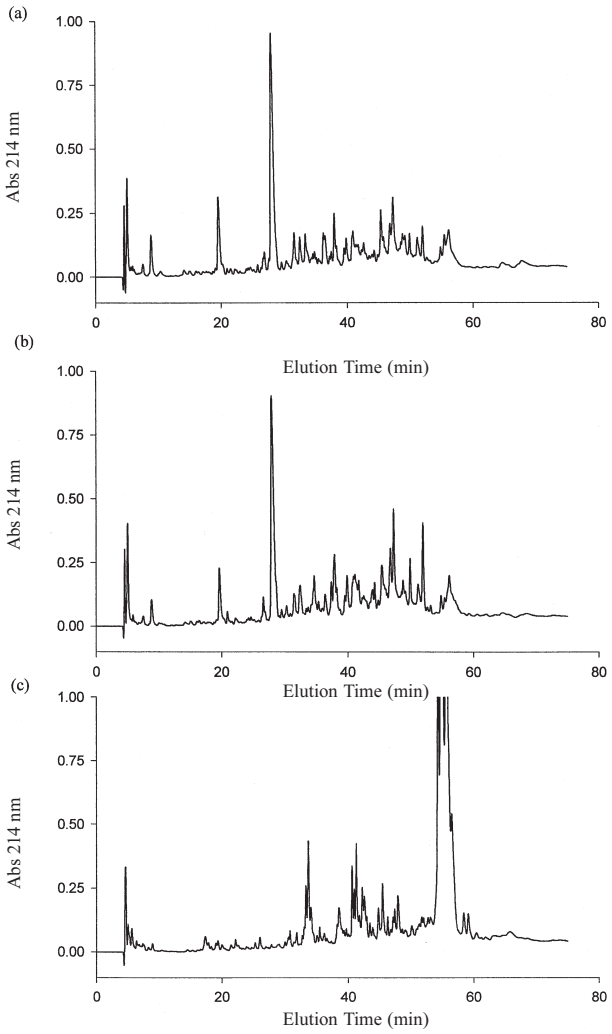

Figure 5. RP-HPLC chromatograms of water-soluble extracts of cheese made from milk (**a**) pasteurised at 63 °C × 30 min and cooked at 38 °C, (**b**) pasteurised at 75 °C × 1 min and cooked at 38 °C and (**c**) pasteurised at 63 °C × 30 min and cooked at 55 °C, all after 3 months of ripening.

inhibitors in whey during cooking, or to increased expulsion of plasmin from cheese, although the pattern of increase noted in cheese suggests that the former mechanism may be more likely. Plasmin activity in whey was quantitatively lower than that in either milk or the cheese, presumably due to its efficient incorporation into the cheese caused by its binding to casein micelles [3].

Increasing severity of milk heat treatment decreased plasmin activity both in milk samples and the cheese manufactured therefrom. This agrees with Benfeldt et al. [4], who found that plasmin activity decreased with increased temperature and holding time. The principal reason for the decrease in plasmin activity with increasing severity of heat treatment is the formation of heat-induced thiol-disulphide complexes between plasmin and β-lactoglobulin [16]. Pasteurisation of the milk at 63 °C for 30 min marginally increased plasmin activity both in milk and cheese relative to raw milk, presumably due to inactivation of

heat-labile inhibitors of milk PA [25]. Pasteurisation using high temperature short time conditions (HTST; 72 °C for 15 s) may have more significant effects on milk plasmin activity, but this treatment was not peformed due to the small volumes of milk being studied.

Urea-PAGE analysis showed less breakdown of β-casein to γ-caseins in cheese made from more severely heat-treated milk. This is consistent with reports by Benfeldt et al. [4] and Kelly [15], but disagrees with the findings of Calvo et al. [5] that the rate of proteolysis of β-casein was faster in cheese made from overheated milk compared to cheese made from pasteurised milk. Enright and Kelly [7] reported that increased severity of heat treatment resulted in less breakdown of β-casein in milk samples to which high levels of exogenous plasmin had been added, and concluded that this effect may result from binding of denatured whey protein to the surface of the casein micelle, with concomitant steric hindrance of the approach of the enzyme to the peptide bonds within the substrate molecule.

Increasing cooking temperature had a greater effect on plasmin-induced proteolysis of β-casein to γ-caseins than increasing the severity of the milk heat treatment. In agreement with Farkye and Fox [8] and Delacroix-Buchet and Fournier [6], plasmin activity was significantly increased at higher cooking temperatures. With increasing cooking temperature, the rate of plasminogen activation during ripening also increased, possibly due to inhibitors of PA and plasmin being lost in the whey during cheesemaking [8] or thermal inactivation of inhibitors of PA.

Increasing cooking temperature decreased chymosin activity (probably due to thermal inactivation of the enzyme), as was apparent from the reduced degradation of α_{s1}-casein to α_{s1}-I casein. This is in agreement with Delacroix-Buchet and Fournier [6] who found that increasing the cooking temperature from 52 °C to 56 °C decreased chymosin activity. Inactivation was gradual, however, as the degradation of α_{s1}-casein to α_{s1}-I casein slowly lessened over the range of cooking temperatures. There was no significant effect of severe heat treatment on chymosin activity, which may be due to the fact that the chymosin is not directly affected by heat treatment of the cheese milk although the accessibility of its substrates, mainly κ-casein and α_{s1}-casein, may be altered [4]. Lo and Bastian [19] investigated the influence of native or heat-denatured α-lactalbumin and β-lactoglobulin on chymosin activity against α_{s1}-casein in various model systems and found that both native and heat-denatured β-lactoglobulin inhibited chymosin activity. However, in this trial, the incorporation of β-lactoglobulin due to heat treatment did not have an inhibitory effect on chymosin activity. Indeed, from Urea-PAGE analysis, slightly greater hydrolysis of α_{s1}- to α_{s1}-I-casein was seen in the cheeses manufactured from the milk which had been more severely heat-treated.

The role of the indigenous milk acid proteinase cathepsin D, which also degrades α_{s1}-casein to α_{s1}-I casein [23], should be considered, as it has recently been shown that it is relatively stable under high temperature cheese cooking conditions (~45% survival after heating at 55 °C for 30 min [13]). However, it is generally regarded that its relative contribution to cheese ripening is masked by the much higher activity of chymosin in curd, although no studies have directly compared the relative activities and extents of inactivation of the two enzymes during cheese manufacture, which makes direct interpretation of its contribution to the results shown, although potentially important, difficult.

HPLC analysis of lyophilised WSN samples showed differences between the peptide patterns in cheeses cooked at different temperatures, also illustrating the importance of a balance between plasmin

activity and chymosin activity on proteolysis during ripening. Examination of the regions of the chromatograms affected by plasmin activity (55–58 min), where the authors have previously shown proteose peptones to elute, showed dramatically that increased cooking temperatures increased the relative contribution of plasmin to cheese ripening, while numbers and levels of more hydrophilic peptides, perhaps products of chymosin and/or starter bacterial enzymes, were reduced markedly. There were, overall, fewer differences in elution patterns between cheeses made from milk heated at different temperature and time combinations. It must be acknowledged that the model system used, where mesophilic starter cultures were used irrespective of cooking regime applied during cheese manufacture, will lead to reduced contribution of starter enzymes to proteolysis during ripening with increased cooking temperature, and simplify peptide profiles. However, the principal objective of this study was to study the relative stabilities and contributions of chymosin and plasmin during cheese manufacture and ripening, and the HPLC profiles confirm the great changes in activity of the latter due to increasing cheese cooking temperature.

5. CONCLUSION

In summary, cooking temperature applied during cheese manufacture had a more significant effect on primary proteolysis during ripening than milk heat treatment, by affecting both activities of chymosin and plasmin, while milk pasteurisation largely influenced plasmin activity alone.

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