Original article

Effect of ripening temperature on the quality of low moisture Mozzarella cheese: 1. Composition and proteolysis

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Abstract — Low-moisture Mozzarella cheese, in duplicate trials, was ripened at 0, 4, 10 or 15 ºC for 70 d. For all temperature treatments, increasing ripening time resulted in significant ($P < 0.05$) increases in the level of proteolysis as measured by the levels of pH 4.6 soluble-N (pH 4.6SN) and 5% phosphotungstic acid-soluble N (PTA-N). Urea-PAGE electrophoretograms of the pH 4.6-insoluble cheese extracts showed that increasing the storage temperature resulted in an increase in the extent of degradation of α_{s1}-CN but had little effect on the degradation of β-casein. Increasing the ripening temperature resulted in a significant increase in the mean level of pH 4.6SN and PTA-N over the 70 d ripening period. Reverse-phase HPLC (RP-HPLC) of the pH 4.6-soluble and 70% ethanol-soluble fractions of the pH 4.6-soluble cheese extracts indicated a heterogeneous array of peptides. The concentration of early-eluting peptides, representing amino acids and small peptides, increased as the ripening temperature was increased.

Ripening / temperature / Mozzarella / composition / proteolysis

Résumé — **Effets de la température d'affinage sur les fromages de Mozzarella à faible teneur en eau. 1. Composition et protéolyse**. Deux lots de fromages de Mozzarella à faible teneur en eau ont été fabriqués et ont été maturés à 0, 4, 10 ou 15 ºC pendant 70 jours. À toutes les températures, l'augmentation du temps de maturation a entraîné une augmentation significative ($P < 0,05$) du niveau de protéolyse comme mesuré par les niveaux d'azote soluble à pH 4,6 (pH 4,6SN) et d'azote soluble dans l'acide phosphotungstique à 5 % (PTA-N). Les électrophorégrammes urée-PAGE des fractions de fromage insolubles à pH 4,6 ont montré que l'augmentation de la température de conservation provoquait une augmentation du niveau de dégradation de la caséine α_{s1} mais avait peu d'effet sur la dégradation de la caséine β. L'augmentation de la température de maturation a entraîné une augmentation significative des niveaux moyens de pH 4,6SN et PTA-N sur toute la période de

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maturation de 70 jours. L'HPLC en phase inverse de la fraction pH4,6SN et de la fraction soluble dans l'éthanol à 70 % obtenue à partir de pH4,6SN a montré un ensemble hétérogène de peptides. La concentration de peptides élués en premier, représentant des acide aminés et petits peptides, a augmenté quand la température de maturation augmentait.

Maturation / température / Mozzarella / composition / protéolyse

1. INTRODUCTION

In recent years, the extent and depth of proteolysis which occurs in low-moisture (LM) Mozzarella cheese has become an area of considerable interest and is now thought to play a major role in the development of the functional properties of the cheese [30, 34]. Initial breakdown of caseins to large peptides in LM Mozzarella cheese occurs mainly through the action of the coagulant [7, 8], as is the case for most rennet-coagulated cheeses [19, 20, 22]. The starter culture contributes primarily to secondary proteolysis, i.e., degradation of the products of primary proteolysis to small peptides and amino acids [32]. However, the starter culture may contribute to the initial hydrolysis of intact caseins [7]. There is also evidence of plasmin activity, the indigenous alkaline milk proteinase, in Mozzarella cheese during ripening, i.e., the degradation of β-casein with concomitant formation of γ-caseins [11, 23].

The initial breakdown of the intact caseins by the coagulant is thought to be one of the main factors affecting the age-related changes which occur in LM Mozzarella cheese [31, 34]. During the first few weeks after manufacture, Mozzarella undergoes large changes in texture and melting properties, as reflected by increases in flowability and stretchability and decreases in melt time and apparent viscosity [25, 31, 33]. These changes in functionality coincide with increased proteolysis and an increase in the water binding capacity of the casein [20, 32]. During storage at 4 °C, ~ 36% of α_{s1}-CN and ~ 10% β-CN are de-

graded in Mozzarella cheeses made using chymosin [49]. Degradation of casein results in a concomitant increase in the level of pH 4.6-soluble N, which typically increases from ~ 2% of total N at 1 d to ~ 8% at 50 d [20, 31]. However, the breadth and depth of proteolysis in Mozzarella is affected by many parameters, including the curd manufacturing procedure, the plasticization conditions and the type of coagulant [10, 30, 33, 42, 48, 49]. Considering the influence of proteolysis on the functionality in Cheddar [2, 26], any factor which influences proteolysis in Mozzarella is expected to influence functionality and the functional shelf life of the cheese.

The use of an elevated ripening temperature to accelerate the maturation of cheese has been studied extensively. Ripening temperature influences the rate of proteolysis, the composition of cheese microflora [12], cheese texture and quality [3–5, 12, 16, 17, 21, 46]. However, most studies have concentrated on Cheddar and Dutch varieties [15].

The objectives of this study were to evaluate the effect of ripening temperature on proteolysis in LM Mozzarella cheese, which does not appear to have been studied previously.

2. MATERIALS AND METHODS

2.1. Cheese manufacture

Milk was standardized to a casein: fat ratio of 0.95, stored overnight at 8 °C,

pasteurized (72 °C, 15 s), cooled to the renneting temperature (36 °C) and divided into four 470 L quantities per cheese vat. Analysis of samples of pasteurized milk taken from the cheese vat, prior to starter addition, indicated that the mean composition (g·kg^{-1}) was: fat, 28.1; protein, 33.0; lactose, 4.55. The milk was inoculated at 15 g·kg^{-1} with a starter culture consisting of *Streptococcus thermophilus* and *Lactobacillus helveticus* (Chr. Hansen's Laboratory (Ireland) Ltd, Little Island, Cork, Ireland), at a ratio of 2:1; each strain was grown separately overnight (38 °C) in reconstituted low-heat skim milk powder (10 g·100 g^{-1}). After ripening for 31 min, the milk, at 36 °C, was set with chymosin (Double Strength Chy-max, 50 000 MCU·mL^{-1}; Pfizer Inc, Milwaukee, WI, USA), which was added at a level of 0.077 mL·kg^{-1} milk.

After cutting (~ 31 min later), the curd/whey mixture was cooked to 42 °C and drained when the pH of the whey, expressed manually from the curd, reached pH 6.1. The curds from each vat were cheddared and the blocks of curd were milled when the pH reached ~ 5.15. The milled curd was dry-salted at a level of 4.6%, w/w, and then allowed to mellow for 20 min. The salted curd was plasticized as described previously [25], moulded into rectangular 2.3 kg blocks which were cooled in water at ~2 °C to a surface temperature of 24 °C and a core temperature of 50 °C. The salted cheeses from each vat were vacuum wrapped and stored for 70 d at 0, 4, 10 or 15 °C. The ripening temperature formed the treatments and are referred to as T0, T4, T10 and T15, respectively. Cheesemaking trials were performed in duplicate.

2.2. Cheese composition

Samples of cheese were grated finely and analysed in duplicate for salt, fat, protein, moisture, pH, calcium and phosphorus, as described by Guinee et al. [26].

2.3. Proteolysis

The levels of cheese nitrogen soluble in water at pH 4.6 (WSN) or in 5% phosphotungstic acid (50 g·L^{-1}) (PTA-N) were measured as described by Guinee et al. [24]

Urea-polyacrylamide gel electrophoresis (urea-PAGE) was performed on pH 4.6-insoluble and -soluble fractions of the cheese using a protean IIxi vertical slab gel unit (Bio-Rad Laboratories Ltd., Watford, HP2 7TD, UK) and the stacking gel system described by Andrews [1]. The gels were stained directly by the method of Blakesley and Boezi [9] with Coomassie brilliant blue G250.

Reverse-phase HPLC was carried out on the lyophilized pH 4.6SN extract and the ethanol soluble fraction there of. The ethanol soluble fraction was prepared as follows: absolute ethanol was added to an aliquot of the pH 4.6-soluble extract to a final ethanol concentration of 700 ml·L^{-1}. The mixture was held for 31 min at 20 °C and centrifuged at 3100 × *g*. The supernatant was filtered through Whatman No. 1 filter paper and the ethanol was removed from the filtrate by rotary evaporation (Model No. RE100, Bibby Sterelin Ltd., Stone, ST15 0SA, UK) at 31 °C under vacuum. The lyophilized fractions were dissolved in solvent A (1 mL·L^{-1} trifluoroacetic acid (TFA) in HPLC grade water) and analyzed by RP-HPLC, using a Varian Prostar 231 HPLC equipment, as described by Shakeel-Ur-Rehman et al. [40]. Samples in buffer A (41 μL) were applied to the column and eluted at a flow rate of 0.75 mL·min^{-1}. The gradient was started with 100% solvent A for 5 min and continued with a linear gradient to 50% (v/v) solvent B (1 mL·L^{-1} TFA in acetonitrile) over 55 min, 50% B (v/v) for 6 min and finally a linear gradient to 60% B (v/v) for 3 min. The column was washed with 95% B (v/v) for 5 min, followed by equilibration

with 100% A for 5 min before injecting the next sample.

2.4. Effect of plasticization temperature on the level of residual coagulant in Mozzarella cheese curd

Four vats of cheese were manufactured on the same day using the method described above except that plasticization was performed at temperatures of 55, 58, 62 and 66 °C. The cheeses were salted, vacuum wrapped and stored at 4 °C. The 1 day-old cheeses were analysed for residual coagulant activity. One day-old Cheddar cheese was also analysed for residual coagulant activity.

Residual coagulant activity was determined according to the method of Hurley et al. [28].

2.5. Statistical analysis

Duplicate cheesemaking trials were undertaken. In each trial, four vats of cheese curd were manufactured and the cheeses were ripened at 0, 4, 10 or 15 °C, designated T0, T4, T10 and T15, respectively.

A randomized complete block design, which incorporated the four treatments and 2 blocks (duplicate trials), was used for analysis of the response variables relating to the composition of cheese milk and cheese. Analysis of variance (ANOVA) was carried out using a SAS procedure [39] where the effects of treatment and replicates were estimated for all response variables. Duncan's multiple-comparison test was used as a guide for pair comparisons of the treatment means. The level of significance was determined at $P < 0.05$.

A split plot design was used to monitor the effects of treatment, ripening time and their interaction on the response variables measured at regular intervals during ripen-

ing, i.e., pH 4.6SN and PTA-N. Analysis of variance for the split plot design was carried out using a general linear model (GLM) procedure of SAS [39]. Statistically significant differences ($P < 0.05$) between different treatments were determined by Fisher's least significant difference.

The four cheeses from each of the 2 trials were analyzed by PAGE at 1, 12, 25, 48 and 70 d, and RP-HPLC at 1, 25 and 70 d; the results are presented as observations and supportive data but were not statistically analysed.

3. RESULTS AND DISCUSSION

3.1. Cheese composition

The composition of the four sets of cheese are presented in Table I. The composition was similar to those reported elsewhere for low-moisture part-skim Mozzarella cheese [25, 27, 48, 49]. Similar to previous findings [25] a large increase in pH occurred between curd milling (5.15) and 1 d, at which stage the pH was ~ 5.4. Factors, which may have contributed to the increase in pH, include the loss of lactic acid and changes in the calcium-phosphate moiety of the curd during plasticization. It is likely that the loss of soluble calcium and phosphate is accompanied by the subsequent solubilization of micellar calcium phosphate during cooling of the cheese and the neutralization of the H^+ ions by the phosphate anions [43]. It is noteworthy that approximately 36% of calcium phosphate in cheese is soluble at pH 5.15 [27].

There were no significant differences between any of the compositional parameters, apart from the salt-in-moisture content, which was significantly higher in T0 than in the other cheeses. The absence of differences in the main compositional parameters, such as protein and fat, is expected as the milk for all treatments was

Table I. Composition of low-moisture Mozzarella cheese.

Cheese composition	Ripening temperature (ºC)			
	0	4	10	15
Moisture (g·kg^{-1})	484.7[a]	489.9[a]	487.6[a]	486.7[a]
Fat (g·kg^{-1})	199.6[a]	194.2[a]	201.5[a]	200.9[a]
Protein (g·kg^{-1})	268.6[a]	272.8[a]	266.2[a]	267.1[a]
MNFS (g·kg^{-1})	605.4[a]	607.9[a]	610.5[a]	608.9[a]
FDM (g·kg^{-1})	387.1[a]	380.8[a]	393.0[a]	391.2[a]
S/M (g·kg^{-1})	37.1[a]	32.1[b]	31.7[b]	33.4[b]
Ca (mg·100 g^{-1})	673[a]	686[a]	681[a]	686[a]
P (mg·100 g^{-1})	475[a]	481[a]	487[a]	488[a]
pH-at 1 d	5.45[a]	5.43[a]	5.41[a]	5.41[a]

MNFS: moisture in nonfat substance; FDM: fat-in-dry matter; S/M: salt in moisture.
[a,b] Values within a row without a common superscript differed significantly: $P < 0.05$.

Figure 1. Level of pH 4.6 soluble N (pH4.6SN), expressed as a percentage of total N (TN), in low-moisture Mozzarella cheese ripened at 0 ºC (●), 4 ºC (○), 10 ºC (▲) or 15 ºC (△).

3.2. Changes in nitrogen fractions

The pH 4.6SN, as a percentage of total N, increased in all cheeses during maturation (Fig. 1), to an extent dependent on the ripening temperature. The interaction between ripening temperature and time had a significant ($P < 0.001$) effect on the level of pH 4.6SN, with the rate of increase over time being least for T0 and highest for T15. At most ripening times > 15 d, the level of pH 4.6SN in T10 and T15 were similar and significantly higher ($P < 0.05$) than those in T0 and T4, which also had similar levels. A similar trend was noted for PTA-N, for which the values for T15 were significantly

standardized to a fixed protein-to-fat ratio and subjected to a similar cheesemaking process.
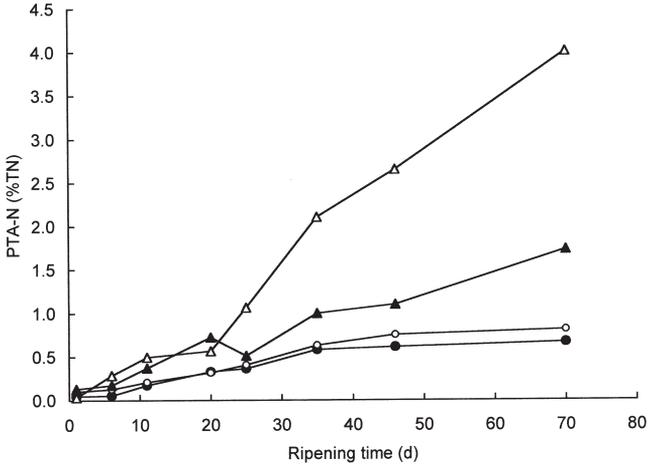
Figure 2. Level of 5% phosphotungstic acid-soluble N (PTA-N), expressed as a percentage of total N (TN), in low-moisture Mozzarella cheese ripened at 0 °C (●), 4 °C (○) 10 °C (▲) or 15 °C (△).

higher than those of all other cheeses at times ≥ 25 d (Fig. 2). The level of PTA-N in the T10 cheese was numerically higher than those in the T0 and T4 cheeses at most times and significantly higher at 70 d.

The increased rate of proteolysis with ripening temperature is consistent with pervious studies [16, 17, 24, 47]. The values for pH 4.6SN and PTA-N are similar to those reported elsewhere for low-moisture part-skim Mozzarella cheese ripened at 4/5 °C for similar times [45, 48, 49]. However, in general, the levels for pH 4.6SN in the T4, T10 and T15 cheeses were somewhat lower than those of Cheddar after a similar ripening time at a similar temperature, e.g., at 70 d, the level of pH 4.6 SN for Cheddar ripened at 10 or 15 °C is typically ~ 17 and 22.5% of total N [16, 24, 47]. This is perhaps surprising, considering the higher moisture and lower salt-in-moisture levels of LM Mozzarella cheese. The relatively low level of pH 4.6SN may be attributed the inactivation of the residual chymosin due to a combination of the relatively high cook temperature (42 °C) and the high temperature (58 °C) during plasticization [41]. Hence, a comparison of 1 day-old Cheddar cheese with T4 cheese at 1 d indicated a much higher level of residual chymosin activity in the Cheddar cheese

(Tab. II). Moreover, increasing the plasticization temperature resulted in a progressive decrease in chymosin activity at all stages of ripening (Tab. II).

3.3. Urea-polyacrylamide gel electrophoresis (urea-PAGE)

Urea-polyacrylamide gel electrophoretograms of the pH 4.6 insoluble (pH 4.6 ISN) fraction of cheese ripened at the different temperatures are shown in Figures 3a and 3b. Overall, the trends observed with PAGE are consistent with those for pH 4.6SN which showed that proteolysis was relatively low and significantly affected by ripening temperature. Increasing the ripening temperature resulted in an increase in the rate of degradation of α_{s1}-CN, especially at 48 and 70 d, when most of this casein was degraded at 10 and 15 °C. The increased hydrolysis of α_{s1}-CN at the higher temperatures was paralleled by a concomitant increase in the intensity of α_{s1}-CN (f24-199) and resulted in a slight increase in the intensity of α_{s1}-CN (f102-199). In support of this, analysis of the lyophilized pH 4.6SN extracts showed higher intensities of peptides with an electrophoretic mobility greater than α_{s1}-CN at

Table II. Residual levels of coagulant in 1 d old Cheddar and low-moisture Mozzarella cheeses plasticized at different stretch temperatures.

Cheese	Stretch[1] temperature (°C)	Residual coagulant activity (Rennet units/kg)
Cheddar	NA[2]	15.3
Mozzarella	55	5.9
	58	5.3
	62	3.6
	66	1.2

[1] Stretch temperature refers to the temperature reached by the curd during plasticization in hot water at different temperatures.
[2] NA, not applicable.
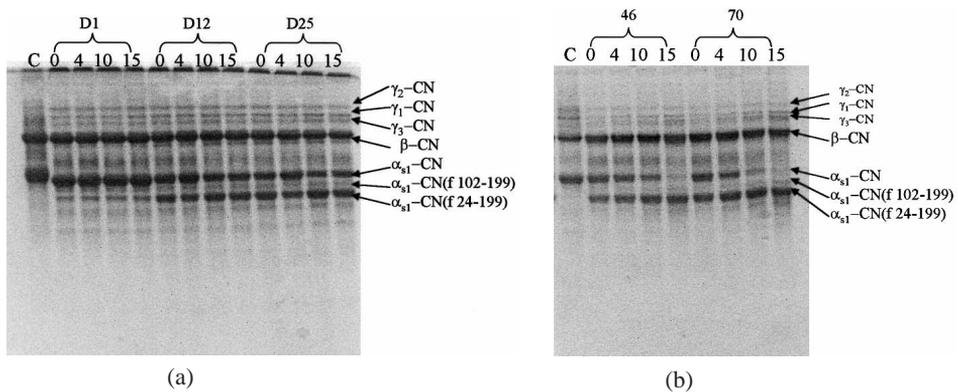
Figure 3. Urea-PAGE analysis of sodium caseinate (C) and the pH 4.6-insoluble fraction of low-moisture Mozzarella cheese ripened at 0, 4, 10 or 15 °C at 1, 12, 25, 48 or 70 d, respectively.

the higher ripening temperatures (f24-199) results not shown.

In agreement with previous studies on Mozzarella [11, 48, 49], there was very little hydrolysis of β-casein over the 70 d period, especially in cheeses ripened at 4 °C. Concomitant with the slightly more extensive degradation of β-casein in the T15 cheese, an increase in the intensity of γ-caseins, especially γ_2-casein, was evident in the T15 cheese, especially at 48 and 70 d. The relatively low degree of age-related degradation of β-casein, compared to α_{s1}-CN, has also been observed for other cheese varieties (made using chymosin or pepsin

as coagulant) such as Cheddar, Gouda and Blue-cheese [18], and is surprising considering that β-casein is readily hydrolyzed by chymosin and pepsin in dilute solutions (< 100 g·L^{-1} sodium caseinate) [35–38, 44]. It has been suggested that the relatively low degree of proteolysis of β-casein in cheese may be due to the concentration-dependent intermolecular association of the hydrophobic C-terminal region of β-casein which contains the chymosin/pepsin sensitive bonds [18, 38].

The alkaline milk proteinase, plasmin, which has a high specificity for β-casein
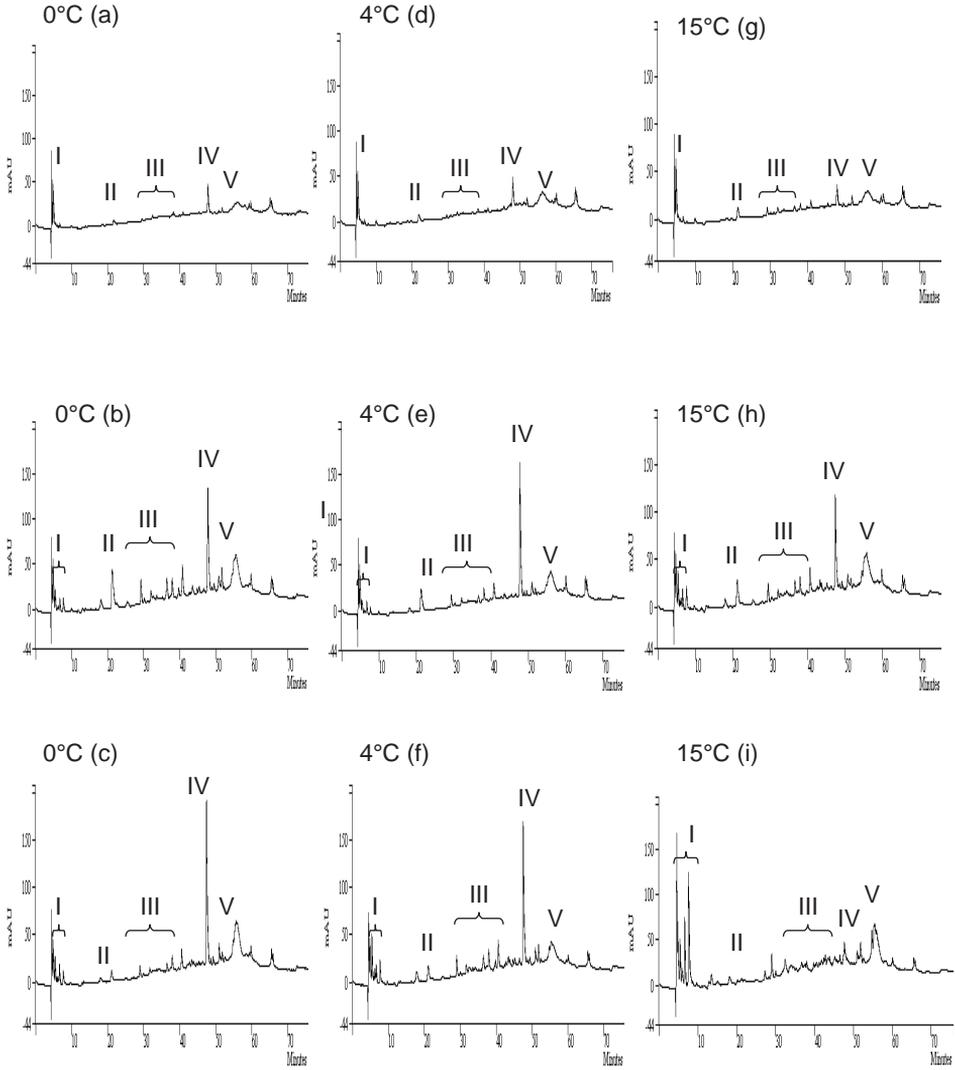
Figure 4. Elution profiles from reverse-phase (C$_8$) HPLC of the pH 4.6-soluble extract of low-moisture Mozzarella cheese ripened at 0 ºC (a–c), 4 ºC (d–f) or 15 ºC (g–i) for 1 (a, d, g), 25 (b, e, h) or 70 (c, f, i) days.

[23] is considered to be the major enzyme contributing to degradation of β-casein in cheese. Plasmin is heat stable and not inactivated by the time/temperature conditions used during plasticization of Mozzarella cheese curd [14]. Studies have also shown that plasmin activity in cheese increases with increased cook temperature, due to the increased conversion of plasminogen to plasmin [15]. Therefore, it is expected that that the high plasticization temperature may result in increased plasmin levels in Mozzarella cheese. However, the pH of all the cheeses in this study was < 5.6 which is
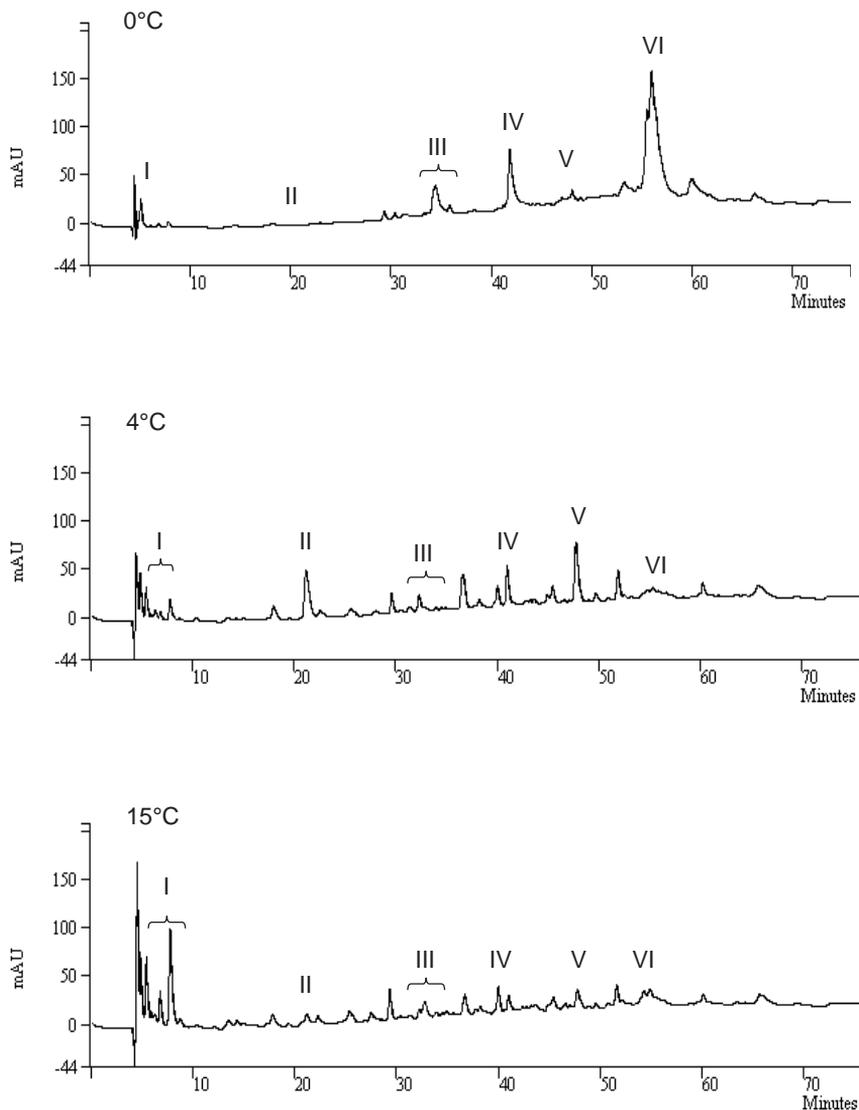
Figure 5. Elution profiles from reverse-phase (C$_8$) HPLC of 70% ethanol-soluble extract of low-moisture Mozzarella cheese ripened at 0, 4 or 15 ºC for 70 d.

much lower than the optimum of plasmin (~ pH 7.5).

3.4. Reverse phase-HPLC

RP-HPLC profiles of the pH 4.6-soluble extracts (pH 4.6-SE) of the cheeses at 1, 25 and 70 d are shown in Figure 4. The chromatograms for all cheeses showed a large number of peaks, indicating a heterogeneous mixture of proteolysis products. While the number of peaks remained fairly constant throughout ripening, there were age-related changes in the height of different peaks, indicating a change in the molecular mass distribution and/or

hydrophobicity of peptides [6]. In all cheeses, there was an increase in the height and areas of peaks, denoted II, IV, V and peaks in zone III during the first 25 d ripening. This trend is expected, as peptides in the water extractable phase of the cheese accumulate, due to the progressive breakdown of caseins and peptides by various enzyme systems in the cheese, including residual rennet, plasmin, starter cell proteinases and peptidases leading to concomitant increases in the concentrations of peptides of different molecular mass and amino acids [13, 19, 20]. Between 25 and 70 d, there was little change in the peak heights and distribution in the cheeses ripened at 0 and 4 °C but in the cheese ripened at 10 °C (results not shown) or 15 °C, there was a notable decrease in the areas of peaks II, IV and peaks in zone III, and an increase in the peaks in zone I. According to Altemueller and Rosenberg [6], the early-eluting peaks (zone I) contains N compounds with molecular mass of up to $1 \ kg \cdot mol^{-1}$ and hence include small peptides and amino acids. The increase in the area of peaks in zone I with elevation of ripening temperature is consistent with the results of Folkertsma et al. [17] and concurs with the trend noted for PTA-N, the concentration of which was highest in the T15 cheese at times ≥ 25 d (Fig. 2); PTA-N is comprised mainly of small peptides and amino acids [29]. The increase in free amino acids and small peptides, as measured by increases in N soluble in various solvents (e.g., 5% phosphotungstic acid, 12% trichloroacetic acid or 70% ethanol) is also in agreement with the results of other groups [3, 4, 24, 47].

RP-HPLC chromatograms of the 70% ethanol-soluble N at 70 d (Fig. 5) indicated a less heterogeneous mixture of peptides than in the corresponding pH 4.6-SE. However, similar to the pH 4.6-SE, ~ 5 major peak zones were present. Increasing the ripening temperature resulted in a decrease in area of peaks III, IV and VI and a notable

increase in the early-eluting peaks, denoted as I. The increase in the latter peak with elevated ripening temperature is consistent with the results for PTA-N solubility and RP-HPLC analysis of the pH 4.6-soluble cheese extracts.

4. CONCLUSION

Increasing the ripening temperature resulted in the extensive degradation of α_{s1}-casein and significant ($P < 0.05$) increases in the level of pH 4.6-soluble N and 5% phosphotungstic acid-soluble N at most times over the 70 d ripening period. RP-HPLC analysis of pH 4.6-soluble and 70% ethanol-soluble extracts indicated the presence of a heterogeneous mixture of peptides. Increasing the ripening temperature resulted in an increase in the area of early-eluting peaks, identified elsewhere as containing low molecular mass nitrogenous material, $< 1 \ kg \cdot mol^{-1}$. The study indicated that changing the ripening temperature provides a convenient means of controlling the extent of proteolysis without altering the type of proteolysis, as identified by PAGE of cheese and RP-HPLC.

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