Effect of pH and Calcium Concentration on Proteolysis in Mozzarella Cheese

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ABSTRACT

Low-moisture Mozzarella cheeses (LMMC), varying in calcium content and pH, were made using a starter culture (control; CL) or direct acidification (DA) with lactic acid or lactic acid and glucono- δ -lactone. The pH and calcium concentration significantly affected the type and extent of proteolysis in Mozzarella cheese during the 70-d storage period at 4°C. For cheeses with a similar pH, reducing the calcium-to-casein ratio from ~29 to 22 mg/g of protein resulted in marked increases in moisture content and in primary and secondary proteolysis, as indicated by polyacrylamide gel electrophoresis and higher levels of pH 4.6- and 5%-PTA-soluble N. Increasing the pH of DA cheeses of similar moisture content, from ~5.5 to 5.9, while maintaining the calcium-to-casein ratio almost constant at ~29 mg/g, resulted in a decrease in primary proteolysis but had no effect on secondary proteolysis. Comparison of CL and DA cheeses with a similar composition showed that the CL cheese had higher levels of α_{s1} -CN degradation, pH 4.6- and 5%-PTA-soluble N. Analysis of pH 4.6-soluble N extracts by reverse-phase HPLC showed that the CL cheese had higher concentrations of compounds with low retention times, suggesting higher concentrations of low molecular mass peptides and free amino acids. (**Key words:** pH, calcium, Mozzarella, proteolysis)

Abbreviation key: CL = control, DA = directly-acidified, ESF = ethanol soluble fraction, GDL = glucono- δ -lactone, pH4.6SN = pH 4.6 water-soluble nitrogen, PTAN = 5% (wt/wt) tungstophosphoric acid soluble N, PAGE = polyacrylamide gel electrophoresis, RP-HPLC = reverse-phase HPLC, TFA = trifluoroacetic acid.

INTRODUCTION

Proteolysis plays a major role in the development of flavor and texture in most rennet- curd cheese varieties.

Small peptides, amino acids, and especially products of amino acid catabolism, e.g., amines and thiols, contribute directly to cheese flavor (Fox et al., 1996; McSweeney and Sousa, 2000). Proteolysis is a major determinant of the intact casein content which has a large impact on the texture of the unheated Cheddar cheese (Creamer and Olson, 1982) and on the functionality of heated cheese (Guinee et al., 2000a).

Owing to its importance in cheese ripening, proteolysis and factors affecting it have been investigated extensively in different cheese varieties, especially Cheddar (O'Keeffe et al., 1975; Lane and Fox, 1997). α_{s1} -Casein is the principal target of chymosin and most other commercial rennets in rennet-curd cheese varieties. The early hydrolysis of α_{s1} -CN at the Phe₂₃-Phe₂₄ peptide bond by residual chymosin results in a marked weakening of para-casein matrix and decreases in fracture stress and firmness (Creamer and Olson, 1982; Fenelon et al., 2000). β -Casein generally undergoes markedly less breakdown than α_{s1} -CN during storage of most cheeses, including Cheddar, Gouda, and Mozzarella (Visser and de Groot-Mostert, 1977; Yun et al., 1993; Fox et al., 1996).

A recent survey (Guinee et al., 2000b) indicated marked intravarietal differences in the Ca-to-casein ratio and pH of retail Cheddar and Mozzarella, which undoubtedly reflect the application of different cheese-making protocols at a commercial level for the manufacture of cheese with different heat-induced functionality. Functional attributes of cheese, such as flow and stretchability, are influenced by pH and Ca content (Kimura et al., 1992; Guinee et al., 2002).

Little information is available on the direct effects of pH and Ca content on proteolysis in cheese, including Mozzarella. However, studies indicate that proteolysis of CN in dilute systems depends on the reaction pH and the state of aggregation of the substrate, as affected by pH and Ca content (Mulvihill and Fox, 1977). Fox (1970) reported that the individual CNS in milk, especially α_{s1} -CN, become progressively more susceptible to rennet-induced proteolysis at pH 6.6 as the level of micellar calcium phosphate is reduced. This effect was

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attributed to the increased accessibility of individual CNS to rennet, owing to the disruption of the micelles on removal of colloidal calcium phosphate. Similarly, reducing the level of calcium phosphate in Cheddar cheese curd (by a rapid decrease in pH), while in contact with the whey containing the full amount of rennet, results in increased susceptibility of the CN to proteolysis and a higher degree of CN degradation in 1-d-old cheese (O'Keeffe et al., 1975).

Tam and Whitaker (1972) found that the extent of hydrolysis in dilute (~0.5%, wt/vol) aqueous dispersions of whole CN and α_{s1} - or β -CN by chymosin generally decreased as the pH was increased from 3.5 to 6.0. Mulvihill and Fox (1977) reported that in the pH range 4.0 to 7.0, the hydrolysis of α_{s1} -CN (2%, wt/vol, aqueous dispersion) to α_{s1} -CN (f24–199) by chymosin was optimal at pH 5.8 and minimal at pH 4.6, where the α_{s1} -CN (f24–199) is aggregated. Moreover, the rate of degradation of α_{s1} -CN (f24–199) and the profile of its products are influenced by reaction pH, with the degradation of α_{s1} -CN (f24–199) to α_{s1} -CN (f102–199) being optimal at pH 5.8 (Mulvihill and Fox, 1977). However, the influence of pH on the proteolytic activity and specificity of chymosin on α_{s1} -CN (f24–199) is influenced by NaCl (Mulvihill and Fox, 1980). At a rennet level typical of that used in cheese manufacture (i.e., ~2.2 CU/ml), hydrolysis of α_{s1} -CN to α_{s1} -CN (f24–199) and further hydrolysis of α_{s1} -CN (f24–199) is markedly inhibited at 5% NaCl in the pH range 5.8 to 7.0. In contrast, at pH 5.2 the addition of NaCl to 5%, wt/vol, had little effect on the degree of chymosin-induced degradation of α_{s1} -CN to α_{s1} -CN (f24–199) but strongly inhibited further proteolysis of α_{s1} -CN (f24–199). Owing to the pH dependence of the inhibitory effect of NaCl, the degree of overall hydrolysis of α_{s1} -CN in the presence of 5%, wt/vol, NaCl increased as the pH was reduced in the range 6.4 to 5.2.

The objective of this study was to investigate the effect of pH and Ca content and their interaction on proteolysis in low-fat Mozzarella cheese.

MATERIALS AND METHODS

Cheese Manufacture

The manufacturing procedures for the experimental cheeses have been described in detail by Guinee et al. (2002). Milk was standardized to a casein:fat ratio of 0.95, pasteurized (72°C, 15 s), cooled to 36°C, and divided into four 450-L quantities per vat. The manufacturing procedures for the four cheeses differed with respect to: 1) method of acidification, i.e., a starter culture (15 g/kg) consisting of *Streptococcus thermophilus* and *Lactobacillus helveticus* for the control (CL) cheese; direct acidification by the addition of lactic acid (5 g/

100 ml) to milk at 36°C (DA1 cheese); or direct acidification by the addition of lactic acid to the milk and glucono- δ -lactone (GDL) as a powder mixed with the salt and added to the curd at a level of 1.6 or 3.6 g/100 g curd for the DA2 and DA3 cheeses, respectively. 2) pH of milk at rennet addition (setting), pH of the curd at whey drainage and at milling, i.e., 5.6, 5.6, and 5.6, respectively, for DA1; 6.55, 6.15, and 5.15 for CL and DA3; 6.5, 6.15, and 5.60 for DA2.

The CL cheese was manufactured using a dry-salting procedure as described by Guinee et al. (2000c). The manufacture of the DA cheeses was similar to that of the CL cheese except for the differences noted above. For all treatments, the salted curd was mellowed for 20 min, kneaded, and heated to 58°C in hot water, and molded into rectangular 2.3-kg blocks. The blocks were cooled in dilute brine (10 g of NaCl/100 g, 0.2 g CaCl₂/100 g, pH 5.1) at ~-4°C to a core temperature of < 45°C for 30 min, vacuum-wrapped, and stored at 4°C. Cheesemaking was performed in triplicate.

Cheese Composition

Grated cheese was analyzed in duplicate for fat, protein, moisture, pH and Ca, as described by Guinee et al. (2000 c).

Proteolysis

The level of cheese nitrogen soluble in water at pH 4.6 (pH4.6SN; Kuchroo and Fox, 1982) or in 5% (wt/wt) tungstophosphoric acid (PTAN; Guinee et al., 2000b) was measured.

Urea-polyacrylamide gel electrophoresis (urea-PAGE) was performed on whole cheese samples and the pH 4.6-soluble fraction of the cheeses using a protean Ixi vertical slab gel unit (Bio-Rad Laboratories, Ltd., Watford, UK) and a stacking gel system, as described by Lane and Fox (1997). The gels were stained directly with Coomassie brilliant blue G250, as described by Lane and Fox (1997).

The lyophilized pH4.6SN and the ethanol-soluble fraction thereof were analyzed by reverse-phase HPLC (RP-HPLC). The ethanol-soluble fraction was prepared as follows: Absolute ethanol was added to an aliquot of the pH 4.6-soluble fraction to a final ethanol concentration of 700 ml/L. The mixture was held for 30 min at 20°C and centrifuged at 3000 $\times$ g for 30 min. 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, UK) at 30°C under vacuum; the residue was lyophilized. The lyophilized sample was dissolved in Solvent A [1 ml/L of trifluoroacetic acid

Table 1. Compositional analysis of low-moisture Mozzarella cheese made using starter culture (CL) or direct acidification (DA) with different pH values at setting, whey drainage, and plasticization. Values presented are the means of three replicates.

Composition	CL	Cheese code		
		DA1	DA2	DA3
Moisture (% wt/wt)	48.00 ^b	56.73 ^a	49.35 ^b	50.78 ^b
Fat (% wt/wt)	20.33 ^a	15.94 ^b	16.89 ^b	16.16 ^b
Protein (% wt/wt)	27.00 ^a	23.52 ^b	28.51 ^a	27.61 ^a
MNFS ¹ (% wt/wt)	60.25 ^b	67.43 ^a	59.38 ^b	60.57 ^b
Ca (mg/g protein)	27.7 ^a	21.8 ^b	29.6 ^a	28.7 ^a
pH at 1d	5.42 ^b	5.96 ^a	5.93 ^a	5.58 ^b

^{a,b}Values within a row not sharing a common superscript differed significantly ($P < 0.05$).

¹MNFS = Moisture-in-nonfat substances.

(TFA) in HPLC-grade water] and a sample (40 μ l) was injected onto a Nucleosil C₈ reversed phase column (250 $\times$ 4.6 mm, 5 μ m), equilibrated with solvent A on a Varian Prostar 230 HPLC unit, as described by Lane and Fox (1997). The sample was 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% (vol/vol) solvent B (1 ml/L of TFA in acetonitrile) for 55 min, 50% B (vol/vol) for 6 min, and a linear gradient to 60% B (vol/vol) for 3 min. The column was washed with 95% B (vol/vol) for 5 min, followed by equilibration with 100% A for 5 min before the next injection.

Statistical Analysis

A randomized complete block design, which incorporated the four treatments and three blocks (replicate trials), was used for analysis of the response variables related to the composition of the cheeses (Table 1). A split plot design was used to monitor the effects of treatment, storage time, and their interaction on pH4.6SN and PTAN. Analysis of variance for the split plot design was carried out using a general linear model procedure (SAS, 1995). Statistically significant differences ($P <$

0.05) between different treatment levels were determined by Fisher's least significant difference.

The four cheeses from each of the three trials were analyzed by PAGE after different storage times; similarly, the pH 4.6-soluble extracts and the 75% ethanol soluble extracts were lyophilized and examined by RP-HPLC. The latter results are presented as observations and supportive data but were not statistically analyzed.

RESULTS AND DISCUSSION

Cheese composition

The compositions of the cheeses, which have been described previously (Guinee et al., 2002), are summarized in Table 1. Compared to the other cheeses, DA1 had a significantly higher moisture content and moisture in nonfat substances, and lower concentrations of Ca and protein. The fat content in CL was significantly higher than that of the other cheeses. The pH of the CL and DA3 cheeses at 1 d was significantly lower than that of the DA1 and DA2 cheeses.

Table 2. Mean squares (MS) and probabilities (P) for changes in proteolysis in low-moisture Mozzarella cheeses made using different procedures and with different pH and calcium levels.¹

Factors	pH 4.6-soluble N (% of total N)			5% (wt/wt) Phosphotungstic acid-soluble N (% of total N)		
	df	MS	P	df	MS	P
Main plot						
Make procedure	3	19.4	<0.001	3	0.53	<0.001
Error	6	0.59		6	0.01	
Sub-plot						
Time	6	33.7	<0.001	6	0.03	<0.001
Interaction (temp $\times$ time)	18	0.54	<0.001	18	0.03	<0.001
Error	48	0.17		48	0.02	

¹Differences in make procedure are given in text.

Figure 1. Age-related changes in the concentration of pH 4.6-soluble N (a) and 5% (wt/wt) phosphotungstic acid soluble N (b) in low-moisture Mozzarella cheeses made using conventional starter culture acidification (●) or direct acidification (○, ▲, △). The cheese-making procedure was altered to give cheeses of different pH and Ca content (mg/g protein): 5.42 and 27.7 (control; ●); 5.96 and 21.8 (DA1; ○); 5.93 and 29.6 (DA2; ▲), and 5.58 and 28.7 (DA3; △). Details of make procedures and composition are given in the text. Values presented are the means from three replicate trials.

pH4.6SN and PTAN

The concentration of pH4.6SN as a percentage of total N increased significantly in all cheeses during maturation (Figure 1a), with the level in the CL cheese at 50 d (~6% total N) being typical of that reported previously by other Irish workers (Guinee et al., 1998; Walsh et al., 1998; Guinee et al., 2000c; Feeney et al., 2001) and somewhat higher than the mean level (4.7% total N) found in retail and wholesale samples of Mozzarella in

Ireland, UK, and Denmark (Guinee et al., 2000b). In contrast, the level of pH4.6SN in the CL cheese was lower than typical values (~8 to 10% total N at 50 d) reported for Mozzarella in studies from the United States (Yun et al., 1993a, 1993b; Kindstedt et al., 1995; Renda et al., 1997). The comparatively low level of pH4.6SN in the CL cheese compared with that in Mozzarella cheeses in the latter studies may be attributed to the higher pH of the CL curd during plasticization and subsequent ripening (e.g., 5.45 vs. typical values of 5.1 to 5.2 at 1 d; Guinee et al., 2002), and the higher temperature used for plasticization of CL (58°C vs. 55°C in some studies in the United States). A higher pH would result in greater inactivation of rennet during heating and plasticization of the curd (Thunell et al., 1979) and would be less favorable for the proteolysis in the curd by residual rennet during storage (Tam and Whitaker, 1972; Mulvihill and Fox, 1980).

The mean concentration of pH4.6SN was significantly affected by the interaction of make procedure and storage time (Table 2). At most analysis times, the mean level of pH4.6SN in the CL and DA1 cheeses was similar and significantly higher than that in the DA2 and DA3 cheeses. The higher level of proteolysis in the DA1 cheese, compared with the DA2 and DA3 cheeses, may be attributed to a number of factors: (1) the higher content of moisture-in-nonfat substance, which favors proteolysis (Creamer, 1971); (2) increased susceptibility of casein to proteolysis by residual rennet due to the lower calcium content while the curd is in contact with the whey and during subsequent storage (Fox, 1970; O'Keeffe et al., 1975); and (3) an expected greater retention of rennet in the cheese due to the lower pH at setting and at whey drainage (Creamer et al., 1985). The higher level of proteolysis in the CL cheese compared to DA2, despite the similar value for pH at setting and at draining, may be related to its lower final pH, which should increase the stability of retained rennet (Thunell et al., 1979) and proteolysis by rennet (Tam and Whitaker, 1972; Mulvihill and Fox, 1977, 1980). The greater proteolysis in the CL cheese compared to DA3 may be due to its slightly lower pH and to the use of a starter culture, which makes a relatively small (compared to chymosin) but significant contribution to the formation of pH4.6SN in Cheddar (O'Keeffe et al., 1978) and Gouda (Visser, 1977) cheeses.

The level of secondary proteolysis in the CL cheese was significantly higher than in the DA cheeses, as reflected by its significantly higher level of PTAN (Figure 1b), which is comprised of amino acids and small peptides with a molecular mass <1.5 kDa (Jarrett et al., 1982). The higher concentrations of PTAN in the CL cheese is attributed to the activity of starter proteinases

Figure 2. Urea-PAGE of sodium caseinate (C) and Mozzarella cheese made using conventional starter culture acidification (pH 5.42, Ca 27.7 mg/g of protein) (control; lanes, 1, 5, 9, 13 and 17) or direct acidification to give cheeses with pH and Ca levels (mg/g protein) of 5.96 and 21.8 (DA1; lanes 2, 6, 10, 14, and 18); 5.93 and 29.6 (DA2; lanes, 3, 7, 11, 15, and 19), or 5.58 and 28.7 (DA3; 4, 8, 12, 16, and 20) after ripening for 1, 12, 21, 46, and 70 d at 4°C. Details of make procedures and composition are given in the text.

and peptidases (Visser, 1977; O'Keeffe et al., 1978), which were not present in the DA cheeses.

Urea-PAGE

Cheese. Urea-PAGE gel electrophoretograms of the different cheeses are shown in Figure 2. The overall degradation pattern of the control cheese was similar to that reported elsewhere for low-moisture Mozzarella (Creamer, 1976; Kindstedt and Guo, 1997). In general, the trend observed with urea-PAGE was consistent with those for pH4.6SN, with the overall level of proteolysis decreasing in the order: CL > DA1 > DA3 >> DA2.

Storage resulted in a decrease in the concentration of α_{s1} -CN in all cheeses and a concomitant increase in the concentration of α_{s1} -CN (f24–199) and its degradation product, α_{s1} -CN (f102–199). The intensity of α_{s1} -CN (f24–199) at 1 d was highest for the DA1 cheese,

which could be due to its higher moisture content and lower calcium-to-casein ratio. It is noteworthy that a reduction in the level of micellar calcium phosphate in milk at pH 6.6 results in greater rennet-induced proteolysis, an effect attributed to the disruption of the casein micelle into subunits, which allows easier access of rennet to the caseins (Fox, 1970). A higher level of residual chymosin activity in the curd, due to the lower pH at whey drainage, may possibly contribute also to the higher intensity of α_{s1} -CN (f24–199) at 1 d in DA1 (Creamer et al., 1985). The concentration of α_{s1} -CN (f24–199) at 12 to 46 d was highest in the CL and DA1 cheeses, and at 70 d was similar in the CL, DA1, and DA3 cheeses. Compared with the other cheeses, there was very little degradation of α_{s1} -CN in the DA2 cheese over the 70-d storage period. The low level of degradation in the DA2 cheese compared with the CL and DA3 cheeses, despite the similar make procedures, gross composition (apart from level of fat), and Ca content, may be attributed to its relatively high pH, which induces a high ratio of colloidal-to-soluble Ca (Guinee et al., 2000b) and a low degree of proteolysis by rennet (Fox, 1970), a higher degree of casein aggregation, and a lower degree of casein hydration (Sood et al., 1979; Creamer, 1985). It is noteworthy that in the presence of 5%, wt/vol, salt-in-moisture, the degradation of α_{s1} -CN (2%, wt/vol) by chymosin decreased as the pH was increased from 5.2 to 5.8 (Mulvihill and Fox, 1980); it is probable that a similar effect occurred at the low salt-in-moisture concentrations (~3.0 to 3.5% w/w) in the cheeses in the current study.

β -Casein was degraded in all cheeses during storage, to an extent depending on Ca level and pH. At most analysis times, the overall level of proteolysis decreased in the order: DA1 > DA2 >> CL > DA3. Degradation of β -CN in the DA1 cheese coincided with the formation of γ -CNs, the concentration of which increased during storage, suggesting that β -CN was degraded primarily by the indigenous milk plasmin, which has a high specificity for β -CN. The high level of β -CN degradation in the DA1 and DA2 cheeses may be attributed to their relatively high pH, which would favor the activity of plasmin, which is optimally active at pH ~7.5 (McSweeney and Sousa, 2000). The high level of β -CN breakdown observed in DA2 also concurs with the observations of Creamer (1976), who noted that the level of β -CN degradation in Gouda cheese, which has a higher moisture content and pH than Cheddar, was substantially higher than that in Cheddar.

There was also an increase in the concentration of β -CN (f1–192), which is formed by chymosin during storage, especially in DA1, due perhaps to the low drain pH (5.6), which would result in a high level of chymosin in the curd (Creamer et al., 1985).

Figure 3. Urea-PAGE of sodium caseinate (C) and the pH 4.6-soluble fraction of Mozzarella cheese made using conventional starter culture acidification (pH 5.42; Ca 27.7 mg/g protein; control; lanes, 1, 5, 9, 13, and 17) or direct acidification to give cheeses with pH and Ca levels (mg/g protein) of 5.96 and 21.8 (DA1; lanes 2, 6, 10, 14, and 18); 5.93 and 29.6 (DA2; lanes 3, 7, 11, 15, and 19), or 5.58 and 28.7 (DA3; 4, 8, 12, 16, and 20) after ripening for 1, 12, 21, 46, and 70 d at 4°C. Details of make procedures and composition are given in the text.

pH4.6-soluble cheese fractions (pH4.6SF). The urea-PAGE electrophoretograms of the pH4.6SF showed notable differences in the banding pattern between the CL and the DA cheeses (Figure 3). The pH4.6SF of the DA cheeses had a number of bands of high intensity, which were scarcely evident in the pH4.6SF of CL, especially after storage for ≥ 46 d. These bands may correspond to large peptides which were degraded in CL to small peptides and amino acids by the action of starter cell proteinases and peptidases and which were, therefore, undetected by urea-PAGE. This trend is consistent with the significantly higher level of PTAN in the CL cheese (Figure 1a).

Figure 4. Reversed-phase (C_8) HPLC profiles of the pH 4.6-soluble fraction of Mozzarella cheese made using conventional starter culture acidification (pH 5.42; Ca 27.7 mg/g protein; control, a, b, c) or direct acidification to give cheeses with pH and Ca levels (mg/g protein) of 5.96 and 21.8 (DA1; d, e, f); 5.93 and 29.6 (DA2; g, h, i), or 5.58 and 28.7 (DA3; j, k, l) after ripening for 1 (a, d, g, j), 21 (b, e, h, k), or 70 d [c, f, i, l (days)] at 4°C. Details of make procedures and composition are given in the text.

RP-HPLC

pH 4.6-soluble fractions. RP-HPLC profiles of the lyophilized pH4.6SF of the cheeses at 1, 21, and 70 d are shown in Figure 4; for convenience, the chromatograms were divided into zones I, II, III, and IV, each of which contained one or more peaks. Similar to previous findings for Cheddar cheeses (Altemueller and Rosenberg, 1996; Lane and Fox, 1997), the chromatograms for all cheeses showed a large number of peaks, indicating a heterogeneous mixture of proteolysis products. In general, the number of peaks remained relatively constant for all cheeses during storage, but there were age-related changes in the area and distribution of different peaks, indicating a transition in the hydrophobicity of peptides. This trend is consistent with the increase in pH4.6SN in all cheeses and suggests the progressive breakdown of casein by residual coagulant, plasmin, and microbial proteinase, and the resultant formation of peptides of different molecular mass and free amino

acids. The total peak area, and in particular that of the late eluting peaks, increased during storage.

At all stages of storage, the RP-HPLC profile for the pH4.6SF of CL differed markedly from those of the DA cheeses. The pH4.6SF of CL cheese had a greater number and wider distribution of peaks, including the early-eluting peaks (retention time <30 min), denoted collectively as zone I, and which probably correspond to small peptides and free amino acids (Lemieux and Simard, 1992; Altemueller and Rosenberg, 1996). In agreement with the trend noted for PTAN, which is comprised mainly of amino acids and small peptides with a molecular mass <1.5 kDa (Jarrett et al., 1982), the total area of peaks in zone I increased during storage in the CL cheese but scarcely changed in the DA cheeses. In contrast, the total area of late-eluting peaks (zones II, III, and IV) was generally greater in the DA than in the CL cheese at all analysis times, especially at 46 and 70 d. Therefore, the results suggest a higher concentration of more hydrophobic peptides in the pH4.6SF of DA cheeses compared to the CL cheese. Peptides rich in hydrophobic amino acid residues have higher retention times on RP-HPLC columns than more hydrophilic peptides (Cliffe and Law, 1990). The larger area of late-eluting peaks in the DA cheeses may reflect the accumulation of rennet-produced peptides, which are not degraded further to peptides of lower hydrophobicity and molecular mass, due to the absence of starter culture peptidases (Lowrie and Lawrence, 1972; Lemieux and Simard, 1992).

Comparison of the pH4.6SF of the different DA cheeses indicated that the areas of peaks in zones II and III were generally lower for the DA2 cheese than for the DA1 and DA3 cheeses. In contrast, the areas of peaks in zone IV were larger for the DA2 cheese than either the DA1 or DA3 cheeses. The differences in the RP-HPLC profiles between the DA2 and other DA cheeses probably reflect the effects of moisture content, pH, and Ca level on proteolysis; the DA2 cheeses had lower moisture and a higher Ca content than the DA1 cheese and had a higher pH than the DA3 cheese. These differences in composition between the DA2 and the other DA cheeses would be conducive to a lower activity by residual rennet in the former (Fox, 1970; Tam and Whitaker, 1972; Mulvihill and Fox, 1980).

Ethanol soluble fraction. The lyophilized 70% ethanol-soluble fractions (ESF) were analyzed by RP-HPLC. The chromatograms of the ESFs from 1-, 21- and 70-d-old cheeses indicated a heterogeneous mixture of peptides (Figure 5). Similar to the trend noted for the RP-HPLC profiles of the pH4.6SF, the total area and number of early-eluting peaks in zone I was higher for the CL cheese than for the DA cheeses, while the total area of the peaks in zones II, III, and IV was

markedly higher for the DA cheeses. The relatively large areas of early-eluting peaks indicate a higher proportion of free amino acids in the ESF of the CL cheese, a trend which correlates with its higher concentration of PTAN. The latter trend was expected since the percentage of total N soluble in 70% ethanol or 5% PTA tends to be very similar for different cheeses, including Cheddar of different ages (Kuchroo and Fox, 1982) and Romano-type cheese (Fox and Guinee, 1987). The absence of the early-eluting peaks in the RP-HPLC chromatograms of the ESF of the DA cheeses also agrees with the trend noted for PTAN, the concentration of which remained essentially unchanged at a very low level in DA cheeses during maturation.

CONCLUSIONS

Low-moisture Mozzarella cheeses (LMMC), differing in Ca content and pH, were made using a starter culture (CL) or direct acidification with lactic acid (DA1) or lactic acid and GDL (DA2, DA3). The pH and Ca level significantly affected the moisture content and the type and extent of proteolysis in Mozzarella cheese during the 70-d storage period at 4°C. For cheeses with a similar pH (i.e., DA1 and DA2), reducing the calcium-to-casein ratio from ~29 to 22 mg/g of protein resulted in marked increases in moisture content and in primary and secondary proteolysis, as evidenced by the higher levels of pH4.6SN, PTAN, and degradation of α_{s1} - and β -CN. This trend concurs with the observations of Fox (1970), who found that reducing the calcium-to-casein ratio in milk resulted in a higher degree of rennet-induced proteolysis of casein, an effect attributed to the lower degree of casein aggregation (or higher degree of casein hydration). Increasing the pH from ~5.5 (as in DA3) to 5.9 (as in DA2), while maintaining the calcium-to-casein ratio relatively constant at ~29 mg/g resulted in a decrease in primary proteolysis but had no effect on secondary proteolysis. However, the extent of β -casein degradation increased upon raising the pH, probably due to plasmin activity.

Comparison of Mozzarella cheeses with a similar composition but made using a starter culture or direct acidification (i.e., CL and DA3) showed that the CL cheese had higher levels of pH4.6SN and greater degradation of α_{s1} -CN (especially at storage for ≥ 46 d). The level of secondary proteolysis in the CL cheese, as monitored by PTAN and RP-HPLC of the 70% ethanol-soluble fraction, was significantly higher than that in the DA cheeses, suggesting that the starter culture is the main agent responsible for the formation of small peptides and amino acids in low-moisture Mozzarella cheese.

Figure 5. Reversed-phase (C_8) HPLC profiles of the ethanol-soluble fraction of Mozzarella cheese made using conventional starter culture acidification (pH 5.42; Ca 27.7 mg/g of protein; control; a, b, c), or direct acidification to give cheeses with pH and Ca levels (mg/g of protein) of 5.96 and 21.8 (DA1; d, e, f); 5.93 and 29.6 (DA2; g, h, i), and 5.58 and 28.7 (DA3; j, k, l) after ripening for 1 (a, d, g, j), 21 (b, e, h, k), or 70 d [c, f, i, l (days)] at 4°C. Details of make procedures and composition are given in the text.

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