J. Dairy Sci. 88:3101–3114


# Chymosin-Mediated Proteolysis, Calcium Solubilization, and Texture Development During the Ripening of Cheddar Cheese

**J. A. O'Mahony,[1,2] J. A. Lucey,[2] and P. L. H. McSweeney[1]**
[1]Department of Food and Nutritional Sciences, University College, Cork, Ireland
[2]Department of Food Science, University of Wisconsin-Madison, 1605 Linden Drive, Madison 53706

## ABSTRACT

Full fat, milled-curd Cheddar cheeses (2 kg) were manufactured with 0.0 (control), 0.1, 1.0, or 10.0 $\mu$mol of pepstatin (a potent competitive inhibitor of chymosin) added per liter of curds/whey mixture at the start of cooking to obtain residual chymosin levels that were 100, 89, 55, and 16% of the activity in the control cheese, respectively. The cheeses were ripened at 8°C for 180 d. There were no significant differences in the pH values of the cheeses; however, the moisture content of the cheeses decreased with increasing level of pepstatin addition. The levels of pH 4.6-soluble nitrogen in the 3 cheeses with added pepstatin were significantly lower than that of the control cheese at 1 d and throughout ripening. Densitometric analysis of urea-PAGE electrophoretograms of the pH 4.6-insoluble fractions of the cheese made with 10.0 $\mu$mol/L of pepstatin showed complete inhibition of hydrolysis of $\alpha_{S1}$-casein (CN) at $Phe_{23}$-$Phe_{24}$ at all stages of ripening. The level of insoluble calcium in each of 4 cheeses decreased significantly during the first 21 d of ripening, irrespective of the level of pepstatin addition. Concurrently, there was a significant reduction in hardness in each of the 4 cheeses during the first 21 d of ripening. The softening of texture was more highly correlated with the level of insoluble calcium than with the level of intact $\alpha_{S1}$-CN in each of the 4 cheeses early in ripening. It is concluded that hydrolysis of $\alpha_{S1}$-CN at $Phe_{23}$-$Phe_{24}$ is not a prerequisite for softening of Cheddar cheese during the early stages of ripening. We propose that this softening of texture is principally due to the partial solubilization of colloidal calcium phosphate associated with the *para*-CN matrix of the curd.
(**Key words:** proteolysis, texture, Cheddar cheese, insoluble calcium)

**Abbreviation key: CCP** = colloidal calcium phosphate, **FAA** = free amino acids, **LAB** = lactic acid bacteria, **NSLAB** = nonstarter lactic acid bacteria, **PC** = principal component, **RP** = reversed phase, **SN** = soluble nitrogen, **TPA** = texture profile analysis.

## INTRODUCTION

The ripening of Cheddar cheese involves a concerted series of microbiological, biochemical, and physicochemical changes that are collectively responsible for the development of its characteristic texture, flavor, and aroma (Fox, 1993; McSweeney, 2004). Cheddar cheese ripening is widely considered an enzymatic process (Fox and Law, 1991), being mediated, to varying extents, by each of the following agents: residual coagulant (usualy chymosin in the case of Cheddar cheese), indigenous milk enzymes, starter bacteria and their enzymes, nonstarter lactic acid bacteria (**NSLAB**) and their enzymes, secondary microorganisms and their enzymes and, in some cases, exogenous enzymes (Fox et al., 2000). Texture is one of the primary quality attributes of Cheddar cheese, with its development being inextricably linked with the biochemical and physicochemical changes that occur during ripening (see Lucey et al., 2003). To date, the exact mechanisms responsible for texture development in Cheddar cheese during ripening have not been elucidated fully.

The development of Cheddar cheese texture during ripening has been thought to depend upon the extent of proteolysis and classically has been divided into 2 phases (Lawrence et al., 1987, 2004). The primary phase occurs during the first 2 to 4 wk and involves weakening of the *para*-casein network of the curd, and ultimately results in softening of texture. The secondary phase occurs for the duration of ripening and involves more gradual changes in texture due to change in pH (if there is a change) and continued proteolysis (Lawrence et al., 1987, 2004; Guinee, 2003). One of the more salient effects of proteolysis on cheese texture is that hydrolysis of peptide bonds releases 2 new charged groups ($NH_3^+$/$COO^-$) that compete for water, reducing the "free" water content of maturing Cheddar cheese curd (Creamer and Olson, 1982; Lawrence et al., 1987, 2004; Irudayaraj et al., 1999; Guinee, 2003; Lucey et al., 2003).

Received February 3, 2005.
Accepted April 30, 2005.
Corresponding author: Paul McSweeney; e-mail: p.mcsweeney@ucc.ie.

More than 20 yr ago, it was hypothesized that chymosin-mediated cleavage of the $Phe_{23}$-$Phe_{24}$ peptide bond of $\alpha_{S1}$-CN was responsible for the significant softening (decreased elasticity, hardness, and force at yield point) observed in Cheddar cheese texture during the early stages of ripening (Creamer and Olson, 1982). Both the N- and C-terminal regions of the $\alpha_{S1}$-CN molecule are strongly hydrophobic (Horne, 1998; De Kruif and Holt, 2003); thus, hydrolysis of $Phe_{23}$-$Phe_{24}$ would be expected to decrease the surface hydrophobicity of the $\alpha_{S1}$-CN molecule by removal of the hydrophobic peptide $\alpha_{S1}$-CN (f1-23). Creamer et al. (1982) suggested that the loss of a hydrophobic interaction site on the $\alpha_{S1}$-CN molecule between residues 14 and 24, caused by cleavage at $Phe_{23}$-$Phe_{24}$, might be responsible for the initial softening of Cheddar cheese texture. Such hydrolysis of $\alpha_{S1}$-CN yields 2 peptides; $\alpha_{S1}$-CN (f1-23) and $\alpha_{S1}$-CN (f24-199) (Carles and Ribadeau-Dumas, 1985; McSweeney et al., 1993). The former peptide is rapidly hydrolyzed by proteinases of the starter microorganisms (Visser, 1993), whereas the latter peptide undergoes further hydrolysis, initially at $Leu_{101}$-$Lys_{102}$ (McSweeney et al., 1993).

Applying the model proposed by Horne (1998) for CN micelle structure to cheese, it appears that the *para*-CN matrix of Cheddar cheese is stabilized by the combined effects of hydrophobic interactions between groups on different CN molecules and chain crosslinking mediated by colloidal calcium phosphate (**CCP**) nanoclusters (Horne, 1998; Lucey et al., 2003). The $\alpha_{S1}$-CN molecule has 8/9 phosphoserine residues with 2 phosphate centers at residues 41-51 and 61-70 (Davies and Law, 1977; De Kruif and Holt, 2003); thus, the peptide $\alpha_{S1}$-CN (f1-23) is devoid of potential CCP crosslinking sites. Consequently, the interactions (primarily hydrophobic in nature) responsible for association of the N-terminal region of $\alpha_{S1}$-CN with the *para*-CN matrix of the curd may not be sufficiently strong to retain the associations when $\alpha_{S1}$-CN is hydrolyzed at $Phe_{23}$-$Phe_{24}$. Evidence of this may be drawn from the rapid rate with which lactococcal proteinases are capable of hydrolyzing $\alpha_{S1}$-CN (f1-23) (once liberated) in cheese, with the accumulation of hydrolysis products $\alpha_{S1}$-CN (f1-9) and $\alpha_{S1}$-CN (f1-13) in water-soluble extracts of Cheddar cheese (Singh et al., 1994).

Calcium associated with the CN particles (i.e., CCP) is considered an important structural component in Cheddar cheese (Lucey and Fox, 1993) and any reduction in the concentration of this form of calcium would be expected to alter cheese texture (Lucey et al., 2003, 2005). It has recently been reported that there is an appreciable reduction in the amount of calcium associated with the CN particles of Cheddar cheese during the early stages of ripening (Hassan et al., 2004).

We propose that hydrolysis of $\alpha_{S1}$-CN at $Phe_{23}$-$Phe_{24}$ by chymosin and the softening of Cheddar cheese texture early in ripening are concurrent, rather than interdependent, processes. We believe that some physicochemical change, such as a reduction in the amount of calcium associated with CN particles, may be responsible for this initial softening. To investigate this hypothesis, full fat, milled-curd Cheddar cheeses were manufactured with 0.1, 1.0, or 10.0 $\mu$mol of pepstatin (a potent competitive inhibitor of chymosin) added per liter of curds/whey mixture at the start of cooking to inhibit hydrolysis of $\alpha_{S1}$-CN at $Phe_{23}$-$Phe_{24}$. Proteolysis and changes in the type of calcium (conversion of insoluble to soluble form) in these cheeses, and their textural properties were then determined to evaluate if texture changes would still occur when chymosin-mediated hydrolysis of $\alpha_{S1}$-CN was completely inhibited.

## MATERIALS AND METHODS

### Cheese Manufacture

Whole milk (3.5% fat; CN:fat = 0.70:1.0) was pasteurized (72°C for 15 s), cooled to 4°C, and stored at 4°C for 18 h before cheese making. Cheddar cheeses were manufactured according to a standard protocol (Fox et al., 2000) on a 20-L pilot-scale in the food processing facilities at University College, Cork, Ireland. *Lactococcus lactis* spp. *lactis* UC317, obtained from the Microbiology Department, University College, Cork and grown in sterile, reconstituted (10% wt/vol) skim milk, was used as the starter culture at a level of 2% (vol/vol). Chymosin (Maxiren-180, DSM Food Specialties, Delft, Holland) at 180 IMCU/mL was added to the cheesemilk at a level of 0.3 mL/L. Pepstatin (Peptide Institute, Osaka, Japan) was added at 3 levels (0.1, 1.0, or 10.0 $\mu$mol/L) to the curds/whey mixture at the start of cooking and evenly distributed by continuous stirring during cooking. The whey was drained at pH 6.2 approximately 45 min after pepstatin addition. After pressing at 150 kPa for 18 h, the cheeses were vacuum packaged and ripened at 8°C for 180 d.

### Chemical Composition

The composition and pH of Cheddar cheeses made with 0.0 (control), 0.1, 1.0, or 10.0 $\mu$mol/L of pepstatin was determined 21 d postmanufacture as described by O'Mahony et al. (2003); moisture was determined by oven drying at 102°C (IDF, 1982), protein by macro-Kjeldahl (IDF, 1986), fat by Gerber (IIRS, 1955), and salt by potentiometric titration (Fox, 1963). The total calcium content of the cheeses was determined using atomic absorption spectroscopy as described by IDF (2003). All analyses were conducted in triplicate.

### Determination of Residual Chymosin Activity

The level of residual chymosin activity in the cheeses was determined using a synthetic heptapeptide as substrate (Pro-Thr-Glu-Phe-[$NO_2$-Phe]-Arg-Leu) as described by Hurley et al. (1999), with one minor modification. The time allowed for incubation of substrate with a citrate dispersion of the cheese was increased from 4 to 24 h to facilitate accurate quantification of the product peptide ([$NO_2$-Phe]-Arg-Leu). The area of the peak corresponding to the product peptide was used to express the residual chymosin activity of the pepstatin-treated cheeses as a percentage of that in the control cheese (0.0 $\mu$mol/L of pepstatin). The stability of the chymosin-pepstatin complex in the experimental cheeses during ripening was evaluated by determining the residual chymosin activity in the control and pepstatin-treated cheeses at 1, 21, 42, 90, 120, and 180 d of ripening. All analyses were conducted in triplicate.

### Proteolysis

The pH 4.6-soluble and -insoluble fractions of the cheeses were prepared by the method of Kuchroo and Fox (1982), as modified slightly by Sousa and McSweeney (2001). The N content of the pH 4.6-soluble fraction was determined by the macro-Kjeldahl method (IDF, 1986). Ethanol (70%)-soluble subfractions of the pH 4.6-soluble extracts were prepared according to the method described by Sousa and McSweeney (2001). Urea-PAGE (12.5% T, 4% C, pH 8.9) of the pH 4.6-insoluble fractions of the cheeses was performed using the procedure of Andrews (1983), as modified by Shalabi and Fox (1987). The gels were stained directly with Coomassie Brilliant Blue G250, as described by Blakesley and Boezi (1977), destained in several changes of distilled water, and scanned on a flatbed scanner (Scanjet 6300C, Hewlett Packard, Singapore). Densitometric analysis was performed on the scanned image using gel analysis software (TotalLab 1D, Nonlinear Dynamix, Newcastle upon Tyne, UK). Peptide profiles of the ethanol-soluble fractions of each of the cheeses were determined by reversed-phase (**RP**) HPLC according to the method described by Sousa and McSweeney (2001). Total free amino acids (**FAA**) were determined by the trinitrobenzenesulfonic acid assay (Polychroniadou, 1988). Individual FAA were determined using the method described by Fenelon et al. (2000).

### Determination of the Proportions of Soluble and Insoluble Calcium

The proportion of total calcium in the insoluble form (i.e., CCP) in Cheddar cheese made with 0.0 (control), 0.1, 1.0 or 10.0 $\mu$mol/L pepstatin was determined at 1, 21, 42, 90, 120, and 180 d of ripening using the acid-base titration method described by Hassan et al. (2004).

### Measurement of Texture Properties

Texture profile analysis (**TPA**) of the cheeses was performed using a TA-XT2i texture analyzer (Stable Micro Systems, Godalming, Surrey, UK). Two cylindrical cores (height ~60 to 70 mm, diameter 20 mm), obtained from each cheese using a stainless steel borer, were placed in airtight plastic bags and equilibrated at 8°C for 18 h. Three cylindrical samples (height 10 mm, diameter 20 mm) were cut from each of the 2 original cheese cylinders using a stainless steel wire cutter and equilibrated at 8°C for a further 30 min before analysis. Samples were removed from the incubator and immediately compressed to 25% of the original height in 2 consecutive cycles (i.e., double compression) at a rate of 1 mm/s. Hardness was defined as the force required to compress the cheese sample to 25% of its original height during the first compression cycle. Cohesiveness was defined as the ratio of the area under the positive region (during application of force) of the second compression curve to that of the first compression curve. Springiness was defined as the ratio of the time taken to compress the sample to 25% of its original height during the second compression cycle to that of the first compression cycle. Chewiness was defined as the product of gumminess and springiness, where gumminess is defined as the product of hardness and cohesiveness (Bourne, 1978).

### Statistical Analyses

One-way ANOVA of data for the composition, levels of pH 4.6-soluble nitrogen (**SN**), total FAA, insoluble calcium, and TPA analysis of the cheeses was conducted using SPSS Version 11.0 for Windows XP (SPSS Inc., Chicago, IL). Pearson's correlation coefficients were determined between the response variables (i.e., intact $\alpha_{S1}$-CN, intact $\beta$-CN, levels of pH 4.6-SN, total FAA, insoluble calcium, and hardness) using SAS, version 8.02 (SAS Institute, 1999). To illustrate the trends in selected response variables measured over time (i.e., levels of pH 4.6-SN, total FAA, and hardness) for each of the 3 trials, the experimental design was a split plot with 3 replicates for each variable except for hardness, which had 5 replicates. The main plot factor was treatment (i.e., level of pepstatin addition) and the subplot factor was ripening time. The data for each trial were analyzed separately. The ANOVA for the split-plot design was performed using a GLM procedure of SAS. The data from RP-HPLC chromatographic analysis of the ethanol-soluble fractions of the cheeses were ana-

**Table 1.** Residual chymosin activity (expressed as a % of that in the control cheese) for Cheddar cheeses made with 0.0 (control), 0.1, 1.0, or 10.0 μmol/L of added pepstatin at 21 d of ripening (data from trials 1, 2, and 3).

| Trial | Pepstatin added (μmol/L) | Product peptide peak area | | Residual chymosin activity (% of control) |
|---|---|---|---|---|
| | | Mean | SD[1] | |
| 1 | Control (0.0) | 116,971 | 3802 | 100[a] |
| | 0.10 | 104,231 | 11,345 | 89[a] |
| | 1.00 | 64,451 | 4389 | 55[b] |
| | 10.0 | 19,065 | 382 | 16[c] |
| 2 | Control (0.0) | 161,393 | 7171 | 100[a] |
| | 0.10 | 129,650 | 5420 | 80[b] |
| | 1.00 | 104,580 | 10,367 | 65[c] |
| | 10.0 | 21,147 | 2921 | 13[d] |
| 3 | Control (0.0) | 163,308 | 2796 | 100[a] |
| | 0.10 | 128,566 | 7123 | 79[b] |
| | 1.00 | 77,616 | 4895 | 48[c] |
| | 10.0 | 20,206 | 1311 | 12[d] |

[a,b,c,d]Means within the residual chymosin activity column with different superscripts are significantly different (Tukey's HSD; $P \leq 0.05$).

[1]SD = Standard deviation (n = 3).

lyzed using multivariate statistical techniques to evaluate the effect of pepstatin addition on liberation of peptides during ripening. The variables (peak height data) were preprocessed according to the method of Piraino et al. (2004). The output from this preprocessing consisted of classes of retention time within which peak heights were accumulated using the distance from center of class as a weight. Principal component (**PC**) analysis and hierarchical cluster analysis were then performed on the data using a covariance matrix and the between-groups linkage cluster method, respectively, using SPSS. When treatment effects were significant ($P \leq 0.05$), the differences between means were analyzed using Tukey's HSD posthoc test.

## RESULTS AND DISCUSSION

### Residual Chymosin Activity and Stability of Chymosin-Pepstatin Complex

Reversed phase-HPLC analysis of citrate dispersions of cheeses incubated with the synthetic heptapeptide substrate showed that residual chymosin activity decreased significantly ($P \leq 0.05$) as the level of pepstatin addition increased in each of the 3 trials (Table 1). It is clear from Table 1 that there was some variation between trials in the extent of inactivation of chymosin achieved in the cheeses with added pepstatin, which was most likely due to differences in the dispersion or diffusion of pepstatin into the curd particles before whey drainage on different days of manufacture. For this reason, unless otherwise stated, the data presented in each of the following tables and figures pertain to trial 1 only. Cheddar cheeses made with 0.1, 1.0, or 10.0 μmol/L of pepstatin in trial 1 had levels of residual chymosin activity that were 89, 55, and 16% of the activity in the control cheese, respectively, at 21 d of ripening (Table 1). The ANOVA results (i.e., mean squares and probability values) for the split-plot experimental design confirmed that the trends observed for the response variables were identical in each of the 3 trials (data not shown). Pepstatin forms a 1:1 complex with pepsin by binding to the active site of the enzyme (McKown et al., 1974); presumably the same mechanism is true for the pepstatin-mediated inhibition of chymosin. Inhibition of aspartyl proteinases by pepstatin involves 2 distinct phases: (1) rapid formation of an enzyme-inhibitor complex (collision complex) followed by (2) slow transformation of the collision complex to a more tightly bound complex (tightened complex) (Rich and Sun, 1980). The formation of the tightened complex is reversible with both formation and dissociation reactions obeying first-order kinetics (Rich and Sun, 1980). In this study, by measuring residual chymosin activity in the control and pepstatin-treated cheeses at 1, 21, 42, 90, 120, and 180 d of ripening, it was shown that the chymosin-pepstatin complex was extremely stable during ripening, at all levels of pepstatin addition to the curds/whey mixture (data not shown).

### Cheese Composition

The composition and pH of Cheddar cheeses made with 0.0 (control), 0.1, 1.0, or 10.0 μmol/L of pepstatin are shown in Table 2. The values for pH, moisture, protein, and salt contents were in the ranges 5.20 to 5.27, 37.8 to 39.5%, 21.1 to 22.6%, and 1.13 to 1.31%, respectively. The salt content of each of the 4 cheeses

**Table 2.** Composition and pH of Cheddar cheeses made with 0.0 (control), 0.1, 1.0, or 10.0 μmol/L added pepstatin at 21 d of ripening.

| | Pepstatin addition (μmol/L) | | | |
|---|---|---|---|---|
| Parameter | 0.0 | 0.1 | 1.0 | 10.0 |
| pH | 5.20[a] | 5.22[a] | 5.22[a] | 5.27[a] |
| Moisture (%) | 39.5[a] | 38.5[ab] | 38.4[b] | 37.8[b] |
| Protein (%) | 21.1[a] | 22.4[b] | 22.1[ab] | 22.6[b] |
| Fat (%) | 30.7[a] | 30.3[ab] | 29.2[b] | 29.3[b] |
| Salt (%) | 1.13[a] | 1.31[b] | 1.26[b] | 1.26[b] |
| S/M[1] (%) | 2.85[a] | 3.40[b] | 3.27[b] | 3.33[b] |
| F/DM[2] (%) | 50.7[a] | 49.4[ab] | 47.3[b] | 47.1[b] |
| Ash (%) | 3.86[a] | 3.78[ab] | 3.65[b] | 3.82[a] |
| Calcium (mg/100g) | 852[ab] | 811[a] | 812[a] | 862[b] |

[a,b]Means within a row with different superscripts are significantly different (Tukey's HSD; $P \leq 0.05$).

[1]S/M = Salt in moisture.

[2]F/DM = Fat in dry matter.

was slightly lower than that typical of Cheddar cheese (Fox et al., 2000); however, there were no significant ($P > 0.05$) differences in the salt content between any of the cheeses made with added pepstatin (0.1, 1.0, or 10.0 μmol/L). Interestingly, the moisture content of the cheeses decreased with increasing level of pepstatin addition. The moisture content of the cheeses made with 1.0 and 10.0 μmol/L of pepstatin was significantly ($P \leq 0.05$) lower than that of the control cheese. Shakeel-Ur-Rehman et al. (1998) manufactured miniature Cheddar cheeses with 0.0, 7.5, 15.0, or 30.0 μmol/L of pepstatin and the results showed no gross differences between the moisture content of any of the cheeses. The lower moisture content of the cheeses manufactured with added pepstatin was possibly due to the enhanced syneresis properties of Cheddar cheese curd made with added pepstatin. Syneresis involves contraction of the *para*-CN matrix of the gel/curd (due to protein rearrangement), with concomitant expulsion of whey (Walstra et al., 1987). The proteolytic action of chymosin on the caseins (i.e., in control cheese) may have reduced the ability of the *para*-CN matrix of the gel/curd to contract and thus retain more moisture than the pepstatin-treated cheeses. As a consequence of the decrease in moisture, the levels of fat-in-dry-matter decreased with increasing level of pepstatin addition. The ash and calcium levels ranged from 3.65 to 3.86% and 811 to 862 mg/100 g of cheese, respectively, and were typical of Cheddar cheese (Fox et al., 2000; Hassan et al., 2004). The salt-in-moisture content of each of the 4 cheeses was slightly lower than that typical of Cheddar cheese (Fox et al., 2000); however, there were no significant ($P > 0.05$) differences in the salt-in-moisture content between any of the cheeses made with added pepstatin. There were no significant ($P > 0.05$) changes in pH during ripening for any of the 4 cheeses (not shown).

## Proteolysis

***pH 4.6-SN as a percentage of total nitrogen.*** The level of pH4.6-SN/total N (**TN**) in each of the 3 cheeses made with added pepstatin was significantly ($P \leq 0.05$) lower than that of the control cheese at 1 d of ripening (Table 3). It appears that extensive inactivation of chymosin, after completion of the rennet coagulation step of cheese manufacture, was responsible for the ~24% reduction in the level of pH 4.6-SN/TN at 1 d postmanufacture. This highlights the importance of chymosin in CN hydrolysis in these cheeses during the interval between completion of rennet coagulation of the milk and vacuum packaging of the cheese, which was approximately 20 h for this pilot-scale process. Indeed, the presence of the peptide $\alpha_{S1}$-CN (f24 -199) in some hard cheese varieties (e.g., Grana Padano), early in ripening, has been attributed to chymosin action early in manufacture but before inactivation by high cooking temperatures (Gaiaschi et al., 2000). The level of pH 4.6-SN/TN increased with increasing ripening time in each of the 4 cheeses. The extent of the increase in pH 4.6-SN/TN levels during ripening was greatest in the control cheese. In fact, the level of pH 4.6-SN/TN in the control cheese was approximately twice that of the cheese made with 10.0 μmol/L pepstatin after only 21 d of ripening. It is clear that the greatest divergence between the cheeses in terms of development of pH 4.6-SN occurred during the first 21 d of ripening and was critical in determining the ultimate (180 d) level of pH 4.6-SN in the cheeses. During the first 21 d of ripening, the level of pH 4.6-SN/TN increased by 6.06, 2.96, 1.22, and 0.76% in cheeses made with 0.0 (control), 0.1, 1.0, or 10.0 μmol/L pepstatin, respectively. The corresponding values for the increase in the level of pH 4.6-SN/TN between 21 and 180 d of ripening were 13.4, 9.50, 6.66, and 5.38%, respectively. These results show that the extent of the increase in pH 4.6-SN/TN level in the control cheese was approximately 2, 5, and 8 times that in cheeses made with 0.1, 1.0, or 10.0 μmol/L pepstatin during the first 21 d of ripening. The levels of pH 4.6-SN/TN increased at a constant rate in each of the 4 cheeses between 21 and 180 d of ripening, which supports the earlier results showing that the chymosin-pepstatin complex was extremely stable throughout ripening, irrespective of the level of pepstatin added. Similar trends were evident for development of pH 4.6-SN in each of the other 2 trials, with pH 4.6-SN/TN levels being significantly ($P < 0.0001$) affected by level of pepstatin addition and ripening time. The interaction between level of pepstatin addition and ripening time was also significant ($P < 0.0001$) in determining the levels of pH 4.6-SN/TN in the 4 cheeses across each of the 3 trials. The level of residual chymosin activity remained

**Table 3.** Levels of pH 4.6-soluble nitrogen (SN) expressed as a percentage of total nitrogen (TN) and total free amino acids for Cheddar cheeses made with 0.0 (control), 0.1, 1.0, or 10.0 μmol/L added pepstatin at 1, 21, 42, 90, 120, and 180 d of ripening.[1]

| | Ripening time (d) | Pepstatin addition (μmol/L) | | | |
|---|---|---|---|---|---|
| | | 0.0 | 0.1 | 1.0 | 10.0 |
| pH 4.6-SN/TN (%) | 1 | 4.94[a] (0.08) | 3.74[b] (0.00) | 3.66[b] (0.11) | 3.77[b] (0.04) |
| | 21 | 11.0[a] (0.17) | 6.70[b] (0.17) | 4.88[c] (0.12) | 4.53[d] (0.03) |
| | 42 | 12.5[a] (0.11) | 7.73[b] (0.07) | 5.88[c] (0.24) | 5.00[d] (0.10) |
| | 90 | 17.2[a] (0.33) | 10.1[b] (0.08) | 7.83[c] (0.14) | 7.21[d] (0.16) |
| | 120 | 19.9[a] (0.19) | 12.5[b] (0.34) | 8.47[c] (0.46) | 7.99[c] (0.11) |
| | 180 | 24.4[a] (0.11) | 16.2[b] (0.27) | 11.1[c] (0.07) | 9.91[d] (0.13) |
| Total free amino acids (mg of Leu/g of cheese) | 1 | 1.96[a] (0.17) | 1.61[a] (0.13) | 1.83[a] (0.16) | 1.70[a] (0.17) |
| | 21 | 4.11[a] (0.10) | 2.87[b] (0.07) | 2.72[b] (0.30) | 2.58[b] (0.17) |
| | 42 | 4.35[a] (0.08) | 3.08[b] (0.14) | 2.75[c] (0.05) | 2.55[d] (0.02) |
| | 90 | 6.70[a] (0.09) | 5.00[b] (0.26) | 4.52[c] (0.10) | 3.73[d] (0.09) |
| | 120 | 7.73[a] (0.07) | 6.66[b] (0.04) | 5.21[c] (0.04) | 4.71[d] (0.14) |
| | 180 | 11.2[a] (0.05) | 9.78[b] (0.51) | 7.22[c] (0.04) | 6.20[d] (0.02) |

[a,b,c,d]Means within a row with different superscripts are significantly different (Tukey's HSD; $P \leq 0.05$).

[1]Values represent means (±SD; n = 3).

constant in each of the 4 cheeses throughout ripening; however, the rate of primary proteolysis (as monitored by levels of pH 4.6-SN/TN) varied greatly with stage of ripening, with the rate being greatest during the early stages of ripening. This pattern in the rate of development of pH 4.6-SN/TN in the control cheese during ripening is in agreement with other authors (Lane et al., 1997; Fox et al., 2000; Lucey et al., 2005).

## Urea-PAGE

Altering the level of residual chymosin activity by addition of pepstatin caused large quantitative differences in the degree of hydrolysis of $\alpha_{S1}$-CN in the experimental cheeses, whereas the breakdown of $\beta$-CN was unaffected. The levels of intact $\alpha_{S1}$- and $\beta$-CN in cheeses made with 0.0 (control), 0.1, 1.0, or 10.0 μmol/L pepstatin at 1, 21, 42, 90, 120, and 180 d of ripening are shown in Figure 1. $\beta$-Casein was hydrolyzed to approximately the same extent in each of the 4 cheeses (Figure 1b), with levels of intact $\beta$-CN ranging from 70.1 to 75.9% for cheeses made with 1.0 or 10.0 μmol/L pepstatin, respectively, at 180 d of ripening. As expected, the 3 principal breakdown products of $\beta$-CN were $\beta$-CN (f29-209), $\beta$-CN (f106-209), and $\beta$-CN (f108-209); that is,$\gamma_1$-, $\gamma_2$-, and $\gamma_3$-CN, respectively. These peptides are liberated from $\beta$-CN by plasmin (Bastian and Brown, 1996), the activity of which is unaffected by pepstatin.

In the control cheese, $\alpha_{S1}$-CN was extensively hydrolyzed during ripening, with the rate of hydrolysis being most rapid during the first 21 d (Figure 1a). The primary cleavage site of chymosin on $\alpha_{S1}$-CN in Cheddar cheese during ripening is the $Phe_{23}$-$Phe_{24}$ peptide bond, releasing $\alpha_{S1}$-CN (f1-23) and $\alpha_{S1}$-CN (f24-199) (Carles and Ribadeau Dumas, 1985; McSweeney et al., 1993). The former peptide is rapidly hydrolyzed by proteinases of starter microorganisms (Visser, 1993), whereas the latter peptide undergoes further hydrolysis, initially at $Leu_{101}$-$Lys_{102}$, producing the peptide $\alpha_{S1}$-CN(f102-199) (McSweeney et al., 1993). In this study, $\alpha_{S1}$-CN (f24-199) accumulated in the control cheese up to 42 d of ripening (Figure 1c), after which the rate of breakdown was greater than the rate of formation, whereas the intensity of the $\alpha_{S1}$-CN (f102-199) band increased progressively during ripening (data not shown). The pattern of hydrolysis of $\alpha_{S1}$-CN was identical in cheese made with 0.1 μmol/L pepstatin and in the control cheese, except that the level of intact $\alpha_{S1}$-CN was 44.9% for the former compared with 12.0% for the latter by 180 d of ripening. The peptide $\alpha_{S1}$-CN (f24-199) continued to accumulate in cheeses made with 0.1 or 1.0 μmol/L pepstatin for up to 180 d (Figure 1c). The level of intact $\alpha_{S1}$-CN in cheese made with 10.0 μmol/L pepstatin was 91% at 180 d. This would indicate that approximately 9% of total $\alpha_{S1}$-CN was hydrolyzed in cheese made with 10.0 μmol/L pepstatin during the 180-d ripening period. However, it is important to note that there were no detectable levels of $\alpha_{S1}$-CN (f24-199) in this cheese during ripening. This would indicate a complete inhibition of chymosin-mediated hydrolysis of $\alpha_{S1}$-CN at $Phe_{23}$-$Phe_{24}$ in cheese made with 10.0 μmol/L pepstatin throughout ripening. The absence of $\alpha_{S1}$-CN(f24-199) in the cheese made with 10.0 μmol/L pepstatin (coupled with the differences in levels of pH 4.6-SN/TN, see Table 3) at 1 d illustrates that inhibition of residual chymosin activity occurred extremely rapidly after pepstatin addition to the curds/whey mixture. The limited hydrolysis of $\alpha_{S1}$-CN that occurred during ripening in the cheese made with 10.0 μmol/L pepstatin may

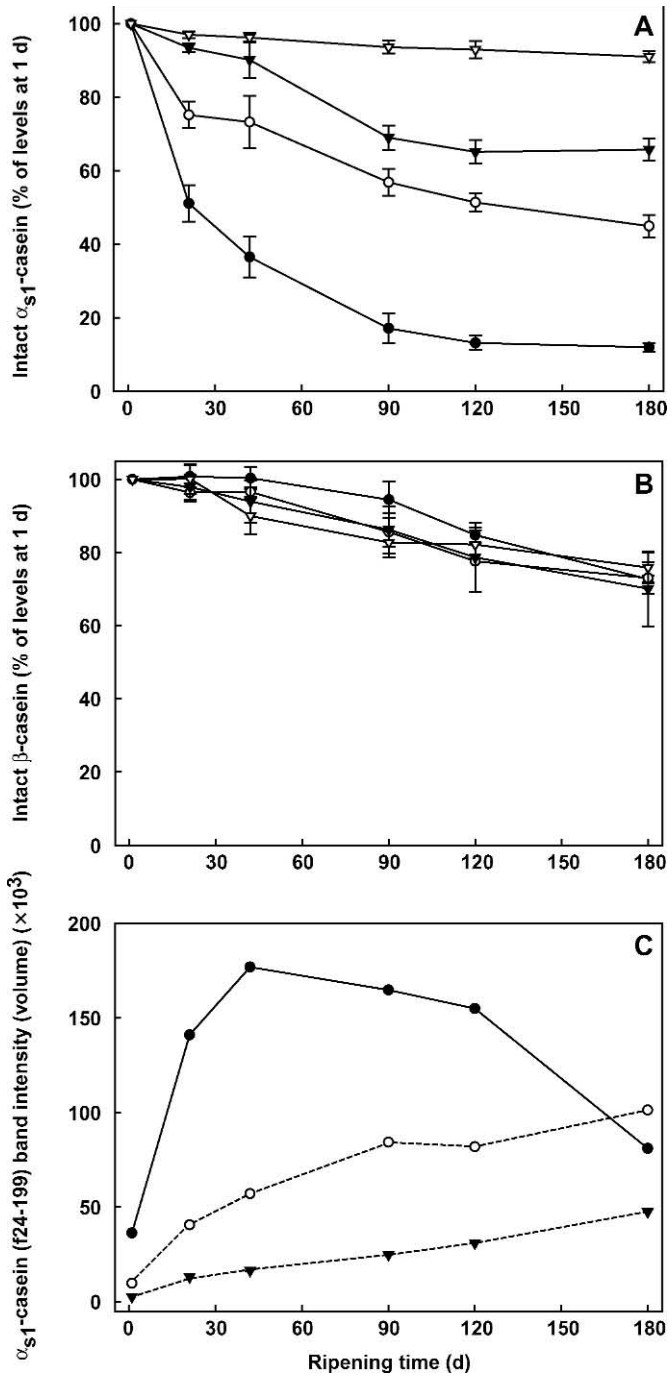


**Figure 1.** Levels of intact $\alpha_{S1}$-casein (A) and $\beta$-casein (B) in Cheddar cheeses made with 0.0 (control) (●), 0.1 (○), 1.0 (▲), or 10.0 (△) $\mu$mol/L added pepstatin at 1, 21, 42, 90, 120, and 180 d of ripening. Also shown (C) is the pixel intensity (volume) of the band corresponding to the $\alpha_{S1}$-CN (f24-199) peptide for Cheddar cheeses made with 0.0 (control), 0.1, or 1.0 $\mu$mol/L added pepstatin at 1, 21, 42, 90, 120, and 180 d of ripening. The peptide $\alpha_{S1}$-CN (f24- 199) was not detected in Cheddar cheese made with 10.0 $\mu$mol/L added pepstatin at any stage of ripening.

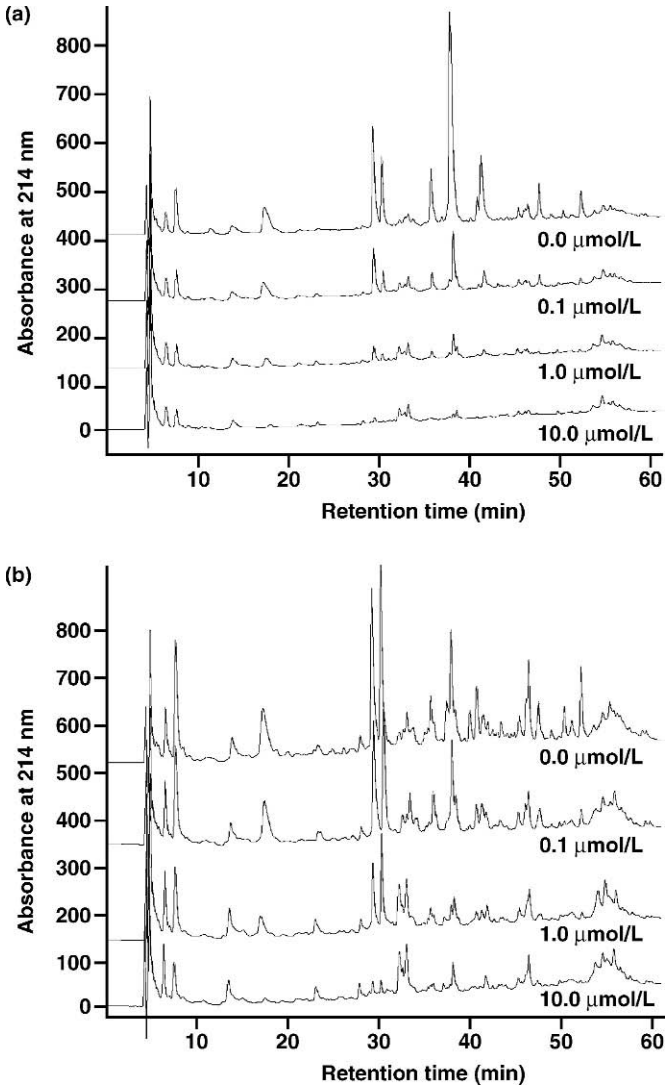


**Figure 2.** Reversed-phase HPLC chromatograms of the ethanol (70%)-soluble subfractions of pH 4.6-soluble extracts of Cheddar cheeses made with 0.0 (control), 0.1, 1.0, or 10.0 $\mu$mol/L added pepstatin at 21 (a) and 180 (b) d of ripening.

have been due to activity of starter or NSLAB proteinases and peptidases or plasmin.

## RP-HPLC Peptide Profiles

The RP-HPLC peptide profiles of the ethanol (70%)-soluble subfractions of the pH 4.6-soluble fractions of cheeses made with 0.0 (control), 0.1, 1.0, or 10.0 $\mu$mol/L pepstatin at 21 and 180 d of ripening are shown in Figures 2a and b, respectively. There were large quantitative differences between the peptide profiles of the 4 cheeses at both 21 and 180 d of ripening. It appears that at 21 d, the greatest differences between the peptide profiles of the cheeses were in the retention time

interval 28 to 45 min, whereas by 180 d of ripening, substantial differences also occurred in the longer retention time interval 45 to 60 min—possibly due to production of hydrophobic peptides. For the control cheese, the greatest quantitative change during ripening occurred in the 2 peptides eluting between 28 and 32 min. It is likely that these peptides are $\alpha_{S1}$-CN (f1-9) and $\alpha_{S1}$-CN (f1-13), which are breakdown products of $\alpha_{S1}$-CN (f1-23) and have been identified in the 10-kDa ultrafiltration permeates of pH 4.6-soluble fractions of 9-mo-old Cheddar cheese (Singh et al., 1994). The small quantitative changes that occurred in the peptide profiles of the cheese made with 10.0 $\mu$mol/L pepstatin during ripening occurred mainly in those peptides eluting between 45 and 60 min and may have been due to plasmin-mediated hydrolysis of $\beta$-CN. Similar changes in peptide profiles in the long retention time region have been reported for Cheddar cheeses with elevated plasmin activity using the same HPLC method (Upadhyay et al., 2004b). The results are consistent with the observation that levels of pH 4.6-SN/TN in the cheese made with 10.0 $\mu$mol/L added pepstatin increased by only ~2.6-fold between 1 and 180 d of ripening (compared with ~5-fold for control cheese). Hydrolysis of $\beta$-CN was the principal primary proteolytic event occurring in the cheese made with 10.0 $\mu$mol/L pepstatin during this time.

The score plot obtained from PC analysis of the peak height data from RP-HPLC of the ethanol (70%)-soluble subfractions of pH 4.6-soluble extracts from the cheeses is shown in Figure 3. Principal components 1 and 2 explained 41.1 and 21.5% of the total variation between the peptide profiles of the cheeses, respectively. Both PC1 and PC2 appear to have separated the samples on the basis of level of pepstatin addition and age; in general, the scores for both PC1 and PC2 increased with decreasing level of pepstatin addition and increasing age. Assuming an arbitrary linkage distance (squared Euclidean distance) cut-off of 8, then hierarchical cluster analysis arranged the cheeses into 5 distinct clusters on the basis of similarities in their peptide profiles (clusters are shown on the score plot). All cheeses had very similar peptide profiles at 1 d (chromatograms not shown); hence, all 4 cheeses were grouped within the same cluster ($C_1$) at 1 d of ripening. Interestingly, cheese made with 10.0 $\mu$mol/L pepstatin was grouped within this cluster ($C_1$) irrespective of age, reflecting the lack of primary (and consequently, secondary) proteolytic activity during ripening. The control cheese was grouped within separate clusters at 42, 90, and 180 d of ripening and the RP-HPLC peptide profiles of this cheese exhibited little similarity with those of cheeses made with 0.1, 1.0, or 10.0 $\mu$mol/L pepstatin from 42 d of ripening onwards. This reflects the substantial qualitative and quantitative differences observed between the peptide profiles of the control cheese and cheese made with 10.0 $\mu$mol/L pepstatin after only 42 d of ripening (Figure 3).

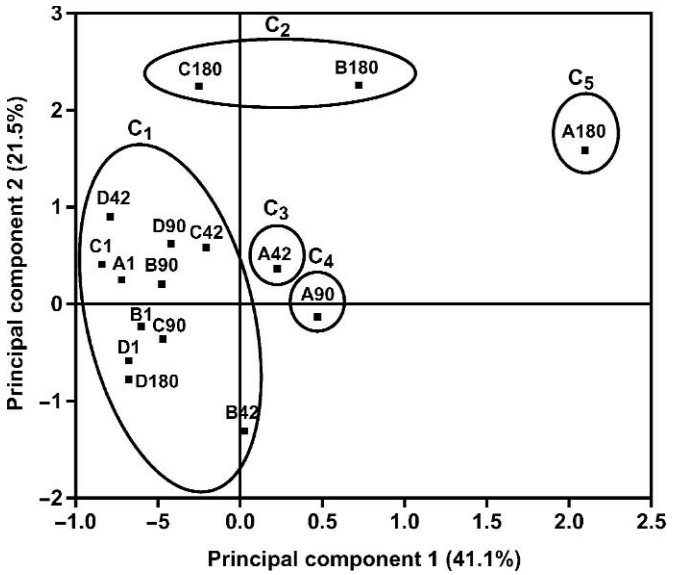


**Figure 3.** Score plot obtained from principal component analysis of processed peak height data from reversed phase-HPLC of the ethanol (70%)-soluble subfractions of pH 4.6-soluble extracts of Cheddar cheeses made with 0.0 (control) (A), 0.1 (B), 1.0 (C), or 10.0 (D) $\mu$mol/L added pepstatin at 1, 42, 90, and 180 d of ripening. Groupings on score plot ($C_1$ to $C_5$) indicate clusters as determined by hierarchical cluster analysis using an arbitrary linkage distance cut-off of 8.

The factor loadings for each of the retention time classes on the PC1 and PC2 axes are shown in Figures 4a and b, respectively. All of the retention time classes were positively correlated with the PC1 axis, whereas only 4 of the retention time classes were negatively correlated with the PC2 axis. This corresponds well with the earlier observation that the scores for the cheeses on the PC1 and PC2 axes generally increased with decreasing level of pepstatin addition and increasing age. The retention time classes most highly correlated (factor loading ≥0.90) with the PC1 and PC2 axes were those corresponding to peptides eluting at approximately 8, 30, 37, 38, 39, 43, and 47 min, respectively. Thus, while peptides across the entire profile were responsible for the differences between the cheeses, many of the peptides that differentiated the RP-HPLC profiles of the cheeses were eluted in the short- to intermediate-retention time classes (~25 to 45 min).

## Total and Individual FAA

There were no significant ($P > 0.05$) differences between the levels of total FAA of any of the 4 cheeses at 1 d postmanufacture, with levels ranging from 1.61 to

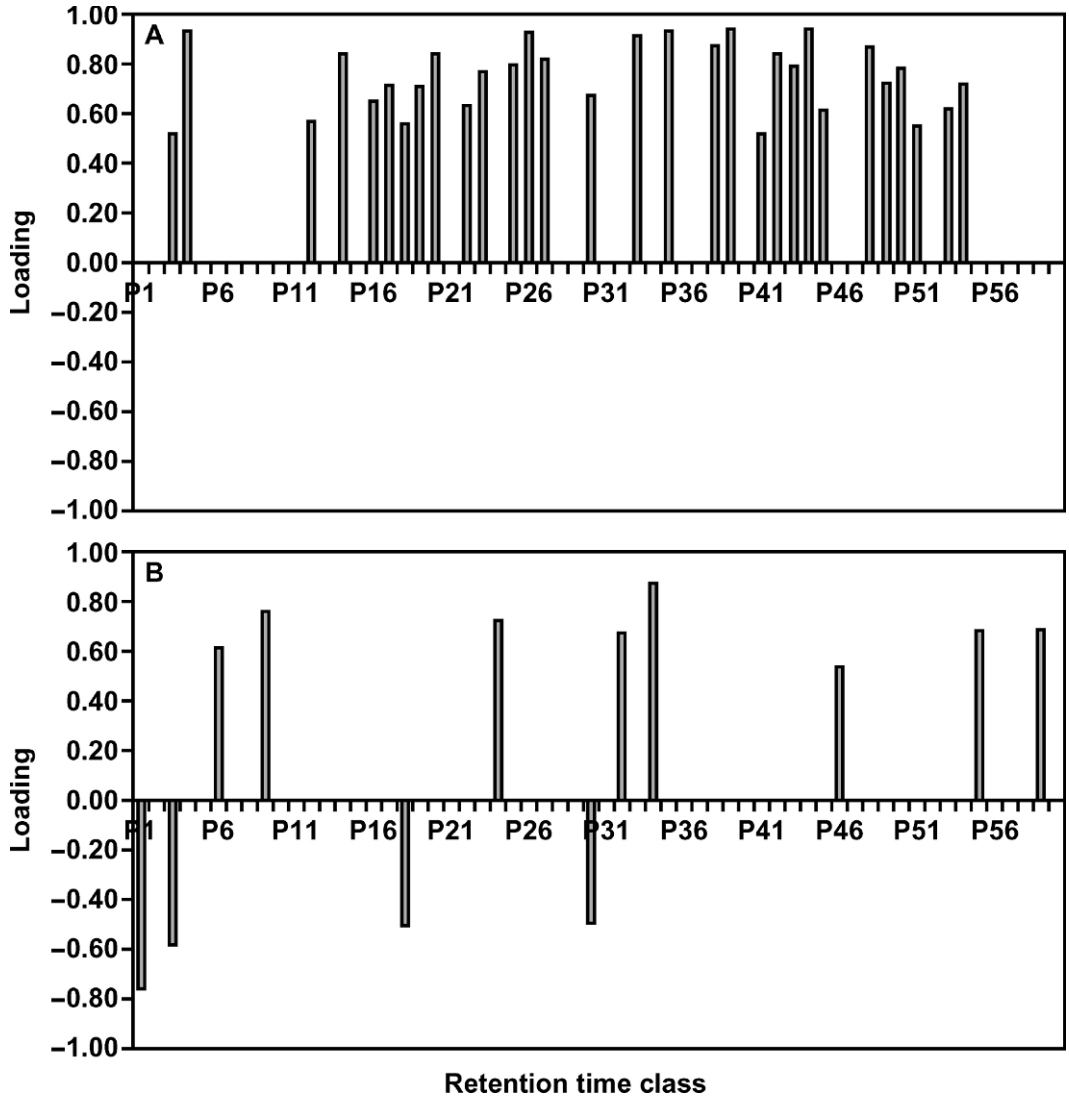


**Figure 4.** Factor loadings calculated from correlation analysis of each of the retention time classes (x-axis) with PC1 (A) and PC2 (B) from principal component analysis of peak height data from reversed phase-HPLC of the ethanol (70%)-soluble subfractions of pH 4.6-soluble extracts of Cheddar cheeses made with 0.0 (control), 0.1, 1.0, or 10.0 $\mu$mol/L added pepstatin at 1, 42, 90, and 180 d of ripening.

1.96 mg of leucine/g of cheese (Table 3). The FAA levels increased in all 4 cheeses during ripening, reaching levels of 11.2, 9.78, 7.22, and 6.20 mg of leucine/g of cheese at 180 d for cheeses made with 0.0, 0.1, 1.0, or 10.0 $\mu$mol/L pepstatin, respectively. Significant ($P \leq 0.05$) differences were evident in FAA levels between the 4 cheeses from 21 d of ripening onwards—the levels decreased with increasing level of pepstatin addition. Pepstatin addition had a significant ($P < 0.0001$) effect on FAA levels in each of the 3 trials, as did ripening time. The interaction between level of pepstatin addition and ripening time was also significant ($P < 0.0001$) for all 3 trials. Similar patterns in FAA levels were also reported by Lane et al. (1997) for Cheddar cheeses in which residual chymosin was partially inactivated by raising pH of curd/whey mixture to pH 7.0 after cutting the coagulum.

The principal amino acids present in each of the 4 cheeses at 180 d of ripening were glutamic acid, valine, leucine, phenylalanine, and lysine (Figure 5). In agreement with these results, glutamic acid, leucine, and phenylalanine were also reported to be the most abundant FAA in miniature Cheddar-type cheeses ripened at 8°C for 2 mo (O'Mahony et al., 2003). Shakeel-Ur-Rehman et al. (2004) also reported glutamic acid, valine, leucine, and phenylalanine to be the most abundant FAA in Cheddar cheeses ripened at 8°C for 180 d. With the exception of threonine, the levels of each of

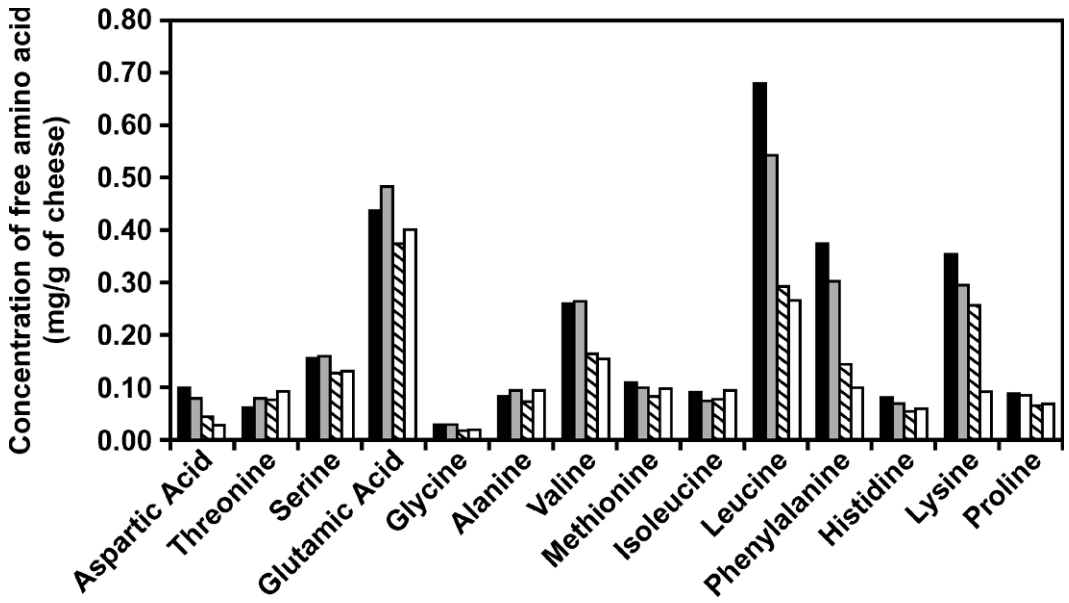


**Figure 5.** Concentration of individual free amino acids in Cheddar cheeses made with 0.0 (control) (black), 0.1 (gray), 1.0 (diagonal lines), or 10.0 (white) $\mu$mol/L added pepstatin at 180 d of ripening.

the individual FAA generally decreased with increasing level of pepstatin addition. This trend was most evident for aspartic acid, valine, leucine, phenylalanine, and lysine.

It is well known that peptidases of starter and NSLAB microorganisms are the principal agents responsible for liberation of FAA in Cheddar cheese during ripening (O'Keeffe et al., 1978; Fox and McSweeney, 1996; Lane and Fox, 1996, 1997). Incorporation of pepstatin into Cheddar cheese curd, at levels of up to 10.0 $\mu$mol/L of curds/whey mixture, has no effect on growth or survival of starter and NSLAB (J. A. O'Mahony and P. L. H. McSweeney, unpublished data, 2003). It should also be noted that microbial proteinase and peptidase activities in Cheddar cheese are not directly affected by pepstatin addition, as these enzymes are not aspartyl proteinases (Upadhyay et al., 2004a). Therefore, it may be inferred from the results that the lower levels of FAA in cheeses made with added pepstatin during ripening was due to limited availability of substrates for lactic acid bacteria (**LAB**) proteinase/peptidase activities. Such substrates normally constitute large and intermediate sized peptides produced by chymosin and plasmin (McSweeney, 2004), and are acted upon by LAB proteinases and peptidases in a sequential manner (Lane and Fox, 1997).

## Insoluble Calcium Content of Cheeses

The insoluble calcium content (expressed as % of total calcium) of the cheeses at 1 d of ripening ranged from 65.6 to 74.4% for the control cheese and the cheese made with 10.0 $\mu$mol/L added pepstatin, respectively (Table 4). The slight differences in the insoluble calcium content between the cheeses at 1 d may have been due to the differences in moisture; the insoluble calcium content of the cheeses decreased with increasing moisture content. However, irrespective of the level of pepstatin addition, there was a significant ($P \leq 0.05$) reduction in the insoluble calcium content of each of the 4 cheeses during the first 21 d of ripening. The insoluble calcium content of the cheeses remained relatively constant for the remainder of the ripening period. These results are in agreement with previous studies involving quantification of the changes in the insoluble calcium concentration of Cheddar cheese during ripening (Hassan et al., 2004; Lucey et al., 2005). The partial solubilization of CCP during the early stages of ripening is thought to be due to the attainment of pseudoequilibrium between the insoluble and soluble forms of calcium in cheese.

It is likely that solubilization of calcium associated with the *para*-CN matrix of the cheese curd (i.e., CCP) during this time (Table 4) increases the susceptibility of casein to proteolytic attack by chymosin. This phenomenon has been illustrated in milk (Fox, 1970) and has been suggested as a possible explanation for accelerated proteolysis in Mozzarella cheese with a reduced ratio of calcium to protein (Feeney et al., 2002; Joshi et al., 2003). In addition, rapid demineralization of curd during the cooking stage of manufacture (i.e., before whey drainage) has been associated with decreased lev-

**Table 4.** Insoluble calcium (expressed as a % of total calcium) content of Cheddar cheeses made with 0.0 (control), 0.1, 1.0, or 10.0 μmol/L added pepstatin at 1, 21, 42, 90, 120, and 180 d of ripening.[1]

| Ripening time (d) | Pepstatin addition (μmol/L) | | | |
|---|---|---|---|---|
| | 0.0 | 0.1 | 1.0 | 10.0 |
| 1 | 65.6[a] (1.90) | 71.3[a] (1.78) | 71.5[a] (1.43) | 74.4[a] (2.65) |
| 21 | 55.8[b] (0.60) | 63.2[b] (1.15) | 59.7[b] (0.21) | 59.8[b] (0.91) |
| 42 | 55.0[b] (2.18) | 61.8[b] (1.27) | 58.4[b] (0.45) | 58.7[b] (3.33) |
| 90 | 54.4[b] (0.53) | 61.6[b] (4.38) | 58.6[b] (1.03) | 57.9[b] (1.89) |
| 120 | 54.3[b] (3.26) | 61.7[b] (0.84) | 58.3[b] (0.60) | 56.9[b] (1.22) |
| 180 | 53.3[b] (1.33) | 60.9[b] (1.60) | 57.3[b] (1.80) | 55.3[b] (0.30) |

[a,b]Means within a column with different superscripts are significantly different (Tukey's HSD; $P \leq 0.05$).
[1]Values represent means (±SD; n = 3).

els of intact CN in 1-d-old Cheddar cheese (O'Keeffe et al., 1975). These authors postulated that the increased CN hydrolysis might be caused by solubilization of some CCP, which facilitates conformational change in the CN substrates, allowing greater access of chymosin to potential cleavage sites.

## TPA

In general, the values for the TPA parameters hardness, cohesiveness, springiness, and chewiness decreased as ripening progressed (Table 5). Cheeses manufactured with 0.0 (control), 0.1, 1.0, or 10.0 μmol/L pepstatin had hardness values of 189, 204, 207, and 233 N at 1 d of ripening, respectively. The higher initial (1 d) hardness, and indeed, springiness and chewiness values for Cheddar cheeses made with added pepstatin compared with the control cheese may be attributed to the lower moisture content of these cheeses (Table 2) or to differences in the levels of primary proteolysis (Table 3). It is known that moisture content and extent of primary proteolysis influence the rheological properties of cheese (Creamer and Olson, 1982; Fox et al., 2000; Watkinson et al., 2002; Guinee, 2003).

There was a significant ($P \leq 0.05$) reduction in the hardness values for all 4 cheeses between 1 and 21

**Table 5.** Texture profile analysis parameters hardness, cohesiveness, springiness, and chewiness for Cheddar cheeses made with 0.0 (control), 0.1, 1.0, or 10.0 μmol/L added pepstatin at 1, 21, 42, 90, 120, and 180 d of ripening.[1]

| Texture parameter | Ripening time (d) | Pepstatin addition (μmol/L) | | | |
|---|---|---|---|---|---|
| | | 0.0 | 0.1 | 1.0 | 10.0 |
| Hardness (N) | 1 | 189[a] (8.08) | 204[a] (8.73) | 207[a] (8.23) | 233[a] (14.9) |
| | 21 | 115[b] (5.58) | 139[b] (15.2) | 163[b] (7.11) | 187[b] (8.66) |
| | 42 | 98.2[bc] (5.89) | 135[b] (9.41) | 146[c] (7.00) | 182[b] (9.73) |
| | 90 | 110[bc] (8.49) | 129[b] (5.71) | 133[cd] (2.43) | 155[c] (10.1) |
| | 120 | 102[bc] (4.26) | 132[b] (7.77) | 145[c] (2.78) | 156[c] (10.9) |
| | 180 | 95.1[c] (9.71) | 125[b] (3.01) | 133[d] (5.83) | 145[c] (6.24) |
| Cohesiveness | 1 | 0.56[a] (0.03) | 0.56[a] (0.02) | 0.47[a] (0.05) | 0.52[a] (0.05) |
| | 21 | 0.29[b] (0.01) | 0.35[b] (0.02) | 0.31[b] (0.02) | 0.39[b] (0.02) |
| | 42 | 0.30[b] (0.03) | 0.32[c] (0.02) | 0.30[b] (0.03) | 0.33[c] (0.03) |
| | 90 | 0.22[c] (0.01) | 0.27[cd] (0.02) | 0.25[c] (0.01) | 0.27[d] (0.02) |
| | 120 | 0.25[c] (0.01) | 0.29[de] (0.01) | 0.26[bc] (0.01) | 0.30[cd] (0.02) |
| | 180 | 0.23[c] (0.01) | 0.25[e] (0.01) | 0.24[c] (0.02) | 0.29[cd] (0.04) |
| Springiness | 1 | 0.75[a] (0.04) | 0.79[a] (0.03) | 0.80[a] (0.02) | 0.82[a] (0.03) |
| | 21 | 0.59[b] (0.06) | 0.63[b] (0.03) | 0.65[b] (0.06) | 0.68[b] (0.03) |
| | 42 | 0.56[bc] (0.07) | 0.60[b] (0.04) | 0.65[b] (0.07) | 0.65[bc] (0.05) |
| | 90 | 0.49[cd] (0.04) | 0.57[c] (0.07) | 0.59[bc] (0.03) | 0.62[cd] (0.02) |
| | 120 | 0.45[d] (0.05) | 0.53[c] (0.03) | 0.58[c] (0.09) | 0.57[d] (0.05) |
| | 180 | 0.44[d] (0.04) | 0.51[c] (0.02) | 0.53[c] (0.02) | 0.55[d] (0.03) |
| Chewiness (N) | 1 | 61.0[a] (2.31) | 61.8[a] (10.1) | 72.1[a] (10.6) | 74.1[a] (6.15) |
| | 21 | 14.0[b] (2.07) | 16.5[b] (1.49) | 24.8[b] (1.73) | 33.8[b] (5.02) |
| | 42 | 11.8[bc] (1.63) | 17.2[bc] (1.46) | 19.1[b] (2.14) | 20.4[c] (3.86) |
| | 90 | 9.89[cd] (3.12) | 13.0[c] (1.35) | 15.4[b] (1.78) | 17.7[c] (2.58) |
| | 120 | 10.0[cd] (1.43) | 15.5[c] (2.37) | 16.9[b] (1.67) | 20.3[c] (1.88) |
| | 180 | 8.48[d] (1.24) | 11.4[c] (0.89) | 13.1[b] (1.05) | 16.8[c] (1.43) |

[a,b,c,d]Means within a row with different superscripts are significantly different (Tukey's HSD; $P \leq 0.05$).
[1]Values represent means (±SD; n = 5).

**Table 6.** Pearson's correlation coefficients between hardness and intact $\alpha_{S1}$-CN, intact $\beta$-CN, pH 4.6-soluble nitrogen, total free amino acids, and insoluble calcium levels of Cheddar cheeses made with 0.0 (control), 0.1, 1.0, or 10.0 $\mu$mol/L added pepstatin at 2 different intervals during ripening.

| Ripening time interval | Pepstatin addition ($\mu$mol/L) | Intact $\alpha_{S1}$-CN[1] | Intact $\beta$-CN[1] | pH 4.6-SN/TN[1,2] | Total FAA[1,3] | Insoluble calcium[1] |
|---|---|---|---|---|---|---|
| 1 to 42 d | 0.0 | 0.92*** | 0.47$^{NS}$ | (0.86)** | (0.82)** | 0.95*** |
| | 0.1 | 0.71* | 0.72* | (0.84)** | (0.71)* | 0.93*** |
| | 1.0 | 0.73* | 0.75* | (0.80)** | (0.63)$^{NS}$ | 0.95*** |
| | 10.0 | 0.63$^{NS}$ | 0.56$^{NS}$ | (0.76)* | (0.76)* | 0.92*** |
| 42 to 180 d | 0.0 | (0.12)$^{NS}$ | 0.25$^{NS}$ | (0.17)$^{NS}$ | (0.40)$^{NS}$ | (0.09)$^{NS}$ |
| | 0.1 | 0.84*** | 0.42$^{NS}$ | (0.63)* | (0.55)$^{NS}$ | 0.25$^{NS}$ |
| | 1.0 | 0.23$^{NS}$ | 0.39$^{NS}$ | (0.30)$^{NS}$ | (0.27)$^{NS}$ | 0.05$^{NS}$ |
| | 10.0 | 0.73** | 0.80** | (0.73)** | (0.78)** | 0.52$^{NS}$ |

[1]Combined data from all 3 trials.

[2]pH 4.6-SN/TN = pH 4.6-Soluble nitrogen as a percentage of total nitrogen.

[3]Total FAA = Total free amino acids.

*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; NS = nonsignificant ($P > 0.05$); parentheses indicate negative correlation.

d of ripening. The significant ($P \leq 0.05$) reduction in hardness (i.e., softening) of the control cheese during this time was expected in accordance with the hypothesis from the work of Creamer and Olson (1982). Interestingly, the hardness, and indeed cohesiveness, springiness, and chewiness values of each of the 3 cheeses made with added pepstatin significantly ($P \leq 0.05$) decreased during the first 21 d of ripening. It is clear from these results that even when hydrolysis of $\alpha_{S1}$-CN at $Phe_{23}$-$Phe_{24}$ was completely inhibited (i.e., cheese made with 10 $\mu$mol/L pepstatin), there was still a significant softening of Cheddar cheese texture during the early stages of ripening. Hardness was much more highly correlated with the level of insoluble calcium than with that of intact $\alpha_{S1}$-CN during the early stages of ripening (Table 6). In the case of the cheese made with 10.0 $\mu$mol/L pepstatin, hardness was not significantly correlated with the level of intact $\alpha_{S1}$-CN, whereas there was a strong positive correlation between hardness and the level of insoluble calcium ($r = 0.92$, $P \leq 0.001$). Assessment of the mean squares values showed that ripening time (subplot factor) was more important than level of pepstatin addition (main-plot factor) on influencing the TPA parameter hardness for cheeses made in each of the 3 trials.

Lane et al. (1997) reported that TPA hardness (i.e., force at 70% compression) of Cheddar cheese made using a protocol modified to inactivate chymosin partially, with a water-SN level of 17%, was approximately 72 N compared with the control cheese, which had a water-SN level of 21% and a hardness value of 58 N at 180 d of ripening. These authors reported that the values for hardness, distance to fracture, force to fracture, and cohesiveness all decreased for each of the cheeses between 60 and 180 d of ripening. Irudayaraj et al. (1999) reported that hardness (at 20% compression) of full- (31%) and reduced- (21%) fat Cheddar cheese decreased during the first 30 d and then increased steadily during the remainder of ripening (210 d). Benech et al. (2003) also reported decreases in TPA fracturability and cohesiveness (at 20% compression) of Cheddar cheese during ripening. Sallami et al. (2004) reported that TPA hardness, fracturability, and springiness (at 20% compression) all decreased with progressive ripening of Cheddar cheese made with or without autolytic, proteolytic, or nisin-producing adjunct starter cultures. Interestingly, the results of Benech et al. (2003) and Sallami et al. (2004) show that the greatest decrease in each of the TPA parameters hardness, cohesiveness, fracturability, and springiness occurred during the first 30 to 60 d of ripening.

For this study, although the greatest change in hardness occurred during the first 21 d of ripening in all 4 cheeses, minor, but important changes in texture occurred throughout the remainder of the ripening period. For the control cheese and cheese made with 0.1 $\mu$mol/L pepstatin, there was no significant ($P > 0.05$) change in hardness between 42 and 180 d of ripening. However, for cheeses made with 1.0 or 10.0 $\mu$mol/L pepstatin, the softening of texture continued throughout ripening. Hardness values decreased significantly ($P \leq 0.05$) from 146 and 182 N at 42 d to 133 and 145 N at 180 d for cheeses made with 1.0 or 10.0 $\mu$mol/L pepstatin, respectively. Overall, the percentage decrease in hardness between 42 and 180 d was 3.1, 7.4, 8.9, and 20.3% for control cheese and cheeses made with 0.1, 1.0, or 10.0 $\mu$mol/L pepstatin, respectively. It appears that there was a significant ($P \leq 0.05$) softening of Cheddar cheese texture between 42 and 180 d only when $\alpha_{S1}$-CN hydrolysis was strongly retarded (or inhibited completely). This can also be seen from the negative correlation between level of intact $\alpha_{S1}$-CN and hardness for

the control cheese in the latter ripening time interval (42 to 180 d; Table 6). These findings are interesting in the context of the work conducted by Benech et al. (2003), who reported that cohesiveness decreased by 45 and 32% during 180 d of ripening in Cheddar cheeses made without nisin- (control) or with nisin-producing starter cultures, respectively. The corresponding values for decrease in fracturability during ripening were 64 and 46%, respectively. The control and nisin-containing cheeses had TCA-soluble nitrogen (as % of total nitrogen) levels of 10.4 and 14.2%, respectively, at 180 d of ripening. The level of phosphotungistic acid-soluble nitrogen (as % of total nitrogen) in the nisin-containing cheese was also approximately 1.6-fold higher than that in the control cheese at 180 d. The authors attributed the differences in textural properties to the increased levels of secondary proteolysis in the nisin-containing cheese; with each peptide bond hydrolyzed releasing 2 new charged groups ($NH_3^+/COO^-$). It is believed that these ionic species compete for available water, reducing the "free" water content and restricting solvation of the para-CN matrix of the cheese resulting in increased hardness, cohesiveness, springiness, and fracturability over ripening (Creamer and Olson, 1982; Lawrence et al., 1987; Irudayaraj et al., 1999; Benech et al., 2003; Guinee, 2003; Lucey et al., 2003).

## CONCLUSIONS

Addition of pepstatin to the curds/whey mixture at the start of cooking was a very effective means of reducing the level of residual chymosin activity in the resultant cheese and inhibiting chymosin-mediated hydrolysis of $\alpha_{S1}$-CN. The chymosin-pepstatin complex was extremely stable during ripening at all levels of pepstatin addition. Increasing the level of pepstatin addition retarded primary and secondary proteolysis during ripening; however, pepstatin addition had a greater influence on primary than secondary proteolysis. The solubilization of CCP (insoluble form of calcium in cheese) during the first 21 d of ripening was unaffected by level of pepstatin addition. Hydrolysis of $\alpha_{S1}$-CN at $Phe_{23}$-$Phe_{24}$ was not a prerequisite for the early softening of Cheddar cheese texture. The softening of Cheddar cheese texture during the early stages of ripening was more highly correlated with the concentration of insoluble calcium than with the level of intact $\alpha_{S1}$-CN. It is concluded that softening of Cheddar cheese texture can occur early in ripening without $\alpha_{S1}$-CN being hydrolyzed at $Phe_{23}$-$Phe_{24}$. We propose that this softening of texture is due largely to solubilization of some of the residual CCP associated with the *para*-CN matrix of the cheese.

## ACKNOWLEDGMENTS

J. A. O'Mahony gratefully acknowledges the financial support of a travel bursary from the National University of Ireland, which facilitated part of this research at the University of Wisconsin-Madison. The authors wish to thank John Hannon for his assistance with statistical analysis of the data and to acknowledge financial support from Dairy Management Inc.

## REFERENCES

Andrews, A. T. 1983. Proteinases in normal bovine milk and their action on caseins. J. Dairy Res. 50:45–55.

Bastian, E. D., and R. J. Brown. 1996. Plasmin in milk and dairy products. Int. Dairy J. 6:435–457.

Benech, R. O., E. E. Kheadr, C. Lacroix, and I. Fliss. 2003. Impact of nisin producing culture and liposome-encapsulated nisin on ripening of *Lactobacillus* added-Cheddar cheese. J. Dairy Sci. 86:1895–1909.

Blakesley, R. W., and J. A. Boezi. 1977. A new staining technique for proteins in polyacrylamide gels using Coomassie Brilliant Blue G250. Anal. Biochem. 82:580–588.

Bourne, M. C. 1978. Texture profile analysis. Food Technol. 32:62–66, 72.

Carles, C., and B. Ribadeau-Dumas. 1985. Kinetics of the action of chymosin (rennin) on a peptide bond of bovine $\alpha_{S1}$-casein. Comparison of the behaviour of this substrate with that of $\beta$- and $\kappa$-casein. FEBS Lett. 185:282–286.

Creamer, L. K., and N. F. Olson. 1982. Rheological evaluation of maturing Cheddar cheese. J. Food Sci. 47:631–646.

Creamer, L. K., H. F. Zoerb, N. F. Olson, and T. Richardson. 1982. Surface hydrophobicity of $\alpha_{S1}$-I, $\alpha_{S1}$-casein A and B and its implications in cheese structure. J. Dairy Sci. 65:902–906.

Davies, D. T., and A. J. R. Law. 1977. An improved method for the quantitative fractionation of casein mixtures using ion exchange chromatography. J. Dairy Res. 44:213–221.

De Kruif, C. G., and C. Holt. 2003. Casein micelle structure, functions and interactions. Pages 233–276 in Advanced Dairy Chemistry. Vol. 1, 3rd ed. P. F. Fox and P. L. H. McSweeney, ed. Kluwer Academic/Plenum Publishers, New York, NY.

Feeney, E. P., P. F. Fox, and T. P. Guinee. 2002. Effect of pH and calcium level on proteolysis in Mozzarella cheese. J. Dairy Sci. 85:1646–1654.

Fenelon, M. A., P. O'Connor, and T. P. Guinee. 2000. The effect of fat content on the microbiology and proteolysis in Cheddar cheese during ripening. J. Dairy Sci. 83:2173–2183.

Fox, P. F. 1963. Potentiometric determination of salt in cheese. J. Dairy Sci. 46:744–745.

Fox, P. F. 1970. Influence of aggregation on the susceptibility of casein to proteolysis. J. Dairy Res. 37:173–180.

Fox, P. F. 1993. Cheese: Biochemistry of cheese ripening. Pages 389–438 in Cheese: Chemistry, Physics and Microbiology. Vol. 1, 2nd ed. P. F. Fox, ed. Chapman and Hall, London, UK.

Fox, P. F., and J. Law. 1991. Enzymology of cheese ripening. Food Biotechnol. 5:239–262.

Fox, P. F., and P. L. H. McSweeney. 1996. Proteolysis in cheese during ripening. Food Rev. Int. 12:457–509.

Fox, P. F., T. M. Cogan, T. P. Guinee, and P. L. H. McSweeney. 2000. Fundamentals of Cheese Science. Aspen Publishers Inc., Gaithersburg, MD.

Gaiaschi, A., B. Beretta, C. Poiesi, A. Conti, M. G. Giuffrida, C. L. Galli, and P. Restani. 2000. Proteolysis of $\alpha_S$-casein as a marker of Grana Padano cheese ripening. J. Dairy Sci. 83:2733–2739.

Guinee, T. P. 2003. Role of protein in cheese and cheese products. Pages 1083–1174 in Advanced Dairy Chemistry. Vol. 1, 3rd ed. P. F. Fox and P. L. H. McSweeney, ed. Kluwer Academic/Plenum Publishers, New York, NY.

Hassan, A., M. E. Johnson, and J. A. Lucey. 2004. Changes in the proportions of soluble and insoluble calcium during the ripening of Cheddar cheese. J. Dairy Sci. 87:854–862.
Horne, D. S. 1998. Casein interactions: Casting light on the black boxes, the structure in dairy products. Int. Dairy J. 8:171–177.
Hurley, M. J., B. M. O'Driscoll, A. L. Kelly, and P. L. H. McSweeney. 1999. Novel assay for the determination of residual coagulant activity in cheese. Int. Dairy J. 9:553–558.
IDF. 1982. Cheese and processed cheese. Determination of the total solids content. 4A:1982. International Dairy Federation, Brussels, Belgium.
IDF. 1986. Determination of the nitrogen content (Kjeldahl method) and calculation of crude protein content. 20A:1986. International Dairy Federation, Brussels, Belgium.
IDF. 2003. Determination of calcium, sodium, potassium and magnesium contents–Atomic absorption spectroscopic method. 119:2003. International Dairy Federation, Brussels, Belgium.
IIRS. 1955. Determination of the percentage of fat in cheese. Institute for Industrial Research and Standards, Dublin, Ireland.
Irudayaraj, J., M. Chen, and D. J. McMahon. 1999. Texture development in Cheddar cheese during ripening. Can. Agric. Eng. 41:253–258.
Joshi, N. S., K. Muthukumarappan, and R. I. Dave. 2003. Understanding the role of calcium in functionality of part-skim Mozzarella cheese. J. Dairy Sci. 86:1918–1926.
Kuchroo, C. N., and P. F. Fox. 1982. Soluble nitrogen in Cheddar cheese: Comparison of extraction procedures. Milchwissenschaft 37:331–335.
Lane, C. N., and P. F. Fox. 1996. Contribution of starter and adjunct lactobacilli to proteolysis in Cheddar cheese during ripening. Int. Dairy J. 6:715–728.
Lane, C. N., and P. F. Fox. 1997. Role of starter enzymes during ripening of Cheddar cheese made from pasteurised milk under controlled microbiological conditions. Int. Dairy J. 7:55–63.
Lane, C. N., P. F. Fox, D. E. Johnston, and P. L. H. McSweeney. 1997. Contribution of coagulant to proteolysis and textural changes in Cheddar cheese during ripening. Int. Dairy J. 7:453–464.
Lawrence, R. C., L. K. Creamer, and J. Gilles. 1987. Texture development during cheese ripening. J. Dairy Sci. 70:1748–1760.
Lawrence, R. C., J. Gilles, L. K. Creamer, V. L. Crow, H. A. Heap, C. G. Honore, K. A. Johnston, and P. K. Samal. 2004. Cheddar cheese and related dry-salted cheese varieties. Pages 71–102 in Cheese: Chemistry, Physics and Microbiology. Vol. 2, 3rd ed. P. F. Fox, P. L. H. McSweeney, T. M. Cogan, and T. P. Guinee, ed. Elsevier Academic Press, London, UK.
Lucey, J. A., and P. F. Fox. 1993. Importance of calcium and phosphate in cheese manufacture: A review. J. Dairy Sci. 76:1714–1724.
Lucey, J. A., M. E. Johnson, and D. S. Horne. 2003. Perspectives on the basis of the rheology and texture properties of cheese. J. Dairy Sci. 86:2725–2743.
Lucey, J. A., R. Mishra, A. Hassan, and M. E. Johnson. 2005. Rheological and calcium equilibrium changes during ripening of Cheddar cheese. Int. Dairy J. 15:645–653.
McKown, M. M., R. J. Workman, and R. I. Gregerman. 1974. Pepstatin inhibition of human renin: Kinetic studies and estimation of enzyme purity. J. Biol. Chem. 249:7770–7774.
McSweeney, P. L. H. 2004. Biochemistry of cheese ripening. Int. J. Dairy Technol. 57:127–144.
McSweeney, P. L. H., N. F. Olson, P. F. Fox, A. Healy, and P. Hojrup. 1993. Proteolytic specificity of chymosin on bovine $\alpha_{S1}$-casein. J. Dairy Res. 60:401–412.
O'Keeffe, A. M., P. F. Fox, and C. Daly. 1978. Proteolysis in Cheddar cheese: Role of coagulant and starter bacteria. J. Dairy Res. 45:465–477.
O'Keeffe, R. B., P. F. Fox, and C. Daly. 1975. Proteolysis in Cheddar cheese: Influence of the rate of acid production during manufacture. J. Dairy Res. 42:111–122.
O'Mahony, J. A., M. J. Sousa, and P. L. H. McSweeney. 2003. Proteolysis in miniature Cheddar-type cheeses made using blends of chymosin and *Cynara cardunculus* proteinases as coagulant. Int. J. Dairy Technol. 56:52–58.
Piraino, P., E. Parente, and P. L. H. McSweeney. 2004. Processing of chromatographic data for chemometric analysis of peptide profiles from cheese extracts: A novel approach. J. Agric. Food Chem. 52:6904–6911.
Polychroniadou, A. 1988. A simple procedure using trinitrobenzenesulphonic acid for monitoring proteolysis in cheese. J. Dairy Res. 55:585–596.
Rich, D. H., and E. T. O. Sun. 1980. Mechanism of inhibition of pepsin by pepstatin; Effect of inhibitor structure on dissociation constant and time-dependant inhibition. Biochem. Pharmacol. 29:2205–2212.
Sallami, L., E. E. Kheadr, I. Fliss, and J. C. Vuillemard. 2004. Impact of autolytic, proteolytic and nisin-producing adjunct cultures on biochemical and texture properties of Cheddar cheese. J. Dairy Sci. 87:1585–1594.
SAS Institute. 1999. SAS Software, release 8.0. SAS Institute, Inc., Cary, NC.
Shakeel-Ur-Rehman, D. Waldron, and P. F. Fox. 2004. Effect of modifying lactose concentration in cheese curd on proteolysis and in quality of Cheddar cheese. Int. Dairy J. 14:591–597.
Shakeel-Ur-Rehman, E. P. Feeney, P. L. H. McSweeney, and P. F. Fox. 1998. Inhibition of residual coagulant in cheese using pepstatin. Int. Dairy J. 8:987–992.
Shalabi, S. I., and P. F. Fox. 1987. Electrophoretic analysis of cheese: Comparison of methods. J. Food Sci. Technol. 11:135–142.
Singh, T. K., P. F. Fox, P. Hojrup, and A. Healy. 1994. A scheme for the fractionation of cheese nitrogen and identification of principal peptides. Int. Dairy J. 4:111–122.
Sousa, M. J., and P. L. H. McSweeney. 2001. Studies on the ripening of Cooleeney, an Irish farmhouse Camembert-type cheese. Irish J. Agric. Food Res. 40:83–95.
Upadhyay, V. K., P. L. H. McSweeney, A. A. A. Magboul, and P. F. Fox. 2004a. Proteolysis in cheese during ripening. Pages 391–433 in Cheese: Chemistry, Physics and Microbiology. Vol. 1, 3rd ed. P. F. Fox, P. L. H. McSweeney, T. M. Cogan, and T. P. Guinee, ed. Elsevier Academic Press, London, UK.
Upadhyay, V. K., M. J. Sousa, P. Ravn, H. Israelsen, A. L. Kelly, and P. L. H. McSweeney. 2004b. Use of exogenous streptokinase to accelerate proteolysis in Cheddar cheese during ripening. Lait 84:527–538.
Visser, S. 1993. Proteolytic enzymes and their relation to cheese ripening and flavor: An overview. J. Dairy Sci. 76:329–350.
Walstra, P., H. J. M. van Dijk, and T. J. Geurts. 1987. The syneresis of curd. Pages 135–177 in Cheese: Chemistry, Physics and Microbiology. Vol. 1, 1st ed. P. F. Fox, ed. Elsevier Applied Science Publishers, Essex, UK.
Watkinson, P. J., R. A. Crawford, and C. C. Dodds. 2002. Effect of moisture on instrumentally measured textural properties of model cheese. Aust. J. Dairy Technol. 57:153. (Abstr.)