Development of a Quantitative Model for Enzyme-Catalyzed, Time-Dependent Changes in Protein Composition of Cheddar Cheese During Storage

J. J. BASCH, H. M. FARRELL, JR., R. A. WALSH, R. P. KONSTANCE, and T. F. KUMOSINSKI
US Department of Agriculture, Agricultural Research Service
Eastern Regional Research Center
Philadelphia, PA 19118

ABSTRACT

To determine changes in protein composition in long-term storage of cheese, two types of cheese, traditional Cheddar and stirred curd Cheddar, were studied. The degradation of caseins in both cheeses during the process of ripening was evaluated by SDS-PAGE and densitometry. Relationships between the method of cheese manufacture and the effects of freezing and thawing of cheese at selected ages were studied. During the storage of cheese in the coolers, the ripening process continued, and as the age of the cheese increased, there were partial breakdowns of caseins into fragmentary products and peptides. Nonlinear regression analysis was used to model the time-dependent changes in casein components. From these analyses, "half-lives" for casein changes could be calculated. Notably, α_{s1}-casein disappears with a half-life of 2 wk; half-life of β-casein is 37 wk. Neither young nor aged Cheddar cheese was affected by differences in freezing temperatures, nor were there differences between samples collected before and after freezing, nor were there significant differences between the stirred curd and traditional Cheddars.

INTRODUCTION

Millions of pounds of Cheddar cheese have been purchased under government price support programs. This high quality product is stored and often used in process cheese manufacture for the school lunch and other food donation programs. The cheese is stored as 40-lb blocks or in 500-lb barrels at temperatures of -2.2 to $0^{\circ}C$ in warehouse coolers. Because the purchase rate on occasion exceeds rate of utilization, available cooler storage space may become scarce. Therefore, a recommendation has been made to utilize available freezers for cheese storage.

Very little is known about the effects of freezing and thawing on the functionality of previously frozen curd when used for process cheese manufacture, and storage life of frozen curd is unknown. Time of ripening before freezing, effects of freezing on ripening, and effects of curd manufacture (stirred or cheddared) to ensure the best storage life for subsequent process cheese manufacture have not been studied in great detail. Previous research on adulteration of NDM with whey protein concentrate (1) led to the development of an SDS-PAGE system capable of visualizing all milk proteins on a single gel. It seemed this method could be adapted to follow proteolytic degradation of caseins in cheese under various conditions.

This paper addresses these factors by examining electrophoretic and densitometric data on freezing and thawing of Cheddar cheese at selected ages under commercial storage conditions. Information on the degradation of proteins in the Cheddar during the storage time was also gathered. The relationships among freezing temperature and samples collected before and after freezing are evaluated for conventional Cheddar and compared at selected ages with those of a commercial stirred curd cheese. Finally a quantitative model for the observed time-dependent changes in protein content was

Received May 9, 1988.
Accepted August 26, 1988.

developed by application of thermodynamic linkage concepts (12).

MATERIALS AND METHODS

Acquisition, Identification, and Preparation of Cheese Samples

Two and five 40-lb blocks of Cheddar cheese and stirred curd cheese, respectively, were received from a local commercial dairy. These cheeses were manufactured according to government specifications. The Cheddar cheese was cut as shown in Figure 1. Figure 1A depicts a 3-D view of a 40-lb block. Stirred curd cheese was cut similarly to the Cheddar cheese except that the fifth layer served as the bottom layer of the block. The dimensions of the bottom layer were the same as the top layer. Each block was cut into smaller subblocks, 115-mm long, 115-mm wide, and either 51-mm or 13-mm thick. Several subblocks were used for immediate sampling, and the others were stored for the ripening process. The number of weeks in cooler storage ranged between 3 and 52 wk; temperatures of the frozen cheese were 0°F (−17.8°C), −20°F (−28.9°C), and −40°F (−40°C); and condition of cheese was prefrozen (PF) or after being frozen (AF).

After a determined time of cheese ripening, a sample subblock was removed from cooler storage. In the center of a subblock, a cylinder, 100-mm in diameter and 50-mm thick, was cut out with a cork borer. Thermocouples were inserted at 5 locations [near edge, .75 radius (R), .5 R, .25 R, and center] at the end of the cylinder (Figure 1B), which was then placed in a freezer at −17.8, −28.9, or −40°C. The time of freezing was only that time required to freeze to the selected temperature based on freezing curves. When all temperatures (thermocouples) were below the selected freezing point, the cylinder (AF) was removed from the freezer, thawed, and finally sampled for the extraction of proteins for electrophoresis. The remaining subblock piece was sampled as PF cheese.

[1] Reference to brand or firm names does not constitute endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

Extraction of Proteins from Cheeses

Approximately 2 g of cheese were solubilized in 5 ml of protein solvent (.166 *M* Tris/1 m*M* EDTA/pH 8.0) by homogenization with a Polytron ST-20.[1] Five milliliters of 7% SDS were added and the sample was gently homogenized by hand to minimize foaming. For protein reduction, 2 ml of 10 m*M* dithiothreitol were added to the homogenized sample, which was then stirred for 15 min and centrifuged for 1 h at 40,000 × *g* at 5°C. Three fractions were obtained: a lipid layer, supernatant, and a pellet. The chilled lipid plugs were carefully removed with a spatula and the clear orange-yellow supernatant was decanted through wiping tissue paper to entrap pieces of lipid plug. The lipid plugs and the pellets were washed with the same solvents (for the pellet only, the volume was reduced 1/10), homogenized, and centrifuged in order to obtain additional supernatants from both fractions. All three supernatants from the lipid layer (fs), original supernatant (cs), and from the pellet (ps) were lyophilized. The washed pellet (pp) was resuspended by homogenization in a minimum amount of deionized water before lyophilization.

Polyacrylamide Gel Electrophoresis

Proteins derived from the fractions of cheese were examined by PAGE by the discontinuous system described by Laemmli (8), as modified for an E-C Vertical Slab Gel Apparatus by Basch et al. (1). The stacking and separating gels were 4 and 15% acrylamide (% T), respectively, crosslinked with bisacrylamide (2.67% C). Gels were run for 13 h and stained for protein with Coomassie blue R250.

Gel Scanning

Quantitative analyses of electrophoretic separations were accomplished by scanning the gels at 550 nm in a Gelman Model 18 automatic computing densitometer. Total milk protein concentrates (TMP) were used as a standard for every gel. Graphical analysis of the protein composition data was carried out on an IBM-XT computer (Armonk, NY) using Lotus 1-2-3 program (Lotus Development Corp., Cambridge, MA).

Theory and Data Analysis

Preliminary analysis of the disappearance of total casein with time showed good correspondence with first order decay. However, inspection of the changes of individual components with time showed more complex patterns. In the conversion to the log form some of these inflections are suppressed. To analyze these time-dependent changes more accurately by nonlinear regression, the following theory was developed. It was first assumed that decreases in individual components (α_{s1}-, α_{s2}-, β-casein, etc.) are due to proteolytic cleavages; this of course is substantiated by the data of Fox (6). Furthermore, these changes could be due to the time-dependent action of different enzymes [e.g., chymosin (rennin), plasmin, acid proteases, etc.]. Thus, for such consecutive events the change in content of a given component would be:

$$\begin{array}{ccccc} P_0 + nE_1 & \overset{k_1}{\rightarrow} & P_1 + mE_2 & \overset{k_2}{\rightarrow} & P_2 \\ (C_0) & & (C_1) & & (C_1 - C_2) \\ & & + & & + \\ & & qE_3 & & qE_3 \\ & & \vert & & \vert \\ & & \vert k_3 & & \vert k_3 \\ & & \downarrow & & \downarrow \\ & & P_4 & & P_3 \\ & & \text{(Not observed)} & & (C_3) \end{array} \qquad [1]$$

Here P_1 represents various possible reactions undergone by a casein component P_0. The quantities in parentheses represent the concentration of these components, E_i represents different proteolytic enzymes responsible for observable changes in molecular weight such that the SDS-gel mobility of the component is changed, and k_i is the rate for the conversion carried out by enzyme i. In all these experiments, the cheese blocks are considered to be homogeneous and representative due to the sampling as described. Because the reaction is occurring in a solid state matrix the actual change in C_0 will depend not only on the actual catalytic rate constant, k_{cat}, but also on the diffusion coefficient of the enzyme in the matrix. Although such relationships are complex, data relating changes in C_0 with time can be thought of as linked thermodynamically through diffusion, so that the equations developed by Wyman (12) could be tested here. Thus, the apparent concentration of a casein component at any time can be given as:

$$C_{app} = C_0 f(P_0) + C_1 f(P_1) + (C_1 - C_2) f(P_2) + C_3 f(P_3) \qquad [2]$$

where f represents a "collisional binding function" for the enzyme with the component fixed in the matrix and the following relationship for reaction of enzyme with protein can be empirically derived from Wyman's binding isotherms (12) as previously shown (5):

$$C_{App} = \frac{C_0 P_0}{P_0 + P_0 E_1^n} + \frac{C_1 P_0 E_1^n}{P_0 + P_0 E_1^n} + \frac{(C_1 - C_2) P_1 E_2^m}{P_1 + P_1 E_2^m} + \frac{C_3 P_1 E_2^m E_3^q}{P_1 + P_1 E_2^m + P_1 E_3^q + P_1 E_2^m E_3^q} \qquad [3]$$

Cancellation and collection of terms yields:

$$C_{App} = \frac{C_0}{1 + E_1^n} + \frac{(C_1) E_1^n}{1 + E_1^n} + \frac{(C_1 - C_2) E_2^m}{1 + E_2^m} + \frac{(C_3) E_2^m E_3^q}{(1 + E_2^m)(1 + E_3^q)} \qquad [4]$$

In such experiments E would represent a concentration term. Indeed, Fox (6) has shown that added plasmin, for example, increases the rate of disappearance of β-casein. But as noted, diffusion through the matrix must occur first; in ordinary enzyme kinetics the latter is neglected; here it cannot be. According to Bull (2):

$$D = \frac{dQ}{A \cdot dt \cdot dC/dx} \qquad [5]$$

where D is the diffusion constant, dQ the quantity of material (enzyme) that diffuses across a plane surface A, in time dt under a concentration gradient dC/dx. If we assume that at the time of cheese manufacture the residual

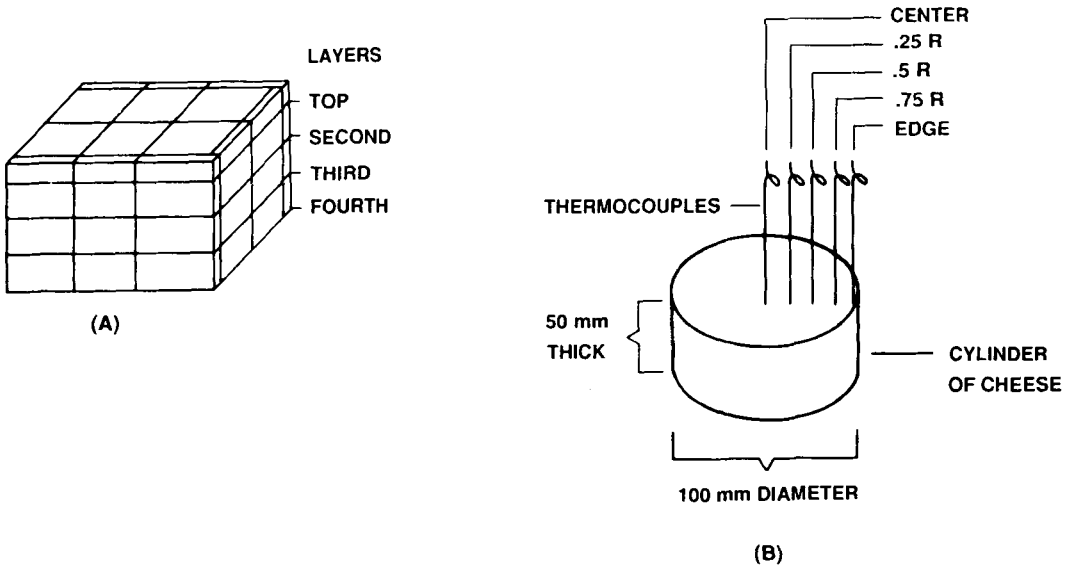

Figure 1. A. Diagram of subblock divisions in a 40-lb block of cheese. B. Location of thermocouples used to determine freezing time of cheese. R = Radius.

chymosin, plasmin, or other enzymes are uniformly distributed throughout the block, then there may be no concentration gradient and dC/dx is a constant. Therefore:

$$\frac{dC}{dx} = \text{constant} = \frac{g}{V \cdot \ell} \qquad [6]$$

where C is replaced by g the grams of enzyme present in volume V, and x by length ℓ (the length of the sample). So combining Equations [5] and [6] we get:

$$dQ = \frac{g}{V \cdot \ell} \cdot A \cdot D \cdot dt \qquad [7]$$

Thus Q, the amount of enzyme, diffusing through the matrix toward a fixed substrate at any time (t) can be given by:

$$Q = E = \frac{gAD}{V \cdot \ell} t \qquad [8]$$

if we consider:

$$\frac{gAD}{V \cdot \ell}$$

as a lumped diffusion constant for a given block of cheese then:

$$E = k_{diffusion} \cdot t \qquad [9]$$

So far we have considered only physical diffusion but the observed conversion will also include factors for the rate constant $k_{kinetic}$ so in reality:

$$E = (k_{diffusion} + k_{kinetic})\, t \qquad [10]$$

Therefore, in our analysis, k_1 or k_2 in Equation [1] represents the quantity in parentheses in Equation [10]. The final equation used to analyze the data then is found by substituting Equation [10] into Equation [4] and using k_i:

$$C_{app} = \frac{C_0}{1 + k_1^n t^n} + \frac{C_1 k_1^n t^n}{1 + k_1^n t^n} + \frac{(C_1 - C_2) k_2^m t^m}{1 + k_2^m t^m} + \frac{(C_3) k_2^m t^m k_3^q t^q}{(1 + k_2^m t^m)(1 + k_3^q t^q)} \qquad [11]$$

It should be stressed here that the latter expression is valid only for sequential binding (reaction), i.e., $k_1 > k_2 > k_3$ and where n sites react prior to the m sites. Also for n or m values greater than 1, k_1, k_2, and k_3 represent an average value for each of the n, m, or q sites.

The time-dependent profiles were directly analyzed using a Gauss-Newton nonlinear regression analysis program developed at this laboratory by William Damert. All profiles were analyzed by fixing the values of n and m and calculating the best least squares fit for the optimum evaluated k_1 and k_2 values. The n and m values were then fixed to new values and the whole procedure repeated. The n and m values, which yielded the minimum root-mean-square and lowest error values for k_1 and k_2, were then reported (5).

Protein Analysis

Protein in the supernatants obtained by the fractionation of cheese was determined by the Coomassie blue assay (1).

RESULTS AND DISCUSSION

Extraction and Sodium Dodecyl Sulfate-Gel Electrophoresis

Previous investigators have applied both SDS-PAGE and two-dimensional electrophoresis to the study of soft cheese ripening (11), whereas Fox (6) has studied Cheddar development using alkaline-urea PAGE. In preliminary experiments both fat pellicles and pellets were observed after homogenization of cheese in protein solvent (reducing agent added). Both of these fractions were re-extracted to ensure more complete recovery of protein as described in Materials and Methods. The three supernatants (cs. fs, and ps) and the final pellet (pp) samples were electrophoresed and the gels were scanned. In this way it was possible to visualize all of the proteins present in cheese.

Typically, electrophoretic patterns of cheese could illustrate as many as 24 protein bands. Electrophoretic separation of a standard total milk protein concentrate contains lactoferrin (MW 86,000), bovine serum albumin (67,000), immunoglobulin, heavy chain (~55,000), α_{s2}-casein (25,200), α_{s1}-casein (23,600), β-casein (24,000), κ-casein (19,000), γ_1-casein (20,600), β-lactoglobulin (18,400), α-lactalbumin (14,200), γ_2-casein (11,800), and γ_3-casein (11,600) (4). The caseins exhibit abnormal behavior in the SDS-PAGE (1). It is known that plasmin, a protease in milk, cleaves α_{s2}-, α_{s1}, and β-caseins into smaller components, which migrate faster than the parent caseins in the SDS-PAGE system (4). In a mature cheese or to a lesser extent in a younger cheese (Figure 2), proteolytic changes can be seen; cleavage of β-casein leads to formation of γ_1-, γ_2, and γ_3-caseins and also protease-peptone components 5, 8-fast, and 8-slow, which are at the front. Rennin hydrolyzes κ-casein into para-κ-casein and the macropeptides (4). In addition, microbial proteases may cause other changes (6). Because all the bands were too numerous for preliminary significant analysis, it was decided that the proteins of the cheese be grouped initially on the basis of protein classes. Figure 3 shows similar changes for stirred curd Cheddar at later stages of its development. A typical densitometric scan of the SDS-PAGE of a stirred curd cheese sample is shown in Figure 4. Each peak is identified as indicated in the figure. Other unidentified peaks are the result of breakdown of several larger proteins by enzymes. The integrator of the densitometer measures the area under the curve and the percent of each peak in the total 100% of all the peaks in the scan is

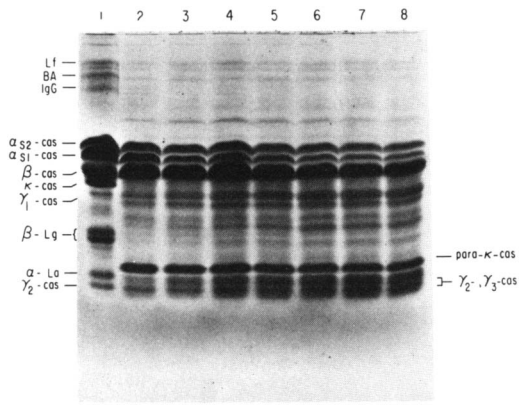

Figure 2. The SDS-PAGE of proteins extracted from Cheddar cheeses that had been ripened in storage at different lengths of time. 1, Total milk proteinate; 2, 3 wk; 3, 9 wk; 4, 14 wk; 5, 20 wk; 6, 26 wk; 7, 39 wk; and 8, 52 wk. Lf = Lactoferrin, BA = bovine serum albumin; IgG = heavy chain Ig; cas = casein; Lg = lactoglobulin, La = lactalbumin.

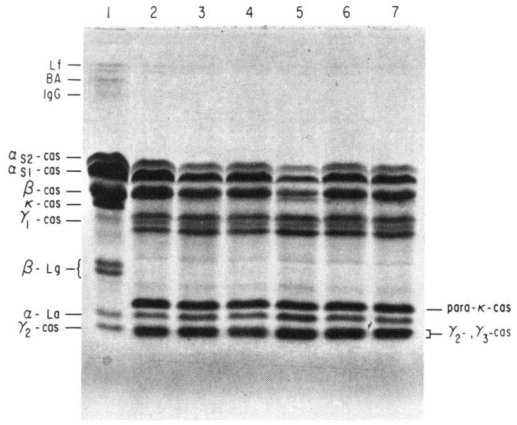

Figure 3. The SDS-PAGE of proteins extracted from stirred curd cheeses that had been ripened in storage at different lengths of time. 1, Total milk proteinate; 2, 22 wk; 3, 22 wk (after freezing); 4, 30 wk; 5, 36 wk; 6, 42 wk; and 7, 52 wk. Lf = Lactoferrin; BA = bovine serum albumin; IgG = heavy chain Ig; cas = casein; Lg = lactoglobulin; LA = lactalbumin.

computed. Peaks 1 to 6 include all the protein bands, which migrate in the gel slower than α_{s2}-casein. The α_{s2}-, α_{s1}, and β-caseins comprise peaks 7 to 9. Peaks 10 to 12 contain γ_1-casein and other proteins that migrate ahead of β-casein. Peaks 13 to 15 include the proteins that migrate between γ_1-casein and para-κ-casein. Several bands of low intensity migrating slower than para-κ-casein (peak 19) are included in peaks 16 to 18. Peaks 20 to 24, which migrate faster than para-κ-casein, may include γ_2- and γ_3-caseins, fragmentary products of β-casein.

Comparisons of four fractions (cs, fs, ps, and pp) of young Cheddar cheese (9 wk), both before and after freezing at 0, −20, and −40 are shown in Table 1. With the exception of peaks 1 to 6, for each protein group the percent of total protein value for each fraction is similar and not statistically different. For 26-week-old cheese there are no significant differences among the fractions except for peaks 20 to 24. Analysis of aged Cheddar cheese (52 wk) is also shown in Table 1; as before, there are few significant differences among the major proteins in the four fractions with the exception of four values randomly distributed in pp and ps. Coomassie blue dye binding assay showed that the cheese supernatants represent 89 ± 5% of the total extractable cheese protein. Therefore, during the rest of the study, the supernatant from the cheeses was used for data, graphical comparisons, and interpretations.

Analysis of Stored Cheddar

Gel electrophoresis patterns of supernatants of traditional Cheddar cheese (PF) that had been ripened for 3, 9, 14, 20, 26, 39, and 52 wk are shown in Figure 2. Intact κ-casein is not observed in any of the cheese samples due to the addition of rennin along with the starter culture during its earliest stage of manufacture. It is likely that para-κ-casein is a normal constitutent of cheese and not a product of storage, since the amount of para-κ-casein detected is constant throughout the ripening ages of cheese as measured by the gel scans. This result agrees with Grappin et al. (7) who reported that para-κ-casein, unlike other caseins from cheese, was not degraded during ripening. It is of interest that when the cheeses are young, the percent total casein (peaks 7–9) are high (∿60%) and other protein groups are low. As the cheeses became older, the percent total casein decreases, whereas the other two groups (peaks 13 to 15 and peaks 20 to 24) increase.

Of special note in the gel is the increase in number of protein bands and in their intensity in the regions between β-casein and para-κ-casein and that below para-κ-casein. These data indicate that as the ripening age of cheese in-

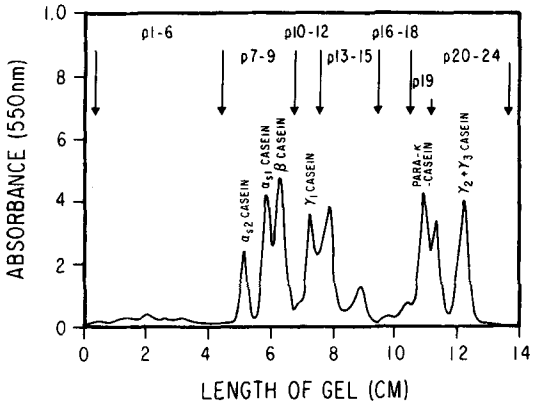

Figure 4. Typical densitometric scan of the SDS-PAGE gel of stirred curd cheese. Peaks are identified; unidentified peaks are the result of breakdown of caseins by the enzymes.

TABLE 1. Comparison of the protein distribution among fractions extracted from the same cheeses: cheese supernatant (cs), fat supernatant (fs), pellets (pp), and pellet supernatant (ps).

Storage and fraction	% of Total peak area						
	Peaks 1–6	Peaks 7–9	Peaks 10–12	Peaks 13–15	Peaks 16–18	Peak 19	Peaks 20–24
9 wk							
cs	1.8	62.6	8.6	3.3	.5	10.8	12.9
fs	14.2*	55.6	4.1	3.8	0	12.4	10.1
pp	17.3*	50.1	7.5	6.3	0	8.1	10.2
ps	9.8	57.5	6.9	7.4	0	8.4	10.2
26 wk							
cs	6.8	38.2	14.5	8.8	0	12.3	19.7
fs	13.4	41.9	5.8	6.8	1.1	11.6	10.4*
pp	13.2	35.7	8.8	8.9	1.3	10.8	9.4*
ps	21.6	35.7	9.2	14.4*	.6	7.5	9.8*
52 wk							
cs	9.8	27.0	7.6	13.5	3.0	9.3	28.4
fs	12.5	26.3	7.0	13.2	2.8	9.1	27.7
pp	14.6	26.0	2.1*	10.4	.7*	6.1	23.7
ps	12.3	28.8	6.7	14.1	.5*	8.7	15.8*

*Means followed by asterisk are significantly different from cs fraction at that age; n = 6 for each fraction (cs, fs, pp, ps) at each age, 3 prefrozen, and 3 after frozen samples.

creases, there is breakdown of major caseins, i.e., β-casein into γ_2- and γ_3-caseins and also α_{s2}- and α_{s1}-caseins into large fragmentary proteins (4, 7).

Analysis of Stirred Curd Cheddar

Protein band patterns of stirred curd cheese that had been ripened for 22, 30, 36, 42, and 52 wk are in Figure 3. Lane 2 illustrates a pattern of a cheese sample that was electrophoresed immediately after a block of stirred curd cheese was received from a local dairy. It was found that this sample was already 22 wk old when we received it. The gel indicates, in contrast with traditional Cheddar, no significant changes in banding patterns as the ripening age increased. Comparison of the data from the two gels (Figures 2 and 3) shows that fewer changes occur in the protein groups below γ_1-casein with stirred curd cheese of the same ages as traditional Cheddar cheese.

Effect of Freezing

The effects of freezing temperatures and of conditions (PF and AF) were studied on young Cheddar cheese. In each case, three PF samples were compared to one each AF at 0, −20, and −40°F freezing temperatures. The similarity of the data indicated that different freezing temperatures did not affect the composition of cheese with 9 wk ripening age. In addition, the protein composition of cheese before freezing compared with that after freezing shows no significant difference between the samples when the AF data were pooled to yield n = 3 (Table 2). Effects of freezing temperatures and conditions on middle aged cheese (26 wk) showed no significant differences among protein groups. A similar trend occurred for Cheddar cheese aged to 52 wk (Table 2).

Because of the nearly identical values in terms of freezing temperature and condition (PF and AF) the following general observation was made: the casein content (peaks 7 to 9) decreased steadily while protein groups (peaks 13 to 15 and peaks 20 to 24) increased as the cheese aged. This clearly indicates that the caseins degrade while the cheese ripens.

TABLE 2. Effects of freezing and thawing on the gel protein patterns of cheese supernatants (cs) at selected ages.

Storage time	n and conditions	% Total peak area (upper); σ (lower)						
		Peaks 1–6	Peaks 7–9	Peaks 10–12	Peaks 13–15	Peaks 16–18	Peak 19	Peaks 20–24
9 wk	3-PF	2.1	63.1	8.3	3.6	0	11.0	12.0
		.6	2.3	1.5	.4		.6	1.1
	3-AF	1.5	62.1	8.9	3.0	.7	10.5	13.8
	0, −20, −40	.6	2.1	.9	.5	.5	.6	.8
26 wk	3-PF	7.0	37.8	13.0	10.1	0	11.4	20.9
		1.2	2.6	1.1	1.8		1.1	.6
	3-AF	6.7	38.5	16.0	7.3	0	13.3	18.5
	0, −20, −40	2.0	1.8	2.4	1.4		1.3	.8
52 wk	3-PF	11.3	27.1	7.0	13.9	3.4	8.9	27.8
		.8	2.6	.4	1.4	1.3	.7	2.1
	3-AF	8.2	26.9	8.2	13.0	2.6	9.7	29.0
	0, −20, −40	1.1	2.1	.6	1.1	.6	.6	2.1

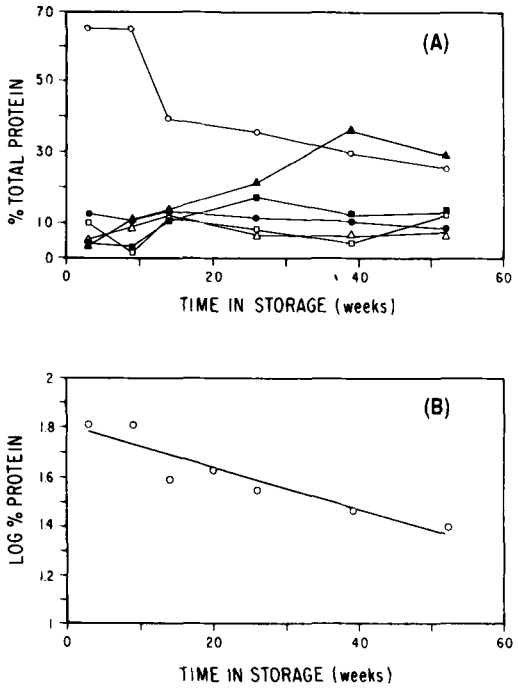

Figure 5. A. Relationship of the percent total protein of each protein group to storage time. Peaks 1 to 6 (□); peaks 7 to 9 (○); peaks 10 to 12 (△); peaks 13 to 15 (■); peak 19 (●); and peaks 20 to 24 (▲). Data were pooled from CS fraction of Cheddar cheese frozen at −17.8°C using both PF and AF data. B. Semilog plot of disappearance curve of casein group (peaks 7 to 9) of Cheddar cheese. Half-life or time of half-disappearance was calculated to be 35.5 wk.

Effects of Ripening

Figure 5A shows a plot of percent total protein versus ripening ages of traditional Cheddar cheese; PF data were considered (n = 3 for each point). The percent of casein group (peaks 7 to 9) decreases abruptly between 9 and 14 wk while that of the degraded products (peaks 20 to 24) increases until about 39 wk when it starts to decline. The percentages of the rest of the protein groups, especially para-κ-casein (peak 19), were consistent over the time studied.

To make the data more meaningful, a semilog plot of the disappearance curve of casein group (peaks 7 to 9) of Cheddar cheese before freezing was constructed, as shown in Figure 5B with the regression line drawn through the data points. The correlation coefficient of the curve was .9323 with a confidence level of about 97%. Using the slope of the regression line, the decay constant λ was calculated and used for the half-life equation,

$$t_{1/2} = \frac{.693}{\lambda}.$$

Half-life, or time of half-disappearance, was 35.5 wk for the casein groups. The casein groups declined rapidly between 9 and 14 wk, but it took about 35 wk of ripening for the group as a whole to decrease 50%.

Application of Nonlinear Regression Analysis

Because the logarithmic form used in Figure 5B can obscure subtle changes, it was decided

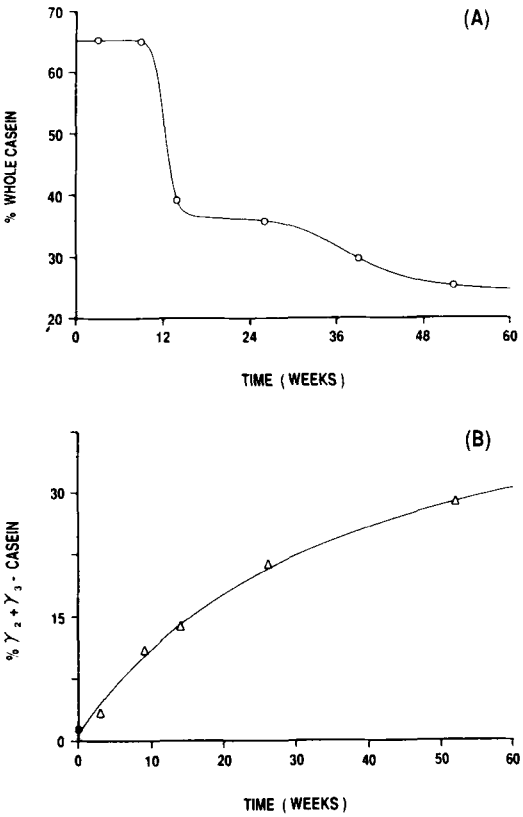

Figure 6. Nonlinear regression plot of A) the disappearance of whole casein (peaks 7 to 9) (○) and of B) the appearance of degraded products (peaks 20 to 24 group which contains γ_2- and γ_3-caseins) (△) for Cheddar cheese. Filled circle represents percent present in caseinate before cheese making. Data were analyzed by Equation [11]; results are given in Table 3.

to analyze the decay of the caseins directly using nonlinear regression analysis. Equations for this method have been presented (Materials and Methods). Data for the disappearance of whole casein (Figure 6A) and the appearance of γ_2- and γ_3-caseins (Figure 6B) were analyzed by Equation [11]. From analysis of the whole casein curve, four parameters are obtained: k_1, k_2, n, and m (results of all analyses are given in Table 3). Parameters k_1 and k_2 represent the summed k of Equation [10] and, although $k_{kinetic}$ plays a role, $k_{diffusion}$ predominates. Hence, the bimodality of Figure 6A argues for either two widely different enzymes with different diffusability or two very different enzyme concentrations since E (Q) of Equation [8] contains the grams of enzyme present as well as the diffusion constant of the enzyme. Thus, for example, residual starter enzyme (chymosin) could be present at high initial concentrations and produce a rapid change (k_1 and n) while more dilute plasmin, could produce the secondary effects (k_2 and m). In terms of cheese storage, it may be more effective to discuss these constants (k) in terms of their reciprocals, which can be thought of as the half-times for the transitions or the half-life of the casein components (see Table 3). Whole casein decays in a bimodal form with inflections at 12 and 38 wk while γ_2- and γ_3-caseins appear with a half-time of 33 wk. It thus appears that major changes in cheese composition occur early with α_{s1}-caseins (and possibly α_{s2}-caseins), decaying rapidly, followed by the breakdown of β-casein around 37 wk.

In order to test this hypothesis, the whole casein group (peaks 7 to 9) was subdivided into α_{s1}-, α_{s2}-, and β-caseins and the gel data replotted for disappearance of each casein. Data for changes in peaks 13 to 15 and para-κ-casein were also plotted. In viewing the data for α_{s1}-casein (Figure 7A), it became apparent that even at the earliest times (3 wk) a substantial portion of this component, based upon its normal relative distribution in milk (3), was degraded. It was therefore decided to fix the zero time, y-axis, as the percent of the component present in acid caseins electrophoresed and scanned by the same method. The values for five acid caseinates, each scanned twice, are given in Table 4. These values were then used as the zero time data point for all the nonlinear regression analysis of casein component appearance or disappearance. The computer fitted plots for these data are given in Figures 7 through 9; results are in Table 3.

Separation of the data showed interesting results. The most rapidly cleaved casein is α_{s1}-casein, which disappears with a half-life of 1.95 wk based upon extrapolation to α_{s1}-casein in caseinate (Figure 7A). The extrapolation to zero time may prejudice the data somewhat, but the implication is that this protein rapidly degrades (without extrapolation the half-life is still less than 8 wk). It has long been suggested that curd formation may be related to α_{s1}-casein (9). However necessary α_{s1}-casein is for curd formation, its rapid degradation points to the fact that loss of structure may be necessary for proper cheese formation.

TABLE 3. Nonlinear regression coefficients for time-dependent changes in casein components in Cheddar cheese supernatant (cs).

Casein component	k Subscript	k_i Weeks^{-1}	SE of k_i	Half-time in weeks $1/k_i$	Exponent	Change
Peaks 7–9	k_1	.0818	.0002	12.2	16	Decrease
	k_2	.0263	.0002	38.0	8	Decrease
γ_2- + γ_3-	k_1	.0270	.0033	37.0	1	Increase
para-κ-	k_1	.0140	.0027	71.4	1	Decrease
α_{s2}-	k_1	.0132	.0032	75.2	1	Decrease
α_{s1}-	k_1	.5124	.0229	1.95	1	Decrease
Peaks 13–15	k_1	.0613	.0033	16.3	1	Increase
β-	k_1	.0530	...	18.8	16	Increase
	k_2	.0991	...	10.0	1	Decrease
	k_3	.0265	...	37.7	12	Decrease
β- + β_0-	k_1	.0237	.0033	36.6	8	Decrease

TABLE 4. Densitometer averages for percentage composition of sodium caseinates made from pooled whole milk samples.

Casein component	% of Total[1]	
	Area	σ
α_{s2}-	9.23	1.90
α_{s1}-	33.42	1.66
β-	30.56	.81
κ-	14.51	2.20
γ_1-	2.91	.52

[1] Average ± σ of five different preparations each scanned twice.

The larger proteolytic fragments peaks 13 to 15 arise with a half-time of 16 wk; the nature of these fragments are unknown but may represent α_{s1}-casein fragments (Figure 7B).

Separation of the data produced a decay curve for α_{s2}-casein with a theoretical inflection of 75 wk predicting an extremely long persistance of this protein as a major cheese component (Figure 8A). Similar results were obtained for para-κ-casein with $1/k_1$ equal to 71 wk (Figure 8B). Here to obtain a zero time estimate, the average κ-content of acid caseinate was used on the y-axis. These two cysteine (or cystine)-containing components persist for the

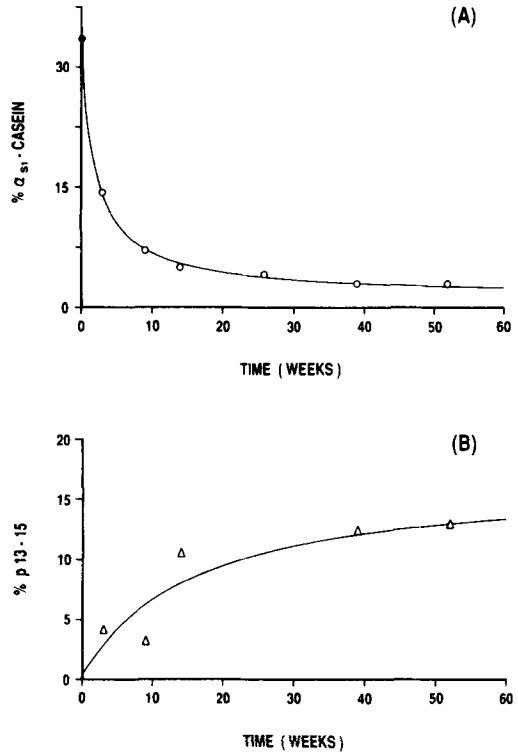

Figure 7. Nonlinear regression plot of A) the disappearance of α_{s1}-casein (○) and of B) the appearance of degraded products (peaks 13 to 15) (△) for Cheddar cheese. Filled circle represents percent present in caseinate before cheese making. Data were analyzed by Equation [11]; results are given in Table 3.

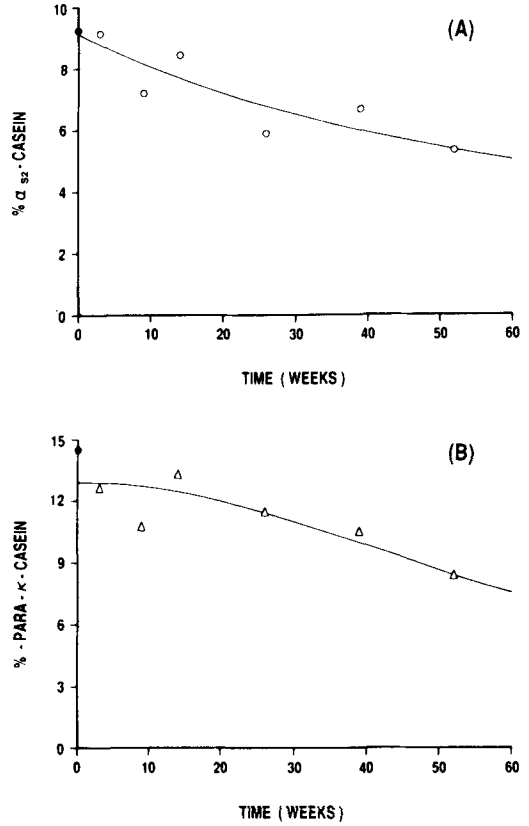

Figure 8. Nonlinear regression plot of A) the disappearance of α_{s2}-casein (○) and of B) the disappearance of para-κ-casein (△) for Cheddar cheese. Filled circle represents percent present in caseinate before cheese making. Data were analyzed by Equation [11]; results are given in Table 3.

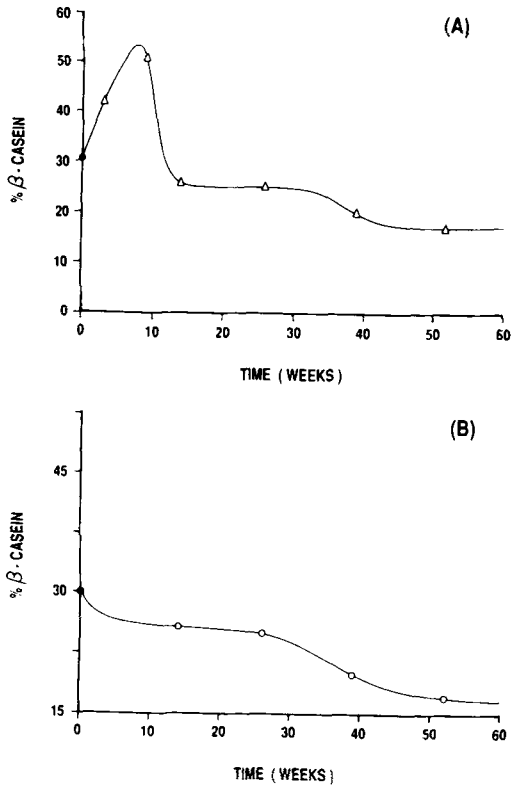

Figure 9. Nonlinear regression plot of A) the apparent disappearance of β-casein (peak 9) (Δ); B) replot subtracting theoretically skewed data due to α_{s1}-I (○). Filled circle represents percent present in caseinate before cheese making. Data were analyzed by Equation [11]; results are given in Table 3.

longest periods of time with predicted half-lives beyond the scope of most normal cheese storage.

Analysis of the changes in β-casein produced at first a curve (Figure 9A) which mirrored that of whole casein (Figure 6A). When the zero time value for β-casein was added to the y-axis then the data appear to have three inflections. Analysis by nonlinear regression gave these as 18.8, 10.0, and 37.7 wk. The apparent increases in β-casein followed by a decrease is apparently due to the rapid conversion of α_{s1}- to α_{s1}-I casein.

It has been shown by several investigators (9) that one of the first significant events in the cheese ripening process is the proteolytic breakdown of α_{s1}-casein to form the α_{s1}-I fragment. This cleavage is thought to be predominately due to chymosin action. In the electrophoretic system employed in this work, α_{s1}-I comigrates with β-casein as is the case for the SDS-PAGE system used by Trieu-Cuot et al. (11). Conversion of the α_{s1}-casein occurs predominately in the first 7 to 14 d of ripening. Thus, at 3 wk the α_{s1}-casein bands represent only 14% of the total caseinate, but β-casein has apparently risen to nearly 50% at 9 wk. As noted, we have included the data from Table 4 for α_{s1}-caseinate at the zero time axis for ripening. Using these data as a starting point half-lives of the α_{s1}- and α_{s1}-I caseins can be calculated and these transitions separated from that of β-casein. The half-life found for α_{s1}-casein was 1.95 wk in excellent agreement with data reviewed by Lawrence et al. (9). The rapid degradation of α_{s1}-casein probably produces α_{s1}-I (and others peaks 13 to 15). If one regraphs the β-casein without the data at 3 and 9 wk, then a single decay is observed (Figure 9B) and the half-time for disappearance is 36.6 wk, which corresponds nicely to the 37.0 wk half-time for appearance of $\gamma_2 + \gamma_3$ caseins. Correspondingly, the optimal concentration for α_{s1}-I appears to be at about 8 wk (Figure 9A).

Finally the exponential parameters n, m, and q need to be considered. In the study of binding isotherms (5) these generally reflect moles of ligand bound per mole of chelator. Here the meaning is somewhat different since the substrate is in a fixed matrix and changes in molecular weight must be due to diffusion of the enzyme followed by an effective proteolytic cleavage. It could reflect the average number of cleavage sites on the protein or the number of collisions required to lead to effective degradation.

Thus, thermodynamic linkage through diffusion may be related by way of Equation [4] to the relative concentration of the different species of enzymes and through Equation [10] to kinetically effective collisions. In the case of k_1 for α_{s1}-casein degradation if k is only diffusion related, then from k_1 a diffusion constant of 8.47×10^{-7} cm^2/s can be calculated. This value is quite close to the diffusion constant for proteins of 45,000 MW (2). All other k are at least 10× lower; thus, only this reaction approaches the correct value for diffusion in solution. All other conversions are much slower. In addition, interactions of the caseins in the solid matrix may play a role (e.g., it could be

speculated that until γ_2- and γ_3-caseins are formed, with resultant changes in cheese structure, few effective collisions of plasmin with α_{s2}-casein can occur). Other speculations could be made but thermodynamic linkage can only quantitate changes, it cannot prove or disprove molecular mechanisms that share a common ΔG of reaction.

The quality of the natural cheese is of prime importance for the properties of the finished process cheese. It has been suggested that the higher the relative casein content of the natural cheese, the better it is for the production of a stable process cheese. The high content of intact casein results in process cheese with a long filamentous structure. This structure is extremely stable against chemical, thermal, or mechanical influences and is hydrophobic, absorbing water slowly and in limited quantities. Young rennet cheese has a relative casein content of 90 to 95%, which decreases during ripening. The present study clearly shows that various caseins are degraded with different half-lives; α_{s1}-casein degrades first while β-casein persists for a longer time and α_{s2}-casein and para-κ-casein extend beyond 70 wk before degrading.

Process cheese is produced from blends of cheeses of different ages. Generally about 75% of the blend consists of 3-mo (12-wk)-old cheeses with the remainder coming from 24 to 52-wk-old cheeses. What appears to be important is a relatively high casein content (10). In the case of the younger Cheddars, our observations would indicate that the presence of α_{s1}-I casein is important. Its half-time for disappearance is 10.0 wk (Figure 9A); thus at 12 wk the majority of this protein (and of course α_{s1}-casein itself) has been converted to lower molecular weight components. Additionally, for the older cheeses, ripening up to the half-life of β-casein (37 wk) may be tolerated in mixtures; beyond this point poorly processed products may result.

Freezing at various temperatures has no effect on cheese. Thomas et al. (10) have indicated, however, that changes in the methods of manufacture of processed cheese from frozen cheeses are necessary. Thus, further experiments on the effects of long-term frozen storage need to be conducted.

ACKNOWLEDGMENTS

The authors wish to thank V. H. Holsinger for her helpful discussions on the cheese project and J. G. Phillips for his advice and contributions to the statistical analyses.

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