Impact of Milk Preacidification with CO₂ on the Aging and Proteolysis of Cheddar Cheese*

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ABSTRACT

To determine the influence of milk preacidification with CO₂ on Cheddar cheese aging and proteolysis, cheese was manufactured from milk with and without added CO₂. The experiment was replicated 3 times. Carbon dioxide (approximately 1600 ppm) was added to the cold milk, resulting in a milk pH of 5.9 at 31°C in the cheese vat. The starter and coagulant usage rates were equal for the control and CO₂ treatment cheeses. The calcium content of the CO₂ treatment cheese was lower, but no difference in moisture content was detected. The higher CO₂ content of the treatment cheeses (337 vs. 124 ppm) was maintained throughout 6 mo of aging. In spite of having almost one and a half times the salt-in-moisture, proteolysis as measured by pH 4.6 and 12% trichloroacetic acid soluble nitrogen expressed as percentages of total nitrogen, was higher in the CO₂ treatment cheeses throughout aging. The ratio of α_s -casein (CN) to para- κ -CN decreased faster in the CO₂ treatment cheeses than in the control cheeses, especially before refrigerated storage. No difference was detected in the ratio of β -CN to para- κ -CN between the control and CO₂ treatment cheeses. Intact α_s - and β -CN were found in the expressible serum (ES) from the CO₂ treatment cheese as well as α_{s1} -I-CN, but they were not detected in the ES from the control cheese. No CN was detected in the ES from the curd before the salting of either the control or CO₂ treatment cheese. Higher proteolysis in the cheese made from milk preacidified with CO₂ may have been due to increased substrate availability in the water phase or increased chymosin activity or retention in the cheese.

(Key words: Cheddar cheese, carbon dioxide, proteolysis)

Abbreviation key: ES = expressible serum, MOSA = method of standard additions, SNPTN = soluble nitrogen as a percentage of total nitrogen, TA = titratable acidity, TN = total nitrogen, USMC = unsalted milled curd.

INTRODUCTION

Carbon dioxide has been shown to extend the shelf life of dairy products (Rashed et al., 1986; King and Mabbitt, 1987; Hotchkiss and Lee, 1996; Ma et al., 2003). King and Mabbitt (1982) reported that CO₂ increased the lag phase of microbiological growth in raw milk. Carbon dioxide directly decreased the colony count of *Pseudomonas fluorescens* in raw milk, not indirectly due to displaced dissolved oxygen or lower pH (King and Mabbitt, 1982). Ma et al. (2003) suggested that in addition to the suppressed microbiological growth, plasmin activity may be inhibited in raw milk with added CO₂ because of the lower milk pH. The physicochemical changes that occur as milk pH changes due to CO₂ content may be as important to cheese making as the antimicrobial effect. Carbon dioxide not only lowers the pH of milk but also lowers the freezing point of milk by increasing the dissolved solutes (Ma and Barbano, 2003).

Lowering the pH of milk increases the soluble calcium and phosphate (Dalgleish and Law, 1989). Casein also dissociated from the micelle when the pH of milk was decreased (Dalgleish and Law, 1988; Law and Leaver, 1998). As temperature was increased from 4 to 30°C at the maximum dissociation pH for each temperature (5.1 and 5.5, respectively), less CN was dissociated (over 50% at 4°C and less than 10% at 30°C) from the micelle (Dalgleish and Law, 1988). Metzger et al. (2001) reported that Mozzarella cheeses manufactured from milk preacidified (pH 5.8 and 6.0) with acetic and citric acids had higher pH 4.6 and 12% TCA soluble nitrogen as a percentage of total nitrogen (SNPTN) contents than the control cheese. Because CO₂ behaves as an acid when dissolved in milk, it may be used to preacidify milk for cheese making instead of using acetic or citric acids. If CO₂ could enhance

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proteolysis in Cheddar cheese similar to acetic and citric acids in Mozzarella cheese and be removed from whey, it could be a valuable processing aide to enhance Cheddar cheese aging.

Of the several reported experiments using CO₂ as an acidulant prior to cheese manufacture, most have focused on the antimicrobial and coagulation firming effects of CO₂ (Calvo et al., 1993; McCarney et al., 1995; Ruas-Madiedo et al., 2003). Others have reported the effects of CO₂ on Cheddar cheese yield (McCarney et al., 1995; St-Gelais et al., 1997). Proteolysis is important to Cheddar cheese flavor development (Aston and Creamer, 1986). Proteolysis data (either CN degradation or an increase in soluble nitrogen) were reported by 2 groups of researchers (Montilla et al., 1995; St-Gelais et al., 1997). Montilla et al. (1995) produced Iberico cheese from milk acidified to pH 6.0 with CO₂ and reduced rennet usage rate by 75% compared with the control cheese. They reported less proteolysis in the cheese from the CO₂ treatment. St-Gelais et al. (1997) produced Cheddar cheese from milk acidified to pH 6.56 with CO₂ and used 25% less coagulant in their CO₂ treatment. St-Gelais et al. (1997) found no difference in pH 4.5 soluble nitrogen between CO₂ treatment and control cheeses and a high TCA soluble nitrogen for the control cheese. The impact that added CO₂ would have on proteolysis without a simultaneous reduction in rennet usage cannot be determined from these studies.

To measure the impact of CO₂ addition to milk on Cheddar cheese proteolysis during aging, we made cheese from milk containing CO₂ and kept the coagulant usage rate the same for both the control and CO₂ treatment cheeses. Grappin et al. (1985) has highlighted the important role that the coagulant plays in the primary proteolysis of cheese. A milk pH closer to the range of 5.8 to 6.0, the range used in Mozzarella cheese by Metzger et al. (2001), was chosen for the CO₂ treated milk for this study of Cheddar cheese.

MATERIALS AND METHODS

Experimental Design

One 18-kg block of milled-curd Cheddar cheese (35 × 29 × 19 cm) was manufactured per treatment (from milk with added CO₂ and without added CO₂) on 3 different days. Pasteurized whole milk was carbonated to approximately 1600 ppm CO₂, which resulted in a milk pH at the vat of 5.93 compared with 6.65, at 31°C, for the control cheese. Cheese manufacturing conditions were kept constant for the 2 treatments, except that the whey from the milk with added CO₂ was drained at pH 5.96 compared with 6.35 for the control cheese. The addition of CO₂ decreased the total

make time because of the shorter stir-out time. The usage rates of chymosin and salt were the same for both treatments. Cheeses were pressed overnight (17 h). When the cheeses were removed from the press, the temperature in the center of the blocks was approximately 29°C. A more detailed description of cheese making conditions is described by Nelson et al. (2004). The CO₂ content, titratable acidity (TA), pH, SNPTN, and CN degradation of the cheeses were monitored over 6 mo of aging at 6°C. Changes in the water phase (monitored by analysis of expressible serum; ES) were determined.

Sampling and Sample Preparation

Unsalted milled curd and cheese sampling. Unsalted milled curd (USMC) samples were taken after milling at pH 5.3, placed in plastic bags, and immediately prepared for removal of ES. Cheeses were sampled by removing 3 cross-sections of cheese, with approximate dimensions of 1 × 28 × 19 cm, from the center of the block immediately after the block was removed from the press. The first cross-section was vacuum packaged for compositional analysis. The second cross-section was used for the ES procedure. The sides of the third section were trimmed to leave a center piece approximately 9 × 15 cm, which was vacuum packaged and used for CO₂ analysis. After the 3 slices were removed from the center of the block, the 2 remaining pieces of the block were placed into a plastic bag and vacuum packaged for further aging. Sampling was done again at approximately 30, 90, and 180 d.

Sample preparation of USMC and cheese. Cheeses and USMC were cut into 2-cm pieces, ground (model 31BL92, Waring, New Hartford, CT) into 2 to 3 mm, packed into 59-mL snap-top vials leaving no head space, and either analyzed fresh or frozen at -80°C until the time of analysis. Cheese slices for CO₂ analysis were not ground but were cut into approximately 3-mm pieces immediately before analysis.

ES preparation. The ES from USMC and cheese immediately after pressing was collected at 25°C, as described in Guo and Kindstedt (1995), except that the samples were centrifuged at 23,500 × g. The ES from several centrifuge bottles for each treatment was combined to obtain enough sample for chemical analyses, placed in 59-mL snap-top vials, and frozen at -80°C.

Chemical Analyses

ES composition. Total nitrogen (TN) content of the ES was determined in duplicate using the Kjeldahl method (AOAC, 2000; 33.2.11, 991.20). Crude protein

was calculated by multiplying the TN by 6.38. Calcium content was determined in duplicate by atomic absorption (Metzger et al., 2000).

USMC and cheese composition and pH. The fat content was determined by the Babcock method (Marshall, 1992; 15.8.A). Moisture was determined gravimetrically by drying in a forced-air oven at 100°C for 24 h (AOAC, 2000; 33.2.44, 990.20) using a 2-g cheese test portion. Salt content was determined using the Volhard method (Marshall, 1992; 15.5.B). The Kjeldahl method (1-g test portion) was used to determine TN (Lynch et al., 2002), and CP was calculated ($\text{TN} \times 6.38$). Fat and salt content were not determined for USMC. Cheese pH was determined using a Xerolyt combination electrode (model HA405; Mettler Toledo, Columbus, OH) with an Accumet pH meter (model AR 25, Fisher Scientific, Pittsburgh, PA) after tempering to 23°C. Titratable acidity (AOAC, 2000; 33.7.14, 920.124) of the cheese was determined as described by Lau et al. (1991). All analyses were carried out in duplicate except TN and fat, which were performed in quadruplicate.

CO₂ content of milk and cheese. A method of standard additions (**MOSA**) was used to determine the CO₂ content of cheese, which was a modification of the method described by Ma et al. (2001) for determining the CO₂ content of milk. The MOSA was selected because the control cheeses contained a small background level of CO₂ (thus, no blank matrix was available) and because the technique is especially useful when an analyte (e.g., CO₂) is present in low concentrations near the level of quantitation, which was the case for the control cheeses. In the MOSA, the sample is tested initially and with increasing added amounts of the analyte, essentially creating a calibration curve using the sample itself (Miller, 1991; González et al., 1999).

For the initial CO₂ determination, cheese was cut into ~3-mm cubes, and 20 ± 0.1 g was weighed into a small, stainless steel blender assembly (catalog number 14-15-18B, mini-sample container, 37 to 110 mL capacity, Fisher Scientific, Pittsburgh, PA). This was followed by the addition of 20 mL of degassed reverse osmosis purified water and 10 mL of 1 N sulfuric acid. The blender was immediately covered with Parafilm M (Pechiney Plastic Packaging, Chicago, IL) and tightly secured with a rubber band. The contents of the jar were blended at low speed for 30 s then for 15 s at 1-min intervals for a total of 5 blends over a 5-min period. At 15 s after the last blend, a sticky nickel (catalog number 380-035, MOCON, Minneapolis, MN) was placed on the Parafilm M cover. The CO₂ content in the headspace was determined by sampling with a gas-sampling needle inserted through the sticky nickel,

taking care to keep the needle out of the cheese slurry. The sampling needle was connected to an infrared CO₂ analyzer (Pac Check 650, MOCON, Minneapolis, MN) previously calibrated with room air (0 CO₂) and 99.8% CO₂ (catalog number 23402, manufactured for Supelco, Bellefonte, PA, by Scott Specialty Gases). A reading of the CO₂ content (% CO₂) of the headspace was taken. After the initial reading was obtained, the same procedure was repeated 5 times using a new 20-g portion of the same cheese sample each time. Carbon dioxide levels were increased in 30 to 50% increments over the previous reading. Carbon dioxide was added to the sample by decreasing the amount of degassed water initially added and substituting a corresponding volume of sodium bicarbonate standard solution (0.5 g/100 g, equivalent to ~2.6 mg CO₂/g or 2600 ppm CO₂) so that the final amount of added standard solution and degassed water totaled 20 mL.

A MOSA linear regression equation ($y = mx + b$) was constructed from the initial and 5 determinations with added sodium bicarbonate standard solution, where y = instrument reading (% CO₂), m = slope, x = sodium bicarbonate added (expressed as ppm CO₂ in cheese) and b = intercept. The concentration of CO₂ in an individual cheese was calculated by extrapolation of the regression equation to $y = 0$ and then using the absolute value of x at $y = 0$. Visual inspection of the experimental data and the resulting coefficients of determination ($R^2 \geq 0.99$) indicated that the resulting regression equations were linear.

Proteolysis

Cheese pH 4.6 and 12% TCA SNPTN contents were determined in duplicate as described by Bynum and Barbano (1985). The SDS-PAGE method was done as described by Neocleous et al. (2002), except that a 7- μ L sample (1 g of cheese per 10 mL of sample buffer) was loaded per lane for all cheese samples, and a constant 15% concentration acrylamide gel was used. Results of the SDS-PAGE analysis were reported as the ratio of α_s -CN and β -CN to para- κ -CN. This was done to normalize the data for small variations in sample loading that can result from sample preparation, since para- κ -CN is not hydrolyzed during aging (Nath and Ledford, 1973). The ratios of α_s -CN and β -CN to para- κ -CN were used by Lau et al. (1991) and Neocleous et al. (2002) in the CN degradation calculations that were reported by those investigators. However, Lau et al. (1991) and Neocleous et al. (2002) used those ratios to calculate the percentage of CN degraded in the cheeses by using the first day of analysis as 0% of CN degraded. In those studies, there was no difference between treatments at time zero. As a result, the data from the

first day of analysis was not reported. In our experiment, the first day of analysis was very important because of the differences between treatments immediately after the cheeses were removed from the press. During aging, α_s -CN and β -CN were hydrolyzed, and their bands on the SDS-PAGE gel became less intense, whereas para- κ -CN remained constant. A decreasing ratio indicates proteolysis of either α_s -CN or β -CN.

Expressible serum from cheese and USMC were prepared using 0.9 mL of the sample buffer containing dithiothreitol as described by Verdi et al. (1987) and 0.1 mL of ES. The SDS was purchased from Sigma-Aldrich Chemical (L-4390; St. Louis, MO). A 10 to 20% SDS-PAGE gradient gel (Verdi et al., 1987) was used for ES electrophoresis. The USMC ES gels were loaded with 16 μ L of sample plus buffer per lane for both the control and CO₂ treatment cheeses. Cheese ES loadings of sample plus buffer for the control and CO₂ treatment samples were 8 and 4 μ L, respectively, because the ES from the CO₂ treatment cheese contained more protein than the control ES.

The presence of α_{s1} -I-CN in the ES of the CO₂ treatment cheeses was confirmed by an additional experiment, where an α_s -CN solution and milk were separately incubated with chymosin. Samples were then analyzed with our SDS-PAGE procedure. After 1 h of incubation, a large protein band was present below the α_{s1} -CN in the α_s -CN solution treated with chymosin. After 2 h, that band was more pronounced. A band in the same location was present in the milk sample after incubation with chymosin. The band appeared below the β -CN. Because α_{s1} -I-CN was found by Creamer and Richardson (1974) to be the primary proteolytic product of chymosin action on α_{s1} -CN, we were confident in identifying the unknown band in the ES of the CO₂ treatment cheese as α_{s1} -I-CN. The presence of the α_{s1} -I-CN band after the β -CN band in our gels differs from the report of Malin et al. (1995). Protein migration patterns can be different due to different sources of SDS (Swaney et al., 1974). Although Malin et al. (1995) did not report their source of SDS, the difference between the SDS used by them and in our laboratory was the likely cause of the α_{s1} -I-CN migration differences.

Statistical Analysis

The PROC GLM procedure of SAS was used for all data analysis (SAS version 8.02, 1999 to 2001, SAS Institute Inc., Cary, NC). The least significant difference test ($P \leq 0.05$) was used to compare treatment means of the compositional data if the F -test for the statistical model was significant ($P \leq 0.05$). One-way ANOVA was used to analyze cheese and USMC compo-

Figure 1. Mean ($n = 3$) CO₂ content of the control and CO₂ treatment cheeses during 6 mo of aging.

sition data. For comparison of the control and CO₂ treatment cheeses at any one sampling period (i.e., 0, 30, 90, and 180 d), a t -test was performed. ANOVA was used to analyze data over the aging period, and least square means are reported in the text for CO₂ content, TA, pH, SNPTN, α_s -CN:para- κ -CN, and β -CN:para- κ -CN of the control and CO₂ treatment cheeses over the 6 mo of aging. Age was analyzed as a continuous variable. A mathematical transformation of the age variable was necessary to minimize multicollinearity of the linear and quadratic forms of the age variable (Glantz and Slinker, 2001). The transformation of age, age = d of storage at 6°C - [(last testing d - first testing day d) / 2], made the data set orthogonal with respect to age. The quadratic form of age and the interaction of age $\times$ treatment were included in the model if significant or to show that the curvature was not detected in the case of CO₂ content during aging (Figure 1).

RESULTS

USMC and Cheese Composition

No difference ($P > 0.05$) was detected between the control and CO₂ treatment cheeses of USMC moisture and CP (Table 1). As expected, the calcium content of the USMC was lower for the CO₂ treatment cheese (Table 1) because of the lower pH at draining (5.96 vs. 6.35). No difference in CP, protein on a dry basis, moisture, and moisture in the nonfat substance was detected between the control and CO₂ treatment cheeses (Table 1). The fat content and fat on a dry basis were higher ($P \leq 0.05$) for the control cheese. The lower ($P \leq 0.05$) calcium content in the CO₂ treatment cheese was expected, but the higher ($P \leq 0.05$) salt

Table 1. Mean (n = 3) unsalted milled curd (USMC) and Cheddar cheese composition.

Component	Control, %	CO ₂ , %	LSD	SEM
USMC				
Moisture	46.58	46.35	NS	0.834
CP	20.29	20.92	NS	0.273
Calcium	0.63 ^a	0.46 ^b	0.063	0.016
Calcium/CP (×100)	3.09 ^a	2.20 ^b	0.344	0.088
Cheddar cheese				
Fat	34.19 ^a	32.83 ^b	1.024	0.261
FDB ¹	54.12 ^a	52.67 ^b	0.859	0.219
CP	23.72	24.02	NS	0.213
PDB ²	37.49	38.09	NS	0.423
Moisture	36.84	37.67	NS	0.285
MNFS ³	55.97	56.08	NS	0.246
Salt	1.44 ^b	2.24 ^a	0.119	0.030
Salt-in-moisture	3.92 ^b	5.96 ^a	0.385	0.098
Calcium	0.69 ^a	0.52 ^b	0.035	0.009
Calcium/CP (×100)	2.91 ^a	2.15 ^b	0.185	0.047

^{a,b}Means within a row without a common superscript differ ($P \leq 0.05$).

¹FDB = Fat on a dry basis.

²PDB = Protein on a dry basis.

³MNFS = Moisture in the nonfat substance.

content (Table 1) of the CO₂ treatment cheese was not expected.

The least square mean CO₂ content of the treatment cheese (337 ppm) was higher ($P \leq 0.01$) than the control cheese (124 ppm) and did not change during aging (Table 2, Figure 1). The least square mean pH of the control cheese, 4.98, was lower ($P \leq 0.01$) than the treatment's pH, 5.14. A linear age × treatment interaction was detected as well as a quadratic function of age ($P \leq 0.01$, Table 2, Figure 2). The least square mean TA of the control cheese, 1.01%, was higher than ($P \leq 0.01$) that of the CO₂ treatment cheese, 0.87%, and was consistent with the difference in pH (Figures 2 and 3). The TA increased as a linear function of cheese age (Table 2, Figure 3), and there was an age × treatment interaction, with the TA of the control

cheese increasing faster with age than the CO₂ treatment cheese (Figure 3).

Proteolysis

The CO₂ treatment cheese had higher (Table 2, $P \leq 0.05$) mean levels of pH 4.6 and 12% TCA SNPTN than the control cheese immediately after pressing, 6.44% vs. 4.79% and 2.71% vs. 2.03%, respectively (Figure 4). During aging, the CO₂ treatment cheese had a higher ($P \leq 0.01$) least square mean content of pH 4.6 SNPTN, 15.31%, than the control cheese, 13.08%. The CO₂ treatment cheese also contained more ($P \leq 0.01$) 12% TCA SNPTN, 6.85%, than the control cheese, 6.28%, during aging. The pH 4.6 and 12% TCA SNPTN increased in both the control and CO₂ treatment

Table 2. Type III SS for cheese CO₂, pH, titratable acidity (TA), soluble nitrogen as a percentage of total nitrogen (SNPTN), and ratios of α_s -CN and β -CN to para- κ -CN at 0, 30, 90, and 180 d of aging.

Factor	df	CO ₂	pH	TA	pH 4.6 SNPTN	12% TCA SNPTN	α_s -CN: para- κ -CN	β -CN: para- κ -CN
Treatment ¹ (T)	1	273494**	0.15**	0.14**	30**	2**	2**	0.1
Age (A)	1	3668	<0.01	0.61**	914**	236**	16**	3.3**
A × A	1	3686	0.02**	...	59**	10**	5**	...
A × T	1	...	0.01**	0.03*	...	...	1*	...
Error	19	...	0.02	...	...	...	3	...
Error	20	27160	...	0.13	8	3	...	...
Error	21	...	...	...	...	...	...	1.9
R ²		0.91	0.89	0.85	0.99	0.99	0.88	0.64

¹Treatments are cheeses manufactured from milk with and without added CO₂.

* $P \leq 0.05$.

** $P \leq 0.01$.

Figure 2. Mean ($n = 3$) pH of the control and CO₂ treatment cheeses during 6 mo of aging.

Figure 4. Mean ($n = 3$) soluble nitrogen as a percentage of total nitrogen (SNPTN) of the control and CO₂ treatment cheeses during 6 mo of aging. Open symbols indicate pH 4.6 SNPTN, and closed symbols indicate 12% TCA SNPTN.

cheeses over the 6-mo aging period (Figure 4) both as a linear and a quadratic function of age (Table 2, $P \leq 0.01$).

No difference ($P > 0.05$) in α_s -CN:para- κ -CN and β -CN:para- κ -CN ratios were detected between the USMC of the control and CO₂ treatment cheeses (data not shown). The difference in the α_s -CN:para- κ -CN ratio between control and CO₂ treatment cheeses was more pronounced at 0 d when the cheeses were removed from the press (Figure 5) than at any other time. When the cheeses were removed from the press, the CO₂ treatment cheese had a lower ($P \leq 0.05$) α_s -CN:para- κ -CN ratio than the control cheese, 2.48 and 3.87, respectively. The least square mean α_s -CN:para- κ -CN ratio of the CO₂ treatment cheese, 1.26, was lower ($P \leq 0.01$) than the control cheese, 1.88, during

6 mo of aging (Figure 5). The α_s -CN:para- κ -CN ratio changed both as a linear and quadratic function of age, and there was a linear age $\times$ treatment interaction (Table 2). No significant difference ($P > 0.05$) in β -CN:para- κ -CN ratio was detected between the control and CO₂ treatment cheeses immediately out of the press or during aging (Figure 5, Table 2). The linear function of age was significant (Table 2), because the β -CN:para- κ -CN ratio decreased in both the control and CO₂ treatment cheeses during the aging period.

USMC and Cheese ES

No difference ($P > 0.05$) in the amount of USMC ES was detected between the control and CO₂ treatment cheeses (Table 3). There was a large decrease in the

Figure 3. Mean ($n = 3$) titratable acidity of the control and CO₂ treatment cheeses during 6 mo of aging.

Figure 5. Mean ($n = 3$) ratios of α_s -CN to para- κ -CN (open symbols) and β -CN to para- κ -CN (closed symbols) in cheeses during 6 mo of aging.

Table 3. Mean ($n = 3$) weights and compositions of expressible serum (ES) from unsalted milled curd (USMC) and cheese, immediately after overnight pressing, removed at 25°C.

Treatment	Control	CO ₂	LSD	SEM
Weight, g ES/100 g of cheese				
USMC ES	17.03	15.74	NS	1.204
Cheese ES	5.65 ^a	2.90 ^b	1.752	0.446
CP, %				
USMC ES	1.40 ^b	1.46 ^a	0.026	0.007
Cheese ES	3.05 ^b	5.67 ^a	1.380	0.352
Calcium, %				
USMC ES	0.36 ^a	0.31 ^b	0.030	0.008
Cheese ES	0.54 ^a	0.36 ^b	0.042	0.011
Calcium/CP ¹ ($\times 100$), %				
USMC ES	25.57 ^a	20.91 ^b	1.809	0.461
Cheese ES	17.57 ^a	6.42 ^b	1.383	0.352

^{a,b}Means within a row without a common superscript differ ($P \leq 0.05$).

¹Calcium expressed as a percentage of CP.

amount of ES for both the control and CO₂ treatment cheeses due to salting and pressing. After salting and pressing, almost twice the amount of ES could be removed from the control cheese compared with the CO₂ treatment cheese (Table 3). The ES from the CO₂ treatment cheese USMC had a slightly higher ($P \leq 0.05$) CP content than the control cheese. After salting and pressing, the CP content of the ES from the CO₂ treatment cheese was much higher than the control cheese (Table 3). The calcium content of the USMC and cheese ES from the CO₂ treatment sample was lower than from the control cheese. Because the CP was higher and calcium was lower in the ES of the CO₂ treatment cheese, the calcium expressed as a percentage of CP was much lower ($P \leq 0.05$) than the control cheese for both the USMC and cheese. Neither the CO₂ treatment nor the control cheese USMC ES contained a detectable amount of CN on an SDS-PAGE gel (data not shown). Casein was found in the CO₂ treatment cheese ES but not in the control cheese ES (Figure 6).

DISCUSSION

Cheese CO₂ Content

A portion of the CO₂ present in milk preacidified with CO₂ remained in the cheese (Figure 1). Does the CO₂ reside in the fat or water phase of the cheese? Ma and Barbano (2003) report that injecting CO₂ into milk at a low temperature (0°C) results in more of the CO₂ dissolving in the water phase of milk. When CO₂ was injected into milk at 40°C, some of the CO₂ dissolved in the fat phase resulting in a higher milk pH when injected with the same level of CO₂ as the cold milk (Ma and Barbano, 2003). The milk in the current study was cold (4°C) when injected with CO₂; however, the milk was warmed to 31°C for starter and coagulant

addition. The CO₂ contents of the control and CO₂ treatment milks, after they had been heated to 31°C, were 88 and 1615 ppm, respectively (Nelson et al., 2004). In total, the milk remained at 31°C for 1 h 20 min, with very little agitation except during starter, color, and coagulant addition. Stirring the milk and whey in the cheese vats likely caused some CO₂ loss to the atmosphere. Agitation of carbonated milk in open containers has been reported to increase the loss of CO₂ from milk, indicated by an increase in milk pH, compared with carbonated milk in an open container without agitation (Champagne et al., 1998). Certainly, during the 1 h 30 min that the milk/curd/whey were in the vat at 31°C, CO₂ was lost to the atmosphere. The temperature of the curds and whey was increased

Figure 6. Proteins in expressible serum (ES; 25°C) of Cheddar cheese, immediately after overnight pressing (approximately 16 h), separated by SDS-PAGE. Lanes 1 to 3 are control cheese ES from 3 cheese makings. Lane 5 is a whole milk reference sample. Lanes 7 to 9 are CO₂ treatment cheese ES from 3 cheese makings. Protein bands are identified on the gel.

from 31 to 38°C for cooking. Total cook time was 30 min (Nelson et al., 2004). Whey was collected, and the curds were packed, at which point the whey was sampled for CO₂ analysis. The CO₂ contents of the whey of the control and CO₂ treatment cheeses was 85 and 1000 ppm, respectively (Nelson et al., 2004). Based on the observations of Ma and Barbano (2003), some of the CO₂ could have moved from the skim portion into the fat of the milk, while the temperature was at or above 31°C when a portion of the fat was liquid. If the system was static with respect to acid production, and no CO₂ was lost from the milk to the atmosphere, the pH of the milk/curds/whey would increase if CO₂ moved into the fat. Because lactic acid was continually produced and CO₂ was also lost to the atmosphere, a milk pH increase due to CO₂ movement into the fat could not be detected. The mean level of CO₂ in the treatment cheese immediately after pressing was 347 ppm, which was more than 3 times that of the control cheese, 101 ppm (Figure 1). The least square mean of the CO₂ content for the control and CO₂ treatment cheeses during aging was 124 and 337 ppm, respectively, and did not change during the 6 mo of aging.

Approximately 33% of the cheese was fat and 38% water (Table 2). The total amount of CO₂ in the cheese immediately after pressing was 337 ppm. Both the fat and water content of the cheese were close to one-third the cheese weight. Because the CO₂ content of the cheese was one-third the level of the whey, 1000 ppm (Nelson et al., 2004), a reasonable assumption would be that the CO₂ was predominantly dissolved in the water phase of the cheese. But the existence of CO₂ in the fat of cheese cannot be ruled out. The analytical method of this study does not separate the CO₂ content of the water phase from the lipid phase of cheese.

Proteolysis. The SNPTN contents in the CO₂ treatment cheese was higher than the control cheese at 0 d after pressing (Figure 4), which was approximately 16 h after the cheeses were placed into the press. The timescale in this study for SNPTN analysis was hours after cheese making, whereas most Cheddar cheese studies do not include SNPTN data that soon after cheese making. Significantly higher levels of pH 4.6 SNPTN in the CO₂ treatment cheeses indicated that primary proteolysis was accelerated by preacidification. Metzger et al. (2001) reported a higher amount of pH 4.6 and 12% TCA SNPTN in Mozzarella cheeses at 2 d of age, where the milk for cheese making was acidified to pH 6.0 and 5.8 with both citric and acetic acids when coagulant addition was constant for all treatments, and the difference in SNPTN increased during the 90 d of storage. Metzger et al. (2001) attributed the slower proteolysis of the control Mozzarella

cheeses to the higher stretching temperature (2 to 3°C); however, the influence of preacidification on physicochemical changes in the equilibrium between the cheese matrix and serum phase that lead to higher SNPTN should not be ruled out. In the study by Metzger et al. (2001), preacidified milk produced cheeses with less ES and more proteolysis. In the current study, given that the coagulant addition rates were the same for both the control and CO₂ treatment cheeses, other factors must have influenced proteolysis to increase the pH to 4.6 and 12% TCA SNPTN contents of the CO₂ treatment cheese over the control cheese at the end of pressing.

Both soluble nitrogen (Figure 4) and data from SDS-PAGE (Figure 5) indicated that the CO₂ treatment cheeses had more proteolysis. This is even more surprising because the salt-in-moisture of the CO₂ treatment cheese was higher than the control cheese (5.96 vs. 3.92). The lower α_s -CN:para- κ -CN ratio in the CO₂ treatment cheese at 0 d indicates that more α_s -CN was degraded before the cheeses were removed from the press than in the control cheese. The α_s -CN:para- κ -CN ratio of the CO₂ treatment cheese was lower than the control cheese at 30 d, but the difference between treatments was greatest at 0 d. A greater extent of CN proteolysis would certainly lead to increased pH 4.6 SNPTN. What caused the more rapid proteolysis in the CO₂ treatment cheese?

Substrate and ionic changes in the water phase due to milk preacidification. Expressible serum removal was used in this experiment to sample the water phase of the cheese. The lower amount of calcium in the matrix at whey draining, indicated by the lower calcium in the USMC before salting for the CO₂ treatment cheese (Table 1), would allow CN to move more easily from the matrix to the water phase of the cheese after salt was added. The CP content of the cheese water phase was higher in the CO₂ treatment cheese than the control cheese (Table 3). Furthermore, the proteins in the ES of the CO₂ treatment cheese after pressing were very different than in the control cheese (Figure 6). Preacidification of milk with CO₂ increased the CN content of the water phase before the cheese was removed from the press. At the end of pressing, no CN bands were visible in the ES of the control cheese (Figure 6). The CO₂ treatment cheese caused intact α_s - and β -CN to move into the water phase of the cheese. Casein has been observed in the ES of nonpreacidified Mozzarella cheese approximately 2 d after salting (Guo et al., 1997). However, in the study of Guo et al. (1997), the cheeses were cooled to 4°C for approximately 2 d before the ES procedure was performed. The decrease in temperature was the likely cause of CN in the water phase. Our ES procedure

was performed before the cheeses were refrigerated, and therefore the temperature effect on CN in the ES was not observed in our control cheese.

Not only were intact α_s - and β -CN in the water phase of the CO₂ treatment cheeses but also a substantial amount of α_{s1} -I-CN. When α_{s1} -CN is cleaved by chymosin, the fragment α_{s1} -I-CN is a product (Creamer and Richardson, 1974). The α_{s1} -I-CN was found in the ES of the CO₂ treatment cheese (Figure 6). Casein micelles that have been disrupted due to a loss of colloidal calcium phosphate undergo more proteolysis by rennet than micelles with their micelle structure intact because the CN substrate was more accessible (Fox, 1970). The presence of intact and hydrolyzed CN in the water phase (measured by ES) of salted but unpressed curd from a preacidified treatment after 1 d of refrigerated storage has been reported (Ramkumar et al., 1997). Normally, α_{s1} -I-CN is rapidly produced from α_{s1} -CN hydrolysis by chymosin within the first few weeks of aging (Creamer and Olsen, 1982). The protein bands shown in Figure 6 indicate that a substantial amount of α_{s1} -I-CN was produced in the CO₂ treatment cheese and was present in the water phase before the cheeses were removed from the press. It is possible that the α_{s1} -I-CN was produced before salting and appeared in the ES after salting or was produced after the intact CN had moved into the serum phase of the cheese after salting. No CN bands were detected under higher sample loading conditions using SDS-PAGE in the USMC ES from either the control or the CO₂ treatment cheese (data not shown).

Influence of milk preacidification on chymosin's activity and retention. Other effects of milk preacidification with CO₂ besides CN migration to the cheese water phase could have aided proteolysis early in cheese making, namely: 1) higher chymosin activity or 2) increased chymosin retention in the cheese. First, chymosin was in an environment with a lower pH in the CO₂ treatment cheese from the start of cheese making. Chymosin is an acid protease with a pH optimum for α_{s1} -CN at 5.8 (Mulvihill and Fox, 1977). It is possible that chymosin was more proteolytic because of the lower pH and that the α_{s1} -CN degradation product (i.e., α_{s1} -I-CN) was created before salting but did not enter the serum phase until after salting. After salting, the high salt contents of the CO₂ treatment cheeses were certainly working against increased chymosin action. The salt-in-moisture content of the CO₂ treatment cheese was almost twice the salt content of the control cheese. Mickelsen and Ernstrom (1967) reported that NaCl caused a substantial loss, 70%, of rennin activity in buffered solutions. Lower soluble nitrogen and α_{s1} -CN degradation have been reported in cheeses with salt-in-moisture of 4.5% compared

with cheeses with 2.7 and 3.7% (Mistry and Kasperson, 1998). It is also possible that the α_{s1} -I-CN was created in the water phase of the cheese after salting because the amount of proteolysis in the CO₂ treatment cheeses remained greater than the control cheeses (Figure 5) in spite of their higher salt-in-moisture (Table 1).

Second, due to the lower draining pH of the CO₂ treatment cheese (5.96 vs. 6.35), the chymosin retention of the curd may have increased over the control cheese. Holmes et al. (1977) indicated that a lower draining pH would result in a greater retention of the total rennin activity. Interpolation of figures included in the publication of Holmes et al. (1977) approximates rennet activity in the curd at a draining pH of 6.4 (about the pH at draining for the control cheese in the current study) at slightly less than 50% and a draining pH of 6.0 (about the pH at draining for the CO₂ treatment cheese in the current study) at approximately 70% of the total rennet activity. The relationship of decreasing pH on increased chymosin association with artificial CN micelles has been reported (de Roos et al., 2000). Creamer et al. (1985) indicated that residual coagulant was higher in curds with more acid at whey draining, but no pH values were reported. Regardless of whether the migration of CN to the water phase was the single reason or was also accompanied by increased chymosin activity, the conditions in the CO₂ treatment cheese were more favorable working conditions for chymosin. The CO₂ treatment cheese must have increased cheese SNPTN content either by increasing chymosin retention and hence more chymosin activity and/or by increasing the substrate availability to chymosin by the movement of CN into the water phase of Cheddar cheese soon after salting.

CONCLUSIONS

The CO₂ content of Cheddar cheese manufactured using milk preacidified with CO₂ was consistently higher during aging than the control cheese. More research is needed to determine if the CO₂ that remained in the CO₂ treatment cheese was predominantly in the lipid or water portion. Milk preacidified with CO₂ to a milk pH of 5.9 changed the water phase of the cheese after salting, as indicated by the composition and amount of ES, namely lowering calcium content, increasing CP content, and lowering ES. The increased CP content was due to the presence of intact CN and partially hydrolyzed CN in the water phase of the cheese. The presence of intact CN in the serum phase of USMC was not detected in the control or CO₂ treatment cheese. Preacidification of milk with CO₂ before cheese making resulted in higher pH 4.6 SNPTN content in the cheese and a lower α_s -CN:para- κ -CN ratio

in the CO₂ treatment cheese when the cheeses were removed from the press. Three possible explanations exist for the increased proteolysis: 1) movement of CN into the water phase of the CO₂ treatment cheese, thereby increasing the accessibility of substrate to chymosin; 2) increased chymosin retention in the cheese due to a lower pH at whey draining; and 3) higher chymosin activity during cheese making due to the lower pH at coagulant addition. Further research is needed to produce control and CO₂ treatment cheeses with the same salt-in-moisture to determine the full effect on milk preacidification with CO₂ on proteolysis.

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