ABSTRACT

This study investigated peptide accumulation and bitterness in reduced- and full-fat Cheddar cheeses that were manufactured with single-strain *Lactococcus lactis* starters that had distinct cell envelope proteinase specificities. Micellar electrokinetic capillary electrophoresis of aqueous cheese extracts detected three large peaks, designated O, P, and Q, that eluted with peptide standards and increased in area during cheese maturation in a pattern that was distinct for each starter. Regression analysis of bitter flavor scores from trained sensory panels and individual O-Q peak areas suggested that peaks P and Q had a negative and positive correlation, respectively, to this defect. Then, HPLC, capillary electrophoresis, peptide sequencing, and mass spectrometry were used to identify five peptides from αS1-casein (CN), one from β-CN, and one from αS2-CN that accumulated in 6-mo-old cheeses. Most of the peptides derived from αS1-CN (f 1–23) accumulated in a manner that corresponded with starter proteinase specificity. All of the peptides identified in the study except αS2-CN (f 1–21) eluted in the O-P-Q region of micellar electrokinetic capillary electropherograms. The αS1-CN (f 1–16), αS1-CN (f 1–17) and β-CN (f 193–209) eluted in peak O, αS1-CN (f 1–13) and αS1-CN (f 1–14) eluted in peak P, and αS1-CN (f 1–9) eluted in peak Q.

*Key words: Lactococcus lactis, proteinase, cheese proteolysis, bitterness*

Abbreviation key: A = absorbance (used with number indicating wavelength), CEP = cell envelope proteinase, FSCE = free solution capillary electrophoresis, MECC = micellar electrokinetic capillary chromatography, PTA N = phosphotungstic acid-soluble N.

INTRODUCTION

Proteolysis in Cheddar cheese is an important and complex process that involves endogenous milk enzymes, chymosin, and microbial proteinases and peptidases. Studies of Cheddar cheese ripening suggest that the hydrolysis of intact caseins is almost exclusively catalyzed by chymosin and endogenous milk enzymes. Proteinases and peptidases from *Lactococcus lactis* starters are principally responsible for the production of water-soluble peptides and free amino acids (9). In Cheddar cheese, the concerted action of these enzymes is widely thought to be one of the most important biochemical events during maturation (8). Proteolysis can also lead to bitterness, however, and this defect is observed in reduced- and full-fat Cheddar cheeses (11, 16, 18). Our group is interested in the contribution of starter lactococci to bitterness in situations in which the production of bitter peptides is thought to be a function of lactococcal cell envelope proteinase (CEP) specificity and activity (17).

The *L. lactis* CEP is a member of the subtilisin family of serine proteases. Lactococcal CEP exhibit an extremely high degree of amino acid sequence identity, but purified enzymes may differ in their relative affinity for individual caseins and their specificity toward αS1-CN (f 1–23) at near neutral pH (3, 6). Most differences in CEP specificity are due to one or more amino acid substitutions in the enzyme substrate-binding regions (6), and this property has recently been used to classify lactococcal CEP (14). Several research groups (14) have investigated the specificity of purified CEP toward individual caseins, but the specificity of purified CEP differs from that of the native cell-bound form; specificity is also affected by concentrations of acid and salt in cheese (4).
though lactococcal CEP is widely thought to have a central role in cheese proteolysis and flavor development (5), relatively little is known about the specificity of native cell-bound CEP during cheese ripening or the effect of CEP specificity on cheese quality.

This study investigated the influence of lactococcal CEP specificity on peptide accumulation and bitterness in full-fat and 50% reduced-fat Cheddar cheeses. Cheeses were manufactured using single-strain L. lactis starters with distinct CEP specificities. Bitterness in the cheeses was evaluated by a trained sensory panel, and a combination of techniques (HPLC, capillary electrophoresis, peptide sequencing, and mass spectrometry) was used to investigate proteolysis.

MATERIALS AND METHODS

Cheese Starter Strains and Growth Conditions

Lactococcus lactis ssp. cremoris S1 and Lactococcus lactis ssp. lactis S3 were acquired from Rhône-Poulenc Dairy Ingredients (Madison, WI). Lactococcus lactis ssp. cremoris S2 was obtained from the University of Wisconsin-Madison culture collection. Lactococci were propagated at 30°C, stored at 4°C, and maintained by biweekly transfer in M17 broth (21).

L. lactis CEP Classification, Specificity, and Activity

The classification of L. lactis ssp. cremoris S1, L. lactis ssp. cremoris S2, and L. lactis ssp. lactis S3 CEP was performed by DNA sequence analysis of substrate-binding regions, and CEP specificity was investigated by incubation of whole cells with αs1-CN (f 1–23) (4, 6). To characterize CEP substrate-binding regions, DNA fragments that included the subtilisin-like binding region (residues 131, 138, 142, 144, 166, and 177) and the distal binding region (residues 747, 748, and 763) were obtained by polymerase chain reaction in a Perkin-Elmer Applied Biosystems thermocycler (model 480; Foster City, CA) using DNA primers described by Exterkate et al. (6). The amplicons were purified with a Bio-Rad Prep-A-Gene kit (Bio-Rad, Hercules, CA) and then sequenced by fluorescent dyeox chain termination on a Perkin-Elmer Applied Biosystems automated DNA sequencer (model 373A). Residues in substrate-binding regions were identified by alignment of the deduced amino acid sequences with the L. lactis ssp. cremoris SK11 group a CEP (6, 22) using GeneWorks version 2.3 software (Intelligenetics, Inc., Mountain View, CA).

Incubations of L. lactis whole cells with αs1-CN (f 1–23) were performed using a peptide that had been isolated from chymosin-treated αs1-CN by preparative HPLC as described by Exterkate and Alting (4). Reactions with lactococci and αs1-CN (f 1–23) were also performed as described by those authors except that cells were grown to an absorbance at 600 nm wavelength (A600) = 0.7 in citrated milk that contained 0.5% β-glycerophosphate (24), and 4% NaCl was added to the 25 mM Tris-NaH2PO4-Na acetate (pH 5.2) reaction buffer to simulate cheese conditions. Samples from each reaction were analyzed by reverse-phase HPLC in a Beckman gradient HPLC system equipped with a 125 dual pump, a 168 diode array detector, and a personal computer-based data system controller (Beckman System Gold version 8.1; Beckman Instruments, Fullerton, CA). The columns were Brownlee Aquapore RP-300 (Perkin Elmer/Applied Biosystems) with 300-Å pores and 7-μm particle size. The column diameter was 2.1 mm for analytical HPLC and 4.6 mm for preparative chromatography; column length was 10 cm. Eluant A was 0.1% (vol/vol) trifluoroacetic acid, and eluant B was 0.085% trifluoroacetic acid in 80% acetonitrile. During each run, the proportion of eluant B in eluant A was increased from 0 to 45% over a 45-min period. Flow rates were 0.2 ml/min for the analytical column and 1 ml/min for the 4.6-mm preparative column. Detection of peptides was performed at 214 nm, and peaks were identified by comparison with the data of Exterkate and Alting (4) and to purified αs1-CN (f 1–9), αs1-CN (f 1–13), αs1-CN (f 1–14), αs1-CN (f 1–16), αs1-CN (f 1–17), and αs1-CN (f 1–23) peptide standards.

Lactococcal CEP activity was measured using the EnzChek protease kit (Molecular Probes, Eugene, OR) with fluorescent-labeled casein as described by the manufacturer. Lactococci were grown to A600 = 0.7 in citrated milk that contained 0.5% β-glycerophosphate; the cells were centrifuged at 6000 × g for 10 min, washed with one volume of ice cold 0.85% saline and 20 mM CaCl2, and then suspended in 0.4 ml of wash solution. Samples (100 μl) of each cell suspension were then transferred to a test tube that contained 1 ml of substrate and 0.9 ml of 10 mM Tris-HCl (pH 7.8). The reactions were incubated for 24 h at 30°C in the dark, and fluorescence was measured (excitation, 480 nm; emission, 520 nm) in a scanning fluorometer (model RF1501; Shimadzu, Columbia, MD). The number of colony-forming units per milliliter in each reaction tube was determined by plate count on Elliker’s agar before the assay, and all reactions were performed in duplicate with sterile
spent culture media included as a negative control. Lactococcal CEP activity per cell was then calculated by dividing the mean relative fluorescence units by the number of colony-forming units per milliliter.

**Cheddar Cheese Manufacture**

Cheddar cheese was manufactured on two separate occasions from 250-kg lots of milk at the University of Wisconsin-Madison. Cheeses made during yr 1 of the study were used to investigate the effect of different cultures on 50% reduced-fat Cheddar cheese; cheeses manufactured during yr 2 were used to compare the effects of those cultures in reduced-fat versus full-fat Cheddar cheese.

Vats of 50% reduced-fat Cheddar cheese were manufactured from raw, whole milk skimmed to 1.3% fat and pasteurized at 73.3°C for 16 s. *Lactococcus lactis* ssp. *cremoris* S1, *L. lactis* ssp. *cremoris* S2, and *L. lactis* ssp. *lactis* S3 were grown separately at 30°C for 12 to 14 h in skim milk that had been steamed for 45 min. Duplicate vats were inoculated with 1.25% (wt/wt) of S1, 2.0% of S2, or 0.5% of S3 to obtain a uniform rate of acid production in each cheese. Some vats of yr 1 reduced-fat cheese were also inoculated with flavor adjunct bacteria at 10⁴ to 10⁵/ml. The identity of those adjuncts and their influence on cheese flavor development were discussed by Weimer et al. (23).

Fifteen minutes after starter inoculation, 49 ml of calcium chloride (Rhone-Poulenc, Madison, WI) and 19 ml of double-strength fermentation-produced chymosin (Pfizer, Inc., Milwaukee, WI) were added. The coagulum was cut at a milk pH of 6.5 with 0.95-cm knives and then allowed to heal for 5 min. After 10 min of gentle agitation, the temperature of the curd and whey slurry was raised from 32 to 38.3°C over 25 min; then the whey was slowly drained. Cheese slabs were milled when the curd reached pH 5.55, and the curd was milled when the curd reached pH 5.95. Fifteen minutes after milling, the curd was salted in three additions, 5 min apart, with 0.275% (wt/wt) flame salt (calculated from the original milk weight). The salted curd was packed into 9-kg rectangular stainless steel hoops, pressed for 4 h at ambient temperature, vacuum-packaged, and stored at 7°C for ripening.

Full-fat Cheddar cheese manufactured in the 2nd yr of the study was produced from pasteurized whole milk essentially as described except that duplicate vats were inoculated with 1.75% (wt/wt) of S1, 2.0% of S2, or 1.0% of S3 to obtain a uniform rate of acid production, the coagulum was cut with 0.63-cm knives, the temperature of the curd and whey slurry was raised from 32 to 38.3°C over 30 min, the cheese slabs were milled when the curd reached pH 5.55, and the curd was salted with 0.3% (wt/wt) flame salt.

**Compositional Analysis**

Fat, moisture, and salt contents and pH of cheese were determined on d 1 as described previously (23). Cheese pH was measured again at 2 mo. Samples were also collected once per month for the enumeration of starter and nonstarter colony-forming units. Cheese for microbiological sampling was homogenized in 2% citrate at 45°C, and then total bacterial counts were collected by pour plating appropriate dilutions in Elliker’s agar (Difco). Nonstarter (or adjunct) lactobacilli were also enumerated using Rogosa SL agar (Difco). Plates were incubated anaerobically for 2 d at 30°C (Elliker’s) or 37°C (Rogosa), and numbers of starter colony-forming units were determined by subtracting the *Lactobacillus* count from the total bacterial count. The production of free amino acids and very small peptides (<600 Da) in cheese was also followed each month by determination of 5% phosphotungstic acid-soluble N (PTA N) (12).

**Sensory Evaluation of Bitterness**

The sensory attributes of cheese samples were evaluated at the University of Wisconsin-Madison by 6 to 10 experienced judges who were sensitive to the bitterness attribute. Experimental cheeses were coded and presented in a randomized order to panelists, to be judged for several attributes, including bitter flavor intensity (where 1 = none, 3 = slight, 5 = definite, and 7 = pronounced). Judges initially met as a group to set consensus ratings for all flavor categories using a full-fat control cheese. Consensus scores were marked on ballot sheets, and judges individually evaluated sample cheeses by category scaling against the reference (control) cheese. Statistical analysis of trained sensory data used a completely randomized split plot with repeated measures design as described by Weimer et al. (23).

**Primary and Secondary Proteolysis**

Cheese proteolysis was monitored by a combination of capillary electrophoresis methods and HPLC. Capillary electrophoresis was performed with a PACE 2100 automated system (Beckman Instruments, Inc.) equipped with an untreated silica capillary (75 μm i.d. × 57 cm) and System Gold software (version 7.11). New capillaries were washed with 1 M NaOH, equilibrated with run buffer until the baseline stabi-
lized (typically overnight), and then dedicated to the equilibration buffer. Voltages were kept within the linear range of an Ohm's law plot of voltage and current at 25°C and were ramped to the desired value in 1 min. Sample detection was achieved at 200 nm with the detector range at 0.02 AUFS (absorbance units full scale) and a data collection rate of 2 Hz. The polarity was set with the positive pole at the capillary inlet.

The hydrolysis of αS1- and β-CN in ripening cheese was followed by free solution capillary electrophoresis (FSCE) at 25°C using 100 mM sodium phosphate and 4 M urea (final pH 3.35) buffer (prepared from a 200 mM sodium phosphate stock solution [pH 2.75]). New capillaries for phosphate-urea FSCE were conditioned in buffer for at least 24 h prior to use and stored in buffer when not in use. Samples were prepared by dissolving 0.5 g of ground cheese in 10 ml of a freshly prepared solution of 10 M urea and 50 mM phosphoric acid. The sample was stirred for approximately 1 h until cheese pieces were dissolved and centrifuged for 10 min at 16,000 × g to remove the lipid fraction. The aqueous layer was collected, filtered through a 0.2-μm low protein-binding filter (Gelman Sciences, Ann Arbor, MI), and then stored in buffer when not in use. Samples were prepared for analysis with 2× sample buffer, followed by a 2-min rinse with 0.2 M sodium phosphate, pH 2.75, and then a 4-min rinse with 100 mM sodium phosphate and 4 M urea.

Secondary proteolysis in the cheeses was studied by a combination of micellar electrokinetic capillary chromatography (MECC), FSCE, and HPLC. Aqueous Cheddar cheese extracts that had been prepared as described by Strickland et al. (20), except that double deionized water was used instead of 100 mM phosphate for extractions in yr 2 of the study. The MECC was performed using 100 mM sodium borate (pH 8.5) with 40 mM SDS as described by Strickland et al. (20). The FSCE in 100 mM sodium phosphate (pH 2.74) buffer was performed as described by Strickland and Strickland (19). Cheese extracts for acid phosphate FSCE were size-fractionated using 3000 molecular mass cutoff membranes (Amicon, Beverly, MA) and washed several times with distilled water. The retentate was collected and stored at −20°C until needed. Samples were run for 30 min at 15 kV followed by a 7-min low pressure rinse. Prior to use, the capillary was conditioned overnight with 500 mM sodium phosphate (pH 2.50). Before each run, a 1-min high pressure rinse with 100 mM sodium phosphate (pH 2.74) was performed. Peptide solutions (ca. 20 μg/ml) in 25 mM sodium phosphate (pH 2.74) were injected with 1 to 5-s injections. At the end of a run, two 1-min forward high pressure rinses were performed with 0.1 M HCl and 500 mM sodium phosphate (pH 2.50).

Cheese samples for reverse-phase HPLC were prepared as described for acid phosphate FSCE. Separations were performed with a Beckman gradient HPLC system as described for the study of CEP specificity.

Peptide Characterization

The peptides that were isolated from sample cheeses were identified by amino-terminal sequencing and mass determination. Samples for each analysis were purified by HPLC as described and then were lyophilized in a benchtop freeze-dryer (model 5L; VirTis, Gardiner, NY). Amino-terminal sequencing was performed by Edman degradation in a protein sequencer (model 477B; Applied Biosystems). Peptides for mass spectrometry were dissolved in 0.1% trifluoroacetic acid and analyzed by matrix-assisted laser desorption ionization time of flight on a ToFSpec (Micromass, Beverly, MA) mass spectrometer with external mass calibration. The spectrometer was set at 20 kV in the linear mode with an N2 laser (337 nm), and the matrix was α-cyano-4-hydroxy-cinnamic acid. Finally, the mean hydrophobicity and molecular mass of each peptide were calculated (PeptID 93 software; University of Minnesota, St. Paul).

RESULTS

Lactococcal CEP Classification and Specificity

Deduced amino acid sequences for the L. lactis ssp. cremoris S1 CEP substrate-binding regions showed that this enzyme belonged to CEP group e (Table 1) (14). Lactococcus lactis ssp. cremoris S2 was originally designated as strain SK11 (23), but SK11 has a group a CEP (14), and DNA sequence analysis of S2 CEP substrate-binding regions showed it was a group b enzyme (Table 1). As is also shown in Table 1, DNA sequence analysis of the S3 CEP substrate-binding regions revealed this enzyme had a distal binding region that was identical to that found in CEP groups f and g, but the subtilisin-like binding region was unlike any previously described CEP group.
TABLE 1. Classification of *Lactococcus lactis* ssp. *cremoris* S1, *L. lactis* ssp. *cremoris* S2, and *Lactococcus lactis* ssp. *lactis* S3 cell envelope proteinase (CEP) from deduced amino acid sequences for substrate binding regions. 1

<table>
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<th>Strain</th>
<th>131</th>
<th>138</th>
<th>142</th>
<th>144</th>
<th>166</th>
<th>177</th>
<th>747</th>
<th>748</th>
<th>763</th>
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<td>Thr</td>
<td>Ser</td>
<td>Leu</td>
<td>Asp</td>
<td>Leu</td>
<td>Leu</td>
<td>Thr</td>
<td>Asn</td>
</tr>
<tr>
<td>S1</td>
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<tr>
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<td>Thr</td>
<td>Ala</td>
<td>Leu</td>
<td>Asp</td>
<td>Leu</td>
<td>Arg</td>
<td>Lys</td>
<td>Asn</td>
</tr>
<tr>
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<td></td>
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<tr>
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<td>Leu</td>
<td>Thr</td>
<td>His</td>
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<td>S3</td>
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</table>

1Determined by comparison to the data of Exterkate et al. (8).

2A new group designation was assigned because CEP with identical substrate binding regions have not been previously described.

Differences in the specificity of S1, S2, and S3 CEP were evident in peptide profiles obtained after brief (15 to 30 min) and prolonged (120 to 180 min) incubations under cheese-like conditions (pH 5.2, 4% NaCl) of whole cells with αS1-CN (f 1–23) (4). Brief incubations with S2 indicated the group b CEP of this bacterium had a strong affinity for the Leu<sub>16</sub>-Asn<sub>17</sub> and Asn<sub>17</sub>-Glu<sub>18</sub> bonds of αS1-CN (f 1–23), but αS1-CN (f 1–13) and some αS1-CN (f 1–9) were also detected (Figure 1A). Similar experiments with S1 suggested that the group e CEP of this bacterium had a specificity toward αS1-CN (f 1–23) that resembled strain HP group g CEP (6, 14). Each of these enzymes preferentially hydrolyzed αS1-CN (f 1–23) at the Gln<sub>13</sub>-Glu<sub>14</sub> position, but αS1-CN (f 1–16) and αS1-CN (f 1–9) were also formed (Figure 1, B and D). Thirty-minute incubations with S3 indicated its CEP had the greatest affinity for the Leu<sub>16</sub>-Asn<sub>17</sub> and Gln<sub>13</sub>-Glu<sub>14</sub> bonds of αS1-CN (f 1–23), and cleavage at Gln<sub>9</sub>-Gly<sub>10</sub> as well (Figure 1C).

Prolonged incubations of S1 or S2 with αS1-CN (f 1–23) showed little change in the peptide patterns for these strains (Figure 2, A and B), but similar experiments with S3 gave a new pattern that was apparently due to efficient secondary conversion of αS1-CN (f 1–16) to αS1-CN (f 1–9) by the S3 CEP (Figures 1C and 2C).

**Cheese Composition**

Percentages of fat, moisture, and salt in moisture contents were very similar among reduced-fat (13.5 ± 0.2, 48.2 ± 0.9, and 3.4 ± 0.2, respectively) or full-fat (31.7 ± 0.5, 38.4 ± 0.5, and 4.2 ± 0.3) Cheddar cheeses that had been manufactured with S1, S2, or S3 single-strain starters. Cheese pH at 2 mo was also similar (range = pH 5.0 to 5.2).

Reduced-fat Cheddar cheese made in yr 1 with S1 or S2 contained approximately 10<sup>8</sup> cfu/g of starter at pressing, and the numbers of these bacteria declined about 1 log during the 1st mo of ripening (Table 2). In contrast, yr 1 cheese that was made with S3 contained well over 10<sup>9</sup>/g of starter at d 1, and starter numbers remained high even after 1 mo of ripening. As shown in Table 2, the trend in starter numbers for reduced-fat cheese made with S2 in yr 2 was similar to that of cheese made in yr 1, but numbers of starter bacteria in all other yr 2 cheeses did not show any decrease during the 1st mo of ripening.
Incubations with whole cells and fluorescent casein indicated that strains S1, S2, and S3 had relative CEP activities of 15.4, 10.1, and 3.7 RFU/cfu, respectively. If one assumes that the differences in relative CEP activity for strains grown in citrated milk are essentially conserved during growth in regular milk, initial starter numbers for each cheese (Table 2) suggest that total CEP activity probably was at least 10-fold higher in reduced-fat cheeses made in yr 1 with S3 versus that made with S1 or S2. A similar disparity was calculated for total CEP activity in reduced-fat and full-fat cheeses made in yr 2 with S3 versus S1, although total CEP activity in yr 2 cheeses made with S2 was approximately 5-fold less than that in S3 cheeses.

The differences that were noted in total CEP activity were not reflected in PTA N concentrations. The PTA N in reduced-fat cheeses made in both years of the study was highest for cheese made with S2, followed by S1, and then S3. The PTA N concentrations in full-fat Cheddar were highest in S1 cheese, followed by S3 and then S2 cheese (data not included).

Primary Proteolysis in Reduced-Fat Cheddar Cheese

Phosphate-urea FSCE was used to investigate primary proteolysis in reduced-fat Cheddar cheese made in this study. In full-fat Cheddar cheese, hydrolysis of intact αS1- and β-CN during ripening occurs primarily through the action of residual chymosin and native plasmin, respectively (10). Coinjection studies with purified αS1- and β-CN (20), purified αS1-CN (f 1–9) and αS1-CN (f 1–13), and partially purified αS1-CN (f 24–199) showed that, at press, reduced-fat Cheddar cheeses made with S1 or S3 single-strain starters contained three large peaks, which corresponded to intact αS1- and β-CN, as well as a small peak, which comigrated with the chymosin-derived peptide αS1-CN (f 24–199) (Figure 3). After 2 mo of ripening, concentrations of intact β-CN had decreased only slightly, but αS1-CN was almost completely hydrolyzed, and the peak for αS1-CN (f 24–199) had become several times larger. Those changes indicated that residual chymosin had a more active role than plasmin in primary proteolysis during the first 2 mo of ripening. Two-month-old cheese also contained two small peaks that comigrated with αS1-CN (f 1–9) and αS1-CN (f 1–13). Those peptides are produced from the chymosin-derived peptide αS1-CN (f 1–23) by starter bacteria (5). By 4 mo, intact αS1-CN had essentially disappeared, and β-CN peaks were approximately one-half of the area that was noted at press. Peaks for αS1-CN (f 24–199), αS1-CN (f 1–9), and αS1-CN (f 1–13) showed only minor changes from 2 to 4 mo, which suggested that the production and conversion of these peptides was approaching equilibrium.

Table 2. Numbers of viable Lactococcus lactis ssp. cremoris S1, L. lactis ssp. cremoris S2, or Lactococcus lactis ssp. lactis S3 starter bacteria in reduced-fat and full-fat Cheddar cheeses.

<table>
<thead>
<tr>
<th>Single-strain starter</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
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<tr>
<td>yr 1</td>
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<tr>
<td>d 1 (press)</td>
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<td>2 × 10⁸</td>
<td>7 × 10⁸</td>
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<tr>
<td>&gt;1 mo</td>
<td>7 × 10⁸</td>
<td>2 × 10⁸</td>
<td>3 × 10⁸</td>
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<td>d 1 (press)</td>
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<td>3 × 10⁸</td>
<td>3 × 10⁸</td>
</tr>
<tr>
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<td>7 × 10⁸</td>
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<tr>
<td>Full-fat cheese</td>
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<tr>
<td>yr 2</td>
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<tr>
<td>d 1 (press)</td>
<td>4 × 10⁸</td>
<td>2 × 10⁸</td>
<td>3 × 10⁸</td>
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<tr>
<td>&gt;1 mo</td>
<td>1 × 10⁹</td>
<td>2 × 10⁹</td>
<td>6 × 10¹⁰</td>
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</table>

Figure 2. Reversed-phase HPLC of the products of αS1-CN (f 1–23) after prolonged incubation with whole cells of Lactococcus lactis ssp. cremoris S1 (A), L. lactis ssp. cremoris S2 (B), or Lactococcus lactis ssp. lactis S3 (C). Incubations were performed at 30°C for 180 (S1) or 120 min (S2, S3) in 25 mM Tris-NaH₂PO₄-Na acetate (pH 5.2) (4) with 4% NaCl. Peptides identified in the chromatograms include peak 1, αS1-CN (f 1–9); peak 2, αS1-CN (f 1–13); peak 3, αS1-CN (f 1–14); peak 4, αS1-CN (f 1–17); peak 5, αS1-CN (f 1–16); peak 6, αS1-CN (f 17–23); peak 7, αS1-CN (f 18–23); peak 8, αS1-CN (f 14–23); and peak 9, αS1-CN (f 1–23). A₂₁₄ = Absorbance at 214 nm wavelength.
Figure 3. Free solution capillary electrophoresis in phosphate-urea of reduced-fat Cheddar cheese made in yr 2 with Lactococcus lactis ssp. cremoris S1 (upper panel) or Lactococcus lactis ssp. lactis S3 (lower panel) single-strain starters. Peaks identified by coinjection with purified standard peptides or caseins include peak 1, αS1-CN (f 1–9); peak 2, αS1-CN (f 1–13); peak 3, αS1-CN; peak 4i, β-CN A1; peak 4ii, β-CN A2; and peak 5, αS1-CN (f 24–199).

Bitter Flavor Intensity

Evaluation by the trained sensory panel showed that yr 1 cheeses, made with S1 or S2, did not develop bitter flavors during 6 mo of ripening (maximum bitter flavor intensity score = 1.3), but cheese made with S3 was slightly bitter by 2 mo (bitter flavor intensity = 2.0 ± 0.1) and definitely bitter after 4 and 6 mo of ripening (bitter flavor intensity = 4.4 ± 0.6 and 5.4 ± 0.8, respectively). The ANOVA showed that starter choice was the only factor that significantly influenced bitterness in Cheddar cheese (α = 0.05; P < 0.0005), and that effect was clearly due to S3. Cheeses made with S3 single-strain starters in the 2nd yr of the study were also bitter. Six-month-old reduced- and full-fat Cheddar cheeses made with S3 each received mean scores for intensity of bitter flavor of 4.5. In comparison, 6-mo-old reduced-fat cheese made with S1 or S2 received bitter flavor scores of 2.7 and 1.7, respectively; full-fat cheese made with those starters had bitter flavor scores of 1.5 and 2.1.

MECC Profile and Bitterness

The MECC electropherograms of aqueous cheese fractions are complex, but characteristically contain three prominent peaks, which elute just before the micelle marker (20). As shown in Figure 4, the total peak area for these peaks, designated O, P, and Q, increased during maturation in a pattern that was characteristic for each starter culture. Correlation analysis between individual O-P-Q peak areas and bitter flavor scores from 16 cheeses made with each starter in yr 1 showed no obvious correlation to peak O, but peaks P and Q gave a negative and positive correlation with bitterness, respectively, that increased as ripening time increased. The correlation coefficient between peak P area and bitterness at 2, 4, and 6 mo in reduced-fat Cheddar made in yr 1 was −0.58, −0.73, and −0.81, respectively; for peak Q and bitterness, r = 0.66, 0.79, and 0.83. Similar results were obtained for Cheddar cheeses made in duplicate with each starter in the 2nd yr of the study. For peaks P and Q with bitterness in 6-mo-old Cheddar made in yr 2, r = −0.80 and 0.83, respectively, for reduced-fat cheese, and r = −0.88 and 0.77 for full-fat Cheddar.

Peptide Identification and Relative Abundance in Cheese

As shown in Figure 5, aqueous extracts of cheese that were made from each starter also had characteristic HPLC and acid phosphate FSCE profiles. The HPLC peaks A through G (Figure 5) were individually collected from 6-mo-old reduced-fat cheese and then were analyzed by MECC and acid phosphate FSCE to evaluate fraction purity and to ascertain electrophoretic mobility on capillary electropherograms. Analysis of the amino-terminal peptide sequence and mass spectrometry of HPLC fractions identified five peptides from αS1-CN, one peptide from αS2-CN, and one from β-CN, which accumulated in reduced-fat Cheddar cheese (Table 3). As shown in Figure 5 and Table 4, levels of individual peptides in reduced- or full-fat Cheddar cheese differed in a manner corresponding with the starter bacterium that was used to produce each cheese. Except for the αS2-CN fragment, all of the peptides that were identified
in this study were relatively hydrophobic molecules (Q >1300) (17), which eluted in the MECC O-P-Q peak region.

**DISCUSSION**

This study investigated peptide accumulation and bitterness in reduced- and full-fat Cheddar cheeses that were manufactured with single-strain *L. lactis* starters that produce distinct CEP. Two of the starters, S1 and S2, are used commercially in the US for Cheddar cheese manufacture; the third, S3, is known to produce bitter flavors in milk consistently (J. Kondo, 1993, personal communication). As shown in Table 1, S1 and S2 produced group e and group b CEP, respectively. Characterization of the S3 CEP showed this enzyme had substrate binding regions that were unlike any previously described CEP group (Table 1), and its specificity toward αS1-CN (f 1–23) under cheese-like conditions was also unique (Figures 1 and 2). These data showed that the S3 enzyme represented a new CEP group, which was designated in this study as group h.

Cheddar cheese manufactured with single-strain starters S1, S2, or S3 had similar moisture, fat, and salt in moisture contents and pH values. Phosphate-urea FSCE electropherograms of ripening reduced-fat cheese (Figure 3) suggested that, like full-fat Cheddar cheese isolated in this study from Cheddar cheese.

<table>
<thead>
<tr>
<th>Peptide1</th>
<th>Elution peak or time</th>
<th>Hydrophobicity3</th>
<th>Mass4</th>
</tr>
</thead>
<tbody>
<tr>
<td>αS1-CN (f 1–9)</td>
<td>A</td>
<td>Q</td>
<td>1422</td>
</tr>
<tr>
<td>αS1-CN (f 1–13)</td>
<td>B</td>
<td>P</td>
<td>II</td>
</tr>
<tr>
<td>αS1-CN (f 1–14)</td>
<td>C</td>
<td>P</td>
<td>III</td>
</tr>
<tr>
<td>αS1-CN (f 1–16)</td>
<td>F</td>
<td>O</td>
<td>IV</td>
</tr>
<tr>
<td>αS1-CN (f 1–17)</td>
<td>E</td>
<td>O</td>
<td>V</td>
</tr>
<tr>
<td>αS2-CN (f 1–21)</td>
<td>D</td>
<td>–12 min</td>
<td>&gt;30 min</td>
</tr>
<tr>
<td>β-CN (f 193–209)</td>
<td>G</td>
<td>O</td>
<td>&gt;30 min</td>
</tr>
</tbody>
</table>

1Identified by amino terminal sequence analysis and mass spectrometry.
2MECC = Micellar electrokinetic capillary chromatography; FSCE = free solution capillary electrophoresis.
3Peptide average hydrophobicity as determined by PeptID software (University of Minnesota, St. Paul).
4Peptide mass as determined by mass spectrometry and as calculated from amino acid composition.
5Mass spectrometry performed after peptide dephosphorylation with calf intestine alkaline phosphatase (1).
Figure 5. Reversed-phase HPLC chromatograms (upper panel) and acid phosphate-free solution capillary electropherograms (lower panel) of size-fractionated aqueous extracts of 6-mo-old reduced-fat Cheddar cheese manufactured in yr 1 with *Lactococcus lactis* ssp. *cremoris* S1, *L. lactis* ssp. *cremoris* S2, or *Lactococcus lactis* ssp. *lactis* S3 single-strain starters. Peptides identified in the chromatogram include peak A, αS2-CN (f 1–9); peak B, αS2-CN (f 1–13); peak C, αS1-CN (f 1–14); peak D, αS2-CN (f 1–21); peak E, αS1-CN (f 1–17); peak F, αS1-CN (f 1–16); and peak G, β-CN (f 193–209). Peptides in the electropherogram were identified as peak I, αS1-CN (f 1–9); peak II, αS2-CN (f 1–13); peak III, αS1-CN (f 1–14); peak IV, αS1-CN (f 1–16); and peak V, αS1-CN (f 1–17).

A200 or A214 = Absorbance at 200 or 214 nm wavelength.

To our knowledge, αS2-CN (f 1–21) has not been previously isolated from cheese, but plasmin is known to cleave αS2-CN at the Lys21-Gln22 position (9). The αS2-CN (f 1–21) peptide was detected in all cheeses, but concentrations were characteristically low in cheeses made with S2 (Figure 5 and Table 4). This observation indicated that the group b CEP or intracellular peptidases from S2, or both, served to limit accumulation of αS2-CN (f 1–21) in S2 cheese.

The peptides αS1-CN (f 1–9), αS1-CN (f 1–13), and αS1-CN (f 1–14) that were identified in this study were also found by Kaminogawa et al. (13) to accumulate in Gouda cheese. Those authors (13) also showed these peptides were produced from purified αS1-CN (f 1–23) by proteases in a cell-free extract of *L. lactis* ssp. *cremoris* H61. That work was followed by a study by Exterkate and Alting (5), who showed that peptides derived from αS1-CN (f 1–23) in 2-wk-old Gouda cheese reflected the in vitro CEP specificity of the starter that had been used to produce the cheese; those researchers (5) concluded that CEP specificity during early proteolysis might be important for the course of gross proteolysis during the later stages of ripening. Comparisons between the relative abundance of peptides derived from αS1-CN (f 1–23) in 6-mo-old Cheddar cheese that was manufactured in this study with S1, S2, or S3 single-strain starters showed that starter CEP specificity was heavily reflected in the pool of peptides that accumulated in ripened cheese. Peptides derived from αS1-CN (f 1–23) accumulated in 6-mo-old Cheddar cheeses made with S1, S2, or S3 (Figure 5 and Table 4) in a manner that was consistent with results from in vitro studies of CEP specificity that were performed under cheese-like conditions (Figure 2). One exception to this observation was αS1-CN (f 1–14), which accumulated in some cheeses even though it
TABLE 4. Relative abundance of individual peptides in size-fractionated aqueous extracts of 6-mo-old reduced-fat and full-fat Cheddar cheeses manufactured in yr 2 with *Lactococcus lactis* ssp. *cremoris* S1, *L. lactis* ssp. *cremoris* S2, or *Lactococcus lactis* ssp. *lactis* S3 single-strain starters.  

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Reduced-fat cheese</th>
<th>Full-fat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S1</td>
<td>S2</td>
</tr>
<tr>
<td>aS1-CN (f 1–9)</td>
<td>9.7</td>
<td>1.6</td>
</tr>
<tr>
<td>aS1-CN (f 1–13)</td>
<td>11.7</td>
<td>10.2</td>
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<tr>
<td>aS1-CN (f 1–14)</td>
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<td>22.8</td>
</tr>
<tr>
<td>aS1-CN (f 1–16)</td>
<td>1.6</td>
<td>1.0</td>
</tr>
<tr>
<td>aS1-CN (f 1–17)</td>
<td>ND</td>
<td>2.8</td>
</tr>
<tr>
<td>aS2-CN (f 1–21)</td>
<td>5.7</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>b-CN (f 193–209)</td>
<td>3.0</td>
<td>ND</td>
</tr>
</tbody>
</table>

1Numbers depict the percentage of the total peak area on respective HPLC chromatograms represented by the peak area for each peptide. Peak areas for each peptide were obtained from the HPLC chromatogram of a representative cheese made with each starter and divided by the total peak area for that chromatogram.

2Not detected.

was not a major product of S1, S2, or S3 CEP (Table 4; Figures 1 and 2). The appearance of aS1-CN (f 1–14) in cheese can be explained by the work of Baankreis et al. (2), which demonstrated that aS1-CN (f 1–23), aS1-CN (f 1–16), and aS1-CN (f 1–17) were hydrolyzed to aS1-CN (f 1–13) and aS1-CN (f 1–14) by the lactococcal neutral oligoendopeptidase, PepO. The activity of PepO, an intracellular enzyme, in the cheese matrix arose as a direct consequence of starter lysis (2, 5).

A contribution by PepO to peptide conversion in Cheddar cheese was evident from comparisons between the peptide content of reduced-fat versus full-fat Cheddar cheese made with *L. lactis* ssp. *cremoris* S2. As shown in Table 2, numbers of viable starter bacteria in full-fat S2 cheese remained constant during the 1st mo of ripening, so starter autolysis (and thus PepO activity) was probably less significant in those cheeses than in S2 reduced-fat cheeses for which starter numbers had declined by approximately 1 log during the same period. As would be expected in a cheese with lower PepO activity, full-fat S2 cheese contained much higher concentrations of aS1-CN (f 1–16) and substantially lower concentrations of aS1-CN (f 1–13) and aS1-CN (f 1–14) than reduced fat cheese made with S2 (Table 4). Full-fat S2 cheese also contained higher levels of b-CN (f 193–209), which can be efficiently hydrolyzed by PepO but not by CEP (2, 7).

The b-CN (f 193–209) peptide is a product of chymosin (9) and has been associated with bitterness in cheese (17). Although b-CN (f 193–209) was detected in bitter S3 cheese, the peptide that accumulated to highest concentration in S3 cheese was aS1-CN (f 1–9) (Figure 5 and Table 4). Because total CEP activity was always several -fold higher in S3 than in S1 or S2 cheeses (see Results), this result was probably a consequence of both group h CEP specificity (Figure 2C) and high total CEP activity. The aS1-CN (f 1–9) peptide has not been linked with bitterness, but the following evidence suggests that aS1-CN (f 1–9) may contribute to that defect. First, peak Q provided good correlation to bitter flavor intensity, and aS1-CN (f 1–9) was the only peptide isolated in the study that eluted in peak Q (Table 3). Second, aS1-CN (f 1–9) has a mean hydrophobicity that is greater than 1400 and a mass that is less than 6000. These properties, according to Ney’s Q rule (20), predict this peptide to be bitter. Third, aS1-CN (f 1–9) was detected in nearly all cheeses, its concentration was always highest in S3 cheese (Figure 5 and Table 4). This observation is consistent with the hypothesis that bitterness develops when the level of a constituent bitter peptide exceeds its taste threshold (17). Finally, Lee et al. (15), recently found that hydrolysis products of aS1-CN (f 1–23), including aS1-CN (f 1–7) and aS1-CN (f 1–13), contributed to bitterness in Cheddar cheese. Sensory studies are underway in our laboratories to establish the bitter taste threshold for aS1-CN (f 1–9) in cheese and to determine whether it contributes to bitterness in Cheddar cheese.

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REFERENCES


