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Relationship between MALDI-TOF Analysis of β-CN f193−209 Concentration and Sensory Evaluation of Bitterness Intensity of Aged Cheddar Cheese

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An internal standard method was previously developed to measure the concentration of a synthetic bitter peptide, β-CN f193−209, by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. The objective of this study was to evaluate the relationship between β-CN f193−209 concentration in an aqueous extract of aged Cheddar cheese and bitterness intensity of the cheese. Concentrations of β-CN f193−209 in cheese extracts were determined by MALDI-TOF at 0, 120, 180, and 270 days. Trained panels evaluated the bitterness intensity of the cheeses at 180 and 270 days. Correlation coefficients between MALDI and sensory data at 180 and 270 days were 0.803 and 0.554, respectively. The decreased correlation may be due to the presence of other bitter peptides more responsible for bitterness at longer aging or the production of compounds that mask bitterness intensity.

KEYWORDS: Bitter peptide; β-CN f193−209; cheese; MALDI; matrix-assisted laser desorption/ionization

INTRODUCTION

Aged Cheddar cheese often develops bitterness due to the accumulation of hydrophobic peptides, consisting of 2−23 amino acids or in the molecular weight range of 500−3000 Da (*1*, *2*). Bitterness is detected when the concentration of bitter peptide exceeds the detection threshold (*3*). Although bitter taste is considered a normal component of cheese taste (*4*), excessive bitterness may limit consumer acceptance of the cheese (*3*). The bitter defect must be controlled to increase the marketability of the cheese.

According to Lowrie and Lawrence (*5*), lactococcal proteinases and rennet are responsible for the formation of bitter peptides from caseins in Cheddar cheese. Fast acid production during cheese manufacture increases rennet retention in the curd and hence increases bitterness (*6*). Bitter peptides can be degraded to nonbitter peptides and amino acids by peptidases. The overall bitterness intensity of cheese depends on the rate of formation and degradation of the bitter peptides. Adjunct cultures, nonstarter lactic acid bacteria, have been used to prevent bitterness in Cheddar cheese as well as to shorten ripening time and increase cheese flavor (*7*).

General aminopeptidase (PepN and PepC) and X-prolyl dipeptidyl aminopeptidase (PepX) are commonly associated with the degradation of bitter peptides from caseins. PepN has specificity for peptides containing basic, hydrophobic/uncharged, or aromatic residue at the N terminus (*8*). Additionally, PepC specificity also includes acidic residue at the N terminus. PepX cleaves the N-terminal X-Pro-containing peptide, with the highest activity observed when X is an uncharged or a basic residue. PepX is essential for the degradation of proline-rich β-casein due to the inability of PepN and PepC to hydrolyze the N-terminal or penultimate proline residue.

Evaluation of bitterness intensity of cheese by sensory panels is subjective and labor-intensive (*7*). Although chromatographic analysis of cheese peptides is a useful technique (*9*), sample preparation and elution steps can be time-consuming. Hence, it is desirable to develop a rapid and sensitive quantitative method for determining bitter peptide concentration in cheese. Quantification of a bitter peptide marker may enable detection of the bitter defect without sensory evaluation.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry is commonly used for qualitative analysis of peptides and proteins (*10*−*12*). Soeryapranata et al. (*13*) reported using an internal standard for quantitative analysis of a synthetic bitter peptide, β-CN f193−209, by MALDI-TOF. Despite poor peak-height reproducibility, the concentration ratio of synthetic β-CN f193−209 to an internal standard was linearly correlated to the peak-height ratio obtained from MALDI-TOF analysis (*13*). The sequence of β-CN f193−209 is YQQPVLGPVRGPFPIIV. The internal standard developed in that study was asparagine (N) substitution for glutamine (Q) at residue

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195 in the β-CN f193−209. The sensitivity of MALDI-TOF, in the picomole range, may be useful for early detection of bitter defect in cheese.

β-CN f193−209, an important bitter peptide in Gouda cheese, is formed by the action of rennet and lactococcal proteinases on β-casein (*12*, *14*) and is hydrolyzed by intracellular peptidases from primary and adjunct cultures (*9*, *15*). The bitter recognition level of β-CN f193−209 has been reported to be 0.35 mg/mL (*15*). The objective of the current study was to evaluate the relationship between β-CN f193−209 concentration in an aqueous extract of aged Cheddar cheese measured by MALDI-TOF and bitterness intensity of the aged cheese reported by sensory evaluation.

MATERIALS AND METHODS

Preparation of Aqueous Cheese Extract. Aged Cheddar cheeses manufactured by Fajarrini (*16*) were the source of aqueous cheese extracts. The cheeses were made in duplicate using primary culture alone (three treatments) or in combination with adjunct culture (six combinations). The primary cultures consisted of mixed strains *Lactococcus lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris* (Chris Hansen Laboratories, Milwaukee, WI), designated 56, 98, and 105. Primaries 56 and 98 are slow acid producers and were expected to produce nonbitter cheeses (*17*). Primary 105 is a fast acid producer and more likely to develop bitterness. The adjunct cultures were *Lactobacillus helveticus* strains WSU19 (WSU Creamery, Pullman, WA) and W900R (Waterford Foods Inc., Millville, UT), which represent adjunct culture having high and low debittering activity, respectively (*16*, *18*). The cheeses (14.5 cm in diameter and 4.5 cm in thickness) were aged at 7 °C for up to 270 days.

Aqueous extract of the aged Cheddar cheese was prepared according to the method of Kuchroo and Fox (*19*). Grated Cheddar cheese (20 g) was blended with water (40 g) in a stomacher (Seward Laboratory, London, U.K.) for 10 min and incubated in a 45 °C water bath for 60 min. The mixture was centrifuged for 30 min (3000*g* at 4 °C) using a Beckman J2-HS centrifuge (Beckman Instruments, Palo Alto, CA). Supernatant was filtered through glass wool and stored at −75 °C.

Cheese Analysis. Analyses of cheese components (pH, moisture, protein, salt and fat contents) were conducted the day after the cheese was made (*16*). Enumeration of the primary culture population was conducted on LM17 medium at each sampling time (*16*).

Sensory Analysis of Aged Cheese. Sensory analysis of the bitterness intensity of the aged cheeses was done using six trained panelists (*16*). The panelists were selected from 25 candidates on the basis of low variability (low mean square error) and high sensitivity (high *F* value) to bitterness at a 0.05 significance level. Glycyl-L-leucine solution (Sigma Chemical Co., St. Louis, MO) was used as bitter reference because this bitter peptide was judged to represent the bitterness of casein hydrolysates (*20*).

After 180 and 270 days of aging, the middle portions of the aged cheeses (two replicates) were cut into 2.5 × 2.5 × 3.75 cm pieces and randomly coded using a three-digit number. The freshly cut cheese was placed into Solo souffle containers (Urbana, IL) and immediately covered with lids to avoid drying. The cheese samples were completely randomized when presented to the panelists. The trained panelists tasted three cheese samples per tasting session and rated the perceived bitterness intensity using a 0−15 cm unstructured line scale. The data are reported as centimeters (0 = low bitterness, 15 = extremely bitter). The whole study (duplicate treatments of nine cheeses, duplicate tastings per treatment) required 12 tasting sessions. Sensory data were analyzed in a randomized complete block design with a two-way treatment structure (primary and adjunct) at a 0.05 significance level.

Aminopeptidase Activity of Cheese. Cheese samples were prepared according to the method of Weimer et al. (*21*), and the aminopeptidase (AP) activity was assayed according to the procedure of Pallavicini et al. (*22*), with modifications. Aged Cheddar cheese (2 g) was mixed with 18 mL of citrate buffer (2% trisodium citrate, pH 7.0, 40 °C) for 2 min in a stomacher. The suspension (15 mL) was centrifuged for 30 min (31000*g* at 4 °C). The supernatant (0.2 mL) was incubated at 37 °C with 1.6 mL of 50 mM Tris-HCl buffer (pH 7.0, Sigma Chemical Co., St. Louis, MO) and 0.2 mL of 25 mM lysine- (Lys-) or arginine-proline-*p*-nitroanilide (Arg-Pro-pNa) substrate in methanol for 1 and 2 h. Lys-pNa and Arg-Pro-pNa substrates were used to estimate general aminopeptidase and PepX activities, respectively. A blank reaction was made up of buffer and substrate. After 1 h, 1 mL of the suspension was transferred to a clean test tube containing 1 mL of 30% (v/v) glacial acetic acid (J. T. Baker, Phillipsburg, NJ). The enzyme activity was terminated after 2 h using 1 mL of 30% acetic acid. The absorbance was measured at 410 nm. The enzyme activity was calculated on the basis of the difference between 1 and 2 h absorbance readings.

Quantification of β-CN f193−209 in Aqueous Cheese Extract. MALDI-TOF analysis was performed using a PerSeptive Biosystems (Framingham, MA) DE-RP time-of-flight mass spectrometer. The matrix used for MALDI-TOF analysis was α-cyano-4-hydroxycinnamic acid (Aldrich Chemical Co., Milwaukee, WI). A saturated solution of matrix was prepared in acetonitrile/water (1:1) containing 0.25% (v/v) trifluoroacetic acid. The crystal of matrix−analyte was ionized by a 337-nm nitrogen laser pulse and accelerated under 25000 V before entering the time-of-flight mass spectrometer. The instrument was set in the positive linear mode.

Concentrations of β-CN f193−209 in aqueous extracts of aged cheeses at 0, 120, 180, and 270 days of aging were determined using an internal standard, synthetic β-CN f193−209, in which asparagine was substituted for glutamine 195. The aqueous extract of aged cheese was mixed with the internal standard at a volume ratio that would fit the range of peak-height ratio in a calibration curve, which was constructed following the procedure of Soeryapranata et al. (*13*). The mixture of cheese extract and internal standard was diluted with saturated matrix solution to a constant final volume (40 μL). One microliter of the mixture of cheese extract, internal standard, and matrix was spotted on the sample plate and allowed to air-dry before MALDI-TOF analysis. Each replicate of the cheese extract was analyzed 10 times to obtain the average peak-height ratio of β-CN f193−209 to internal standard. The concentration ratio of β-CN f193−209 to internal standard was calculated using the calibration curve equation

$$y\,[\pm 0.03] = [1.20\ (\pm 0.03)]x + [0.01\ (\pm 0.02)]$$

where y and x are peak-height ratio and concentration ratio of β-CN f193−209 to internal standard, respectively. Conversion from x to molar concentration of β-CN f193−209 was obtained from

$$[\beta\text{-CN f193}-209] = (x)\,(\text{volume ratio of internal standard/cheese extract})\,[\text{internal standard}]$$

where the internal standard concentration is 76.8 μM. Regression analysis was performed to establish the correlation between MALDI-TOF and sensory data. The correlation coefficient, determined as the Pearson correlation coefficient, was analyzed at a 0.05 significance level.

RESULTS AND DISCUSSION

Percentages (wwb) of moisture, salt, protein, and fat of the experimental cheeses were 34.41 ± 0.67, 1.67 ± 0.13, 25.59 ± 0.42, and 34.31 ± 0.48, respectively. The pH of the cheeses was 5.06 ± 0.05. Analyses of the cheese components were done the day after the cheese was made. The composition of the experimental cheeses was similar to that of typical commercial Cheddar cheeses (*23*).

Table 1 shows concentrations of β-CN f193−209 in aqueous extracts of Cheddar cheese measured by MALDI-TOF mass spectrometry. [Please note that the bitter recognition threshold of β-CN f193−209 reported by Koka and Weimer (*15*) is 0.35 mg/mL, equivalent to 186 μM. In our study, the β-CN f193−209 concentration in the aqueous extract of cheese has been diluted 1:3 due to the addition of 2 parts of water to 1 part of cheese when the extract was prepared.] At 180 and 270 days,

Table 1. Concentration of β-CN f193–209 (Micromolar) in Aqueous Extracts of Cheddar Cheese during Aging Measured by MALDI-TOF Mass Spectrometry[a]

	aging time			
cheese	0 days	120 days	180 days	270 days
56	7.48 ± 5.21acd	30.06 ± 1.27d	55.73 ± 3.00e	54.68 ± 1.20d
56-W900R	5.42 ± 0.19ab	27.83 ± 4.43d	22.08 ± 1.24d	41.40 ± 7.00d
56-WSU19	5.90 ± 1.41ab	8.26 ± 4.77c	15.52 ± 2.27cd	10.20 ± 5.20c
98	3.22 ± 2.58bd	79.12 ± 11.65a	108.32 ± 2.27a	87.92 ± 10.71b
98-W900R	4.15 ± 0.18bc	53.24 ± 13.06b	51.97 ± 7.64e	48.05 ± 10.24d
98-WSU19	1.85 ± 1.29b	5.53 ± 6.60c	10.96 ± 6.14cd	10.30 ± 8.97c
105	10.17 ± 2.96a	60.23 ± 6.73b	74.78 ± 5.70b	130.44 ± 4.12a
105-W900R	8.89 ± 0.35 ac	53.80 ± 5.30b	72.61 ± 9.40b	76.32 ± 2.68b
105-WSU19	3.88 ± 0.20bc	7.32 ± 6.92c	5.48 ± 5.52c	8.79 ± 9.54c

[a] Data are the average of duplicate cheeses. Means in a column followed by the same letter are not significantly different ($p < 0.05$) using Fisher's LSD analysis.

the concentrations of β-CN f193−209 in the extracts of cheeses made with primary 56 alone were significantly lower than in the extracts of cheeses made with primary 98 or 105 alone (**Table 1**), in agreement with bitterness intensities of the cheeses (**Figure 1**). With the exception of primary 98, the trend in β-CN f193−209 concentration (**Table 1**) was in agreement with the expected propensity of primary culture to develop bitterness based on the rate of acid production (*17*). The concentration of β-CN f193−209 in the aqueous extract of cheese made with primary 98 alone was significantly higher at 120 and 180 days of aging than in the extract of cheese made with primary 105 (**Table 1**). In agreement with **Figure 1**, these data indicate that primary 98 is a bitter cheese producer, in contrast to the prediction based on the rate of acid production. The sensory data imply that β-CN f193−209 concentration is more accurate than fast/slow acid production in categorizing bitter/nonbitter primary culture.

The accumulation of β-CN f193−209 in cheeses made with primary cultures alone might be associated with the type of cell envelope proteinase (CEP) possessed by the primary cultures or the ability of peptidases of the cultures to break down the β-CN f193−209. Visser et al. (*14*, *24*) reported the production of β-CN f193−209 in Gouda-type cheeses made with *Streptococcus cremoris* strain HP, which has a P_I-type CEP, based on the activity on α_{s1}-, β-, and κ-casein substrates. Broadbent et al. (*12*) confirmed the production of β-CN f193−209 using *Lc. lactis* starter with group h CEP specificity, determined from the substrate binding region and activity on α_{s1}-CN f1−23. However, the current study does not further characterize the type of CEP of the primary cultures.

Lb. helveticus W900R and WSU19 represent adjunct cultures having low and high aminopeptidase (AP) activity, respectively (*18*). The use of either W900R or WSU19 adjunct culture generally decreased the concentration of β-CN f193−209 in the cheese extracts (**Table 1**). However, concentrations of β-CN f193−209 in the extracts of cheeses made with adjunct W900R were not always significantly lower than in cheeses made with primary culture alone. In contrast, the use of WSU19 markedly decreased the concentration of β-CN f193−209, regardless of the type of primary culture. Cheeses made with adjunct WSU19 were perceived to be less bitter than the corresponding cheeses made with adjunct W900R (**Figure 1**).

Figure 2 illustrates general AP activities in cheese during aging, measured using Lys-pNa substrate. Cheeses made with primary culture alone showed an increase in general AP activity during aging (**Figure 2a**). Primary 56 cheeses had higher general AP activity than primary 98 or 105 cheeses. The general AP activity of primary 56 likely relates to the lower accumulation of β-CN f193−209 in primary 56 cheese than in primary 98 or 105 cheese. The addition of adjunct W900R did not markedly increase AP activity in cheese (**Figure 2b**), which might explain the relatively small differences between β-CN f193−209 concentrations in cheeses made with primary alone and with adjunct W900R. In contrast, adjunct WSU19 markedly increased AP activity in cheese (**Figure 2b**).

Figure 3 presents PepX activities in cheese during aging, measured using Arg-Pro-pNa substrate. In contrast to the general AP activity, the primary 56, 98, and 105 cheeses showed a decrease in PepX activity during aging (**Figure 3a**). The addition of adjunct W900R did not markedly increase PepX activity in cheese. The trends of PepX activities in cheeses made with adjunct WSU19 were similar to the corresponding general AP activities (**Figure 3b**). Relationships of β-CN f193−209 concentration and peptidase activities are shown in **Figures 4** and

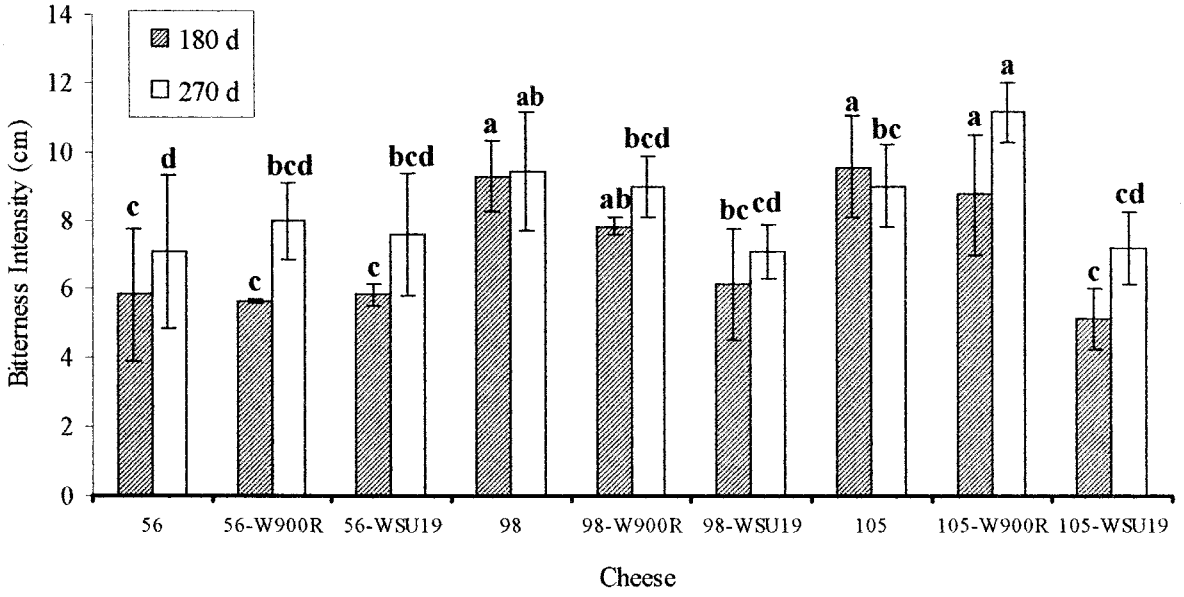

Figure 1. Bitterness intensity (cm) of cheese at 180 and 270 days (0 = low bitterness, 15 = high bitterness). Values in a given aging time followed by the same letter are not significantly different ($p < 0.05$). Vertical bars indicate standard deviation.

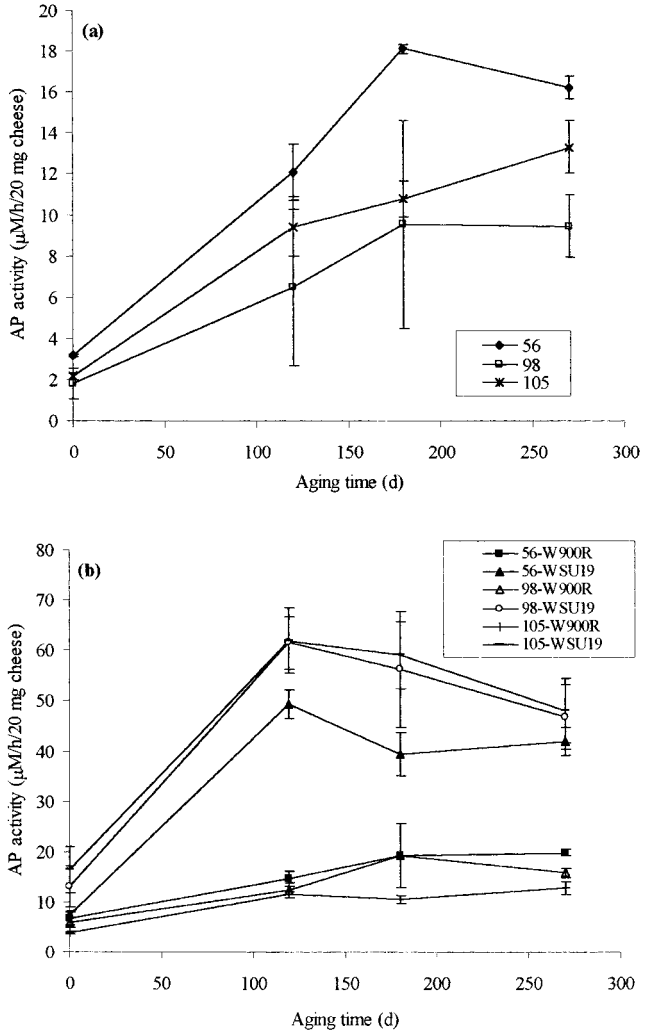

Figure 2. General aminopeptidase activity (μM/h/20 mg of cheese) in cheese as a function of aging time: (a) cheeses made with primary culture alone; (b) cheeses made with the addition of adjunct culture. Each point represents an average of duplicate cheeses. Vertical bars indicate standard deviation.

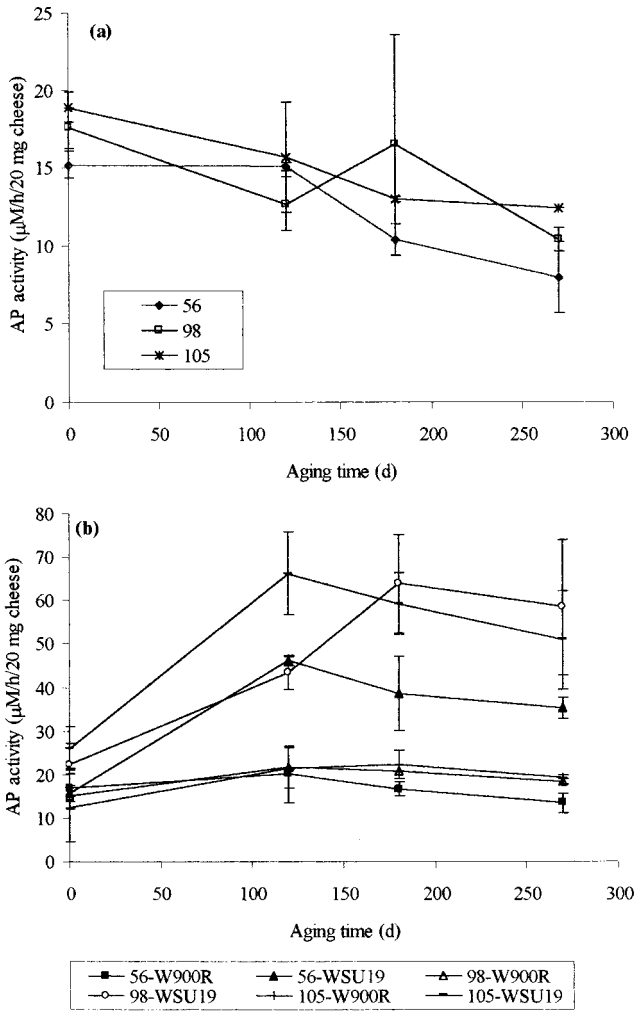

Figure 3. PepX activity (μM/h/20 mg of cheese) in cheese as a function of aging time: (a) cheeses made with primary culture alone; (b) cheeses made with the addition of adjunct culture. Each point represents an average of duplicate cheeses. Vertical bars indicate standard deviation.

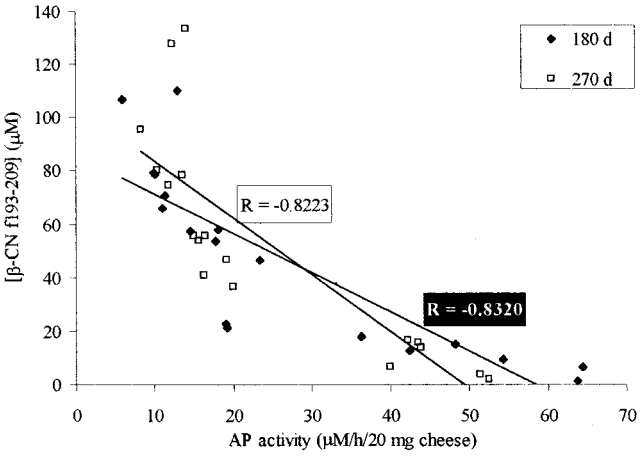

Figure 4. Correlation between β-CN f193–209 concentration in the aqueous extract of cheese and general aminopeptidase activity in cheese at 180 days (■) and 270 days (□).

5. The correlation coefficients between β-CN f193−209 concentration and general AP activity at 180 and 270 days of aging were −0.8320 and −0.8223, respectively, which were greater than the correlation coefficients for β-CN f193−209 concentration and PepX activity at the same aging time. The results of this study were also in agreement with a previous study reported by Baankreis (*25*), who demonstrated an increasing bitterness in cheese made with PepN negative mutants, whereas cheese made with PepX negative mutants exhibited no increase in bitterness.

Besides peptidase activity, the accumulation of β-CN f193−209 during aging might relate to the relative susceptibility of the culture cells to lysis. Kunji et al. (*26*) reported that the oligopeptide transporter of *L. lactis* does not transport the 193−209 fragment of β-casein into the cells of the culture. Because the breakdown of β-CN f193−209 primarily results from the action of intracellular peptidases, cell lysis is important for breakdown of the peptide. In our study, cheeses made with primary 105 had the largest viable cell density throughout aging regardless of the type of adjunct culture, whereas the cell density of primary 56 and 98 cheeses decreased rapidly during ripening. The populations of primaries 56, 98, and 105 in the cheeses at day 0 were 7.69 ± 0.55, 7.40 ± 0.54, and 8.77 ± 0.12 log colony-forming units (CFU)/g, respectively, whereas populations at 270 days were 4.60 ± 0.99, 3.46 ± 0.22, and 6.77 ± 0.48 log CFU/g, respectively. Adjunct W900R has been reported to have a greater viable cell number compared to WSU19 throughout aging, irrespective of the type of primary culture

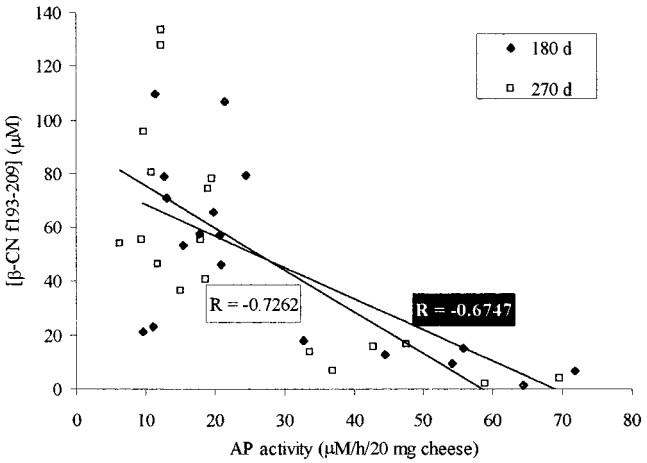

Figure 5. Correlation between β-CN f193–209 concentration in the aqueous extract of cheese and PepX activity in cheese at 180 days (■) and 270 days (□).

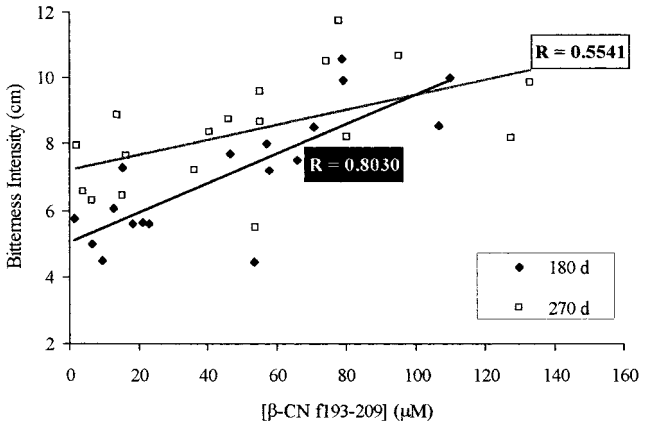

Figure 6. Correlation between bitterness intensity of cheese and β-CN f193–209 concentration in the aqueous extract of cheese at 180 days (■) and 270 days (□).

(*16*). In this study, the low susceptibility of primary 105 to lysis as compared to primaries 56 and 98 might explain the increase of β-CN f193−209 concentration in cheese made with primary 105 alone throughout aging. The low rate of cell lysis would prevent the release of intracellular peptidase that would produce smaller peptide fragments from β-CN f193−209.

The concentration of β-CN f193−209 in cheese extract was significantly correlated to the bitterness intensity of cheese ($p < 0.05$). The correlation coefficients at 180 and 270 days aging were 0.8030 and 0.5541, respectively (**Figure 6**). The relationship of β-CN f193−209 concentration and bitterness intensity is weaker as the aging time progresses, probably due to the presence of other bitter peptides more responsible for bitterness at longer aging and/or the presence of compounds produced during ripening of cheese that mask bitterness perceived by the panelists. The decrease of correlation coefficient between β-CN f193−209 concentration in the cheese extract and bitterness intensity of cheese suggests that β-CN f193−209 is a better marker for bitterness development at early ripening time. Additional study using other potential bitter peptide markers, such as α_{s1}-CN f1−9, is necessary to more fully evaluate the potential of MALDI-TOF to predict bitterness in aged Cheddar cheese. The β-CN f193−209 peptide may be useful not only as a marker for following the development of bitterness in Cheddar cheese but also for categorizing cheese cultures as bitter/nonbitter.

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