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Production and Sensory Characterization of a Bitter Peptide from β-Casein

TANOJ K. SINGH,[†] NIKKI D. YOUNG,[‡] MARYANNE DRAKE,[‡] AND KEITH R. CADWALLADER*,[†]

Department of Food Science and Human Nutrition, 1302 West Pennsylvania Avenue, 205 Agricultural Bioprocess Laboratory, University of Illinois at Urbana−Champaign, Urbana, Illinois 61801, and Department of Food Science, Southeast Dairy Foods Research Center, North Carolina State University, Raleigh, North Carolina 27695-7624

Peptide β-casein fragment 193-209 (β-CN f193-209) was isolated and purified for detailed sensory analysis in different matrices. The purity of the peptide was >98%. The mass of the peptide was 1882.51 Da, which coincided with the expected mass of β-CN f193-209. N-Terminal analysis confirmed that the peptide started at residue 193 on the published sequence of β-casein. Detection thresholds were 0.03, 0.06, and 0.63% (w/w) for water, milk, and cheese, respectively. Descriptive sensory analysis confirmed that the peptide exhibited a bitter taste, which increased with increasing concentrations, with minimal other flavors or tastes detected. The β-CN f193-209 can contribute to bitterness in cheeses.

KEYWORDS: **Caseins; peptide; bitter taste; caffeine; quinine sulfate; sensory threshold analysis; descriptive sensory analysis**

INTRODUCTION

Bitterness is one of the most common off-flavor problems in many cheese varieties, especially those made with mesophilic cultures, and has been associated with the production (by rennet and starter bacteria) of bitter peptides, which predominantly contain hydrophobic amino acid residues (*1*). Aged Cheddar cheese often develops bitterness due to the accumulation of hydrophobic peptides, consisting of 2−23 amino acid residues in the molecular mass range of 500−3000 Da (*2*). A number of bitter peptides, originating from various caseins, in cheeses have been identified. However, the majority of potentially bitter peptides identified in cheeses were found to originate from α_{s1}- and β-casein (*1*). Although some bitterness is considered a normal component of cheese taste, excessive bitterness may limit consumer acceptance of the cheese. The problem of bitterness has been the subject of considerable research, which has been reviewed by Lemieux and Simard (*3*, *4*).

Bitterness appears to be a particular problem in low-fat cheeses (*5*). In normal fat cheese, bitter peptides, being hydrophobic, probably partition into the fat phase where they are less likely to be perceived as being bitter. The activities of both chymosin and starter bacterial proteinases have been implicated in the formation of bitter peptides in cheese (*3*). Most of the chymosin is lost in the whey, and only a small portion (reported values range between 0 and 15% of total coagulant added to cheese milk) is retained in the cheese curd, which continues to play a very important role in the initial degradation of casein during cheese ripening (*1*). The level of chymosin incorporated in the cheese curd is dependent on the initial level of chymosin added and the pH at whey drainage (more rennet is retained in the curd at lower pH) (*6*). In solution, chymosin can hydrolyze seven peptide bonds in β-casein, which are arranged in decreasing order of specificity: Leu^{192}-Tyr^{193} > Ala^{189}-Phe^{190} > Leu^{165}-Ser^{166} = Gln^{167}-Ser^{168} = Leu^{163}-Ser^{164} > Leu^{139}-Leu^{140} = Leu^{127}-Thr^{128} (*7*). During the ripening of Cheddar cheese, β-casein undergoes limited hydrolysis by plasmin but does not appear to be hydrolyzed by chymosin (*8*). Kelly and co-workers (*9*) found that the formation of β-CN f193-209 (a primary product of chymosin action on β-casein and a potentially bitter peptide) is inhibited by increasing NaCl concentrations. This clearly shows that the concentration of NaCl has a major effect on the hydrolysis of β-casein by chymosin in cheese and thus may also be a factor in the control of bitterness. Peptide β-CN f193-209 is a major cause of bitterness in Gouda (*10*) as well as in Cheddar cheese (*9*).

Some authors suggest that bitterness is simply related to starter cell numbers, e.g., fast acid-producing, heat tolerant strains, but others maintain that there are inherent differences between bitter and nonbitter starter strains with respect to proteinase and peptidase profiles (*3*). From experiments on the ability of different cultures to hydrolyze β-CN f193-209 under different growth conditions, Smit and co-workers (*11*) concluded that in general cells grown under pH-controlled conditions have a stronger debittering ability than cells grown in acidifying conditions. These differences were the result of increased

* To whom correspondence should be addressed. Tel: 217-333-5803. Fax: 217-333-1875. E-mail: cadwlldr@uiuc.edu.
[†] University of Illinois at Urbana−Champaign.
[‡] North Carolina State University.

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sensitivity to lysis of cultures grown under pH-controlled conditions than cells grown under acidifying conditions. The aminopeptidase activity appears to be important in reducing the intensity of bitterness (*12*).

There is now a fairly good understanding of mechanisms involved and factors affecting the production of bitter peptides in cheeses. Several peptides have been isolated from different cheese varieties and linked to bitter taste, but previous studies have lacked a detailed sensory characterization. The peptide β-casein fragment 193-209 (β-CN f193-209), produced by chymosin action on β-casein, has been widely reported as the major cause of bitterness in cheeses. The objective of this study was to isolate and purify peptide β-CN f193-209 and perform detailed sensory analysis on the same peptide in different matrices.

MATERIALS AND METHODS

Protein Sample and Chemicals. Purified β-casein used in this experiment was isolated using the procedure of Ward and Bastian (*13*). This sample was supplied by R. Miller (Pilot Plant Manager, Department of Food Science and Human Nutrition, University of Minnesota, St. Paul). Chymosin (Chymax) was obtained from Chr. Hansen Inc. (Milwaukee, WI). Trifluoroacetic acid (TFA) and acetonitrile were purchased from Aldrich (St. Louis, MO) and Fisher Scientific (Hampton, NH), respectively. All other chemicals used were of analytical grade.

Production of Peptide β-CN f193-209. β-Casein was dissolved in deodorized, distilled, deionized water (water prepared by one-third volume reduction of distilled deionized water by boiling) at a concentration of 10 mg/mL. Solubilized protein (100 mL) was treated with chymosin (1 μL enzyme preparation per 1 mL protein solution). Chymosin hydrolysis of β-casein was followed for 17 h at 30 °C (at pH 6.8). The reaction was stopped by heating the reaction mixture in a water bath (70 °C for 20 min). Bitter peptide β-CN f 193-209 was isolated by adjusting the pH of the mixture to 4.6 using 4 M HCl. At pH 4.6, β-casein and the larger polypeptide β-CN f1-192 were precipitated leaving only the small peptide β-CN f193-209 in the solution. The formation of peptide (present in the pH 4.6 soluble material) was monitored by reversed phase high-performance liquid chromatography (RP-HPLC). For the scaled up preparation of peptide, chymosin hydrolysis of β-casein (5 L batches with double the protein and enzyme concentrations mentioned above, pH 6.8) was performed for only 5 h. This hydrolysis time allowed optimal production of the peptide of interest with little or no hydrolysis of any other bonds in β-casein. Peptide preparation was freeze-dried and stored frozen (−70 °C) until analysis.

Compositional Analysis and Preparation of Water Soluble Extract (WSE) of Cheese. Mild nonbitter Cheddar cheese used in threshold studies was analyzed for fat, moisture, salt, and pH using the methods outlined by ur-Rehman et al. (*14*). The WSE of nonbitter Cheddar cheese used as a matrix for studying bitterness of peptide β-CN f193-209 was prepared as outlined by Kuchroo and Fox (*15*). The peptide profile of WSE was analyzed using RP-HPLC.

RP-HPLC Analysis. The production and purity of peptide preparation were monitored by RP-HPLC. The pH 4.6 soluble fraction of hydrolyzate, lyophilized peptide preparation, or water extract of Cheddar cheese was dissolved in 10% solvent B (0.1%, v/v, TFA in acetonitrile) in solvent A (0.1%, v/v, TFA in deionized water) and filtered through 0.2 μm filters for application to the column. HPLC was performed using an automated Agilent HPLC system (consisting of a model 1050 quaternary solvent delivery pump, autosampler; and photodiode array detector; Agilent Technologies Inc., Wilmington, DE) fitted with a Prodigy C18 column (particle size 5 μm, pore size 100 Å, 250 mm × 4.6 mm; Phenomenex, Torrence, CA) and guard column (30 mm × 4.6 mm, Phenomenex). The samples were eluted initially with 10% B for 5 min, then with a gradient from 10 to 70% B over 35 min, and finally % B increased to 95 over 20 min; the flow rate was maintained at 0.75 mL min^{-1}. The eluate was monitored at 214 and 280 nm using a photodiode array detector. The HPLC system was interfaced with a computer using Chemstation software (Agilent Technologies Inc.).

For the characterization of peptide, the peak corresponding to the peptide of interest was collected manually over several runs in eppendorf tubes. The collected peaks from different runs on RP-HPLC were combined, diluted by adding deionized water to reduce the concentration of acetonitrile, and applied on a C8 solid phase extraction cartridge (JT Baker, Phillipsburg, NJ), which was previously washed first with solvent B (5 mL) and then solvent A (10 mL). After application of the peptide solution, the C8 cartridge was washed with 10 mL of solvent A. Finally, peptide was eluted from the cartridge by 5 mL of solvent B. The final eluant was collected and concentrated down to 200 μL under a gentle stream of nitrogen (ultrahigh pure grade). This concentrated solution was used for the characterization of peptides by mass spectrometry and N-terminal amino acid sequencing. The WSE sample of nonbitter Cheddar was analyzed for the presence of peptide β-CN f193-209. RP-HPLC analysis was also preformed on the WSE sample with the addition of purified peptide β-CN f193-209.

Identification of Peptide β-CN f193-209. Three N-terminal amino acid residues of isolated peptide were determined by automated Edman degradation chemistry using a Procise 494 protein/peptide sequencer (Applied Biosystem Inc., Foster City, CA). The mass of the isolated peptide was determined using a Voyager DE STR matrix-assisted laser desorption/ionization time-of-flight (MALDI/TOF) mass spectrometer (Applied Biosystem Inc.). Some details about the mass spectrometry analysis are as follows: mode of operation, linear; polarity, positive; accelerating voltage, 20000 V; acquisition mass range, 500−6000 Da; MALDI matrix, α-cyano-4-hydroxycinnamic acid; calibration, external (using angiotensin and adrinocorticotropic hormone).

Sensory Threshold Analysis. Sensory analysis was conducted in compliance with North Carolina State University Institutional Review Board (NCSU IRB) human subject regulations. A modification of the ASTM procedure E679-79 (*16*), an ascending forced choice method of limits, was used to determine taste threshold values for each compound. Four different matrices were used as carriers: odor-free water (prepared by boiling 4 L of distilled water until its volume was decreased by one-third), skim and whole milk provided from the dairy plant (Department of Food Science at North Carolina State University), and mild Cheddar cheese (purchased from a local grocery store, prescreened to be free from bitterness). Detection thresholds of three bitter compounds were evaluated, caffeine, quinine sulfate, and β-CN f193-209 (**Table 2**).Three samples (two blank and one containing the bitter compound) were presented at six concentration levels. Fluid samples (20 mL) were placed into 4 oz soufflé cups coded with three-digit random codes and were presented at room temperature. For Cheddar cheese, bitter compounds were first dissolved in odor-free water as stock solutions. The solutions were then added using a sterile micropipet to 100 g of manually grated Cheddar cheese (warmed to room temperature) in a plastic Ziploc bag. The grated cheese and bitter compound solutions were kneaded by hand in the bag (approx 5 min) until homogeneous and formed into logs. For blanks (no bitter compound), this procedure was repeated except that a volume of water was added to the cheese shreds. The logs were refrigerated overnight for equilibration. Logs were sliced into 10 g disks and placed into 4 oz soufflé cups coded with three-digit random codes and equilibrated to room temperature before testing. Lids were not used on the cups for testing to minimize the possibility of aroma contributing to threshold detection.

Subjects (n = 35) were instructed prior to testing. Subjects were instructed to taste the solution or cheese in each cup in the series. Subjects were told to thoroughly disperse the samples within their mouths. Subjects rested 3 min between each set of three and were also instructed to rinse their mouths with spring water and to take a bite of unsalted cracker between cups. Subjects were asked to choose the one item from the three that they thought different and to give a certainty judgment (sure/not sure). The individual best estimate threshold was taken as the geometric mean of the last concentration with an incorrect response and the first concentration with a correct response except for the following sequence: if the subject indicated a "not sure" response for the correct choice, that concentration was increased by a factor of 1.41, to adjust for the possibility of a chance correct response (*17*).

Table 1. Lexicon of Flavor Attributes for Casein Peptide

flavor attribute	definition
animal hair/glue [a]	aromatics associated with horse hair or glue
salty [b]	the taste stimulated by sodium salts, such as sodium chloride and sodium glutamate, and in part by other salts, such as potassium chloride
bitter [b]	the taste on the tongue associated with bitter agents such as caffeine or quinine
astringent [b]	the shrinking or puckering of the tongue surface caused by substances such as tannins or alum

[a] Ref *19*. [b] Ref *18*.

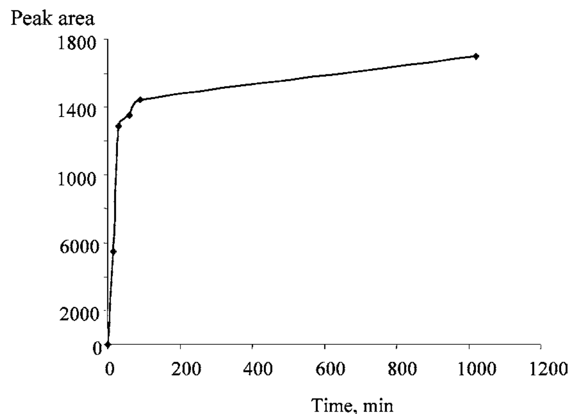

Figure 1. Formation of β-CN f193-209 by chymosin hydrolysis of β-casein.

Threshold testing was conducted in duplicate for each compound on different days. Group thresholds were taken as the geometric mean of the individual best estimate thresholds.

Descriptive Sensory Analysis. Sensory analysis was conducted in compliance with NCSU IRB human subject regulations. Descriptive sensory analysis was conducted to confirm that thresholds were based on bitter taste detection and not some other stimuli provided by the purified peptide. A trained descriptive sensory panel (seven panelists with over 500 h each of descriptive analysis of dairy products, previously screened for the ability to detect bitter taste with caffeine and quinine sulfate solutions) evaluated the sensory attribute intensities of the bitter peptide in the four matrices using the Spectrum intensity scale (*18*). The attributes and definitions for each attribute are provided in **Table 1**. The panel evaluated 50 mL samples of the water and milk stimuli, while 10 g samples of the Cheddar cheese stimuli were evaluated. The three highest concentrations used in threshold detection were evaluated in duplicate by each trained panelist.

RESULTS AND DISCUSSION

Production and Characterization of Purified Peptide. Purified preparation of β-casein dissolved in water was hydrolyzed by chymosin at 30 °C. Under these conditions, the bond of Leu^{192}-Tyr^{193} was rapidly hydrolyzed by chymosin with little or no hydrolysis of any other peptide bond. **Figure 1** shows the peak areas corresponding to the peptide β-CN f193-209, from the RP-HPLC chromatograms of various times during reaction. The β-CN f193-209 reached a plateau level within 90 min of hydrolysis of β-casein by chymosin.

The identity of the peptide was confirmed by N-terminal amino acid sequencing and mass spectrometry. The first three amino acid residues determined using automated Edman degradation chemistry were H.Tyr.Gln.Glu, which perfectly matches with residues Tyr^{193}, Gln^{194}, and Glu^{195} in the published primary amino acid sequence of β-casein. To know the exact length of the peptide, we performed mass spectrometry analysis. The experimental mass obtained using MALDI/TOF-MS was 1882.51 Da, which is an excellent match with the theoretically calculated mass 1881.2336 Da for the β-CN f193-209 (sequence of H.Tyr^{193}.Gln.Glu.Pro.Val.Leu.Gly.Pro.Val.Arg.Gly.Pro.Phe.Pro.Ile.Ile.Val^{209}.OH). The difference of 1.2764 Da (% variation of 0.0678) between experimental and theoretically calculated masses is well within the instrumental error of MS. With the sequence and mass data, we can conclusively say that the N terminus of the isolated peptide started at amino acid number 193 and ended at amino acid number 209 (which is also the C-terminal end of the parent molecule β-casein).

For the scaled up production of the peptide β-CN f193-209, a reaction time of 5 h was chosen, which gave a nearly homogeneous preparation (>98%, based on the peak area from RP-HPLC chromatogram at 214 nm; result not shown) of peptide with optimal production. In buffered solution of Na-caseinate, chymosin hydrolyzes α_{s1}-casein at a much faster rate than β-casein. However, in distilled water solution of the same protein, β-casein was hydrolyzed faster than α_{s1}-casein (Wang, W.; Fox, P. F. Personal communication). Increasing the ionic strength of β-casein solution promotes intermolecular hydrophobic interactions between its hydrophobic C-terminal region, which contains the chymosin sensitive bonds. The protocol developed here in the present study for the production of peptide β-CN f193-209 from a purified preparation of β-casein by chymosin action is based on the observations mentioned above.

The presence of peptide in the nonbitter Cheddar cheese (compositional data: moisture %, 37.8 ± 0.2; fat %, 33.0 ± 0.1; salt %, 1.55 ± 0.4; and pH, 5.08 ± 0.02) used as a matrix in sensory characterization was assessed by peptide profiling of WSE using RP-HPLC. Chromatography results showed that β-CN f193-209 was not present in the Cheddar cheese matrix used in this study (chromatogram not included). This observation was further confirmed by the spiking of above WSE with purified peptide β-CN f193-209 followed by peptide profiling by RP-HPLC (chromatogram not included).

Sensory Analysis of Peptide β-CN f193-209. As expected, best estimated thresholds (BETs) for each compound were distinct. Different bitter compounds exhibit different sensory thresholds and span an array of different chemical and physical properties (*20*). Within each compound, the matrix had a significant impact on the threshold (**Table 2**). In general, the BET increased as the matrix complexity increased. For caffeine, BETs were not different between water and skim milk, but the BET increased by a factor of 10 between these matrices and whole milk and by a factor of 100 between whole milk and Cheddar cheese. This trend was similar with quinine sulfate. In contrast, large differences were not observed in the BETs for the casein peptide between water, skim, and whole milk. The BET did increase, however, by a factor of 10 between these three matrices and Cheddar cheese. Cheddar cheese represents the most complex matrix tested in terms of composition and also in terms of the presence of other basic tastes and volatile flavor compounds.

Descriptive sensory analysis was conducted with the casein peptide across the concentrations that spanned the BETs for each matrix to confirm that bitter taste was the actual stimuli rather than some other volatile or basic taste. Results indicate that the peptide was bitter across the concentration range spanned by the BET in each matrix and provides additional

Table 2. Concentrations Used for Threshold Testing, Best Estimate Thresholds (BETs), and BET Standard Deviations for Caffeine, Quinine Sulfate, and β-CN f193-209 in Different Matrices

compound	carrier	range of threshold concentrations (% w/v)	geometric mean (BET)	standard deviation
caffeine	water	0.00003–0.0073	0.00038	0.002
caffeine	skim milk	0.00003–0.0073	0.00026	0.021
caffeine	whole milk	0.0001–0.0243	0.0025	0.011
caffeine	cheese	0.01 – 2.43	0.1290	0.29
quinine sulfate	water	0.00000033–0.000081	0.0000027	0.000024
quinine sulfate	skim milk	0.00000033–0.000081	0.000011	0.00005
quinine sulfate	whole milk	0.00001–0.00243	0.000125	0.0006
quinine sulfate	cheese	0.0003–0.0243	0.0035	0.013
casein bitter peptide	water	0.0027–0.2187	0.0266	0.067
casein bitter peptide	skim milk	0.003–0.729	0.0620	0.105
casein bitter peptide	whole milk	0.0033–0.81	0.0620	0.12
casein bitter peptide	cheese	0.017–4.05	0.6345	1.021

Table 3. Mean Descriptive Sensory Attributes of the β-CN f193-209 in Water, Skim Milk, Whole Milk, and Cheese[a]

matrix	concn (% w/v)	animal hair/glue	salty	bitter	astringent	bitter detection frequency from panelists
water	blank	0.0	0.0	0.0	0.0	0/7 bitter
	0.0243	1.1	0.0	0.6	0.0	2/7 bitter
	0.0729	2.0	0.0	0.8	0.5	4/7 bitter
	0.2187	2.5	1.5	1.1	0.5	6/7 bitter
	0.656	4.0	2.0	1.8	0.5	7/7 bitter
skim milk	blank	0.0	1.0	0.0	1.1	0/7 bitter
	0.081	0.0	1.5	1.3	2.0	5/7 bitter
	0.243	0.0	2.5	2.2	3.5	6/7 bitter
	0.729	1.0	3.5	3.5	5.1	7/7 bitter
whole milk	blank	0.0	1.1	0.0	0.5	0/7 bitter
	0.009	0.0	1.2	0.5	3.1	2/7 bitter
	0.27	0.0	2.5	1.5	4.0	5/7 bitter
	0.81	0.0	3.5	3.5	6.2	7/7 bitter
cheese	blank	0.0	2.8	0.0	1.1	0/7 bitter
	0.45	0.0	3.8	0.5	1.2	4/7 bitter
	1.35	0.0	3.8	2.2	4.0	5/7 bitter
	4.05	0.0	4.5	3.5	6.3	7/7 bitter

[a] Attributes were scored on a 15 point numerical intensity scale where 0 = absence of the attribute and 15 = extremely high intensity of the attribute (*19*).

confirmation that the peptide was bitter in taste (**Table 3**). A low intensity of volatile flavor (animal hair/glue) was noted by trained panelists in water and skim milk with the added β-CN f193-209. This volatile flavor was not detected in whole milk or Cheddar cheese and was likely masked by other flavors in these matrices. This volatile flavor has been previously described in spray-dried caseins and caseinates (*19*). The salty taste was also detected by trained panelists. The presence of NaCl/salty taste in the bitter peptide preparation can be explained by the neutralization (to pH 7.0 with NaOH) of aqueous suspension of purified β-CN prior to spray drying and followed by acidification of the chymosin hydrolyzate of the same (to pH 4.6 with HCl) for the isolation of bitter peptide. These panelists all had previous experience with descriptive analysis of dairy products (primarily milk and cheese) and had been screened for the ability to detect bitterness using caffeine solutions. Wide variability exists in individual bitter taste sensitivities between individuals and within one individual for different bitter compounds (*21*). None of the trained (and previously screened) panelists had any problem detecting the bitter taste in the β-CN f193-209. However, all panelists noted that the bitterness perception of the β-CN f193-209 was “different” from the bitterness of caffeine. Some described this difference as the bitter intensity seeming to be most intense on a different part of the tongue while others were unable to describe the difference but were certain that it was different. Peptide β-CN f193-209, determined to be bitter by sensory analysis in the present study, with a Q value of 1839 cal mol^{-1} residue^{-1} and a molecular mass of 1882.51 Da can also be classified as potentially bitter in the Q value model proposed by Ney (*22*).

A number of other small molecular mass bitter peptides have been identified from the β-CN region Tyr[193] to Val[209], which were found to be more bitter than β-CN f193-209 (see ref *23*). Relative bitterness of the peptide β-CN f193-209 in comparison with its shorter fragments arranged in ascending order (with threshold in water given in parentheses) is β-CN f196-201 (0.503 mM) < β-CN f193-209 (0.129 mM, present study) < β-CN f196-209 (0.015 mM) < β-CN f200-209 (0.004 mM) = β-CN f202-209 (0.004 mM).

An attempt was made to theoretically quantify the amount of β-casein that needed to be hydrolyzed during ripening of Cheddar cheese to exceed the BET value of 0.45% (w/w) for β-CN f193-209. Cheddar cheese contains about 25.4% protein (*24*). Assuming that all of this protein is casein, then about 40% (by weight) will be β-casein. Therefore, about 60% of the total β-casein would need to be hydrolyzed at the bond Leu[192]-Tyr[193] to exceed the BET for β-CN f193-209. It has previously been reported that nearly 50−60% of total β-casein is hydrolyzed during the 9−12 month ripening of Cheddar cheese (*25*). Lower salt and/or higher moisture in Cheddar cheese could lead to an even higher level of β-CN hydrolysis (*9*), which can easily exceed BET for β-CN f193-209 and result in a bitter taste defect in cheese. In reduced fat cheeses, bitterness may be more pronounced. From the studies on varying fat content Danbo cheeses, Madsen and Ardo (*26*) concluded that about 50% more bitter peptides derived from casein could be added to the normal or reduced fat cheese than to the low-fat cheese before bitterness was noticed, indicating masking of bitterness in cheese containing more fat. Therefore, the BET value for the peptide β-CN f193-209 could be even lower in low or reduced fat cheeses.

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