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Sensomics Mapping and Identification of the Key Bitter Metabolites in Gouda Cheese

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Application of a sensomics approach on the water-soluble extract of a matured Gouda cheese including gel permeation chromatography, ultrafiltration, solid phase extraction, preparative RP-HPLC, and HILIC combined with analytical sensory tools enabled the comprehensive mapping of bitter-tasting metabolites. LC-MS-TOF and LC-MS/MS, independent synthesis, and sensory analysis revealed the identification of a total of 16 bitter peptides formed by proteolysis of caseins. Eleven previously unreported bitter peptides were aligned to β-casein, among which 6 peptides were released from the sequence β-CN(57–69) of the N terminus of β-casein and 2 peptides originated from the C-terminal sequence β-CN(198–206). The other peptides were liberated from miscellaneous regions of β-casein, namely, β-CN(22–28), β-CN(74–86), β-CN(74–77), and β-CN(135–138), respectively. Six peptides were found to originate from α_{s1}-casein and were shown to have the sequences α_{s1}-CN(11–14), α_{s1}-CN(56–60), α_{s1}-CN(70/71–74), α_{s1}-CN(110/111–114), and α_{s1}-CN(135–136). Sensory evaluation of the purified, synthesized peptides revealed that 12 of these peptides showed pronounced bitter taste with recognition thresholds between 0.05 and 6.0 mmol/L. Among these peptides, the decapeptide YPFPGPIHNS exhibited a caffeine-like bitter taste quality at the lowest threshold concentration of 0.05 mmol/L.

KEYWORDS: Gouda cheese; bitterness; bitter peptides; taste dilution analysis; sensomics; sensomeabololome; metabolites

INTRODUCTION

Gouda cheese is highly appreciated by consumers all over the world. Gouda is made from cow's milk that is cultured and heated until the curds separate from the whey. The curds are pressed into circular molds for a couple of hours to give the cheese its traditional shape. Thereafter, the cheese is soaked in a brine solution, which gives the cheese its rind and improves the taste. After the salt soaks in, the cheese is dried for a couple days before being coated to prevent it from drying out. The Gouda cheese is then matured for 6−44 weeks before it is ready to eat. Depending on its degree of ripening, the cheese is well-known to develop a distinct bitter taste, which is desirable when perceived in low intensity and sometimes considered as a flavor defect when too strong.

Whereas multiple studies published in the past 30 years have been performed to identify and quantify the odor-active key volatiles in cheese, knowledge of the nonvolatile taste compounds is still rather fragmentary. Although literature studies suggest the sour-tasting lactic acid, the salty-tasting sodium chloride, and sweet, umami or bitter-tasting free amino acids, as well as bitter peptides of various structures, as the key compounds imparting the typical taste profile of cheese (*1*), the data published on the bitterness-inducing molecules are somehow contradictory (*2–8*). Multiple studies focused on the compounds inducing a bitter flavor defect to Cheddar cheese (*1–3*), Gouda cheese (*4, 5*), Camembert (*6*), Swiss mountain cheese (*7*), butter cheese (*8*), or Ragusano (*9*) provided evidence that peptides play an important role in inducing the bitter taste of cheese (*10*). It is discussed in the literature that the bitter flavor defect results from the accumulation of bitter peptides due to either increased casein proteolysis or inadequate degradation of these peptides during cheese ripening (*11, 12*).

In comparison, knowledge of the peptides inducing a mild and attractive bitterness in premium cheese exhibiting no flavor defect is rather fragmentary, and the contribution of bitter peptides seems to be doubtful. For example, the bitter taste of goat cheese was reported to be due to calcium and magnesium chloride (*13*), and no significant correlations between bitter-tasting cheese fractions and the occurrence of peptides could be found (*14*). Being well in line with these findings, reincorporation of bitter peptide fractions into a Cheddar cheese matrix did not induce any significant change in the sensory profile of the product (*15*). In contradiction, amino acids and biogenic

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amines including cadaverine, ornithine, and citrulline were reported as the bitter compounds in Camembert cheese (*16*), whereas a slight and pleasant bitterness imparted by peptides is believed to be desirable for cheese flavor (*17*).

Driven by the need to discover the key players imparting the attractive taste of foods, the research area of "sensomics" made tremendous efforts in recent years to map the entire sensome-tabolome and to identify and catalog the most intense taste-active metabolites in fresh and processed foods. This approach has been proven successfully to identify cooling compounds in roasted malt (*18*), a taste enhancer in beef bouillon (*19*), and the astringent and bitter key compounds in tea infusions (*20*), as well as in roasted cocoa (*21*) and, recently, the astringent ellagitannins migrating into spirits and wines upon oak treatment (*22*).

The objectives of the present investigation are to screen the metabolome of a Gouda cheese for bitter compounds by means of a sensomics approach, to isolate and identify the most intensely bitter metabolites using sensory directed fractionation, and to determine the human thresholds of the metabolites evaluated with the highest gustatory response.

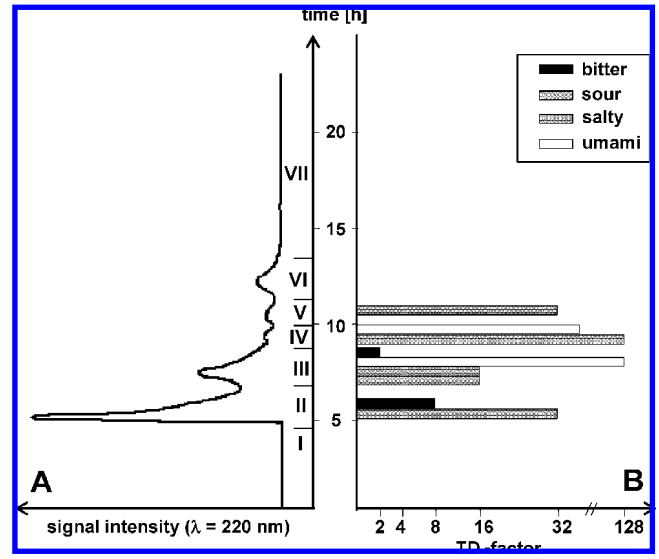

Figure 1. GPC chromatogram (**A**) and taste dilution analysis (**B**) of CPWSE isolated from Gouda cheese.

MATERIALS AND METHODS

Chemicals and Materials. Formic acid was purchased from Grüssing (Filsum, Germany), trifluoroacetic acid was obtained from Fluka (Neu-Ulm, Germany), and solvents were of high-performance liquid chromatography (HPLC) grade (Merck, Darmstadt, Germany). Deionized water used for chromatography was prepared by means of a Milli- Q water gradient A 10 system (Millipore, Billerica, MA). Synthetic peptides were purchased from EZBiolab (Westfield, IN). Standard Gouda cheese samples, which were ripened for 44 weeks under controlled standard conditions and exhibited medium bitterness, were obtained from the Dutch food industry in rindless portions (100 g) freshly cut from a cheese wheel, sealed in nitrogen-flushed packages, and stored at –20 °C until use.

Preparation of the Water-Soluble Extract (WSE) from Gouda Cheese. A sample (60 g) of the Gouda cheese, cut into small pieces with a kitchen knife, and deionized water (240 mL) were placed into a centrifuge beaker, homogenized for 5 min by means of an Ultra-Turrax T 25 basic (Ika Labortechnik, Germany), and then centrifuged at 10000 rpm for 20 min at 4 °C by means of a Varifuge 20 RS (Kendro, Langenselbold, Germany). Three phases were separated: an upper solid fat layer, a liquid fraction including the cheese water-solubles (pH 5.7), and a protein pellet. The protein pellet as well as the fat layer were isolated and re-extracted with deionized water (240 mL) as detailed above. The aqueous layers containing the cheese water solubles were pooled to obtain the WSE. Soluble casein was then precipitated upon adjustment of the pH of the WSE solution to 4.6 by the addition of formic acid (1%, v/v, in water). Centrifugation at 10000 rpm at 4 °C for 20 min, followed by paper filtration (Macherey-Nagel, 615-1/4) and freeze-drying, revealed the casein-precipitated water-soluble extract (CPWSE), which was stored at –20 °C until further analysis.

Gel Permeation Chromatography (GPC). An aliquot (1.0 g) of the CPWSE lyophilysate was dissolved in water (10 mL); the solution was adjusted to pH 4.0 with aqueous formic acid (1% in water) and after filtration was placed on top of a XK 50/100 glass column (Pharmacia, Uppsala, Sweden) filled with a slurry of Sephadex G15 (Amersham Bioscience, Uppsala, Sweden) in water (pH 4.0). Monitoring the effluent (2.5 mL/min) at 220 nm by means of an L-7420-type UV−vis detector (Merck Hitachi, Darmstadt, Germany) allowed the collection of seven fractions (I−VII) as given in **Figure 1**, which, after freeze-drying, were stored at −20 °C until use.

High-Performance Liquid Chromatography (HPLC). For analytical HPLC, a Prominence-type HPLC (Shimadzu, Duisburg, Germany) was equipped with two LC-20AT type pumps, a CBM-20A type communication bus module, an SIL-20A type autosampler unit, a DGU-20A3 type solvent degasser, and a Sedex 75 type evaporative light scattering detector (LT-ELSD, S.E.D.E.R.E., Alfortville Cedex, France) with nebulizer. Data acquisition was performed with the software LabSolutions LCsolution V 1.21 SP1. For analysis of isolated fractions, an aliquot was dissolved in aqueous trifluoroacetic acid (0.1% in water), membrane-filtered (0.45 μm), and analyzed on an RP-18 Microsorb 100-5, 250 × 4.6 mm i.d., 5 μm column (Varian, Darmstadt, Germany) equipped with a guard column of the same type. Chromatography was performed with an isocratic gradient of 0.1% aqueous trifluoroacetic acid and acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 1.0 mL/min.

For preparative HPLC, the HPLC system consisted of S 1122 type pumps (Sykam, Eresing, Germany), an Rh 7125i type Rheodyne injection valve (Bensheim, Germany), an ERC-3215α type solvent degasser (ERC, Riemerling, Germany), a gradient mixer (Sunchrom, Friedrichsdorf, Germany), a splitter (Upchurch, Oak Harbor, WA), and a Sedex 85-type ELSD (LT-ELSD, S.E.D.E.R.E.) with nebulizer. The split ratio was set to a flow of 1 mL/min for the ELSD detector. Data acquisition was performed by means of ChromStar V. 6.2 software.

Ultrafiltration. An aliquot (300 mg) of GPC fraction II was dissolved in water (20 mL) and separated by means of ultrafiltration using a Vivacell 70-type gas-pressure filtration unit (VivaScience AG, Sartorius Group, Hannover, Germany) equipped with a polyether sulfone membrane (5 kDa MW cutoff) and operated with a nitrogen pressure of 5 bar. The high molecular weight fraction (HMW; >5 kDa) obtained was taken up in water and, the ultrafiltration was repeated twice to comprehensively remove low molecular weight compounds (LMW; <5 kDa). The HMW fraction (yield = 54%) and the pooled LMW fractions (yield = 46%) were lyophilized, the yield was determined by weight, and they were stored at –20 °C until further use.

Solid Phase Extraction (SPE). Aliquots (maximum 400 mg) of the LMW fraction (<5 kDa) isolated from GPC fraction II were dissolved in aqueous trifluoroacetic acid (1% in water) and applied onto the top of a Strata C18-E SPE cartridge (10 g/60 mL) (Phenomenex) conditioned with the same solvent. Gradient elution started with a 1% aqueous trifluoroacetic acid (fraction II-LMW/1), followed by the same solvent with stepwise increasing amounts of 10% (fraction II-LMW/2), 15% (fraction II-LMW/3), 20% (fraction II-LMW/4), 30% (fraction II-LMW/5), and 40% acetonitrile (fraction II-LMW/6), respectively. Fractions obtained were freed from solvents under vacuum, taken up in water, and freeze-dried twice. The residue obtained for each fraction was dissolved in bottled water (2.0 mL, pH 5.7) prior to analytical sensory experiments.

Analytical Sensory Experiments. *General Conditions, Panel Training.* To familiarize the subjects with the taste language used by our sensory group and to get them trained in recognizing and distinguishing different qualities of oral sensations in analytical sensory experiments, 11 assessors (4 women and 7 men, ages 23–39 years), who gave

informed consent to participate in the sensory tests of the present investigation and have no history of known taste disorders, participated for at least two years in weekly training sessions. For example, the subjects were trained to evaluate the taste of aqueous solutions (2 mL; pH 5.7) of the following standard taste compounds in bottled water (Evian, low mineralization, 500 mg/L): NaCl (20 mmol/L) for salty taste, lactose (50 mmol/L) for sweet taste, lactic acid (20 mmol/L) for sour taste, and monosodium L-glutamate (3 mmol/L) for umami taste. For training and classification of bitter taste, solutions of $MgSO_4$ (166 mmol/L) representing a short-lasting, metallic bitter taste quality perceived mainly at the anterior part of the tongue, salicin (1.4 mmol/L), imparting a long-lasting bitter taste sensation perceived mainly at the back of the tongue as well as the throat, and caffeine (8.0 mmol/L), providing a long-lasting bitterness perceived all over the oral cavity, were used as references. The sensory sessions were performed at 21 °C in an air-conditioned room with separated booths in three independent sessions. To prevent cross-modal interactions with odorants, the panelists used nose clips.

Precautions Taken for Sensory Analysis of Food Fractions and Taste Compounds. Prior to sensory analysis, buffer compounds and solvent traces were removed from the freeze-dried fractions isolated from cheese. To achieve this, the individual fractions were dissolved in water, and remaining volatiles and solvent traces were removed under high vacuum (<5 mPa, 35 °C) and then again taken up in water and freeze-dried twice. HRGC-MS and ion chromatographic analysis revealed that food fractions treated with that procedure are essentially free of the solvents and buffer compounds used.

Formic acid, which is GRAS listed as a flavoring agent for food and feed applications, was used to adjust the pH value of solutions to be sensorially evaluated, because trace amounts of this acid do not influence the sensory profile of the test solution.

To minimize the uptake of any toxic compound to the best of our knowledge, all of the sensory analyses were performed by using the sip-and-spit method, which means the test materials were not swallowed but expectorated.

Taste Recognition Threshold Concentrations. Eleven panelists determined the threshold concentrations of purified bitter peptides in bottled water adjusted to pH 5.7 with trace amounts of aqueous formic acid (1% in water) using triangle tests with ascending concentrations of the stimulus following the procedure reported previously (*22*). The threshold value of the sensory group was approximated by averaging the threshold values of the individuals in three independent sessions. Values between individuals and separate sessions differed by not more than plus or minus one dilution step; that is, a threshold value of 0.1 mmol/L for the peptide YPFPGPIHN represents a range of 0.05–0.2 mmol/L. The bitter taste quality of each peptide was evaluated in aqueous solutions (pH 5.7) containing 5-fold suprathreshold concentrations of the individual peptide.

Taste Profile Analysis. Gouda cheese samples, cut into cubes (1 × 1 cm), were presented to the panel of 11 trained subjects to evaluate the taste qualities bitter, sour, sweet, salty, and umami on an intensity scale from 0 (not detectable) to 5 (strongly detectable). For taste profile analysis of the water-soluble cheese extract, the CPWSE lyophilysate was dissolved in bottled water in "natural" cheese concentration, and the pH value was adjusted to 5.7 with trace amounts of a 1% aqueous solution of formic acid. A 1:4 dilution of this solution was then presented to the members of the sensory panel, who were asked to rate the intensity of the individual taste qualities on a scale from 0 (not detectable) to 5 (strongly detectable).

Taste Dilution Analysis. The lyophilized GPC fractions were taken up in bottled water (10.0 mL) and adjusted to pH 5.7 with a 1% aqueous solution of formic acid. These stock solutions were diluted stepwise 1:2 with water (pH 5.7), and the series of dilutions were randomly presented to the sensory panel in order of increasing concentration. By means of a duo test with one sample as the blank (water), panelists were asked to determine the dilution step at which a difference between sample and blank water could be detected. These so-called taste dilution (TD) factors (*20–22*) determined by the individuals in separate sessions were averaged.

Sensory Analysis of Individual Fractions. Fractions obtained by means of HPLC or SPE, respectively, were taken up in bottled water (2 mL), the pH value was adjusted to 5.7 with aqueous formic acid (1% in water), and the bitter intensity of the different fractions was judged on a scale from 0 (not detectable) to 5 (strongly detectable).

Isolation of Individual Compounds from GPC, SPE, and HPLC Fractions. The individual fractions obtained from GPC, SPE, and HPLC separations, respectively, were dissolved in aqueous trifluoroacetic acid (0.1% in water) and, after membrane filtration, aliquots (0.5–1.0 mL) were separated by preparative HPLC on a Microsorb 100-5 RP-18, 250 × 21.0 mm i.d., 5 μm column (Varian) using a solvent gradient (18.0 mL/min) of acetonitrile and water, both containing 1% trifluoroacetic acid. The GPC fractions III-1 and III-5, respectively, were separated by hydrophilic interaction liquid chromatography (HILIC) using a TSKgel Amide-80, 300 × 21.5 mm i.d., 10 μm column (Tosoh Bioscience, Stuttgart, Germany) using a solvent gradient (6.0 mL/min) of acetonitrile and water, both containing 1% trifluoroacetic acid. The effluent of several runs was separated into subfractions, which were collected individually, freed from solvent in vacuum, and freeze-dried twice. The individual fractions were stored at –20 °C until use.

Ion Chromatography. Cations and anions were analyzed by means of an ICS 2500 ion chromatography system (Dionex, Idstein, Germany) equipped with an AS 50 thermal compartment, a GS 50 gradient pump, an ED 50 electrochemical detector, an AS 50 A autosampler, a GM-4 gradient mixer, and an Ion Pac ATC-3, 4.0 × 35 mm column. System control and data acquisition were accomplished using Dionex Chromeleon version 6.60 software.

Liquid Chromatography−Mass Spectrometry (LC-MS). LC-MS measurements were acquired on an API 4000 Q-Trap MS/MS system (Applied Biosystems Sciex Instruments, Darmstadt, Germany) connected to an Agilent 1100 HPLC-system (Agilent, Waldbronn, Germany). Instrumentation control and data acquisition were performed with Sciex Analyst software (v 1.4).

Recording of ESI Spectra. The spray voltage was set to 5500 eV, the declustering potential was set between 30 and 80 V, and nitrogen served as curtain gas (20 psi) in electrospray positive (ESI^+) mode. The isolated fractions were dissolved in water acidified with trace amounts of formic acid and directly introduced into the mass spectrometer by loop injection. The mass spectrometer was operated in full-scan mode of Q1/Q3 as well as in enhanced mass scan (EMS) mode (ion trap). Fragmentation of $[M + H]^+$ molecular ions was performed in the ion trap unit with the enhanced product ion (EPI) mode in ESI^+ mode with variable collision energy.

Information Dependant Acquisition (IDA) Experiments. To gain further information on the charge of the peptides as well as the retention time and the purity of the peptide, IDA experiments were performed in ESI^+ mode using the settings for the spray voltage (5500 eV), declustering potential (30 V), scan rate (4000 amu/s), and curtain gas (20 psi) as given in parentheses. The EMS experiment was conducted in the range of *m/z* 100–1100, and the EPI experiments were performed for the two most intense ions in the range of *m/z* 200–1100. An enhanced resolution scan was performed for the determination of the charge state and the isotopic pattern. A Fusion-RP80, 150 × 2.0 mm i.d., 5 μm column (Phenomenex, Aschaffenburg, Germany), equipped with a guard column of the same type, was used for HPLC operated with a solvent gradient (0.2 mL/min) of water and acetonitrile, both containing 1% formic acid.

Analysis of Amino Acids by HPLC-MS/MS. CPWSE was dissolved in aqueous ammonium acetate (0.1% in water, pH 3.0) and membrane-filtered, and then an aliquot (25 μL) was analyzed by HILIC on a TSKgel Amide-80, 300 × 7.8 mm i.d., 10 μm column (Tosoh Bioscience). An aqueous solution of ammonium acetate (0.1% in water, pH 3.0) was used as eluent A, and a mixture (95:5, v/v) of acetonitrile and water containing 0.1% ammonium acetate (pH 3.0) was used as eluent B. The gradient (1.0 mL/min) started with 80% eluent A, held isocratically for 10 min, and then the content of eluent A was decreased to 60% within 40 min and finally to 0% after 60 min. The effluent was split into two aliquots; the major aliquot (80%) went to waste, and the minor aliquot (20%) was introduced into the LC-MS/MS system. The MS system was operated in the ESI^+ mode, the spray voltage was set to 4500 eV, and curtain gas was set to 20 psi. Collision energy as well as the declustering potential was adjusted depending on the amino acid.

Table 1. Taste Profile of Gouda Cheese (GC) and Water-Soluble Extract (WSE) Prepared Thereof

	intensity[a] of the taste qualities				
sample	salty	sour	sweet	bitter	umami
GC	4.0	2.0	0.5	3.0	2.0
WSE	4.5	2.0	0	2.5	2.0

[a] Intensity was rated on a scale from 0 (not detectable) to 5 (strongly detectable) and is given as the mean of triplicates.

The amino acids were determined in the multiple reaction monitoring (MRM) mode and m/z 132.2→ 86.1 were selected as mass transitions for the L-isoleucine and L-leucine eluting with retention times of 26.2 and 28.1 min, respectively.

Liquid Chromatography−Time of Flight Mass Spectrometry (LC-TOF-MS). High-resolution mass spectra were determined by means of a Micro-TOF-MS from Bruker Daltonik GmbH (Bremen, Germany) in the ESI^+ mode. Calibration was performed before each measurement, thus achieving a maximum estimation error of 5 ppm.

Sequence Identification of Peptides. On the basis of the high-resolution MS data obtained for each purified peptide, candidate amino acid sequences from milk proteins were determined. The candidate peptides fitting to the exact masses were fed into the software Protein Prospector MS-Product (http://prospector.ucsf.edu) provided by the University of California. Comparison of the theoretical fragmentation pattern with the recorded fragmentation pattern led to the unequivocal identification of the peptide sequences.

Purification of Synthetic Peptides. Purchased synthetic peptides were purified prior to sensory evaluation by means of preparative RP-HPLC using a Microsorb 100-5, 250 × 21.2 mm; 5 μm column (Varian) equipped with am RP-18, 10 × 10 mm; 5 μm guard column (Varian) operated with a gradient (18.0 mL/min) of water and acetonitrile, both containing 0.1% trifluoroacetic acid. The purified peptides were freed from solvent under vacuum, freeze-dried twice, and stored at –20 °C prior to use. HPLC-MS analysis revealed a purity of >99% for the individual peptides.

RESULTS AND DISCUSSION

Preliminary sensory analysis revealed the overall taste of the Gouda cheese as intense, rich, and complex, centering around umami, saltiness, bitterness, and sourness. To perform a taste profile analysis, small cubes of the cheese were presented to trained sensory panelists, who were asked to judge the intensity of the taste qualities salty, sour, bitter, umami, and sweet on a linear scale from 0 (not detectable) to 5 (strongly detectable). Saltiness was rated with the highest intensity of 4.0, followed by bitter taste (3.0) as well as sour and umami taste, both evaluated with a score of 2.0 (**Table 1**). Sweetness was only slightly perceivable with an intensity of 0.5.

As earlier reports on cheese taste indicated that the key taste molecules of cheese are water-soluble (*23–27*), the hydrophilic taste compounds were comprehensively extracted from the Gouda cheese matrix and separated from a protein fraction as well as the milk fat. The water-soluble extract (WSE; 15.1% in yield) obtained was taken up in bottled water in the same concentration (on mass basis) as present in the cheese, adjusted to the pH value of the cheese (pH 5.7) with trace amounts of formic acid, and was then 1:4 diluted with water prior to taste profile analysis. As given in **Table 1**, the highest intensities were again found for saltiness (4.5), followed by bitter taste (2.5), umami taste (2.0), and sourness (2.0). The protein fraction as well as the milk fat obtained during isolation of the WSE did not show any taste activity. The matrix differences between the cheese and the aqueous solution did not allow a direct comparison of the taste intensities, but the observation that bitterness was perceived as less intense and saltiness was judged as more predominant in the WSE than in the cheese confirms previous literature data (*28, 29*).

To further fractionate the WSE, soluble casein was removed from the WSE by acidic precipitation. Sensory analysis of the casein-precipitated WSE (yield = 14.2%) by means of a triangle test did not show any taste differences to WSE, thus indicating that the key compounds are present in the casein-precipitated water soluble extract (CPWSE). To comprehensively map the taste-active metabolites and to locate potential candidates imparting the bitter taste of the cheese, CPWSE was fractionated by gel permeation chromatography (GPC) and evaluated by means of the taste dilution analysis (TDA).

GPC−TDA. The CPWSE was separated by means of GPC using Sephadex G-15 as the stationary and water (pH 4.0) as the mobile phase. As given in **Figure 1**, the seven GPC fractions I−VII were collected, freeze-dried, dissolved in bottled water in their "natural" concentrations, and then used for TDA. The bitter-tasting metabolites present in CPWSE could be located in GPC fractions II and III, which were obtained in yields of 26.8 and 41.3%, respectively, and evaluated with TD factors of 8 and 2 (**Figure 1**). Besides bitterness, GPC fraction II also imparted some sourness and GPC fraction III induced an umami taste and a salty taste impression. Furthermore, GPC fraction IV imparted umami and salty tastes, and fraction V tasted only salty, whereas GPC fractions I, VI, and VII were entirely tasteless.

Sensory-Directed Fractionation of GPC Fraction II. To sort out the bitter molecules from the less bitter or tasteless compounds, first, GPC fraction II was separated into a low molecular weight fraction (LMW; yield = 54%) and a high molecular weight fraction (HMW; yield = 46%) by means of ultrafiltration using a gas-pressure filtration unit equipped with a 5 kDa cutoff membrane (**Figure 2**). Sensory analysis of aqueous solutions containing the LMW or HMW compounds revealed that the key bitter-tasting metabolites have molecular weights below <5 kDa. This observation is in agreement with the literature reporting that bitter peptides have molecular weights of up to 3000 Da (*15, 29–31*).

To further decrease the enormous complexity of the bitter-tasting LMW fraction, this fraction was further separated by means of SPE using an RP-18 cartridge as stationary phase and a water/acetonitrile gradient as the mobile phase. Six fractions, namely, II-LMW/1−II-LMW/6, were obtained, freed from solvent under vacuum, and then evaluated sensorially in aqueous solution on a five-point intensity scale. As given in **Figure 2**, all of the SPE fractions showed bitter taste with the exception of subfraction II-LMW/6. As the highest bitter intensity of 4.0 was found for fractions II-LMW/2 and II-LMW/5, respectively, these two fractions were further separated by preparative RP-HPLC to give nine subfractions each (**Figures 3** and **4**). HPLC degustation revealed that not only one but six of the nine subfractions 2A−2I or 5A−5I, respectively, collected from fraction II-LMW/2 or II-LMW/5 imparted bitterness (**Figure 2**). Among these, HPLC fractions 2D, 2E, and 5A were evaluated with the highest bitterness scores of 4.0 and 4.5, respectively. Rechromatography of fraction 2D and 2E by means of preparative RP-HPLC yielded nine (2D/1−2D/9) and five subfractions (2E/1−2/E5), respectively. Among the fractions 2D/1−2D/9, only fraction 2D/7 exhibited bitterness, whereas HPLC/degustation of fractions 2E/1−2E/5 revealed two bitter-tasting subfractions, namely, 2E/1 and 2E/3, respectively (**Figure 2**).

To gain first insights into the bitter metabolites present in the most active subfractions, fractions 2D/7 and 2E/3 were then analyzed by means of LC-MS/MS. As an example, the TIC

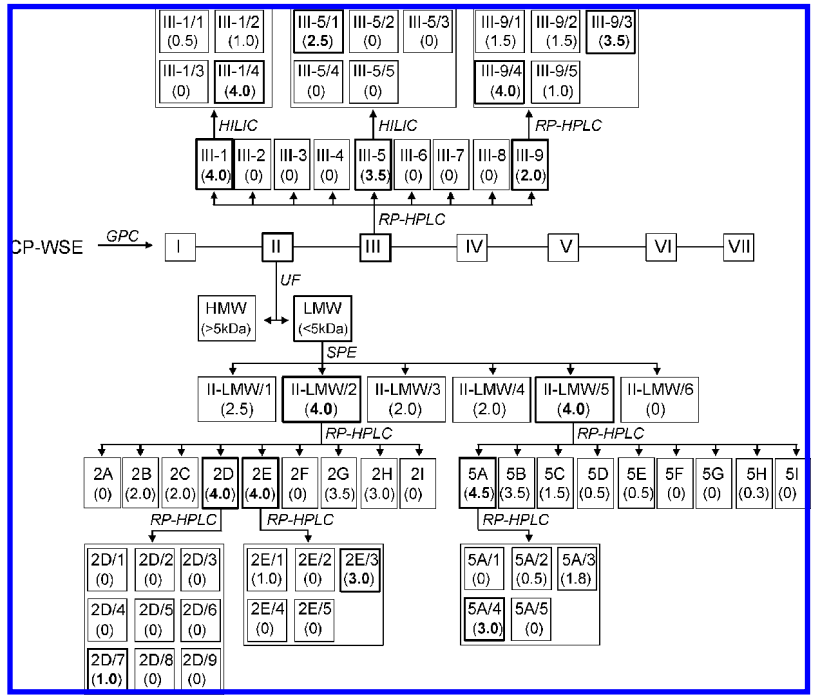

Figure 2. Sensomics mapping of bitter-tasting metabolites in CPWSE. Bitter taste intensities of individual fractions in water (pH 5.7) are given in parentheses.

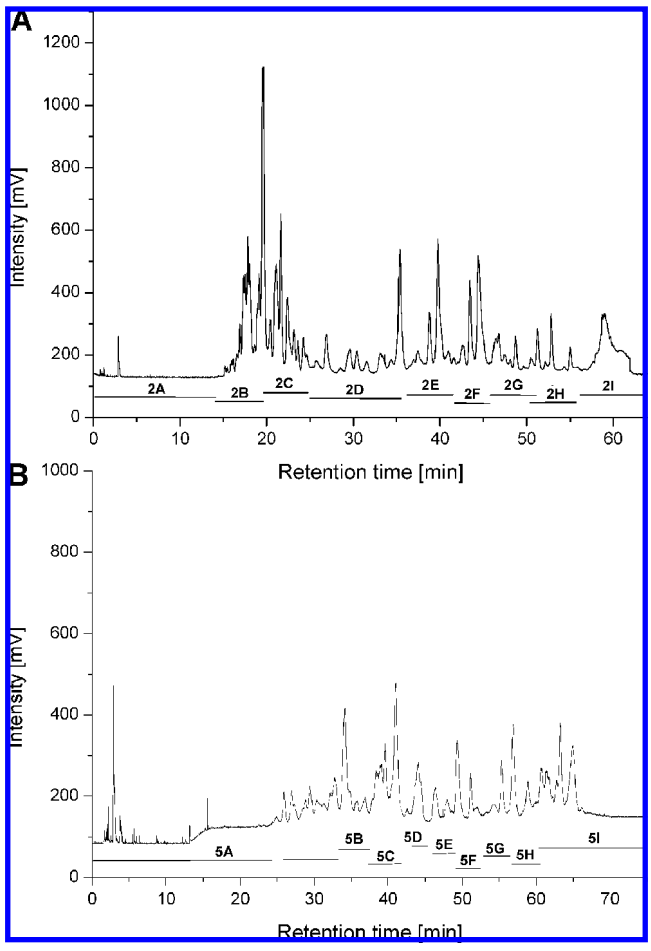

Figure 3. RP-HPLC/ELSD chromatogram of SPE fraction II-LMW/2 (**A**) and SPE fraction II-LMW/5 (**B**), respectively.

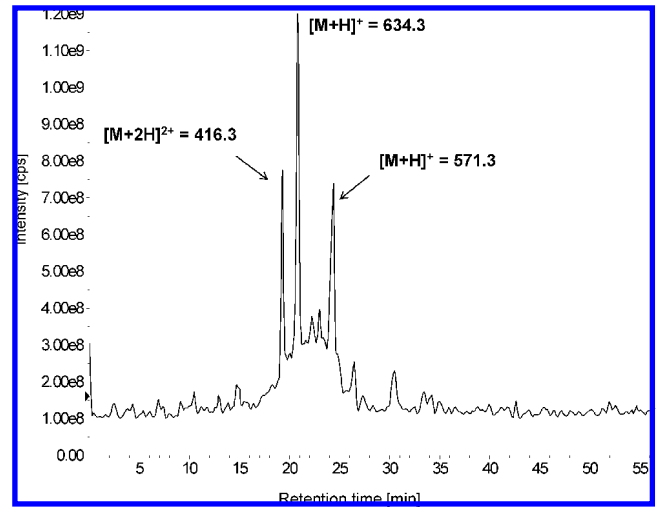

Figure 4. HPLC-MS(TIC) chromatogram of bitter-tasting fraction 2D/7.

chromatogram of fraction 2D/7 is shown in **Figure 4B**. Three main peaks were detectable, showing the pseudomolecular ions *m/z* 634.3 ($[M + H]^+$), 571.3 ($[M + H]^+$), and 416.3 ($[M + 2H]^{2+}$), respectively. Exact mass analysis performed by means of MS-TOF experiments and LC-MS/MS analysis with variable collision energy resulted in the unambiguous identification of the bitter metabolites as peptides. As an example, the daughter ion spectrum recorded for the peptide showing *m/z* 634.2 is given in **Figure 5**. Using the nomenclature for the individual fragments according to Roepstorff (*32*), all of the fragments could be successfully assigned mostly to *y*- and *b*-ions generated by peptide bond cleavage from the pentapeptide DIKQM. Sequence alignment revealed that the peptide DIKQM occurs in the primary structure of α_{s1}-casein, namely, at position α_{s1}-CN(56–60). In addition, the sequences of the peptides detected as $[M + H]^+$ ion with *m/z* 571 and as $[M + 2H]^{2+}$ ion with *m/z* 416.3 were elucidated as the pentapeptide EIVPN and the heptapeptide SITRINK, respectively. Sequence alignment revealed that the peptide EIVPN occurs twice in the primary structure of α_{s1}-casein, namely, at positions aa(70–74) and

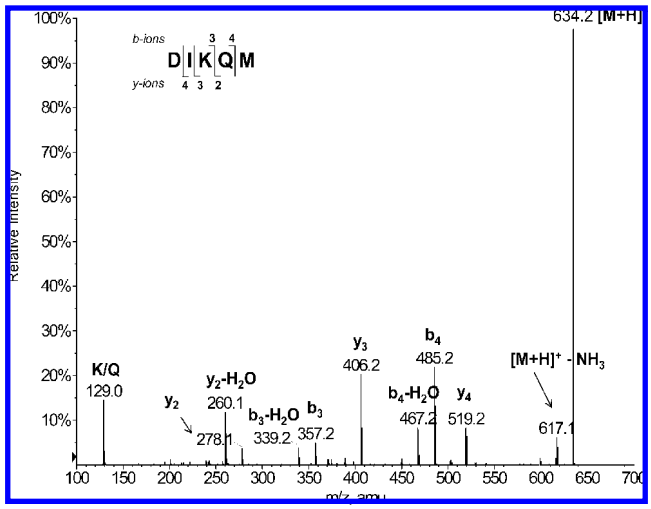

Figure 5. LC-MS/MS spectrum (ESI^+) of the pseudomolecular ion *m/z* 634.3 detected in fraction 2D/7.

aa(110–114), whereas SITRINK seems to originate from β-casein, namely, from the amino acid sequence β-CN(22–28) (**Table 2**).

LC-MS/MS and MS-TOF experiments also enabled the straightforward identification of peptides in the intensely bitter fraction 2E/3 (**Figure 2**). LC-MS analysis of fraction 2E/3 revealed two main compounds showing the pseudomolecular ions *m/z* 486.3 and 442.3, respectively. Exact mass analysis and LC-MS/MS analysis of these peptides resulted in the unambiguous identification of these peptides as IVPN and LPQE, respectively. Sequence alignment gave strong evidence that IVPN originates from α_{s1}-CN(71–74) or α_{s1}-CN(111–114), respectively, whereas the peptide LPQE shows sequence identity with α_{s1}-CN(11–14) (**Table 2**).

Among the subfractions 5A−5I isolated from the fraction II-LMW/5, subfraction 5A exhibited the most intense bitter taste, evaluated with a score of 4.5 (**Figure 2**). By means of RP-HPLC, this fraction was further separated into the five subfractions 5A/1−5A/5, among which subfraction 5A/4 was the most bitter one, judged with an intensity of 3.0 (**Figure 2**). LC-MS analysis of 5A/4 indicated the presence of four peptides showing the doubly charged pseudomolecular ions ($[M + 2H]^{2+}$) *m/z* 627.3, 714.4, 550.8, and 679.4, respectively. MS-TOF and LC-MS/MS analysis of these peptides enabled their sequence identification as LVYPFPGPIHN (1253.7 Da), SLVYPFPGPIHNS (1427.7 Da), VYPFPGPIPN (1100.6 Da), and IPPLTQTPVVVPP (1357.8 Da), respectively. Sequence alignment demonstrated that all four peptides originate from the sequence β-CN(57–90) (**Table 2**).

Sensory-Guided Fractionation of GPC Fraction III. To locate the bitter compounds in GPC- fraction III, this fraction was further separated by means of RP-HPLC to give the nine subfractions III-1−III-9 as given in **Figure 6**. Sensory analysis led to the detection of bitterness in HPLC fractions III-1, III-5, and III-9, evaluated with intensities of 4.0, 3.5, and 2.0, respectively (**Figure 2**). The intensely bitter-tasting fraction III-1 was further separated by means of preparative HILIC to yield the four subfractions III-1/1−III1/4. Fraction III-1/4 exhibited a metallic bitter taste as known for calcium and magnesium salts. Sensory evaluation was confirmed by ion chromatography demonstrating the divalent magnesium and calcium ions as well as chloride in this fraction (**Figure 2**).

The bitter fraction III-5 was separated on a HILIC column to give the five subfractions III-5/1−III-5/5, among which fraction III-5/1 exhibited bitter taste with an intensity of 2.5. LC-MS analysis revealed *m/z* 132.1 as the pseudomolecular ion $[M + H]^+$. Upon fragmentation, the main daughter ion observed was *m/z* 86.1, which could be assigned to the immonium ion of the amino acid isoleucine or leucine. Amino acid analysis confirmed that both amino acids were present in fraction III-5/1 with higher amounts of L-leucine than L-isoleucine. In addition, trace amounts of the dipeptide MI were found and could be assigned to α_{s1}-CN(135–136) (**Table 2**).

To identify the bitter metabolites in fraction III-9, this fraction was separated by means of preparative RP-HPLC. Five bitter subfractions (III-9/1−III/9–5) were obtained, among which fractions III-9/3 and III-9/4 were judged with intensities of 3.5 and 4.0, respectively (**Figure 2**). In fraction III-9/3, LC-MS/MS analysis revealed four peptides, namely, YPFPGPIHNS and YPFPGPIHN assigned to β-CN(60–69) and β-CN(60–68), respectively, as well as GPVRGPFP and VRGPFP corresponding to the sequences β-CN(198–206) and β-CN(200–206) (**Table 2**). The LC-MS scan of fraction III-9/4 revealed the two pseudomolecular ions *m/z* 1001.5 and 439.3, respectively. LC-MS/MS analysis identified the larger peptide as YPFPGPIPN, corresponding to β-CN(60–68), in which the amino acid His^{67} was substituted by Pro^{67}. LC-MS/MS analysis of the smaller peptide identified its structure as LPPL or IPPL, respectively, and could be assigned to β-CN(74–77) or β-CN(135–138), respectively (**Table 2**).

In summary, all of the identified peptides seem to originate from the hydrolytic breakdown of α_{s1}-casein and, in particular, β-casein. In **Figures 7** and **8**, the entire primary structure of α_{s1}- and β-casein, the assignment of the identified peptides, and the potential cleavage sites of the endogenous milk enzymes plasmin, cathepsins, elastase, and the coagulant enzyme chymosin are given for the candidate peptides identified in the present study. It is interesting to note that most of identified peptides were released from the same sequence domains of β-casein. The fact that the central part of β-casein is a sensitive substrate for proteases during ripening of several cow cheeses is well documented in the literature (*33*). For example, five peptides originate from the region aa(57–69) differing in deletion of one or more amino acid residues at both termini of the peptide sequence, thus indicating the activity of bacterial amino peptidases and carboxy peptidases cleaving amino acids from the individual N or C terminus of peptides. Although it is known from the literature that bitter peptides are generated mainly from the breakdown of α_{s1}- and β-casein, to the best of our knowledge the sequences of the peptides summarized in **Table 2** have not been previously reported to contribute to bitter taste.

Several groups identified bitter peptides that were either isolated from cheese and casein hydrolysates or prepared synthetically (*34–37*), for example, β-casein fragments within the sequences β-CN(46–67) and β-CN(196–209) and N-terminal fragments of α_{s1}-casein, namely, α_{s1}-CN(1–9), α_{s1}-CN(14–17), and α_{s1}-CN(23–34) were reported in the literature. Literature studies on matured Gouda cheese revealed the fragments β-CN(84–89), β-CN(193–207) and/or β-CN(193–208), and β-CN(193–209) as bitter peptides (*4, 5, 35*). Among these, the bitter fragment β-CN(193–209) is known as the primary breakdown product released by chymosin (*35*) and is believed to contribute to the bitter flavor defect in Gouda cheese (*5*). It is interesting to note that the identified peptides Y*PFPGPIH*N, Y*PFPGPIH*NS, SLVY*PFPGPIH*NS, VY*PFPGPIP*N, and LVY*PFPGPIH*N contain the common hydrophobic fragment β-CN(61–67) included in several known bitter peptides (*35*). It is also interesting to note that among the β-CN

Table 2. Identified Peptides within Partial α/β-Casein Sequence Isolated from Gouda Cheese

fraction[a]	exact mass m/z[b] obsd	exact mass m/z[b] calcd	sequence alignment[c]	peptide sequence[d]
2D/7	571.3086	571.3086	α_{s1}-CN(70–74)/α_{s1}-CN(110–114)	EIVPN
	634.3220	634.3234	α_{s1}-CN(56–60)	DIKQM
	416.2568**	416.2566**	β-CN(22–28)	SITRINK
2E/3	486.2545	486.2564	α_{s1}-CN(11–14)	LPQE
	442.2642	442.2642	α_{s1}-CN(71–74)/α_{s1}-CN(111–114)	IVPN
5A/4	627.3377**	627.3381**	β-CN(58–68)	LVYPFPGPIHN
	714.3677**	714.3701**	β-CN(57–69)	SLVYPFPGPIHNS
	550.7943**	550.7930**	β-CN-A^2(59–68)	VYPFPGPIPN
	679.4120**	679.4087**	β-CN(74–86)	IPPLTQTPVVVPP
III-5/1	263.1461	263.1430	α_{s1}-CN(135–136)	MI
	132.1033	132.1025		L/I
III-9/3	564.7818**	564.7779	β-CN(60–69)	YPFPGPIHNS
	826.4599	826.4575	β-CN(198–206)	GPVRGPFP
	672.3858	672.3834	β-CN(200–206)	VRGPFP
	521.2650**	521.2619	β-CN(60–68)	YPFPGPIHN
III-9/4	1001.5103	1001.5097	β-CN-A^2(60–68)	YPFPGPIPN
	439.2927	439.2921	β-CN(135–138)	LPPL
			β-CN(73–76)	IPPL

[a] The numbering of the fractions is according to the most bitter fractions outlined in **Figure 2**. [b] Singly charged unless marked with a double asterisk (**), then doubly charged. [c] αs_1-CN variant B; β-CN variant A^1 if not stated otherwise. [d] Sequences given in one-letter code.

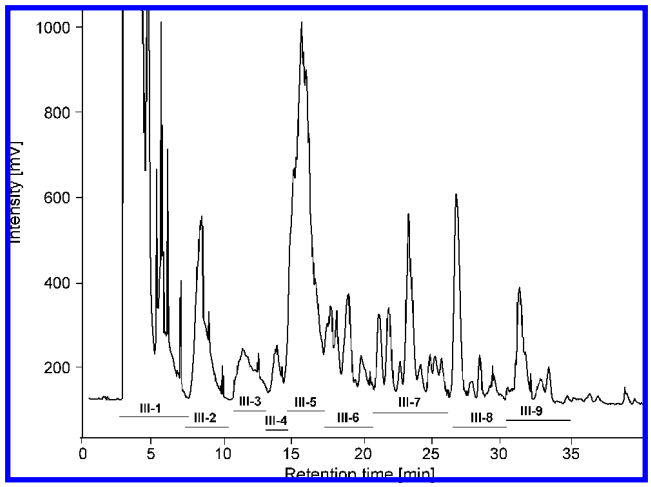

Figure 6. RP-HPLC/ELSD chromatogram of GPC fraction III.

fragments liberated from aa(57–69), the two fragments aa(59/60–68) originate from another genetic variant, namely, variant A^2, in which His^{67} is substituted by Pro^{67}. The occurrence of the two β-casein variants A^1 and A^2 is common in the Western world (*38*) and, therefore, it is not surprising that peptides derived from both genetic variants were found in CPWSE.

Studies on the Bitterness of Peptides. According to the literature (*39, 40*), the degree of hydrophobicity is believed to be a first predictor for the bitterness of a peptide fragment. To address the hydrophobicity of peptide, the so-called Q value was introduced. The average hydrophobicity of a peptide $Q = (\Sigma\Delta f)/n$ is defined as the ratio of the sum of the free energy of transfer (Δf) of the side chains of the amino acid residues divided by the number (n) of amino acid residues. For peptides with a molecular weight of <6000 Da, a Q value of 1400 cal/res is believed to be the lower limit for bitterness. As given in **Table 3**, the metabolites identified in the present study showed Q values between 1200 and 2000 cal/res, among which the peptides DIKQM assigned to α_{s1}-CN(56–60), SITRINK assigned to β-CN(22–28), and LPQE assigned to α_{s1}-CN(11–14) should not exhibit bitter taste according to the Q rule.

To validate the bitter taste of the identified metabolites, the individual peptides were synthesized and purified by means of RP-HPLC, and, prior to sensory analysis, the purity of all compounds was checked by HPLC-MS as well as ^{1}H NMR spectroscopy. In addition, the peptide β-CN(193–209) reported as the "bitter flavor defect" peptide of cheese and the peptide YPFPGPIPNS assigned to β-CN(60–69) were synthesized and purified to obtain first information on the influence of the substitution of His^{67} (genetic variant A^1) by Pro^{67} (genetic variant A^2) on the bitter taste activity. To determine the human threshold concentrations for bitter taste, aqueous solutions (pH 5.7) of the individual peptides were evaluated by means of the three-alternative forced-choice test in ascending concentrations up to a maximum concentration of 6.0 mmol/L. The human taste threshold concentrations determined for the peptides under investigation ranged from 0.05 to 1.18 mmol/L (**Table 3**). The threshold of the "bitter flavor defect" peptide β-CN(193–209) was found to be 0.18 mmol/L, which is well in agreement with previously reported data (*41, 42*). In comparison, the identified peptide β-CN(198–206), which is a segment of β-CN(193–209), showed a 6-fold higher threshold concentration of 1.18 mmol/L. Literature studies on segments within the sequence β-CN(193–209) revealed that the fragment aa(202–209) is required for intense bitter taste; for example, the thresholds reported for the peptides aa(196–209), aa(200–209), and aa(202–209) were found to be below 0.02 mmol/L (*43*).

Interestingly, the peptides IVPN, IPPLTQTPVVVPP, IPPL, and LPPL did not exhibit any bitter taste up to the tested concentration of 6.0 mmol/L, although they showed Q values around 1800 cal/res, and even the highest Q value of 2658 cal/res was found for the tasteless peptides IPPL and LPPL. Surprisingly, LPQE and DIKQM, both showing Q values below 1400 cal/res, exhibited some bitterness at 6.0 mmol/L. Altogether, no clear correlation between the Q value and the bitter threshold of the peptides could be found. For example, the peptide YPFPGPIHNS, for which a high Q value of 1688 cal/

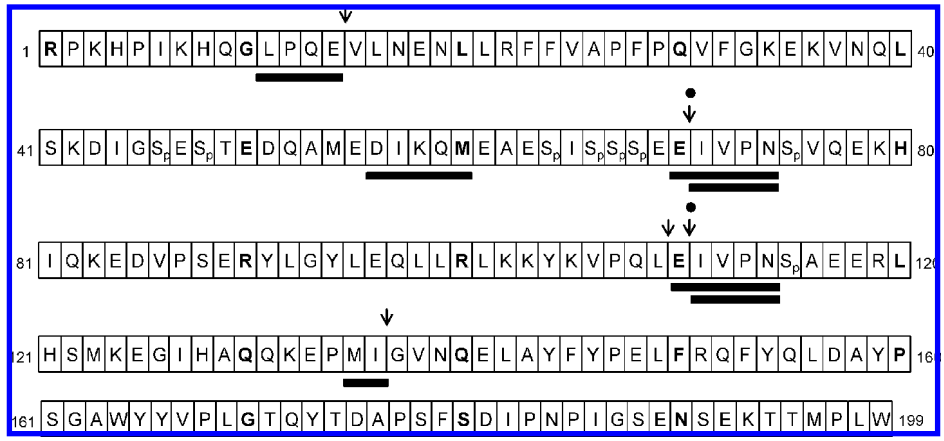

Figure 7. Primary structure of bovine αs_1-casein and assignment of identified peptides (black lines) as well as cleaving sites of cathepsin G (●) and cathepsin B (↓) (adapted from ref *50*).

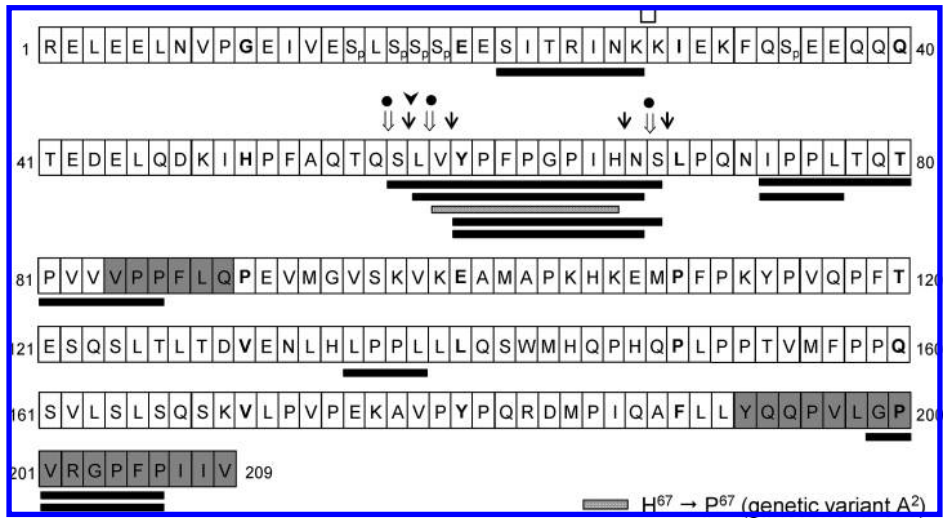

Figure 8. Primary structure of bovine β-casein and assignment of identified peptides (black lines) as well as cleaving sites of cathepsin G (●), cathepsin B (↓), cathepsin D (chevron), elastase ("downward arrow"), plasmin (□) (adapted from ref *50*). Sequences with gray background illustrate bitter peptides earlier identified in Gouda cheese (*5*).

Table 3. Human Threshold Concentrations of Peptides Isolated from Gouda Cheese

peptide sequence	sequence alignment[a]	*Q* value[b] (calcd/res)	threshold concn[c] (mmol/L)	bitterness[d]
SITRINK	β-CN(22–28)	1234	>6.0	nd
DIKQM	α_{s1}-CN(56–60)	1242	6.0	posterior tongue
LPQE	α_{s1}-CN(11–14)	1373	6.0	posterior tongue
EIVPN	α_{s1}-CN(70–74)/α_{s1}-CN(110–114)	1564	0.43	posterior tongue, long-lasting
GPVRGPFP	β-CN(198–206)	1616	1.18	anterior tongue, $MgSO_4$-like
SLVYPFPGPIHNS	β-CN(57–69)	1618	0.06	posterior tongue
YPFPGPIHNS	β-CN(60–69)	1688	0.05	caffeine-like
VRGPFP	β-CN(200–206)	1718	0.42	anterior tongue, $MgSO_4$-like
IVPN	α_{s1}-CN(71–74) α_{s1}-CN(111–114)	1818	>4.0[e]	nd
YPFPGPIHN	β-CN(60–68)	1871	0.10	caffeine-like
IPPLTQTPVVVPP	β-CN(74–86)	1872	>6.0	nd
YPFPGPIPNS		1900	0.33	caffeine-like
LVYPFPGPIHN	β-CN(58–68)	1905	0.08	caffeine-like, slightly salicin-like
VYPFPGPIPN	β-CN-A^2(59–68)	2065	0.17	caffeine-like, slightly salicin-like
YPFPGPIPN	β-CN-A^2(60–68)	2107	0.23	caffeine-like
MI	α_{s1}-CN(135–136)	2135	0.42	posterior tongue
IPPL	β-CN(73–76)	2658	>6.0	
LPPL	β-CN(135–138)	2520	>6.0	
YQQPVLGPVRGPFPIIV	β-CN(193–209)	1762	0.18	caffeine-like

[a] Peptide sequences are given in the one-letter code. [b] *Q* values were calculated according to the literature (*39*). [c] Taste threshold concentrations were determined by means of a triangle test in bottled water (pH 5.7). [d] Bitterness quality or place of perception was evaluated in 5-fold overthreshold concentration of the corresponding peptide in water (pH 5.7); nd, no bitterness detectable up to 6 mmol/L. [e] Solubility maximum of the peptide in water (pH 5.7).

res was calculated, provided a lower taste threshold concentration of 0.06 mmol/L when compared to YPFPGPIPNS, showing a higher taste threshold of 0.33 mmol/L and a higher *Q* value of 1900 cal/res.

Furthermore, the data, presented in **Table 3**, demonstrate that peptides released from the same sequence region of the protein and the same casein variant have comparable threshold values; for example, YPFPGPIHN and its homologues YPFPGPIHN*S*, *LV*YPFPGPIHN, and *SLV*YPFPGPIHN*S* show bitter threshold concentrations in the narrow range between 0.05 and 0.1 mmol/L. The substitution of His^{67} in β-CN(50–69) by Pro^{67} as found in the genetic β-casein variant A^2 increased the bitter taste threshold from 0.05 to 0.17 mmol/L and also the *Q* values of the peptide. The influence of the conformational changes in the peptide skeleton induced by the introduction of L-proline on the taste activity of the peptide is well in line with reports on the importance of the spatial structure as well as the involvement and location of basic as well as hydrophobic side chains in the amino acid sequence on taste activity (*44–47*). It is believed in the literature that a hydrophobic moiety and another hydrophobic or a bulky basic group are needed in concrete steric distance to induce a bitter receptor activation (*44, 45, 48*). On the basis of quantitative structure−activity studies, bulky hydrophobic amino acids at the C terminus and bulky basic amino acids at the N terminus were found to be highly correlated to bitterness (*49*), which is supported by our own findings. The substitution of the bulky basic amino acid L-histidine by L-proline results not only in the loss of the required basic unit but also in a significantly different conformation of the peptide. On the basis of these findings, it can be concluded that the bitter activity of a peptide cannot be predicted simply on the basis of *Q* values, but sensory experiments with purified reference compounds and, even better, bitter receptor studies are essential requirements for future studies.

Besides the taste threshold concentrations, also the bitterness quality of the peptides was sensorially evaluated. To achieve this, aqueous solutions containing the individual peptides in 5-fold threshold concentrations were judged in their bitter quality and intensity and compared to reference solutions of the inorganic salt $MgSO_4$, the alkaloid caffeine, and the phenolic glycoside salicin. Interestingly, the peptides with the sequence aa(60–68/69) including the genetic variants all imparted a caffeine-like bitter quality, which was perceived in the complete oral cavity. Peptides with additional one or two amino acids at the N terminus such as the peptide β-CN(59–68) and β-CN(58–68) imparted the caffeine-like bitter quality but were also somehow reminiscent of the bitter taste induced by salicin. The dipeptide MI, LPQE, and DIKQM as well as EIVPN provided a comparatively long-lasting bitterness perceived solely in the throat. Interestingly, the two peptides VRGPFP and GPVRGPFP originating from the C-terminal part of β-casein exhibited a metallic, $MgSO_4$-like bitter taste that was perceived predominantly on the tip of the tongue.

In summary, 16 peptides were successfully identified in Gouda cheese, among which 12 peptides exhibited bitter taste in concentrations between 0.05 and 6.0 mmol/L. Sequence alignment revealed that 11 of these bitter peptides originate from β-casein and, in particular, from the N-terminal fragment β-CN(57–69) including the very hydrophobic sequence β-CN(61–67). To investigate the contribution of the identified peptides to the bitterness of Gouda cheese, quantitative studies and taste reconstruction as well as omission experiments using these peptides in their "natural" concentrations are currently in progress and will be published elsewhere.

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