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Identification of Bitterness-Masking Compounds from Cheese

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Supporting Information

ABSTRACT: Bitterness-masking compounds were identified in a natural white mold cheese. The oily fraction of the cheese was extracted and further fractionated by using silica gel column chromatography. The four fractions obtained were characterized by thin-layer chromatography and nuclear magnetic resonance spectroscopy. The fatty acid-containing fraction was found to have the highest bitterness-masking activity against quinine hydrochloride. Bitterness-masking activity was quantitated using a method based on subjective equivalents. At 0.5 mM, the fatty acid mixture, which had a composition similar to that of cheese, suppressed the bitterness of 0.008% quinine hydrochloride to be equivalent to that of 0.0049−0.0060% and 0.5 mM oleic acid to that of 0.0032−0.0038% solution. The binding potential between oleic acid and the bitter compounds was estimated by isothermal titration calorimetry. These results suggest that oleic acid masked bitterness by forming a complex with the bitter compounds.

KEYWORDS: *bitterness masking, bitter taste, cheese, fatty acid, isothermal titration calorimetry, oleic acid, sensory test*

INTRODUCTION

The five basic tastes comprise sweet, sour, bitter, salty, and umami.[1] Of these tastes, sour and bitter are generally unfavorable and avoided by humans, because these tastes are associated with spoiled and unripe foods as well as bitter toxins.[2−4] The perception of bitterness may have evolved in humans and animals to avoid the intake of toxins.[5] The origins and structural diversity of bitter compounds are also much greater than those of other substances.[3,6,7] Metal ions, some amino acids, peptides, and the secondary metabolites produced by plants, such as alkaloids, phenols, flavonoids, isoflavones, terpenes, and glucosinolates, are bitter.[3,8,9] These substances are perceived by taste 2 receptors (TAS2R), which are classified as G protein-coupled receptors.[10−13] In human, 25 kinds of TAS2R are reported.[14] The interaction of ligands with these receptors has been studied by some researchers.[15−19]

Bitter-tasting foods are not preferred in most cases; a few exceptions include coffee, beer, and wine.[2,3] Even though humans are averse to bitter taste, pharmaceutical compounds with physiological benefits often taste bitter.[20] Therefore, the masking of bitterness is considered to be important in food processing and pharmacology.

Many bitterness-masking compounds have been identified.[21] Some substances with potent tastes such as salts, acids, and sugars can suppress bitterness.[22] Cyclodextrin includes some bitter substances intramolecularly, thereby inhibiting the binding of these substances to taste receptors.[23] Phosphatidic acid (PA) and its lipoprotein derivative, formed by interactions with β-lactoglobulin, are reported to suppress the bitterness of quinine sulfate.[24] The activation pattern of TAS2Rs with some bitter chemicals, including quinine hydrochloride (QHCl), is now known.[14] Recently, an antagonist for several TAS2R taste receptors was identified by screening a chemical compound library.[25] This compound binds the TAS2R activation pocket to inhibit ligand binding, thereby effectively suppressing bitter taste. In addition, other antagonists for several TAS2R taste receptors have been identified.[26] However, these are not applicable for processed food because the safety of the antagonists is not guaranteed. To utilize these compounds for processed food, it is necessary to confirm the safety of these compounds. Therefore, it is more appropriate to screen for bitterness-masking compounds in conventional foods to meet the requirements of the food-processing industry.

Cheese is one of the most popular fermented foods worldwide. Its unique flavors and tastes are produced by the action of microorganisms such as lactic acid bacteria and fungi.[27,28] During the fermentation process, various compounds not contained in milk are produced. Peptides and free amino acids are generated by the digestion of milk proteins by microbial proteinases and exopeptidases.[29] Bitter- or astringent-tasting peptides are often produced by digestion of the caseins in cheese.[9] Free fatty acids are liberated from triacylglycerol by the lipase produced by microorganisms.[30,31]

Many kinds of tastants are present in foods; they may interact with each other to enhance or suppress the taste. The aim of this study was to purify and identify compounds in cheese with bitterness-masking properties and to reveal the mechanism of their suppression by using isothermal titration calorimetry (ITC).

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MATERIALS AND METHODS

Materials. The following cheeses were purchased from supermarkets in Tokyo, Japan: Baraka cheese (Lincet Saint-Julien, Trappes, France), Gouda (Frico, Wolvega, The Netherlands), Ricotta (Galbani, Milan, Italy), and Brie (Bongrain SA, Viroflay, France). Kirin Lager Beer (Kirin Brewery Co., Ltd., Tokyo, Japan) was purchased in Tokyo and Fukuoka, Japan.

Chemicals were obtained from commercial sources: 9-anthryldiazomethane (ADAM) from Funakoshi Co., Tokyo, Japan; QHCl from Nacalai Tesque Inc., Kyoto, Japan; fatty acids (FAs) including myristic acid, palmitic acid, stearic acid, and oleic acid (OA), glycerides including trioleoylglycerol (TOG), dioleoylglycerol (DOG), and monooleoylglycerol (MOG), and promethazine hydrochloride (PHCl) from Sigma-Aldrich Co., Tokyo, Japan; and caffeine and $CDCl_3$ containing 1 vol % of tetramethylsilane (TMS) from Wako Pure Chemical Industries, Ltd., Osaka, Japan.

Wakogel C-200 (chromatography grade, particle size = 75−150 μm) was purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan, and silica gel $60F_{254}$ (HX953368; 5 × 25 cm) for thin-layer chromatography (TLC) was from Merck KGaA, Darmstadt, Germany. ITC was performed on a MicroCal iTC_{200} instrument (GE Healthcare Japan Corp., Tokyo, Japan).

Extraction of the Oily Fraction from Baraka Cheese. A kitchen knife was used to cut 150 g of cheese into small pieces after removal of the mold-covered surface, prior to the addition of 600 mL of ethanol. The mixture was homogenized at 20000 rpm for 10 min by using a Polytron homogenizer (PT1300D, DA1607/2; Ishii Laboratory Works Co., Ltd., Osaka, Japan) and then centrifuged at 15300*g* for 10 min at room temperature. The liquid layer was separated from the debris and re-extracted twice with 400 mL of ethanol. The liquid layers collected were then concentrated to dryness by using a rotary evaporator. The dried residue was dissolved in 200 mL of ethyl acetate, dehydrated with Na_2SO_4 anhydrate, and filtered with filter paper (Advantec No. 5A). The filtrate was then concentrated and desiccated to obtain the oily fraction.

Thin-Layer Chromatography. The extracted samples were separated and analyzed by TLC. The plate was spotted with the samples, developed with a mixture of *n*-hexane/acetone (2:1), and exposed by spraying with 10% H_2SO_4, prior to heating on a hot plate, to detect the separated samples.

Silica Gel Column Chromatography. Silica gel (80 g) was packed into a glass column (29 × 300 mm) and equilibrated with *n*-hexane. Next, 5−10 g of the oily fraction was applied and eluted with *n*-hexane. The elution was performed with a *n*-hexane/acetone mixture, with the ratio of acetone increasing stepwise.

The typical elution profile in sequence is 200 mL of *n*-hexane, 210 mL of *n*-hexane/acetone (100/5), 220 mL of *n*-hexane/acetone (100/10), 240 mL of *n*-hexane/acetone (100/20), 260 mL of *n*-hexane/acetone (100/30), and 280 mL of *n*-hexane/acetone (100/40). The eluents were collected in appropriate volumes and subjected to TLC. The resulting fractions with the same TLC patterns were gathered, and the four fractions A, B, C, and D were obtained. Fraction A corresponded to the *n*-hexane/acetone mixtures of 100/5 and 100/10, fraction B to 100/20, fraction C to 100/30, and fraction D to 100/40. The separation was performed eight times, and each pooled fraction was further purified by rechromatography, eluting using the *n*-hexane/acetone system. The obtained fractions were then dried and stored at 4 °C until analyzed.

Determination of the Free Fatty Acid Composition of Different Cheeses. Baraka, Ricotta, Gouda, and Brie cheeses were analyzed to determine their free FA composition. The oily fraction from each cheese was prepared according to the above-described method. The free FAs were then analyzed using high-performance liquid chromatography (HPLC) using the ADAM method, as reported previously.[32]

Panel Selection and Training. Panelists (n = 9) were selected using the difference test with five basic tastes and the discrimination test for the differences in the concentrations of four basic tastes[33] and were trained using the methods described below.

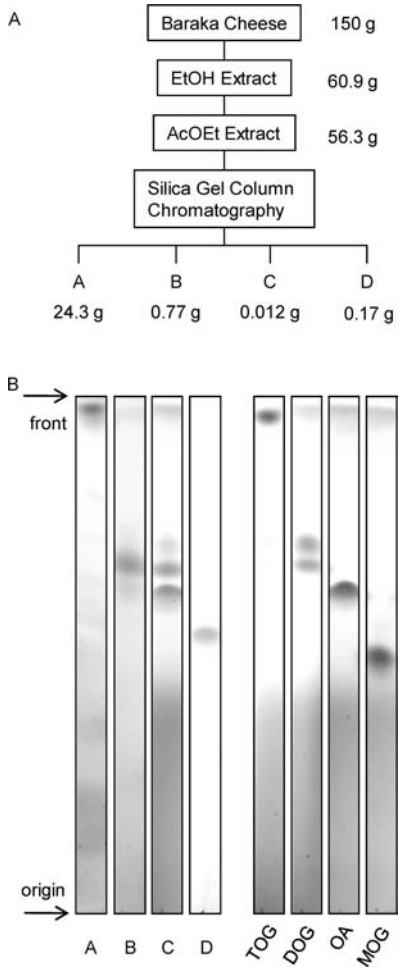

Figure 1. Extraction and separation of oily fraction from Baraka cheese: (A) fractionation scheme; (B) thin-layer chromatography (TLC) analysis. The four fractions were analyzed by TLC, developed using *n*-hexane/acetone (2:1), and detected by spraying with 10% H_2SO_4 and heating on a hot plate. The R_f values for each authentic sample, applied at 1 μg/lane, are as follows: trioleoylglycerol (TOG) (R_f = 0.96); dioleoylglycerol (DOG) (R_f = 0.72, 0.68); OA, oleic acid (OA) (R_f = 0.63); monooleoylglycerol (MOG) (R_f = 0.50).

For the discernment of bitter taste, each panelist was trained with triangular tests to distinguish bitter taste at four concentration levels near the threshold value. In addition, each panelist was trained in the discernment of difference in the concentrations of bitter taste by using a ranking test involving the QHCl solution at seven concentration levels (common ratio 1.1−1.2).

Evaluation of Bitterness-Masking Activity of Each Fraction from Baraka Cheese. In sensory tests with panelists (n = 4), a piece of Baraka cheese was placed on the tongue after peeling off the mold-covered surface and spread over the whole tongue prior to tasting 0.0080% QHCl solution. To estimate the bitterness-masking activity, fractions A, B, C, and D were each solubilized in 0.0080% QHCl containing 1% β-lactoglobulin, at a concentration of 1−2 mg/mL. Because the oily fractions dissolved poorly in water, β-lactoglobulin was added as a solubilizer. β-Lactoglobulin (1%) alone did not possess bitterness-masking activity under these conditions (data not shown). The free FAs were solubilized by the addition of equimolar NaOH and stirred with a magnetic stirrer. Then, 1 mL of bitter-tasting solutions containing each of the four fractions was put in the mouth, and the bitter taste intensity was evaluated on a three-level scale: bitterness equal to 0.0080% QHCl (1), slightly less than 0.0080% QHCl (2), or significantly less bitterness (3). The bitterness score was shown as the average of four trials.

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Table 1. Main Compounds Present in Fractions A, B, C, and D,[a] As Determined by Nuclear Magnetic Resonance

identification	assignment	chemical shifts ^{1}H	chemical shifts ^{13}C
	Fraction A		
triacylglycerol		not analyzed	
	Fraction B		
1,2-diacylglycerol	1	4.10, 4.25	62.1
	2	5.00	71.8
	3	3.61	60.9
	1-OCO−		173.5
	2-OCO−		173.2
1,3-diacylglycerol	1	4.05	64.7
	2	3.97	67.7
	3	4.05	64.7
	1-OCO−, 3-OCO−		173.6
	Fraction C		
1,3-diacylglycerol	1	4.22	62.1
	2	4.1	68.9
	3	4.22	62.1
	1-OCO−, 3-OCO−		173.3
fatty acid	R−CO−OH		179.6
	Fraction D		
1-monoacylglycerol	1	3.60, 3.69	
	2	3.89	not analyzed
	3	4.15, 4.21	
	1-OCO−		

[a]Fraction A, triacylglycerol, was determined by TLC. The compounds containing fractions B, C, and D were identified from ^{1}H and ^{13}C NMR spectra.

Table 2. Evaluation of the Bitterness-Masking Activities of Fractions Separated by Silica Gel Chromatography

sample	sensory test[a]	score	concentration
Baraka cheese	I	3	0.5 g/sip
oily fraction	II	2	1 mg/mL
fraction A	II	1	1 mg/mL
fraction B	II	1.5	1 mg/mL
fraction C	II	3	1 mg/mL
fraction D	II	1.5	2 mg/mL

[a]Sensory test I: 0.5 g of Baraka cheese was put on the tongue and spread over the entire tongue prior to tasting 0.0080% quinine hydrochloride (QHCl). Sensory test II: each fraction was solubilized in 0.0080% QHCl and evaluated for bitterness. The bitterness was evaluated on a 3-score scale: (1) equal to that of 0.0080% QHCl, (2) slightly less bitter than 0.0080% QHCl, or (3) significantly less bitter. Score shows the median of the answers of the panelist. The details are provided under Materials and Methods. Each score is the average of four trials.

Bitterness-Masking Activity of Four Cheeses. Panelists (n = 7) performed sensory tests by using the beer. Here, a piece of Baraka, Gouda, Brie, or Ricotta cheese was put in the mouth, and a sip of beer was consumed. The bitter taste was evaluated using a four-point categorical scale: strong (0), medium (1), weak (2), or very weak (3). For evaluation of scores, the Steel−Dwass test, a nonparametric multiple-comparison method, was applied for detecting between-sample differences.

Quantitation of the Bitterness-Masking Activities of Fatty Acids. Two test solutions were prepared: (A) 0.2 mM OA, 0.2 mM palmitic acid, 0.05 mM myristic acid, 0.05 mM stearic acid, and 0.0080% QHCl in 5 mM sodium phosphate buffer (pH 7.0); (B) 0.5 mM OA and 0.0080% QHCl in 5 mM sodium phosphate buffer (pH 7.0). Standard solutions with seven different concentrations of QHCl, that is, 0.0026, 0.0030, 0.0035, 0.0040, 0.0046, 0.0053, and 0.0060%, in 5 mM sodium phosphate buffer (pH 7.0) were also prepared. Panelists (n = 9) who could discriminate the seven standard solutions in order of concentration participated in this test. Each sample was served at room temperature (20 °C).

Each of the standard solutions (5 mL) was put in a clear plastic cup, whereas each of the test solutions (5 mL) was in a white paper cup because the test solutions were slightly cloudy. First, the panelists tasted the three standard solutions, 0.0030, 0.0040, and 0.0053% QHCl, to remember the bitterness of each solution. Then, 5 mL of the test solution was held in the mouth for 15 s prior to its being spat out. After that, the mouth was rinsed with water to remove any bitter aftertaste, and the panelist waited for 30 s before moving to the next test. An interval of 60 s was provided before and after tasting each FA solution to avoid confusing the tastes. The bitter taste intensities of test solutions A and B were estimated in comparison with seven standard solutions. Panelists were allowed to repeatedly taste the standard solutions before selecting the solution that was the closest to the bitterness of the test solutions. The tests were repeated twice on different days.

The Shapiro−Wilk W test was used to judge the normality of evaluation score distribution. Analysis of variance (ANOVA) was applied to detect the variation of judged sensory scores at significance level α = 0.05. All statistical analyses were carried out using the computer software JMP 9.0.2 (SAS Institute, Inc., Cary, NC, USA).

Bitterness-Masking Activity of Oleic Acid. Bitter taste intensity evaluations were performed by using the following paired difference tests: between 0.22 mM QHCl and 0.22 mM QHCl containing 0.5 mM OA (involving 20 panelists), between 1.5 mM PHCl and 1.5 mM PHCl containing 0.5 mM OA (involving 6 panelists), and between 50 mM caffeine and 50 mM caffeine containing 0.5 mM OA (involving 10 panelists).

Statistical analysis of scores was conducted using a one-tailed binomial test (significance level α = 0.05).

Isothermal Titration Calorimetry. The concentrations of OA, QHCl, and PHCl used in titration were 0.5, 2.2, and 1.5 mM, respectively. They were dissolved in 5 mM sodium phosphate buffer (pH 7.0) containing 5% ethanol. The reference cell was filled with Milli-Q water (Millipore Corp., Billerica, MA, USA). OA solution was titrated into QHCl solution at 1000 rpm and 25 °C. Each titration was carried out with initial injection (0.4 μL) followed by 18 main injections (2 μL each) at intervals of 120 s.

The first titration (0.4 μL) was excluded for the analysis. The dilution calorie of the ligand in the buffer was subtracted from the titration data of QHCl. The titration of OA with PHCl was also performed except for using at 150 s intervals. Although the titration of 0.5 mM OA with 2.2 mM caffeine was performed at 150 s intervals, the titration was dispersed. Therefore, 50 mM caffeine containing 50 mM sodium phosphate buffer (pH 7.0) was adopted. In addition, the concentration of OA was used up to 5 mM.

The data were analyzed according to a model for one set of sites provided in the Origin 7.0 software for MicroCal iTC$_{200}$. The dissociation constant (K_d) and enthalpy change of binding (ΔH) were obtained from the fitted curve. The entropy change of binding (ΔS) and free energy change of binding (ΔG) were obtained from eq 1; R is the gas constant, T, the thermodynamic temperature, and K, the association constant.

$$\Delta G = \Delta H - T\Delta S = -RT \ln K \quad (1)$$

RESULTS

Screening for Cheeses with Bitter-Masking Activity. Seventy-one brands of cheeses were commercially obtained and subjected to sensory tests to identify the cheese with bitterness-masking activity. In addition, panelists were asked to consider

Table 3. Free Fatty Acid Composition of Four Cheese Samples[a]

	cheese							
	Baraka		Brie		Gouda		Ricotta	
fatty acid	mM	%	mM	%	mM	%	mM	%
butyric acid ($C_{4:0}$)	0.14	1.2	0.17	4.3	0.20	8.9	0.01	1.0
valeric acid ($C_{4:0}$)	nd		0.02	0.4	0.10	4.5	0.01	1.2
2-methylbutyric acid ($C_{5:0}$)	nd		nd		nd		nd	
caproic acid ($C_{6:0}$)	0.09	0.7	0.07	1.9	0.07	3.3	0.01	1.2
heptanoic acid ($C_{7:0}$)	nd		nd		nd		nd	
caprylic acid ($C_{8:0}$)	0.09	0.7	0.06	1.6	0.03	1.5	0.02	1.6
nonanoic acid ($C_{9:0}$)	nd		nd		nd		nd	
capric acid ($C_{10:0}$)	0.34	2.8	0.15	3.9	0.10	4.4	0.05	4.1
lauric acid ($C_{12:0}$)	0.55	4.6	0.21	5.4	0.14	6.4	0.07	6.3
myristic acid ($C_{14:0}$)	1.27	10.5	0.46	12.0	0.26	11.6	0.14	12.3
palmitic acid ($C_{16:0}$)	3.70	30.5	1.14	29.5	0.63	28.1	0.35	31.0
palmitoleic acid ($C_{16:1}$)	0.24	2.0	0.10	2.5	0.05	2.3	0.03	2.4
stearic acid ($C_{18:0}$)	0.86	7.0	0.26	6.8	0.15	6.5	0.09	7.8
oleic acid ($C_{18:1}$)	4.29	35.3	1.05	27.2	0.41	18.2	0.29	26.0
linoleic acid ($C_{18:2}$)	0.43	3.5	0.11	2.7	0.05	2.3	0.04	3.5
linolenic acid ($C_{18:3}$)	0.16	1.3	0.07	1.8	0.05	2.2	0.02	1.6
total	12.15	100.0	3.86	100.0	2.25	100.0	1.13	100.0

[a]Free fatty acids (FAs) in the cheese samples were quantitated from their oily fractions. The molar concentration and weight percentage of free FAs in the whole cheeses were calculated. nd, not detected.

which of the 71 cheeses had the strongest bitterness-masking activity. As a result of the screening, Baraka cheese was selected (Supporting Information).

Fractionation and Identification of Bitterness-Masking Compounds in Baraka Cheese. The oily fraction was extracted with ethanol and then with ethyl acetate (Figure 1A), resulting in a yield of 56 g from 150 g of Baraka cheese. It was further separated by silica gel column chromatography by using a *n*-hexane/acetone stepwise elution system. Four fractions, A, B, C, and D, weighing 24.3, 0.77, 0.012, and 0.17 g, respectively, were eluted in this order. TLC was performed with TOG, DOG, MOG, and OA as the references (Figure 1B). The mobilities of TOG, DOG, MOG, and OA are indicated by their R_f values of 0.96, 0.72 and 0.68, 0.63, and 0.50, respectively. The mobilities of the fractions A−D were compared with those of the standard compounds. Fraction A moved to the front of the plate and accounted for 96.2% of the oily fraction. This suggests that fraction A comprised triacylglycerol (TG). The main spot of fraction B had a mobility equal to that of the DOG spots, whereas that of fraction C nearly coincided with those of DOG and OA. The mobility of fraction D was equal to that of MOG.

To identify the substances in the fractions B, C, and D, the samples were analyzed by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy (Varian Inova 500). The methyl, methylene, and olefin signals of the FAs are not shown because these signals were derived by a mixture of many FA molecules.

Fraction B (diacylglycerol (DG) mixture of 1,2-isomer (53%) and 1,3-isomer (47%)): ^{1}H NMR (500 MHz, $CDCl_3$, TMS) δ 3.61 (2, 3-CH_2 of 1,2-isomer), 3.97 (1, 2-CH of 1,3-isomer), 4.05 (4, 1-CH_2 and 3-CH_2 of 1,3-isomer), 4.10 (1, 1-CH_2 of 1,2-isomer), 4.25 (1, 1-CH_2 of 1,2-isomer), 5.00 (1, 2-CH of 1,2-isomer); ^{13}C NMR (125 MHz, $CDCl_3$, TMS) δ 60.9 (3-CH_2 of 1,2-isomer), 62.1 (1-CH_2 of 1,2-isomer), 64.7 (1-CH_2 and 3-CH_2 of 1,3-isomer), 67.7 (2-CH of 1,3-isomer), 71.8 (2-CH of 1,2-isomer), 173.2 (2-CH−O−CO− of 1,2-isomer), 173.5 (1-CH_2−O−CO− of 1,2-isomer), 173.6 (2, 1-CH_2−O−CO− and 3-CH_2−O−CO− of 1,3-isomer). The ratio of the 1,2- to 1,3-isomers was calculated from the integration value of the two signals corresponding to the 2-CH protons.

Fraction C (DG of 1,3-isomer and FAs): ^{1}H NMR (500 MHz, $CDCl_3$, TMS) δ 4.1 (1, 2-CH), 4.22 (4, 1-CH_2 and 3-CH_2); ^{13}C NMR (125 MHz, $CDCl_3$, TMS) δ 62.1 (1-CH_2 and 3-CH_2), 68.9 (2-CH), 173.3 (1-CH−O−CO− and 3-CH−O−CO−), 179.6 (HO−CO−).

Fraction D (1-monoacylglycerol (MG)): ^{1}H NMR (500 MHz, $CDCl_3$, TMS) δ 3.60 (1, 3-CH_2), 3.69 (1, 3-CH_2), 3.89 (1, 2-CH), 4.15 (1, 1-CH_2), 4.21 (1, 1-CH_2).

Fractions B and D were found from their NMR spectra to include DG and MG, respectively (Table 1). Fraction C was determined to mainly include 1,3-DG and free FAs by TLC and NMR analyses. The concentration of free FAs in this fraction was 43.6%, as determined by HPLC by using the ADAM method. The major component in the rest of fraction C was DG.

Next, the bitterness-masking activity of each fraction was analyzed by sensory tests. Using the examination of the Wilcoxon rank-sum test and Steel−Dwass test, fraction C had the strongest bitterness-masking activity compared to the other three fractions with a significant level of 10% (Wilcoxon rank-sum test, $p < 0.025$; Steel−Dwass test, fractions A, B, and D, $p = 0.063$, 0.089, and 0.089) (Table 2). These results suggest that free FAs are the bitterness-masking compounds in cheese.

Bitterness-Masking Activities of Free Fatty Acids in Four Natural Cheeses. To confirm the bitterness-masking activity of free FAs, we determined the free FA content of Baraka cheese. The total concentration of free FAs in Baraka cheese was 12.15 mM, with OA present at the highest concentration (4.29 mM, 35.3%) followed by palmitic acid (3.70 mM, 30.5%), myristic acid (1.27 mM, 10.5%), and stearic acid (0.86 mM, 7.0%) (Table 3).

To examine the correlation of free FA content with bitterness-masking activity, Baraka and the other three cheeses

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with different bitterness-masking activities were analyzed. First, the bitterness-masking activities of Baraka, Gouda, Brie, and Ricotta were estimated using beer. The bitterness-masking activity of Baraka cheese was found to be significantly stronger than that of the other three cheeses by using the nonparametric Steel–Dwass test. The significant differences between Baraka and the other cheeses were as follows: Baraka versus Gouda, p = 0.0159; versus Brie; p = 0.0423; and versus Ricotta; p = 0.0077 (Figure 2).

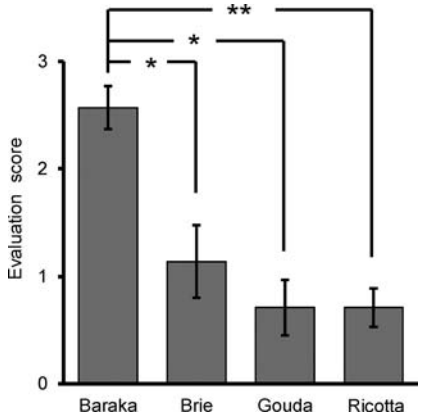

Figure 2. Comparison of the bitterness-masking activity of four cheese samples. The average values of the panelists' answers concerning the masking activity of cheese to the bitter taste of beer are shown (n = 7). The intensity of bitter taste was evaluated using a 4-point categorical scale, as follows: strong (0), medium (1), weak (2), or very weak (3). The Steel–Dwass test, which uses the nonparametric multiple-comparison method, was applied for detecting the between-sample differences. Error bars indicate standard error. The Baraka cheese sample showed significantly higher bitterness-masking activity than the other three samples [Gouda, p = 0.0159 (*); Brie, p = 0.0423 (*); Ricotta, p = 0.0077 (**)].

Next, the concentrations of free FAs in the other three cheeses were analyzed. The total concentrations of free FAs were 1.13 mM in Ricotta, 3.86 mM in Brie, and 2.25 mM in Gouda. Thus, the total free FAs are apparently high in Baraka and low in the other three cheeses. These results suggest that free FA content is correlated with the bitterness-masking activity.

Although the total concentrations of free FAs in the four cheeses are quite different, their free FA compositions are almost identical (Table 3). In all of the cheeses analyzed, four FAs (OA, palmitic acid, myristic acid, and stearic acid) comprised up to 60% of the total free FAs.

Evaluation of Bitterness-Masking Activities of Fatty Acids by Sensory Tests. Next, the bitterness-masking activities of free FAs were quantitated. A 0.5 mM solution of mixed free FAs (equivalent to the average free FA composition of cheese), 0.2 mM OA, 0.2 mM palmitic acid, 0.05 mM myristic acid, and 0.05 mM stearic acid were subjected to a bitterness-masking test. A 0.0080% QHCl solution was used to determine the bitterness-masking activity of the mixed free FAs. A test was constructed using seven concentrations of QHCl solution to evaluate the degree of bitterness-masking activity. The data followed a normal distribution according to the Shapiro–Wilk W test (p = 0.2123 and W = 0.932201) (Figure 3A). On the basis of these results, it was concluded that the bitterness-masking effect existed within the 95% confidence interval of the mean response. We found that the 0.5 mM mixed FA solution

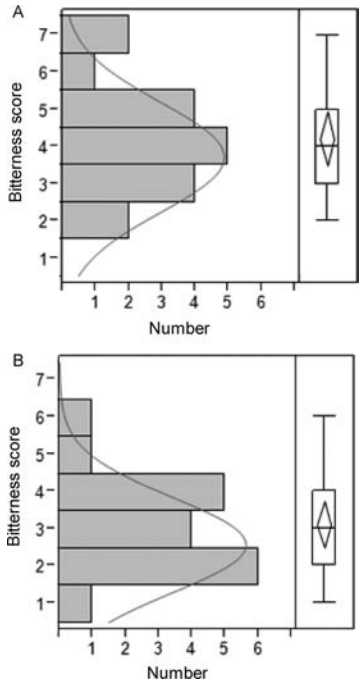

Figure 3. Histograms and box plots of sensory evaluation data. Two sample solutions were prepared: (A) 0.0080% quinine hydrochloride (QHCl) containing 0.2 mM oleic acid (OA), 0.2 mM palmitic acid, 0.05 mM myristic acid, and 0.05 mM stearic acid; (B) 0.0080% QHCl containing 0.5 mM OA. Bitter taste intensity was evaluated by comparing the sample solutions with seven standard QHCl solutions, as follows: 1, 0.0026%; 2, 0.0030%; 3, 0.0035%; 4, 0.0040%; 5, 0.0046%; 6, 0.0053%; and 7, 0.0060%. "Number" indicates the number of panelists. The lines show the normal distribution curves. The right panels are box plots with the smallest observation, lower quartile, median, upper quartile, and largest observation. The rhomb indicates the 95% confidence limit of averages. There is no outlier in these data. The Shapiro–Wilk W test for non-normality showed p = 0.2123 and W = 0.932201 in (A) and p = 0.1376 and W = 0.921529 in (B).

reduced the bitterness of QHCl from 0.0080 to 0.0049–0.0060%. However, there was a significant difference in the evaluation score between the panelists according to the F test of ANOVA (p = 0.001). The mixed free FAs apparently suppressed the bitterness of QHCl, but the data varied among panelists. This could be explained by the fact saturated FAs, myristic acid, palmitic acid, and stearic acid were little dissolved and could not be suspended in buffer and, therefore, the recognition of bitter taste would be variable.

To eliminate the factor of low solubility of these FAs, OA was used for the bitterness-masking test, because it can be suspended in buffer as a sodium salt and it was the predominant FA in Baraka cheese. The normality of distribution of the sensory evaluation score, using the seven concentration levels of QHCl for quantitatively evaluating the degree of bitterness-masking activity, was confirmed by the Shapiro–Wilk W test (p = 0.1376 and W = 0.921529) (Figure 3B). On the basis of these results, it was estimated that the bitterness-masking effect existed within the 95% confidence interval of the mean response. Our findings showed that a 0.5 mM OA solution reduced the bitterness of QHCl from 0.0080 to 0.0032–0.0038%. In addition, there was no significant difference in the evaluation score among the panelists, as demonstrated by the F test of ANOVA (p = 0.131). Thus, in the measurements conducted using the 0.5 mM solution of OA alone, the

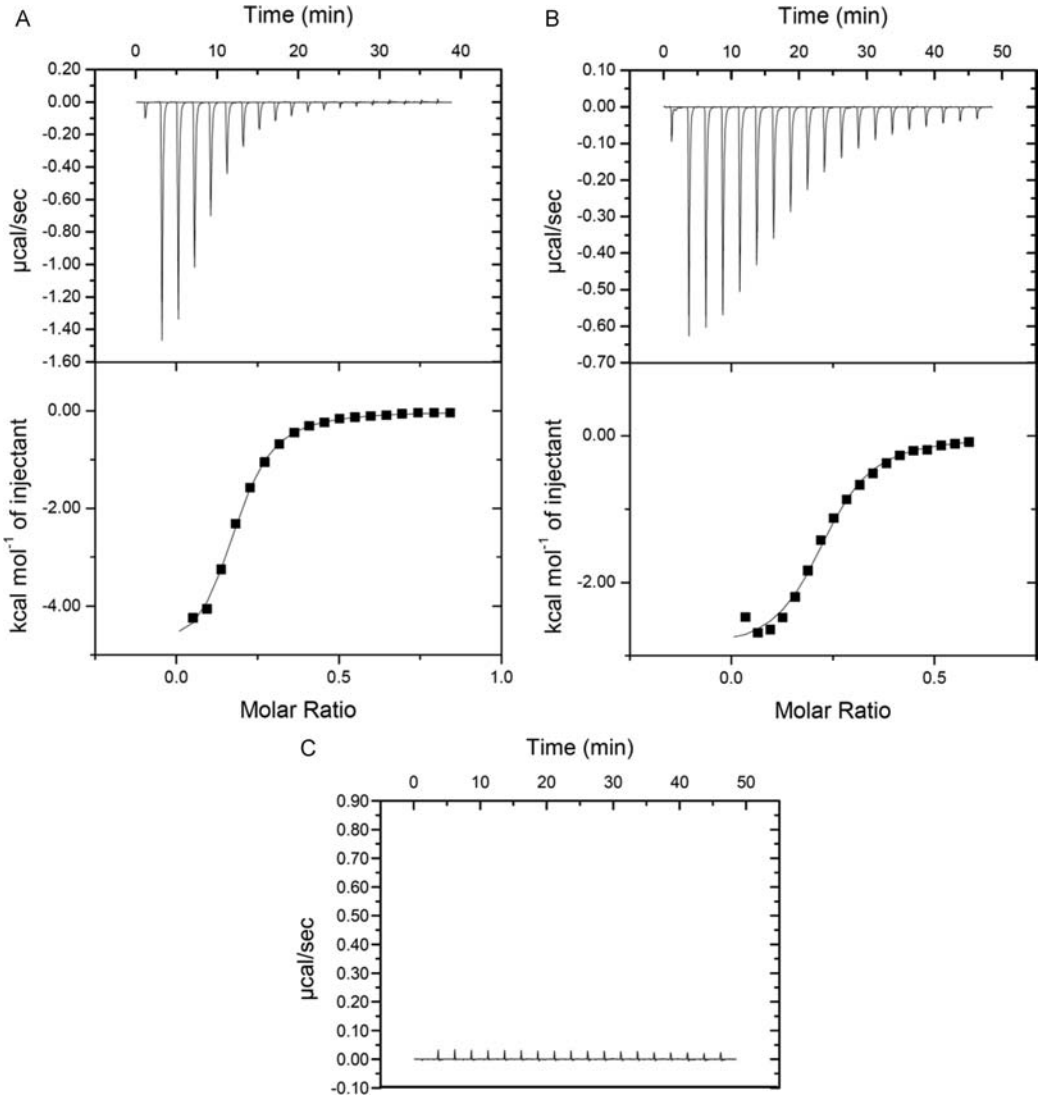

Figure 4. Isothermal titration calorimetry profiles of oleic acid binding to quinine hydrochloride (QHCl) and promethazine hydrochloride (PHCl): (A) 0.5 mM oleic acid (OA) was titrated with 2.2 mM QHCl; (B) 0.5 mM OA was titrated with 1.5 mM PHCl; (C) 0.5 mM OA was titrated with 2.2 mM caffeine. The upper panels show the raw data of titration. The experiments were performed at 25 °C, and the titration was repeated 19 times at (A) 120 s and (B, C) 150 s intervals. In the lower panels, the area of the peak was integrated and plotted against the molar ratio of QHCl or PHCl to OA. The solid line represents the best fit for the experimental data. The raw data of titration are shown in (C).

panelists' evaluations were found to be highly consistent with one another. These results demonstrate that FAs, especially OA, suppress the bitterness of QHCl.

Bitterness-Masking Activity of Oleic Acid and Isothermal Titration Calorimetry Analysis. As already shown, the bitter taste of 0.0080% QHCl was suppressed by 0.5 mM OA. A further study involved the bitterness-masking activity of 0.5 mM OA against 0.22 mM QHCl, 1.5 mM PHCl, and 50 mM caffeine. Because of the sensory tests, the bitterness of QHCl and PHCl was significantly suppressed by OA (one-tailed binomial test: $p = 0.0059$, 0.0156). On the other hand, that of caffeine was not suppressed (one-tailed binomial test: $p = 0.0547$).

To validate the bitterness-masking activity of OA, the binding potential between OA and QHCl was examined by ITC, which analyzes the interaction of two compounds by measuring the change in caloric output when they are mixed. When OA was titrated with QHCl, the reaction was exothermic, and the dissociation constant was 11 ± 1 μM (Figure 4A; Table 4). The interactions between OA and PHCl were also examined, and the dissociation constant was 8.8 ± 1.6 μM (Figure 4B; Table 4). The interactions between OA and caffeine were also examined, but were not detected under the same conditions (Figure 4C).

Table 4. Thermodynamic Parameters by Isothermal Titration Calorimetry[a]

analyte	K_d (μM)	ΔG (kcal/mol)	ΔH (kcal/mol)	ΔS (cal/mol/deg)
QHCl	11 ± 1	−6.8 ± 0.1	−5.1 ± 0.1	5.53
PHCl	8.8 ± 1.6	−6.9 ± 0.1	−3.0 ± 0.1	13.2

[a] K_d and ΔH were obtained from the fitted curve according to a model for one set of sites. ΔS and ΔG were obtained from eq 1. QHCl, quinine hydrochloride; PHCl, promethazine hydrochloride; K_d, dissociation constant; ΔG, free energy change of binding; ΔH, enthalpy change of binding; ΔS, entropy change of binding.

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DISCUSSION

In this study, we have purified a bitterness-masking fraction in cheese, and free FAs were identified as the compounds responsible for the bitterness of this fraction. The total concentration of free FAs in Baraka cheese was about 12 mM, which included 4.3 mM OA. A sensory test showed that both a 0.5 mM free FA mixture (OA, palmitic acid, stearic acid, myristic acid) and 0.5 mM OA alone reduced the bitterness of QHCl. The concentration of OA in Baraka cheese is about 10 times larger than that of OA used for the sensory test, which is adequate for it to develop the bitterness-masking effects.

Several kinds of free FAs other than OA were also contained in the four cheeses analyzed. Although individual free FAs were not examined, a mixture of these FAs showed bitterness-masking activity, proving that they must possess this property. In fact, linoleic acid has been reported to mask the bitterness of caffeine.[34] However, the concentration of linoleic acid used in the previous experiment was ~70 times greater than that of the OA used in the present experiment. PA and PI exhibit a strong bitterness-masking activity against QHCl, although some other bitter substances are not similarly influenced.[35] Bitterness-masking activity can be determined by the affinity of a bitter tastant for a masking compound. On the basis of the ITC analyses, the K_d values between OA versus QHCl and OA versus PHCl were 11 ± 1 and 8.8 ± 1.6 μM, respectively. Several studies have shown that a midmicromolar dissociation range of interaction leads to the formation of a complex between two compounds. Dimyristoylphosphatidylcholine and cationic antimicrobial tripeptide form a complex with a midmicromolar K_d.[36] Similarly, 1-aminoanthracene interacts with horse spleen apoferritin (HSAF) with a dissociation constant of 100 μM, which was shown to be appropriate for binding.[37] Both pairs of compounds form complexes. The affinity of OA for QHCl and PHCl was in the midmicromolar range. When the results of this study are considered comprehensively, the most likely hypothesis is that OA binds to QHCl or PHCl to form complexes in aqueous solution, thereby masking the bitterness of QHCl.

We speculate that a complex may form because of the interaction between alkyl chains in OA or in other free FAs and the hydrophobic partial structure in QHCl and PHCl. The calorimetric parameters as well as the values of ΔH and $T\Delta S$ are in line with the assumption that the suppression is caused by hydrophobic interactions. On the other hand, OA did not suppress the bitterness of caffeine. The interaction between OA and caffeine was not detected by ITC under the present conditions. Even the higher concentrations of 0.5 mM OA and 50 mM caffeine did not show interaction. These results suggest that the binding ability of OA and bitter compounds is a factor determining the bitterness-masking activity. Moreover, it was suggested that the K_d value was related to the strength of the bitter-masking activity.

Manufacturers of processed foods generally use purified oil and fats that contain TG. However, fermented foods contain free FAs produced by the digestion of TG by the lipases secreted from microorganisms.[30,31] Some studies showed that short-chain FAs liberated from milk lipids generate a cheese-specific flavor,[29,38,39] although the bitterness-masking activity of free FAs has not been discussed. The present study found a close relationship between the high bitterness-masking activity of Baraka cheese in sensory tests and its high free FA content. To the best of our knowledge, this study is the first to report the bitterness-masking effect of foods containing free FAs in general and of OA in particular.

Food components interact with each other and change the quality of taste. However, no convincing method for analyzing the interaction of food components at the molecular level is available. In particular, interactions among small molecules have not been detectable in the intact molecular form. In this study, we directly analyzed the interaction between a bitter tastant and its masking compound. Our approach will be useful for studying the interactions concerned with tastants in foods.

ASSOCIATED CONTENT

Supporting Information

Supplemental table: screening for cheeses with strong bitterness-masking activity. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

TAS2R, taste 2 receptor; PA, phosphatidic acid; QHCl, quinine hydrochloride; PI, phosphatidylinositol; ADAM, 9-anthryldiazomethane; FA, fatty acid; TOG, trioleoylglycerol; DOG, dioleoylglycerol; MOG, monooleoylglycerol; PHCl, promethazine hydrochloride; TMS, tetramethylsilane; TLC, thin-layer chromatography; ITC, isothermal titration calorimetry; HPLC, high-performance liquid chromatography; OA, oleic acid; ANOVA, analysis of variance; TG, triacylglycerol; NMR, nuclear magnetic resonance; DG, diacylglycerol; MG, monoacylglycerol.

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