JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Evaluation of the Bitter-Masking Potential of Food Proteins for EGCG by a Cell-Based Human Bitter Taste Receptor Assay and Binding Studies

Maxime C. Bohin,[†] Wibke S. U. Roland,[†] Harry Gruppen,[†] Robin J. Gouka,[‡] Harry T. W. M. van der Hijden,[‡] Peter Dekker,[§] Gerrit Smit,[†,∥] and Jean-Paul Vincken*,[†]

[†]Laboratory of Food Chemistry, Wageningen University, Bornse Weilanden 9, 6708, Wageningen, The Netherlands

[‡]Unilever R&D, Olivier van Noortlaan 120, 3133 AT Vlaardingen, The Netherlands

[§]DSM Food Specialties, Alexander Fleminglaan 1, 2613 AX Delft, The Netherlands

[∥]Valio Limited R&D, Meijeritie 6, 00370, Helsinki, Finland

ABSTRACT: Epigallocatechin gallate (EGCG) has been ascribed to several health benefits, but its bitter taste influences the liking of products with high concentrations of this compound. β-Casein, in particular, and several gelatins are known as strong binders of EGCG, contrary to β-lactoglobulin. The current study aimed at relating the EGCG-binding characteristics of those proteins and their food-grade equivalents to their effects on reducing bitter receptor activation by EGCG in vitro and their bitter-masking potential in vivo. Also in the bitter receptor assay, β-casein showed the strongest effect, with a maximum reduction of hTAS2R39 activation of about 93%. A similar potency was observed for Na-caseinate. β-Lactoglobulin had little effect on bitter receptor activation, as expected based on its low binding affinity for EGCG. The bitter-masking potential of Na-caseinate was confirmed in vivo using a trained sensory panel. β-Lactoglobulin also slightly reduced EGCG bitter perception, which could not be directly related to its binding capacity. The bitter receptor assay appeared to be a valid tool to evaluate in vitro the efficacy of food proteins as complexing agents for masking bitterness.

KEYWORDS: *flavonoid−protein interaction, complexation, β-casein, caseinate, gelatin, EGCG, bitterness, ultrafiltration, sensory, hTAS2R*

INTRODUCTION

Epigallocatechin gallate (EGCG) is known to be the most abundant catechin in green tea (ca. 60% of the total catechins) and has been ascribed to several beneficial health effects (e.g., anticarcinogenic and cardioprotective effects).[1] With respect to taste, tea catechins are known to be astringent and bitter.[2] The mechanism of astringency perception is not yet fully defined but can be partially attributed to the interaction of EGCG with salivary proteins. Astringency seems sensorially coupled with bitterness, although, compared to the latter, it has been usually reported as a secondary attribute in time−intensity experiments.[3,4] On the human tongue, bitter compounds are perceived by bitter taste receptors, referred to as hTAS2Rs, which are part of the family of G-protein-coupled receptors.[5] To date, 21 hTAS2Rs out of the 25 known have identified agonists.[6,7] Among these, hTAS2R39 has been associated with taste perception of green tea catechins.[8] As evaluated in vitro with hTAS2R39, EGCG has a two times lower EC_{50} value (181.6 μM) compared to its nongalloylated equivalent epigallocatechin (EGC; EC_{50} = 395.5 μM). This difference was confirmed in vivo by a higher perceived bitterness for EGCG.[2,8]

Effective health benefits against cardiovascular and metabolic diseases have been associated with a daily intake of green tea containing 200−300 mg of EGCG.[9] Food products with high concentrations of EGCG may have off-tastes and consequently low consumer acceptance.[10] Various approaches to modulate bitterness of bioactive compounds in functional foods have been described, such as the use of sweeteners, blockers for bitter taste receptors, and complexation with other compounds. In the latter approach, cyclodextrins are the most commonly used carriers while other carriers (e.g., proteins) are seldomly reported.[10−13] A typical example of off-taste reduction in food is addition of milk to tea, which has been linked to the interaction of tea catechins with milk proteins[3] without impairing their bioavailability.[14] Milk proteins have also been suggested as carriers for bioactive compounds and, in particular, thermally induced β-lactoglobulin−EGCG complexes.[12,15]

In our previous work,[16] we investigated the potential of food proteins as carriers for flavonoids. On the basis of affinities and binding capacities measured, β-casein and gelatins, in particular, fish gelatin, were found to be the most promising carriers for EGCG. One necessary condition for the applicability of those complexes in food is their effective reduction in bitter taste perception of EGCG. Bitter receptor activation by flavonoids can be evaluated in vitro by a cell-based receptor assay.[8,17] To our knowledge, the present study is the first report using such a setup to evaluate the reduction in activation of bitter receptors by EGCG after forming complexes with proteins. This primary approach can help to predict the outcome of sensory panels.

Received: July 12, 2013
Revised: October 2, 2013
Accepted: October 4, 2013
Published: October 4, 2013

dx.doi.org/10.1021/jf4030823 | *J. Agric. Food Chem.* 2013, 61, 10010−10017

The aim of the present study was to correlate EGCG binding of pure β-casein, food-grade caseinates, and several gelatins to the potential of these proteins for reducing bitterness perception of EGCG. This was first tested in vitro using a cell-based bitter receptor assay and then in vivo with a trained sensory panel in order to evaluate the applicability of those complexes in foods.

MATERIALS AND METHODS

Materials. Bovine β-casein (≥98% of total protein), bovine β-lactoglobulin (≥90% of protein), solid fish gelatin (Gelatin F1) from cold water fish skin, and gelatin type B (Gelatin B1) from bovine skin (75 bloom) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Protein contents of the two gelatins were estimated to be ≥90% as detailed in a previous study.[16] Food-grade EGCG (≥94%), Sunphenon 90LB (>80% catechins including ∼50% EGCG), and food-grade calcium and sodium caseinates (protein content ≥ 90%, N × 6.38) were kindly provided by DSM Nutritional Products (Basel, Switzerland), Taiyo GmbH (Filderstadt, Germany), and DMV International (Veghel, The Netherlands), respectively. Bovine Vinoferm gelatin powder (≥85% of protein; N × 5.55), Vinoferm gelatin liquid (20% (w/v) gelatin (supplier information)), and Vinoferm Isinglass (fish gelatin, 2% (w/v) protein (supplier information)) were food grade and purchased from Brouwland (Beverlo, Belgium). Throughout this study, these gelatins are referred to as gelatin B2, gelatin B3, and gelatin F2, respectively. Food-grade BioPURE-β-lactoglobulin (≥90% of total protein) was kindly provided by Davisco Food International (Eden Prairie, MN, USA). Water for in vitro tests was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). Water (Spa Reine, Spadel Group, Brussels, Belgium) for in vivo tests was obtained from a local supermarket. All other chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany).

Assessment of Binding of Proteins to EGCG by Ultrafiltration (UF Assay). *Determination of Binding Parameters of Food-Grade Proteins for EGCG (UF Assay, Method 1).* All samples were prepared in a 50 mM sodium phosphate buffer, pH 7.0. Protein stock solutions (0.2 mM) were prepared freshly before each experiment. For both caseinates, an average molecular mass of 23.3 kDa was calculated from the protein composition in bovine milk.[18] Gelatins were prepared at a concentration equivalent to 0.2 mM β-casein (i.e., 4.72 mg/mL). Similarly, a stock solution of EGCG (6 mM) was used to obtain a range of dilutions between 0 and 6 mM. EGCG−protein mixtures were prepared and the binding affinities of each protein toward EGCG measured using an ultrafiltration microtiter plate setup (Ultracel 10, Millipore, Cork, Ireland) as described previously.[16]

The protein-bound and free fractions of EGCG at each concentration tested were calculated, and plots of the bound fraction versus the concentration of free EGCG were used to determine the binding parameters. For each binding curve obtained, a linear regression was used on the initial linear increase ($R^2 > 0.8$) in order to estimate the binding affinity (K) of the compounds. A maximal binding capacity (R_{max}) was derived from the plateau value or the highest bound fraction observed at high phenolic compound/protein molar ratios. Binding parameters were reported as mean ± standard deviation (SD) of two replicates.

Determination of $[EGCG]_{free}$ with Increasing Concentrations of Various Proteins (UF Assay, Method 2). An EGCG stock solution (0.5 mM) and solutions of proteins with concentrations ranging from 0.013 to 0.2 mM (EGCG to protein molar ratios from 2.5 to 40) were prepared in a similar way as described above. The concentration of free EGCG remaining in the mixtures after incubation was determined using an ultrafiltration microtiter plate setup as described previously.[16]

In Vitro Assessment of hTAS2R39 Activation by Intracellular Calcium Release. Activation of bitter receptors was investigated by the release of intracellular Ca^{2+} using a fluorescent calcium dye.[19] Expression of hTAS2R39 in HEK293 cells and the detailed procedure for monitoring its activation were performed as reported elsewhere.[17]

All samples were prepared in Tyrode's buffer (140 mM NaCl, 5 mM KCl, 10 mM glucose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 20 mM Hepes, pH 7.4). EGCG stock solution (1 mM) was prepared freshly before each experiment. Similarly, stock solutions of proteins (0.8 mM) were used to obtain a concentration range from 0.006 to 0.8 mM. Protein−EGCG complexes were made in a microtiter plate by mixing protein solutions 1:1 with EGCG solutions. Controls were made by mixing EGCG with buffer without proteins. The microtiter plate was incubated at room temperature under constant shaking (300 rpm, 10 min).

Next, the complexes were loaded (ratio 1:1 (v/v)) in a microtiter plate containing the cells (final concentrations of EGCG of 0.25 mM and of protein between 0 and 0.2 mM) and evaluated for their potential to activate bitter receptor hTAS2R39 at 37 °C with a FlexStation II 384 (Molecular Devices Corp., Sunnyvale, CA, USA) for 90 s as described elsewhere.[17] Prior to addition of the complexes to the cells, the baseline signal was determined in the first 17 s. Then, fluorescence signals (excitation 485 nm/emission 520 nm) were measured until 90 s. As negative control, noninduced cells, which do not express the hTAS2R receptor, were always measured in parallel. Additionally, a dose−response curve of EGCG was determined in the same way by measuring concentrations of EGCG up to 1 mM without proteins. Measurements were performed at least in duplicate on two or more different days.

Sensory Analysis. *Panelists.* The panel consisted of 13 persons (10 males and 3 females), which were part of a larger sensory panel familiar to bitterness rating and selected within Unilever R&D (Vlaardingen, The Netherlands) for their ability to taste and rank bitterness. Panelists could join each session on a voluntary basis and participated in at least one of the sessions described below. Panelists were trained to taste bitterness and rate it on a scale from 1 to 10 using a known reference (Sunphenon 90 LB). Sunphenon 90 LB was sensorially close to EGCG as it was mostly bitter with a low astringency (supplier information).[2] The ratings reported were relative to this EGCG-rich reference and not to pure EGCG. No specific training was conducted to discriminate between astringency and bitterness.

Sample Preparation. Proteins (10 g/L) and EGCG (1 g/L) stock solutions were prepared freshly before each experiment. Compounds were dissolved in water (Spa Reine), known for its neutral pH and low content in minerals. EGCG was mixed 1:1 with each protein solution and incubated at room temperature for at least 10 min. Fifteen milliliters of each sample was poured into a yellow cup. Colored cups were used to limit the visual perception of the differences between samples (e.g., slight haze or color) with or without proteins and thus limit its possible impact on the choice and rating by the panelists. Additionally, three solutions of Sunphenon 90 LB were prepared: Two references known to the panelists (0.3 and 0.8 g/L) and one unknown to the panelists (0.4 or 0.5 g/L). The former were used as a calibration for the panelists prior to and throughout each session. The latter was used by the panelists to check the accuracy of their rating at the beginning of each session.

Selection of Suitable Food-Grade Proteins for Quantitative Sensory Analysis. A preliminary session was organized to select food-grade proteins with the highest potential for bitterness reduction. Volunteers from the panel (n = 8) were allowed to taste a known sample with only EGCG (0.5 g/L) and rate it against the references. Then, each protein−EGCG sample was tasted, described individually, and subsequently discussed with the other panelists. The group rating and the most recurrent descriptors were used to select the most suitable proteins for further experiments.

2-Alternative Forced Choice (2-AFC) Test. EGCG complexed with Na-caseinate and β-lactoglobulin were evaluated in duplicate against a control EGCG sample without proteins. The experiment was conducted on two different days with 12 panelists, and 6 of them were present on both days (n = 36 per sample). Pairs of samples were all provided at once to each panelist. In between samples, panelists were instructed to rinse their mouth with water or milk and eat a piece of cucumber or plain cracker. For each pair, panelists had to indicate

which sample was the most bitter and rate the two samples on a scale from 0 to 10 as described above.

Ranking Test. EGCG (1 g/L) was mixed 1:1 with 4 different concentrations of Na-caseinate (2, 5, 10, and 15 g/L) and prepared as described above. Panelists were provided with a series of 5 samples in duplicate (13 panelists, n = 26 per sample) and instructed to rank them in order of increasing bitterness and rate them on a scale from 0 to 10 as described above. Series included one control containing only EGCG (0.5 g/L) and no proteins. The instructions given to the panelists for rinsing their mouth between samples were the same as for the 2-AFC test.

Data Processing and Statistical Analysis. For hTAS2R39 activation data, SoftMax Pro 5.4 software (Molecular Devices Corp.) was used to plot fluorescence signals. Data processing for the activation curve of hTAS2R39 by EGCG was performed as described previously.[17] Similarly, for EGCG−protein complexes, the fluorescence values ($\Delta F/F_0$) were calculated by subtracting the baseline fluorescence (F_0) prior to loading from the maximum fluorescence (F) after addition of the compounds, divided by the signals of the baseline to normalize to background fluorescence.[17] Besides the response of induced cells, also the response of noninduced cells was measured as negative control for every compound at every concentration on the same plate. In cases that a nonspecific signal occurred with $\Delta F/F_0 >$ 0.25, the corresponding concentration of the protein was not considered for further calculations. Response of noninduced cells was subtracted from its corresponding response of induced cells at all valid concentrations. The activation and decrease of receptor activation were expressed as percentages relative to the maximum response measured (i.e., EGCG control) and plotted versus the protein concentration. Data were reported as the mean value of the replicates, and error bars represented the standard error of the mean (SEM).

The dose−response curve of hTAS2R39 by EGCG was fitted with nonlinear regression curves in Graph Pad Prism (version 4 for Windows, Graph Pad Software, San Diego, CA, USA). The sigmoidal dose−response curve model with variable slope corresponded to the following equation

$$\frac{\Delta F}{F_0} = B + \frac{(T - B)}{1 + 10^{(\log(EC_{50}) - \log([EGCG]) - H}} \quad (1)$$

where B is the bottom plateau value, T the top plateau value, $\log(EC_{50})$ the $\log([EGCG])$ value at which the response is halfway between B and T, and H the Hill slope or steepness of the curve. Best-fit parameters for the activation curve of hTAS2R39 by EGCG were as follows: B = 0.107, T = 1.531, $\log(EC_{50})$ = −3.793, and H = 1.976.

The aforementioned best-fit parameters and dose−response curve equation were used to predict the receptor activation ($\Delta F/F_0$) which should be observed based on the concentration of free EGCG measured in the UF assay, method 2. The theoretical receptor activation was plotted as percentage of reduction of activation versus protein concentration using the following equation

$$\%\text{reduction activation} = \left(1 - \frac{\left(\frac{\Delta F}{F_0}\right)_i}{\left(\frac{\Delta F}{F_0}\right)_{250}}\right) \times 100 \quad (2)$$

where $((\Delta F)/(F_0))_i$ is the theoretical receptor activation at $[EGCG]_{free} = i$ (in μM) and $((\Delta F)/(F_0))_{250}$ the theoretical receptor activation at $[EGCG]_{free}$ = 250 μM. Data were reported as the mean value of the replicates, and error bars represented the SEM.

Averages and confidence intervals (95%) from the 2-AFC and ranking tests were calculated. Significance ($p < 0.05$) for the 2-AFC test was determined based on a minimum number of correct judgments for paired difference using a statistical table reported elsewhere.[20] Significance ($p < 0.05$) for the ranking test was determined by Kramer's rank sum test.[21]

RESULTS

Reduction of Activation of Bitter Receptor hTAS2R39 by Complexing EGCG to Proteins. On the basis of a previous study on common food proteins binding EGCG,[16] β-casein, β-lactoglobulin, gelatin B1, and gelatin F1 were selected and tested for their potential to reduce the activation of the bitter receptor hTAS2R39 by EGCG in a cell-based assay. The test was conducted at a concentration of EGCG of 250 μM, which was about the EC_{70} value (EC_{50} = 161 μM, Figure 1A)

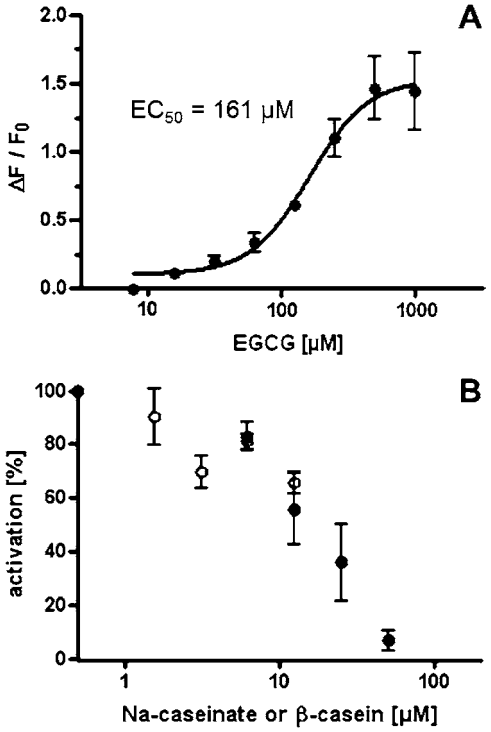

Figure 1. Dose−response curve of hTAS2R39 stimulated with EGCG (A), and example of receptor activation by EGCG (250 μM) complexed with increasing concentrations of β-casein (●) or Na-caseinate (○) (B).

and provided sufficient signal to clearly observe the effect of the proteins, as illustrated in Figure 1B. The decrease of hTAS2R39 activation by EGCG with the various proteins tested is reported in Figure 2. Maximum reductions of receptor activation (as percentages of reduction of activation from control EGCG without protein) for protein−EGCG complexes are summarized in Table 1.

Among the four tested proteins, β-casein showed the strongest concentration-dependent reduction of the receptor activation by EGCG with a decrease of 93.3(±5.3)% at the highest measurable protein concentration applied (i.e., 50 μM). β-Lactoglobulin did not show a clear effect on decreasing the receptor activation, and relatively high variations between replicates were observed. Gelatin F1 was found to have the second strongest reduction of the receptor activation by EGCG (maximum receptor activation decrease measured of 46.0(±2.6)%), whereas its maximum decrease was reached at a lower protein concentration than with β-casein. Gelatin B1 did not reduce the activation of hTAS2R39 by EGCG by more than 23.0(±8.0)% in the measurable protein concentration range, indicating that it had low potential for masking bitterness. The slight trend of decreasing receptor activation

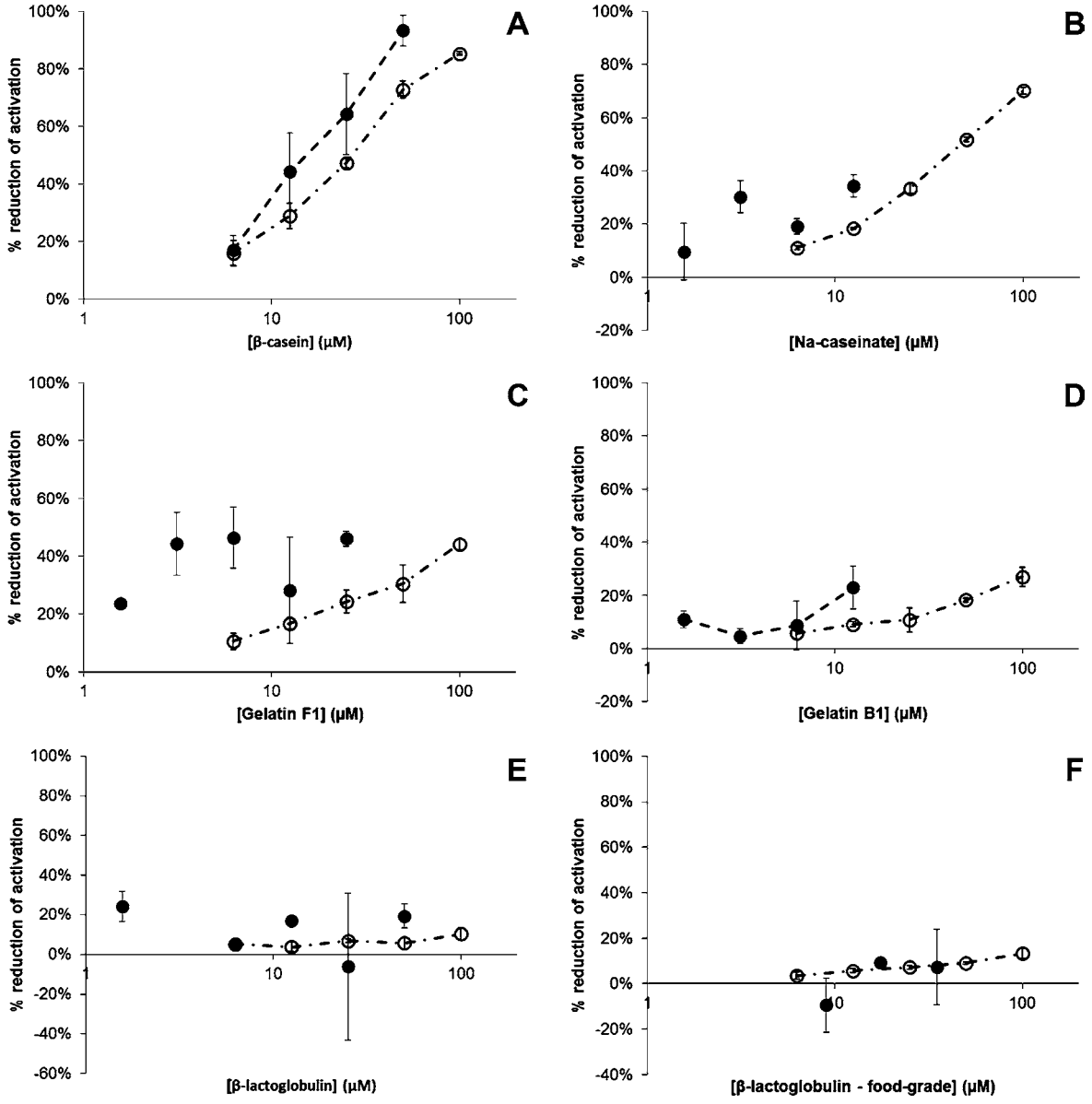

Figure 2. Comparison of percentages of reduction of hTAS2R39 activation by EGCG by various proteins measured experimentally with the bitter receptor assay (●) and predicted with data from the UF assay, method 2 (○) at constant concentration of EGCG (250 μM): (A) β-casein, (B) Na-caseinate, (C) gelatin F1, (D) gelatin B1, (E) analytical-grade β-lactoglobulin, (F) food-grade β-lactoglobulin.

with increasing protein concentrations observed for gelatins B1 and F1, contrary to the clear trend observed for β-casein, might be caused by the turbidity of these samples, affecting the accuracy of the measurements. In fact, the presence of insoluble aggregates might have affected the loading volumes on the cells and interfered with fluorescence readings. The observations made for gelatins suggest that a larger proportion of EGCG remained able to interact with the bitter receptor when applying gelatins compared to β-casein.

Relationship between hTAS2R39 Activation and Binding Characteristics of EGCG to Analytical-Grade Proteins. The binding affinity (K) and maximal binding capacity (R_{max}) of the aforementioned proteins for EGCG were determined in a previous study[16] and are summarized in Table 2. β-Casein and gelatin F1 had similar affinities for EGCG, both about 2 times and 10 times higher than the ones measured with gelatin B1 and β-lactoglobulin, respectively. Those affinities were found to be sufficient to have a strong effect on the reduction of activation of hTAS2R39 (Figure 2A and 2C). The limited effect of β-lactoglobulin on the reduction of receptor activation in the bitter receptor assay is thought to be linked to its low affinity for EGCG. Even though gelatin B1 had an intermediate affinity for EGCG, it had a limited effect on the receptor activation by EGCG, suggesting that a minimum affinity is required for a significant reduction of receptor activation. Binding affinity seemed to be a more important factor than R_{max} as gelatin B1 had a higher R_{max} than β-casein but only showed a limited effect on decreasing receptor activation.

A second ultrafiltration method (UF assay, method 2) was used to mimic the conditions of the bitter receptor assay (i.e., constant [EGCG] and variable [protein]). The concentrations of free EGCG measured in the UF assay, method 2, were used to predict the percentage of reduction of receptor activation using eqs 1 and 2 and compared to the experimental data obtained. β-Casein was used to evaluate the accuracy of this

Table 1. Comparison of the Bitterness Reduction Potential of Proteins Evaluated in Vitro and in Vivo (Based On Preliminary Experiment) at an EGCG-to-Protein Molar Ratio of 5[a]

protein	grade	% reduction of activation (cell assay)	% reduction of activation (UF assay)[d]	reduction of rating (in vivo)[e]
β-casein	analytical	93.3 (±5.3)	72.8 (±2.9)	n.a.
Na-caseinate	food	34.3 (±4.1)[b]	51.9 (±0.9)	3
Ca-caseinate	food	n.d.	49.2 (±0.5)	3.5
β-lactoglobulin	analytical	c	5.8 (±2.9)	n.a.
β-lactoglobulin	food	c	9.0 (±0.5)	1
gelatin B1	analytical	23.0 (±8.0)[b]	18.3 (±0.8)	n.a.
gelatin B2	food	n.d.	n.d.	2.5
gelatin B3	food	n.d.	n.d.	1.5
gelatin F1	analytical	46.0 (±2.6)[b]	30.6 (±6.5)	n.a.
gelatin F2	food	n.d.	n.d.	n.a.[f]

[a]n.a.: not applicable; n.d.: not determined. [b]Percentage of the highest measurable protein concentration in the bitter receptor assay. [c]No clear trend in reduction of receptor activation detected. [d]Percentages calculated based on concentrations of unbound EGCG in UF assay, method 2. [e]Reduction of ratings and percentages calculated using a bitterness score of 7 for the EGCG reference. [f]Strong sour taste overruled bitter taste.

approach (Figure 2A). Data derived from the UF assay, method 2, and the bitter receptor assay were in good agreement, with an underestimation of only 10−20% for the theoretical values compared to the experimental values.

In contrast to β-casein, the theoretical and experimental values showed clear discrepancies for gelatin F1 (Figure 2C). As mentioned earlier, interferences due to aggregates could explain this observation. In the case of gelatin B1 (Figure 2D), a limited amount of experimental data points could be matched but similar trends between the theoretical and experimental values were observed. The theoretical percentages of reduction of receptor activation calculated for gelatin F1 are two times lower than those for β-casein, although their previously reported affinities for EGCG are similar (Table 2).[16] This shows that affinity is not the only parameter for an efficient reduction of receptor activation, although it might be a good first indicator. For example, the flexibility of β-casein and its ability to form micelles which could entrap EGCG might be advantageous characteristics compared to the more rigid structure of gelatins.[16,22,23] Summarizing, the bitter receptor assay was in good agreement with the ultrafiltration assay when applying a protein with a good binding capacity, such as β-casein. Discrepancies, however, could be observed when using proteins forming insoluble aggregates, such as gelatins, or having a low binding capacity, such as β-lactoglobulin.

In the present study, β-casein was confirmed as a promising carrier for EGCG in food as its previously reported high binding affinity and capacity for EGCG could be linked to an effective reduction of bitter receptor activation by EGCG.

Efficiency of Food-Grade Protein Ingredients to Complex EGCG. Highly purified proteins are not commonly used in the food industry and are, in most cases, not commercially available as food ingredients. Hence, the analytical-grade proteins were replaced by common food ingredients containing these proteins (e.g., β-casein replaced by caseinates). These food-grade proteins were evaluated for their binding potential for EGCG by the UF assay, and their binding parameters were compared to the ones of their equivalent analytical-grade proteins as summarized in Table 2. A higher concentration of proteins as used in the current assay (i.e., 100 μM) did not seem to influence the binding affinity as found for β-casein ($(45.0 \pm 7.2) \times 10^3$ M^{-1} at 25 μM versus $(43.3 \pm 2.2) \times 10^3$ M^{-1} at 100 μM). Ca-Caseinate and Na-caseinate were considered as an acceptable replacement for β-casein with good potential for further application. The lower values obtained for the binding parameters of caseinates compared to β-casein might be related to their more heterogeneous protein composition, with a ratio α_{S1}:α_{S2}:β:κ of about 11:3:10:4 on a molar basis.[18] In fact, it has been shown that α-casein had a two times lower binding affinity for EGCG compared to β-casein.[24]

Na-Caseinate was tested for its capacity to reduce hTAS2R39 activation by EGCG (Figure 1B). Although only a narrower range of protein concentration could be measured due to nonspecific signals, Na-caseinate showed a similar trend in receptor activation decrease compared to β-casein at protein concentrations between 1.5 and 6 μM. Theoretical percentages of reduction of receptor activation were calculated for Na-caseinate using the UF assay, method 2, and similar to those obtained for β-casein (Figure 2B), with a maximum reduction of 70.1(±1.2)% at a protein concentration of 100 μM (85.3(±0.6)% for β-casein). Theoretical and experimental values at Na-caseinate concentrations of 6 and 12.5 μM were in good agreement. Ca-Caseinate showed the same theoretical potential of reduction of receptor activation compared to Na-caseinate using the UF assay, method 2, with a maximum reduction of 72.0(±0.9)% (data not shown). Hence, Na-caseinate and Ca-caseinate were confirmed as acceptable food-grade alternatives to analytical-grade β-casein for reducing hTAS2R39 activation by EGCG.

Gelatin B2 showed similar binding potential compared to the model protein gelatin B1, whereas gelatins B3 and F2 displayed lower binding affinities compared to their analytical equivalents.

Table 2. Binding Characteristics (affinity, K; maximum binding capacity, R_{max}) of Analytical-Grade (taken from previously published data[16]) and Food-Grade Proteins (current study) with EGCG by UF assay at pH 7.0, 25 °C[a]

analytical-grade proteins	K (10^3 M^{-1})	R_{max} (mol/mol)	R_{max} (g/100g)	food-grade protein equivalents	K (10^3 M^{-1})	R_{max} (mol/mol)	R_{max} (g/100g)
β-casein	45.0(±7.2)	19.6(±4.9)	38.1(±9.6)	Na-caseinate	22.8(±0.5)	12.8(±0.4)	24.7(±0.7)
				Ca-caseinate	24.8(±0.5)	12.7(±0.0)	24.7(±0.1)
β-lactoglobulin	4.5(±2.3)	6.6(±3.7)	16.5(±9.2)	β-lactoglobulin	3.6(±0.3)	7.5(±0.3)	14.6(±0.5)
gelatin B1	25.4(±4.1)	31.6(±2.1)	57.8(±3.8)	gelatin B2	29.2(±0.6)	16.9(±0.4)	32.8(±0.8)
				gelatin B3	6.9(±1.1)	9.8(±1.0)	19.1(±2.0)
gelatin F1	53.3(±1.8)	57.5(±1.8)	43.9(±1.4)	gelatin F2	7.8(±0.8)	10.8(±0.7)	21.1(±1.3)

[a]n.d.: not detectable; n.a.: not applicable.

This difference compared to the model proteins could be due to variations in the characteristics of the gelatin samples (e.g., amino acid composition, average molecular mass). Food-grade and analytical-grade β-lactoglobulins had similar low binding affinities (Table 2). As shown in Figure 2 and Table 1, the weak binding properties of both β-lactoglobulins to EGCG also did not result in a significant effect in reduction of hTAS2R39 activation. This was also shown by the UF assay, method 2, with a maximum value in reduction of receptor activation below 15% calculated for both proteins (Figure 2E and 2F).

Sensory Analysis of EGCG Complexed with Proteins. As summarized in Table 3, various food-grade proteins were compared in a preliminary sensory experiment for their potential to reduce bitterness of EGCG at a protein to EGCG mass ratio of 10, which was equivalent to an EGCG to protein molar ratio of about 5 using the molecular mass of β-casein. Ca-Caseinate and Na-caseinate had similar effects on the taste of EGCG with a reduction of EGCG bitterness rating by 3.5 and 3 units, respectively. The relatively transparent appearance of Na-caseinate was preferred over the white color of Ca-caseinate in water for further investigation as it offers fewer limitations for applications (e.g., in clear beverages). β-Lactoglobulin had the least effect on the bitterness reduction of EGCG (1 unit). Therefore, it was selected as a negative control for further experiments. The three gelatin samples generally had unpleasant off-tastes, especially gelatin F2. In addition, gelatins B2 and F2 formed visible aggregates with EGCG at the molar ratio used. Taken together, gelatins were considered as unsuitable for further sensory tests and applications as bitter-masking compounds.

In a 2-AFC test, β-lactoglobulin and Na-caseinate significantly reduced the bitter taste of EGCG by 1.4 ± 0.4 and 2.3 ± 0.5 units, respectively (Figure 3). The effect of Na-caseinate on EGCG bitterness perception was in accordance with the expectations based on the reduction of hTAS2R39 activation by Na-caseinate (Figure 2B). A significant, although lower, effect of β-lactoglobulin on bitterness of EGCG was not expected as only a limited effect was observed in a preliminary sensory session (Table 3) and also in the in vitro assays (Figure 2D).

In a ranking test with increasing concentrations of Na-caseinate, it appeared that the lowest bitterness score (~4) was already reached at a concentration of 0.25% (w/v) of Na-caseinate (Figure 4A). This observation concurs with a sensory study on olive oil phenolics binding Na-caseinate, for which a minimum bitterness score was reached with 1% (w/v) protein and did not decrease further with increasing protein concentration.[25] In the current study, 0.25% (w/v) Na-caseinate resulted in a bitterness reduction of 3 units compared to the EGCG reference and the reduction of bitterness ratings reported at lower EGCG to protein molar ratios remained

Table 3. Sensorial Comparison of EGCG (0.5 g/L) Complexed with Various Food-Grade Proteins (5 g/L)

protein	pH in water[a]	rating	aspect	taste attributes (other than bitter)
EGCG control	6.3	7	clear	
Ca-caseinate	6.9	3.5	turbid (milk-like)	milky, astringent
Na-caseinate	6.9	4	slightly turbid	milky, slightly metallic
gelatin B2	6.0	5.5	visible aggregates	astringent, burned, strong off-taste
gelatin B3	4.9	4.5	slightly turbid	off-taste
gelatin F2	2.6	n.a.[b]	visible aggregates	very sour
β-lactoglobulin	6.3	6	clear	slight off-taste

[a]pH of EGCG−protein complexes after incubation. [b]n.a.: not applicable; strong sour taste overruled the bitter taste.

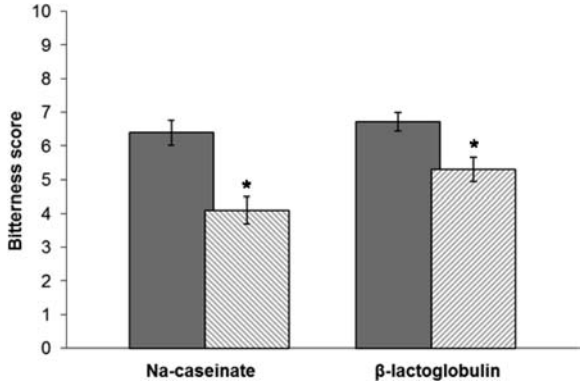

Figure 3. Comparison of perceived bitterness of EGCG, free (plain bars) or complexed with Na-caseinate or β-lactoglobulin (hatched bars), in a 2-AFC sensory test. (*) significant difference ($p < 0.05$).

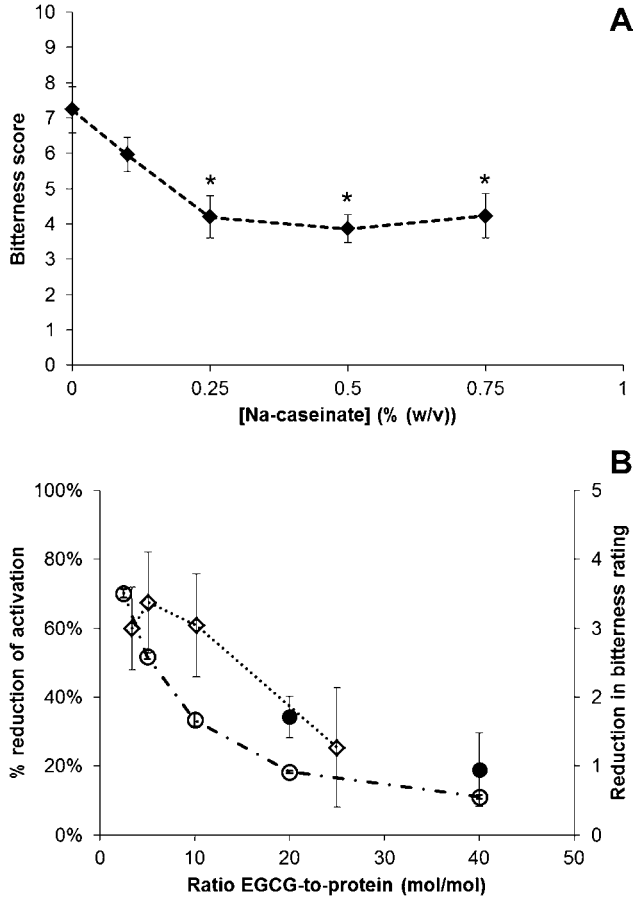

Figure 4. Dose−response curve for perceived bitterness of EGCG (0.5 g/L) with increasing concentration of Na-caseinate ((*) significant difference ($p < 0.05$)) (A), and comparison of the reduction of bitterness ratings in vivo (◊) (right y axis) with percentages of reduction of hTAS2R39 activation by EGCG with Na-caseinate measured experimentally with the bitter receptor assay (●) and predicted with data from the UF assay (○) (left y axis) (B).

between 3 and 3.5 units (Figure 4B). As shown in Figure 4B, the reduction of bitterness ratings measured in vivo at EGCG to protein molar ratios of 10 and 25 followed the trend of the theoretical percentages for the reduction of receptor activation calculated from the UF assay, method 2. At molar ratios lower than 10, however, a plateau was observed in vivo while in vitro theoretical values predicted a continuously increasing percentage of reduction of hTAS2R39 activation.

DISCUSSION

In Vitro Prediction of Bitterness Reduction Compared to in Vivo Sensory Analysis. According to the in vitro assays conducted in this study (Figure 2), the efficacy in reducing bitterness of EGCG of the food-grade proteins tested should be ranked as follows: Ca-caseinate/Na-caseinate ≥ gelatins > β-lactoglobulin. This order was in line with our first sensory experiment (Table 3), confirming the applicability of our in vitro approach for screening the potential of food proteins for bitter masking.

A 2-AFC test demonstrated a decrease of perceived bitterness of EGCG when complexed to Na-caseinate by 2.3 units. The effect of Na-caseinate on the intrinsic bitterness of EGCG in vitro at the same EGCG to protein molar ratio (i.e., 5) was calculated to be a decrease of 50% based on the concentration of free EGCG after binding measured by ultrafiltration and related to the activation curve of hTAS2R39 by EGCG. Although our in vivo and in vitro results match well, it should be noted that the in vitro assay does not take account of actors other than hTAS2R39 in the mouth environment, such as other hTAS2Rs being activated by EGCG (although their response will also be modulated by complexation of EGCG to protein) and salivary proteins which might interact with EGCG and disturb the binding equilibrium. The intrinsic bitterness is calculated under the assumptions that hTAS2R39 is the main bitter receptor sensing EGCG, that complexes remain stable in the mouth, and that the slight off-taste of Na-caseinate does not influence bitterness ratings. In addition, a stronger effect of β-lactoglobulin on EGCG bitterness was observed in vivo compared to the in vitro experiments. This effect is unlikely to have resulted from its binding to EGCG nor from a direct interaction of β-lactoglobulin with the hTAS2R39 receptor, as suggested for another protein.[26] An indirect effect due to the interaction of β-lactoglobulin with the buccal environment could have interfered with the bitter perception (e.g., interaction with saliva and buccal cells).[27,28]

In this study, a maximum reduction in bitterness of EGCG was achieved at 0.25% (w/v) of Na-caseinate, although our in vitro assay predicted a continuous decrease in receptor activation with increasing protein concentrations (Figure 4B). This concurs with a model proposed by Pripp et al., which predicted a minimum bitterness reached at 0.5% (w/v) Na-caseinate for olive oil phenolics, assuming a binding affinity of 10^5 M^{-1}.[25] The bitter receptor assay complemented with ultrafiltration appears as an appropriate tool to evaluate the efficacy of a given macromolecule as a bitter-masking ingredient, although it tends to overestimate its potential (Figure 4B). Discrepancies between in vitro and in vivo evaluation of bitterness have already been reported. For example, higher threshold concentrations and EC_{50} values for bitter hop compounds were found in a sensory test compared to the taste receptor assay, whereas the ranking in order of potency for the compounds was the same.[29]

Bitter Receptor Assay as a Tool To Study Bitterness Masking by Complexing Agents. It has been shown that a cell-based bitter receptor assay can be a valuable tool to predict the intrinsic bitterness of food-related components.[8,17,29] In the present study, we report for the first time its potential in evaluating the modulation of the intrinsic bitterness of bitter tastants, e.g., EGCG, by combining them with complexing agents, such as proteins. Despite some limitations related to the range of protein concentrations that can be used or the influence of turbidity on the measurement, challenging hTAS2R39 with a combination of bitter tastant and protein allowed rapid identification of good candidates for complexing, such as β-casein. Proteins were ranked for their efficacy for reducing receptor activation as follows: β-casein > gelatin F1 ≈ Na-caseinate > gelatin B1 > β-lactoglobulin. This ranking was in good agreement with findings from a complementary ultrafiltration assay relating the concentration of free EGCG with increasing protein concentration to hTAS2R39 activation.

Provided that the hTAS2R activated by the bitter compound of interest is known, the bitter receptor assay seems to be promising for discovery of bitter-masking agents. It has been applied in several instances for high-throughput screening for so-called bitter blockers, i.e., compounds that act antagonistically on the bitter receptor of interest.[30,31] These blockers are thought to be rather specific in reducing bitterness, although their suggested promiscuity (i.e., the bitter blocker inhibits several hTAS2Rs)[31] or their potential agonistic behavior on other bitter receptors might compromise this idea.[32] Besides, some bitter compounds have been described to activate more than one bitter receptor, which might call for more than one blocker for a particular bitter tastant.[6,17,29] Therefore, it might be advantageous to use a more generic approach for masking bitterness, e.g., by applying complexing agents, such as food proteins, which do not act directly at the receptor. We have now shown that the cell-based bitter receptor assay can be used as a tool to study such complexing agents, given that the protein is able to bind a significant amount of the bitter tastant.

AUTHOR INFORMATION

Corresponding Author

*E-mail: jean-paul.vincken@wur.nl.

Funding

This work was financially supported by the Food and Nutrition Delta of the Ministry of Economic Affairs, The Netherlands (FND 08018 and FND 08019).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank the panelists who contributed to this study and Teun De Joode and Max Batenburg (Unilever R&D, Vlaardingen, The Netherlands) for their assistance in setting up the sensory experiments.

REFERENCES

(1) Cabrera, C.; Artacho, R.; Giménez, R. Beneficial effects of green tea - A review. *J. Am. Coll. Nutr.* **2006**, *25*, 79−99.

(2) Narukawa, M.; Kimata, H.; Noga, C.; Watanabe, T. Taste characterisation of green tea catechins. *Int. J. Food Sci. Technol.* **2010**, *45*, 1579−1585.

(3) Rossetti, D.; Bongaerts, J. H. H.; Wantling, E.; Stokes, J. R.; Williamson, A. M. Astringency of tea catechins: More than an oral lubrication tactile percept. *Food Hydrocolloids* **2009**, *23*, 1984−1992.

(4) Brossaud, F.; Cheynier, V.; Noble, A. C. Bitterness and astringency of grape and wine polyphenols. *Aust. J. Grape Wine Res.* **2001**, *7*, 33−39.
(5) Adler, E.; Hoon, M. A.; Mueller, K. L.; Chandrashekar, J.; Ryba, N. J. P.; Zuker, C. S. A novel family of mammalian taste receptors. *Cell* **2000**, *100*, 693−702.
(6) Meyerhof, W.; Batram, C.; Kuhn, C.; Brockhoff, A.; Chudoba, E.; Bufe, B.; Appendino, G.; Behrens, M. The molecular receptive ranges of human TAS2R bitter taste receptors. *Chem. Senses* **2010**, *35*, 157−170.
(7) Thalmann, S.; Behrens, M.; Meyerhof, W. Major haplotypes of the human bitter taste receptor TAS2R41 encode functional receptors for chloramphenicol. *Biochem. Biophys. Res. Commun.* **2013**, *435*, 267−273.
(8) Narukawa, M.; Noga, C.; Ueno, Y.; Sato, T.; Misaka, T.; Watanabe, T. Evaluation of the bitterness of green tea catechins by a cell-based assay with the human bitter taste receptor hTAS2R39. *Biochem. Biophys. Res. Commun.* **2011**, *405*, 620−625.
(9) Wolfram, S. Effects of green tea and EGCG on cardiovascular and metabolic health. *J. Am. Coll. Nutr.* **2007**, *26*, 373S−388S.
(10) Drewnowski, A.; Gomez-Carneros, C. Bitter taste, phytonutrients, and the consumer: a review. *Am. J. Clin. Nutr.* **2000**, *72*, 1424−1435.
(11) Szejtli, J.; Szente, L. Elimination of bitter, disgusting tastes of drugs and foods by cyclodextrins. *Eur. J. Pharm. Biopharm.* **2005**, *61*, 115−125.
(12) Shpigelman, A.; Cohen, Y.; Livney, Y. D. Thermally-induced β-lactoglobulin−EGCG nanovehicles: Loading, stability, sensory and digestive-release study. *Food Hydrocolloids* **2012**, *29*, 57−67.
(13) Sun-Waterhouse, D.; Wadhwa, S. S. Industry-relevant approaches for minimising the bitterness of bioactive compounds in functional foods: A review. *Food Bioprocess Technol.* **2013**, *6*, 607−627.
(14) Van Het Hof, K. H.; Kivits, G. A. A.; Weststrate, J. A.; Tijburg, L. B. M. Bioavailability of catechins from tea: the effect of milk. *Eur. J. Clin. Chem.* **1998**, *52*, 356−359.
(15) Livney, Y. D. Milk proteins as vehicles for bioactives. *Curr. Opin. Colloid Interface Sci.* **2010**, *15*, 73−83.
(16) Bohin, M. C.; Vincken, J.-P.; van der Hijden, H. T. W. M.; Gruppen, H. Efficacy of food proteins as carriers for flavonoids. *J. Agric. Food Chem.* **2012**, *60*, 4136−4143.
(17) Roland, W. S. U.; Vincken, J.-P.; Gouka, R. J.; van Buren, L.; Gruppen, H.; Smit, G. Soy isoflavones and other isoflavonoids activate the human bitter taste receptors hTAS2R14 and hTAS2R39. *J. Agric. Food Chem.* **2011**, *59*, 11764−11771.
(18) Walstra, P.; Wouters, J. T. M.; Geurts, T. J. Milk Components. In *Dairy Science and Technology*, 2nd ed.; CRC Press: Boca Raton, FL, 2006; pp 17−108.
(19) Chandrashekar, J.; Mueller, K. L.; Hoon, M. A.; Adler, E.; Feng, L.; Guo, W.; Zuker, C. S.; Ryba, N. J. P. T2Rs function as bitter taste receptors. *Cell* **2000**, *100*, 703−711.
(20) Roessler, E. B.; Pangborn, R. M.; Sidel, J. L.; Stone, H. Expanded statistical tables for estimating significance in paired-preference, paried-difference, duo-trio and triangle tests. *J. Food Sci.* **1978**, *43*, 940−943.
(21) Lawless, H. T.; Heymann, H. *Sensory Evaluation of Food: Principles and Practices*; Springer Science+Business Media: New York, 1998; p 827.
(22) O'Connell, J. E.; Grinberg, V. Y.; De Kruif, C. G. Association behavior of β-casein. *J. Colloid Interface Sci.* **2003**, *258*, 33−39.
(23) Zanchi, D.; Narayanan, T.; Hagenmuller, D.; Baron, A.; Guyot, S.; Cabane, B.; Bouhallab, S. Tannin-assisted aggregation of natively unfolded proteins. *EPL* **2008**, *82*, 58001.
(24) Hasni, I.; Bourassa, P.; Hamdani, S.; Samson, G.; Carpentier, R.; Tajmir-Riahi, H. A. Interaction of milk α- and β-caseins with tea polyphenols. *Food Chem.* **2011**, *126*, 630−639.
(25) Pripp, A. H.; Busch, J.; Vreeker, R. Effect of viscosity, sodium caseinate and oil on bitterness perception of olive oil phenolics. *Food Qual. Pref.* **2004**, *15*, 375−382.
(26) Maehashi, K.; Matano, M.; Nonaka, M.; Udaka, S.; Yamamoto, Y. Riboflavin-binding protein is a novel bitter inhibitor. *Chem. Senses* **2008**, *33*, 57−63.
(27) Vardhanabhuti, B.; Cox, P. W.; Norton, I. T.; Foegeding, E. A. Lubricating properties of human whole saliva as affected by β-lactoglobulin. *Food Hydrocolloids* **2011**, *25*, 1499−1506.
(28) Ye, A.; Zheng, T.; Ye, J. Z.; Singh, H. Potential role of the binding of whey proteins to human buccal cells on the perception of astringency in whey protein beverages. *Physiol. Behav.* **2012**, *106*, 645−650.
(29) Intelmann, D.; Batram, C.; Kuhn, C.; Haseleu, G.; Meyerhof, W.; Hofmann, T. Three TAS2R bitter taste receptors mediate the psychophysical responses to bitter compounds of hops (*Humulus lupulus L.*) and beer. *Chemosens. Percept.* **2009**, *2*, 118−132.
(30) Fletcher, J. N.; Kinghorn, A. D.; Slack, J. P.; McCluskey, T. S.; Odley, A.; Jia, Z. In vitro evaluation of flavonoids from Eriodictyon californicum for antagonist activity against the bitterness receptor hTAS2R31. *J. Agric. Food Chem.* **2011**, *59*, 13117−13121.
(31) Slack, J. P.; Brockhoff, A.; Batram, C.; Menzel, S.; Sonnabend, C.; Born, S.; Galindo, M. M.; Kohl, S.; Thalmann, S.; Ostopovici-Halip, L.; Simons, C. T.; Ungureanu, I.; Duineveld, K.; Bologa, C. G.; Behrens, M.; Furrer, S.; Oprea, T. I.; Meyerhof, W. Modulation of bitter taste perception by a small molecule hTAS2R antagonist. *Curr. Biol.* **2010**, *20*, 1104−1109.
(32) Brockhoff, A.; Behrens, M.; Roudnitzky, N.; Appendino, G.; Avonto, C.; Meyerhof, W. Receptor agonism and antagonism of dietary bitter compounds. *J. Neurosci.* **2011**, *31*, 14775−14872.