DOI:10.1021/jf803956f

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Interactions of the Sweet-Tasting Proteins Thaumatin and Lysozyme with the Human Sweet-Taste Receptor

Nobuyuki Ide,[†] Eriko Sato,[†] Keisuke Ohta,[†] Tetsuya Masuda,[†,§] and Naofumi Kitabatake*,[†,§]

[†]Division of Food Science and Biotechnology, Graduate School of Agriculture and [§]Department of Natural Resources, Graduate School of Global and Environmental Studies, Kyoto University, Uji, Kyoto 611-0011, Japan

This study investigated the sweetness of the sweet-tasting protein thaumatin and lysozyme by both an in vitro cell-based assay and an in vivo sensory analysis to elucidate the differences between in vitro and in vivo response profiles. Hek293 cells were constructed that stably expressed the human T1R2+T1R3 sweet-taste receptor, and their responses to thaumatin and lysozyme were analyzed by monitoring the levels of intracellular cAMP. The results indicated that thaumatin and lysozyme as well as aspartame induced a decrease in the intracellular cAMP accumulation of the T1R2+T1R3-transfected cells and that EC_{50} values of thaumatin and lysozyme determined by cell-based assay are well-consistent with the results of the sweetness threshold value determined by sensory analysis in the presence of 140 mM NaCl. The results of both in vitro and in vivo experiments confirmed that the sweetness inhibitor lactisole significantly suppressed the sweetness of thaumatin and lysozyme.

KEYWORDS: Thaumatin; lysozyme; sweet taste; T1R2+T1R3 sweet-taste receptor

INTRODUCTION

Most sweet-tasting substances are low molecular mass compounds such as natural sugars (sucrose, fructose, etc.), sweet amino acids (D-tryptophan, D-serine, etc.), and artificial sweeteners (aspartame, saccharin, etc.). Besides these low molecular mass compounds, some proteins are known to elicit a sweet-taste response on the human palate, including thaumatin, monellin, brazzein, mabinlin, curculin (also termed neoculin), and hen egg lysozyme (*1*). No homology has been identified among these sweet-tasting proteins in terms of amino acid sequences and tertiary structures. A noteworthy feature is that all of these proteins have high isoelectric points except for a small protein, brazzein, the isoelectric point of which is 5.4 (*2*). Even in the brazzein molecule, a local positively charged region exists on the surface of the molecule. Although these proteins typically have an extremely low threshold value of sweetness when compared with that of sucrose, these threshold values have a wide range.

Thaumatin, one of the most characterized sweet-tasting proteins, is a 22 kDa plant protein extracted from the aril of the fruit of *Thaumatococcus daniellii* Benth (*3*). This protein's threshold value of sweetness is around 50 nM, which is about 100,000 times sweeter than sucrose on a molar basis (*3*, *4*). Lysozyme obtained from hen egg white has recently been characterized as a sweet-tasting protein (*5*, *6*). Lysozyme consists of 129 amino acid residues and has a molecular mass of 14.5 kDa. The threshold value of the sweetness of lysozyme is around 7 μM (*7*). The sweetness of lysozyme is about 700 times higher than that of sucrose and about 140 times lower than that of thaumatin. These sweet-tasting proteins can be useful tools for studying how the ligand-binding site of the sweet-taste receptor confers a broad and specific receptive range, because sweet-tasting proteins have high and wide ranging affinities to the sweet-taste receptor.

Sweet tastes are detected in large part by G-protein-coupled receptors (GPCRs), which lead to the activation of intracellular signaling cascades (*8*). The T1R family (T1R1, T1R2, and T1R3) is specifically expressed in taste cells and is involved in the signal transduction mechanism of sweet and umami tastes. A heterodimeric receptor composed of T1R1+T1R3 functions as an umami (L-amino acids) receptor, whereas T1R2+T1R3 forms a broadly tuned sweet receptor (*9*, *10*). Heterologous expression and a Ca^{2+} imaging analysis showed that cells cotransfected with T1R2, T1Rm3 and a promiscuous phospholipase C-linked G-protein (Gα15, Gα16, or those mutants) responded to sweet-tasting proteins (thaumatin, monellin, brazzein, and neoculin) as well as low molecular mass sweeteners (*10*−*12*). Thus, it has been suggested that there may be a single receptor (T1R2+T1R3) and one signaling pathway involved in the perception of sweetness. However, these in vitro experiments were conducted at high protein concentrations, and the affinity of sweet-tasting proteins for the T1R2+T1R3 receptor in vitro was extremely lower than that in vivo. In addition, it was reported that a sweetness inhibitor, (±)-2-(*p*-methoxyphenoxy)propionic acid sodium salt (lactisole), did not suppress the sweetness of thaumatin in vivo (*13*), whereas the sweet-taste response of thaumatin and brazzein to T1R2+T1R3 receptor was suppressed by lactisole in vitro

*Corresponding author (telephone +81-774-38-3742; fax +81-774-38-3743; e-mail kitabatake@kais.kyoto-u.ac.jp).

 Published on Web 06/03/2009

(*14*). These discrepancies between the results of in vivo and in vitro experiments suggest that other receptors or other signal transduction mechanisms, which are presumably sweet-tasting protein-specific and lactisole-insensitive, are involved in the elicitation of the sweetness of proteins. The interaction of sweet-tasting proteins with the T1R2 + T1R3 sweet taste receptor is controversial.

In this study, we constructed the cell-based assay and then examined the sweetness of sweet-tasting proteins, thaumatin and lysozyme, by both in vitro (cell-based assay) and in vivo experiments (human sensory analysis) to elucidate the differences between the in vitro and in vivo response profiles. The in vitro Ca^{2+} imaging analysis using the cells expressing a promiscuous G-protein is widely used to monitor the responses of sweeteners to the sweet-taste receptor. However, the response profile is affected by a kind of G-protein transfected (*10*−*12*). The measurement of intracellular cAMP of T1R2 + T1R3-transfected cells has been shown to reflect to the receptor activation quantitatively even in the Gα15-expressing cells (*15*). In addition, it is not known which G-protein(s) can intrinsically couple to the sweet-taste receptor in a taste cell. Therefore, we tried to construct Hek293 cells stably expressing T1R2 + T1R3 without a promiscuous G-protein and monitor the change of intracellular cAMP levels induced by an endogenously expressing G-protein. We found that EC_{50} values of thaumatin and lysozyme determined by our cell-based assay are well-consistent with the results of the sweetness threshold value determined by sensory analysis in the presence of 140 mM NaCl and that lactisole significantly suppressed the sweetness of sweet-tasting proteins in vivo as well as in vitro.

MATERIALS AND METHODS

Materials. Aspartame was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). 3-Isobutyl-1-methylxanthine (IBMX), forskolin, and (±)-2-(*p*-methoxyphenoxy)propionic acid (lactisole) were obtained from Sigma-Aldrich (St. Louis, MO). IBMX and forskolin were dissolved in DMSO at 100 and 50 mM as stock solutions, respectively. Lactisole solution was neutralized to pH 7 by adding 0.1 N NaOH. Thaumatin I was purified from crude thaumatin powder (Wako Pure Chemical Industries Ltd.) as described previously (*16*). This purified thaumatin I (referred to as simply thaumatin hereafter) was used throughout the study. Hen egg lysozyme was purchased from Nacalai Tesque (Kyoto, Japan), and further purification was performed by crystallization (*6*).

Cloning of the T1R2 and T1R3 cDNAs. Human T1R2 cDNA and T1R3 cDNA were amplified from PCR Ready First Strand cDNA of normal human tongue (BioChain Institute, Hayward, CA) by PCR with *LA Taq* DNA polymerase (Takara Bio, Shiga, Japan) and *Pfu* Ultra Hotstart DNA polymerase (Stratagene, La Jolla, CA). The primers including the Kozak consensus sequence were constructed on the basis of the previously deposited sequences for T1R2 and T1R3 (GenBank accession nos. BX537160 and BK000152) (*10*). The PCR primers were as follows: t1r2-5′, 5′-C ACC ATG GGG CCC AGG GCA AAG ACC ATC TCC TCC C-3′; t1r2-3′, 5′-CTA GTC CCT CCT CAT GGT GTA GCC CTG GAT-3′; t1r3-5′, 5′-C ACC ATG CTG GGC CCT GCT GTC CTG GGC CT-3′; t1r3-3′, 5′-TCA CTC ATG TTT CCC CTG ATT TCC TGT GTT-3′. The DNA sequence of the cloned genes was confirmed by an ABI 310 DNA sequencer (Applied Biosystems, Foster City, CA). Then, the resulting cDNA of T1R2 and T1R3 was inserted into a mammalian expression vector pcDNA3.2-TOPO and pcDNA6.2-TOPO (Invitrogen, Carlsbad, CA), respectively (**Figure 1A**).

Cell Culture and Transfection. Human embryonic kidney 293 (Hek293) cells were obtained from American Type Culture Collection (ATCC, CRL-1573) and were grown in low-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with GlutaMAX and 10% dialyzed fetal bovine serum (FBS) (Invitrogen). All cells were cultured at 37 °C under a humidified atmosphere containing 5% CO_2. To generate a stable cell line that expressed the sweet-taste receptor, the Hek293 cells were grown to 80% confluency in noncoated, six-well plates (Nalge Nunc, Roskilde, Denmark) and then transfected with 2 μg each of the *Ssp*I-linearized T1R2-pcDNA3.2 plasmid and T1R3-pcDNA6.2 plasmid by using Lipofectamine2000 (Invitrogen), according to the manufacturer's protocol. Forty-eight hours after transfection, the medium was replaced with the selection medium containing 1.0 mg/mL of G418 (Wako Pure Chemical Industries) for the T1R2-pcDNA3.2 transfectants and 10 μg/mL of blasticidin (Kaken Pharmaceutical, Tokyo, Japan) for the T1R3-pcDNA6.2 transfectants. The medium was exchanged every 3−4 days during the 3 weeks. Resistant colonies were picked and expanded to scale up for further experiments. The expression of T1R2 and T1R3 mRNAs was confirmed by RT-PCR. One clone, expressing mRNA of both T1R2 and T1R3, was designated Hek293-T1R2/T1R3. The cloned stable cell lines expressing the sweet-taste receptor were maintained in culture medium containing 500 μg/mL G418 and 5 μg/mL blasticidin. The cells were passaged every 6−7 days and were not used above passage 20 after transfection.

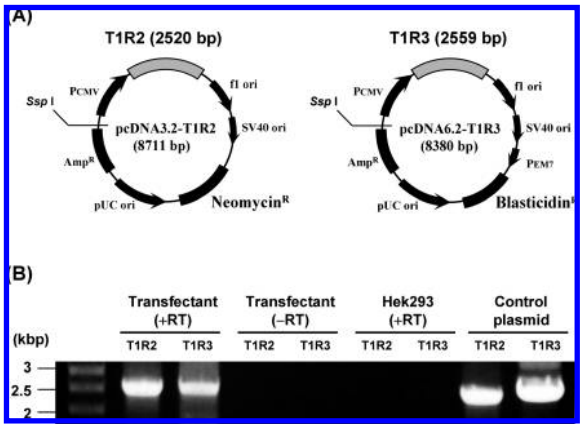

Figure 1. Expression vectors (**A**) and RT-PCR analysis of the expression of human T1R2 and T1R3 mRNA in the cloned cell line (**B**). RT-PCR was performed by using total RNAs after treatment with (+) and without (−) reverse transcriptase (RT).

RT-PCR Analysis of RNA Extracted from Control and Transfected Cells. To determine whether the T1R genes were expressed by the transfected cells, RT-PCR was performed. Total RNA was extracted from the cells by using the Protein and RNA Extraction Kit for mammalian cells (Takara Bio Inc.) and then treated with DNase I (Invitrogen) to degrade any contaminating genomic DNA. First-strand cDNAs were synthesized by using PrimeScript Revese Transcriptase (Takara Bio Inc.) and oligo(dT) primer (Invitrogen). PCR was performed with *LA Taq* DNA polymerase using the resulting cDNAs and specific primers as described above. The thermal cycle program was 1 cycle of 2 min at 96 °C, followed by 30 cycles of 30 s at 96 °C, 30 s at 56 °C, and 3 min at 72 °C, with a final elongation step of 5 min at 72 °C.

Intracellular cAMP Assay. Intracellular cAMP contents were measured by using the cAMP Biotrak enzyme immunoassay (EIA) kit (GE Healthcare Bio-Sciences, Little Chalf-ont, U.K.) according to the nonacetylation EIA procedure. The cells were seeded in noncoated, 96-well culture plates (Nalge Nunc) at a density of 2×10^5 cells/well 24 h prior to the experiment. The medium was aspirated, and 200 μL of prewarmed HBSS (137 mM NaCl, 5.3 mM KCl, 4.2 mM $NaHCO_3$, 0.33 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.25 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 0.1% bovine serum albumin) containing 0.5 mM IBMX was added to each well. After the cells had incubated for 30 min at 37 °C, the buffer was aspirated, and 200 μL of prewarmed agonist solution in D-PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.9 mM $CaCl_2$, and 0.49 mM $MgSO_4$) containing 0.5 mM IBMX and 50 μM forskolin was added to each of the corresponding wells. Plates were incubated for 20 min at 37 °C. The agonist solution was aspirated, and 200 μL of the lysis reagent was added to each well. The cAMP levels were then determined as described by the kit instructions. An independent cAMP standard curve was determined for each 96-well plate. Data were plotted with GraphPad PRISM software version 5 (GraphPad Software, San Diego, CA).

Preparation of R82A-Thaumatin. The R82A-thaumatin, which is a mutant of thaumatin substituting Arg82 with Ala82, was produced by a *Pichia pastoris* expression system as described previously (*17*). The *P. pastoris* harboring mutated thaumatin cDNA was cultured in a 3 L jar fermenter (M-100, Tokyo Rikakikai, Tokyo, Japan). Induction of the production of recombinant protein was initiated by replacing the carbon source from glycerol to methanol. The fermentation was completed 72 h after induction. The supernatant of the culture medium was recovered and exhaustively dialyzed against 5 mM sodium phosphate buffer, pH 7.0, containing 0.1 mM EDTA at 4 °C. Subsequently, the recombinant thaumatin was purified by ion-exchange chromatography and ammonium sulfate precipitation. The purity of the recombinant thaumatin was then confirmed from the band on SDS-PAGE and acetic acid non-denaturing-PAGE.

Sensory Analysis. The sweetness threshold values of the samples were evaluated by human volunteers as previously described (*18*). A well-trained panel of seven subjects participated in this trial. All subjects gave their informed consent to participate in this study. The protocol was approved by the Graduate School of Agriculture, Kyoto University. Five milliliters of test solution that was prepared in 5 mM sodium phosphate buffer, pH 7.0, was put in one of three paper cups. The other two cups contained 5 mM sodium phosphate buffer, pH 7.0, as a blank. When the effect of lactisole or NaCl was examined, 1 mM lactisole or 140 mM NaCl was added to 5 mM sodium phosphate buffer, pH 7.0. These three cups were given to the panelists, who were asked to indicate which cup had the taste-eliciting solution. The panel members were instructed to swirl each sample around in their mouths for 10 s and then to expectorate. After tasting each sample, the subjects rinsed their mouths thoroughly with distilled water or NaCl solution (140 mM) to eliminate any remaining taste. Next, similar experiments were performed with a more concentrated sample solution. The concentrations of samples are given in Tables S1−S3 of the Supporting Information. The sweetness intensity was evaluated on a scale from 0 to 5. A value of 0 indicates that no taste was detected from the test solution, and a value of 1 indicates that the sample solution elicited some taste stimulation. A value of 2 indicates that the subjects detected sweetness from the sample solution; thus, the threshold value of sweetness is the concentration that yielded a value of 2. As the sweetness intensity increased, scores of 3−5 were given. The average concentration of a particular sample that subjects rated "2" was estimated to be the sweetness threshold value of that sample.

RESULTS

Cloning of T1R2 and T1R3 cDNAs and Construction of a Stable Cell Line Expressing Sweet-Taste Receptor. PCR was performed with the first-strand cDNAs from the normal human tongue as a template and a pair of oligonucleotide primers designed on the basis of the 5′- and 3′-nucleotide sequences of the reported sequence (*10*). The nucleotide sequence of the cloned cDNA showed that T1R3 cDNA was the same sequence as previously reported (*10*) except for two silent mutations (G636A and C1788T). For T1R2 cDNA, its DNA sequence was different from the reported sequence at nine positions: G26C (giving Cys9Ser), C231T (silent mutation), G571A (giving Val191Ile), G624T (silent mutation), G882T (silent mutation), G949C (giving Gly317Arg), G1456A (giving Val486Ile), T1929C (silent mutation), and G2358A (silent mutation). Recently, Kim et al. reported single-nucleotide polymorphisms (SNPs) and haplotypes in the human T1R gene family (*19*). Six of the nine mutations (G26C, C231T, G571A, G882T, G949C, and G1456A) in the T1R cDNA cloned in this study corresponded to the SNPs reported by Kim et al. (*19*). The sequence of the cloned T1R2 cDNA was consequently the same as that registered in RefSeq accession no. NM_152232 except for two silent mutations (G624T and G2358A).

T1R2 and T1R3 cDNA were inserted into pcDNA3.2 and pcDNA6.2 vectors, respectively, to produce T1R2-pcDNA3.2 and T1R3-pcDNA6.2 (**Figure 1A**). Each plasmid was cotransfected into Hek293 cells, and stable cell lines containing the transfectants were isolated by antibiotic selection. The presence of T1R2 and T1R3 mRNAs in the stable clones was confirmed by RT-PCR. The PCR product from total RNA extracted from the stable clone gave a positive band of the expected size (**Figure 1B**).

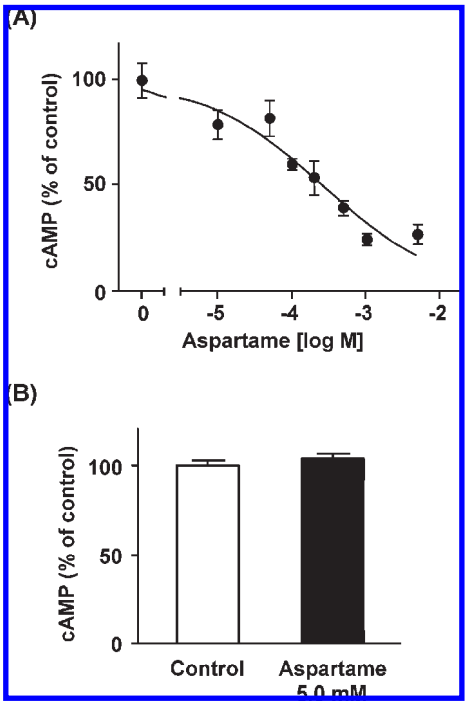

Figure 2. Aspartame inhibits intracellular cAMP accumulation in T1R2 +T1R3 receptor-expressing cells: (**A**) effects of concentrations of aspartame on forskolin-induced cAMP accumulation. Hek293-T1R2/T1R3 cells were incubated with 0.5 mM IBMX and 50 μM forskolin in the presence of increasing concentrations of aspartame (10 μM−5.0 mM). (**B**) Untransfected Hek293 cells were incubated with 0.5 mM IBMX and 50 μM forskolin in the presence and absence of 5.0 mM aspartame. Each point represents the mean ± SD (n = 4).

Characteristics of the Intracellular cAMP Response to Sweet-Tasting Proteins. To confirm the activation of the receptor expressed in Hek293 cells, we measured the intracellular cAMP in cells stimulated by sweeteners. It has been reported that the T1R2+T1R3 sweet-taste receptor expressed in cultured cells is coupled to the $G_{i/o}$ family of G-proteins and that the activation of the receptor leads to a reduction in the intracellular cAMP levels (*15*, *20*). As shown in **Figure 2**, aspartame led to a reduction in the forskolin-induced intracellular cAMP accumulation in a concentration-dependent manner with an EC_{50} of 265 μM. This EC_{50} value for aspartame is comparable to those of other papers (*10*, *15*) and to the physiological threshold value in humans. The effect of aspartame on the intracellular cAMP level is receptor-dependent because the intracellular cAMP of parental Hek293 cells was unchanged by aspartame (**Figure 2B**). This indicates that the receptor expressed in Hek293 cells is functionally active and that this in vitro cell-based assay is valid.

Then, we examined the effect of sweet-tasting protein on the T1R2+T1R3 receptor activity. Thaumatin inhibited the forskolin-induced intracellular cAMP accumulation in a concentration-dependent manner with EC_{50} values of 5.2 μM (**Figure 3A**). We previously reported that Arg82 in the thaumatin molecule plays a key role in the elicitation of the sweetness response to thaumatin. The sweetness threshold value of R82A-thaumatin

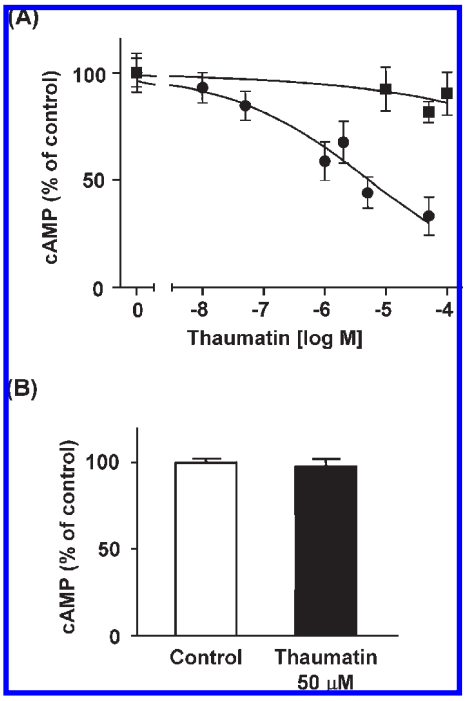

Figure 3. Thaumatin inhibits intracellular cAMP accumulation in T1R2 +T1R3 receptor-expressing cells: (**A**) effects of concentrations of thaumatin or R82A-thaumatin on forskolin-induced cAMP accumulation. Hek293-T1R2/T1R3 cells were incubated with 0.5 mM IBMX and 50 μM forskolin in the presence of increasing concentrations of thaumatin (10 nM−50 μM) (●) or R82A-thaumatin (10−100 μM) (■). (**B**) Untransfected Hek293 cells were incubated with 0.5 mM IBMX and 50 μM forskolin in the presence and absence of 50 μM thaumatin. Each point represents the mean ± SD (n = 4).

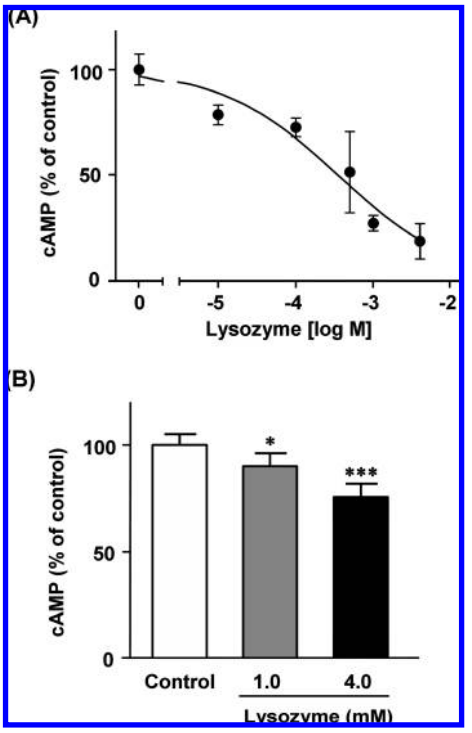

Figure 4. Lysozyme inhibits intracellular cAMP accumulation in the cells: (**A**) effects of concentrations of lysozyme on forskolin-induced cAMP accumulation. Hek293-T1R2/T1R3 cells were incubated with 0.5 mM IBMX and 50 μM forskolin in the presence of increasing concentrations of lysozyme (10 μM−4.0 mM). (**B**) Untransfected Hek293 cells were incubated with 0.5 mM IBMX and 50 μM forskolin in the absence and presence of 1.0 or 4.0 mM lysozyme. Each point represents the mean ± SD (n = 4). Significant differences from the control response were determined by one-way ANOVA with Dunnett's post hoc test; *, $P < 0.05$; ***, $P < 0.001$.

was significantly increased from 50 nM to 1.1 μM (*17*). When the Hek293-T1R2/T1R3 cells were stimulated by the R82A-thaumatin, no significant reduction of the intracellular cAMP level was observed at 100 μM (**Figure 3A**). Furthermore, the intracellular cAMP level of parental Hek293 cells was unchanged by 50 μM thaumatin (**Figure 3B**). Thus, thaumatin specifically stimulates the T1R2+T1R3 sweet-taste receptor and activates the signaling cascade. Lysozyme also inhibited the forskolin-induced intracellular cAMP accumulation in a concentration-dependent manner as well as thaumatin, and its EC_{50} value was 350 μM (**Figure 4A**). However lysozyme slightly inhibited the intracellular cAMP accumulation in the parental Hek293 cells through a T1R2+T1R3 receptor-independent mechanism (**Figure 4B**).

Effects of NaCl on the Sweetness of Sweet-Tasting Proteins. Although thaumatin and lysozyme activated the T1R2+T1R3 sweet receptor in a concentration-dependent manner, their EC_{50} values were much higher than the physiological threshold values of sweetness measured by the sensory analysis. Because the positively charged residues are important for thaumatin and lysozyme to elicit the sweetness response (*7*, *16*−*18*), an electrostatic interaction may contribute to the binding of the sweet-tasting protein to the sweet-taste receptor. This suggests that NaCl in the D-PBS buffer could affect the affinity of the thaumatin and lysozyme for the sweet-taste receptor. We examined whether NaCl suppresses the sweetness of the sweet-tasting proteins by sensory analysis in humans. The results of sweetness threshold values of sweeteners are summarized in **Table 1**. It is noted that the sweetness threshold value of aspartame was not affected by the presence of 140 mM NaCl, whereas those of sweet-tasting proteins thaumatin and lysozyme were considerably increased by 140 mM NaCl. The sweetness threshold of sucrose was not affected by NaCl (data not shown). Thus, NaCl specifically suppressed the sweetness of sweet-tasting protein, but it did not suppress the sweetness of low molecular mass sweeteners. In the presence of NaCl, the sweetness threshold values of thaumatin and lysozyme were comparable to the EC_{50} values determined by the in vitro cell-based assay.

Table 1. Sweetness Threshold Values Measured by Sensory Analysis

	threshold values[a]		
	control	+ NaCl (140 mM)	+ lactisole (1 mM)
aspartame	102.5 ± 31.5 (μM)	116.5 ± 39.6 (μM)	1750 ± 560*** (μM)
thaumatin	47.9 ± 18.1 (nM)	2.7 ± 0.9*** (μM)	285.7 ± 83.3*** (nM)
lysozyme	7.5 ± 2.3 (μM)	307.5 ± 49.5*** (μM)	76.7 ± 35.0*** (μM)

[a] Sweetness threshold value is evaluated with sensory analysis and expressed as mean ± SD. ***, $P < 0.001$ vs control values.

Effects of Lactisole on the Sweetness of the Sweet-Tasting Proteins. To clarify the suppressive effect of the sweetness inhibitor lactisole on sweet-tasting proteins, we examined the cell-based assay and the sensory analysis in humans. In the cell-based assay, lactisole itself led to an increased accumulation of intracellular cAMP in the Hek293-T1R2/T1R3 cells without sweeteners (**Figure 5**). Ca^{2+} imaging analysis using the heterologously expressed cells reported that lactisole reduced the intracellular Ca^{2+} concentration, contrary to sweeteners, and

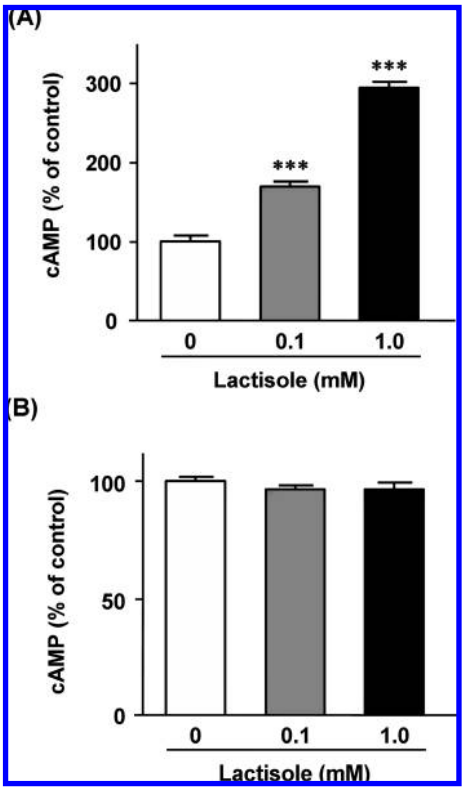

Figure 5. Lactisole increases intracellular cAMP accumulation in T1R2+T1R3 receptor-expressing cells: (**A**) Hek293-T1R2/T1R3 cells were incubated with 0.5 mM IBMX and 50 μM forskolin in the absence and presence of 0.1 or 1.0 mM lactisole. Control response was determined in the absence of lactisole. Significant differences from the control response were determined by one-way ANOVA with Dunnett's post hoc test; ***, $P < 0.001$. (**B**) Untransfected Hek293 cells were treated as described in (**A**). Each point represents the mean ± SD ($n = 4$).

functioned as an inverse agonist for the T1R2+T1R3 receptor (*21*). Thus, it is thought that lactisole itself increased the intracellular cAMP as an inverse agonist like a case of Ca^{2+} imaging analysis. In the presence of 0.1 and 1.0 mM lactisole, the response of the Hek293-T1R2/T1R3 cells to aspartame was significantly suppressed, and the EC_{50} values were increased to 451 μM and 1.8 mM, respectively (**Figure 6A**). Similarly, the response to thaumatin was also suppressed by lactisole, and EC_{50} values in the presence of 0.1 and 1.0 mM lactisole were 17.5 and 68.5 μM, respectively (**Figure 6B**). In the case of lysozyme, because a high concentration of lysozyme led to a slight reduction in the intracellular cAMP accumulation in a T1R2+T1R3 receptor-independent manner (**Figure 4B**), the lysozyme concentration was fixed at 500 μM and the lactisole concentration was changed. Lactisole suppressed the reduction of the intracellular cAMP accumulation induced by 500 μM lysozyme in a concentration-dependent manner (**Figure 6C**). These results indicate that lactisole suppressed the sweet-taste responses of the sweet-tasting proteins thaumatin and lysozyme as well as aspartame.

Next, sensory analysis in humans was performed to examine the suppressive effect of lactisole on the sweetness of sweet-tasting proteins. As shown in **Table 1**, in the presence of lactisole, the sweetness of aspartame was well-suppressed, as previously reported (*13*). Although it has been reported that lactisole did not affect the sweetness of thaumatin (*13*), we confirmed that the sweetness threshold values of both thaumatin and lysozyme were significantly increased by lactisole.

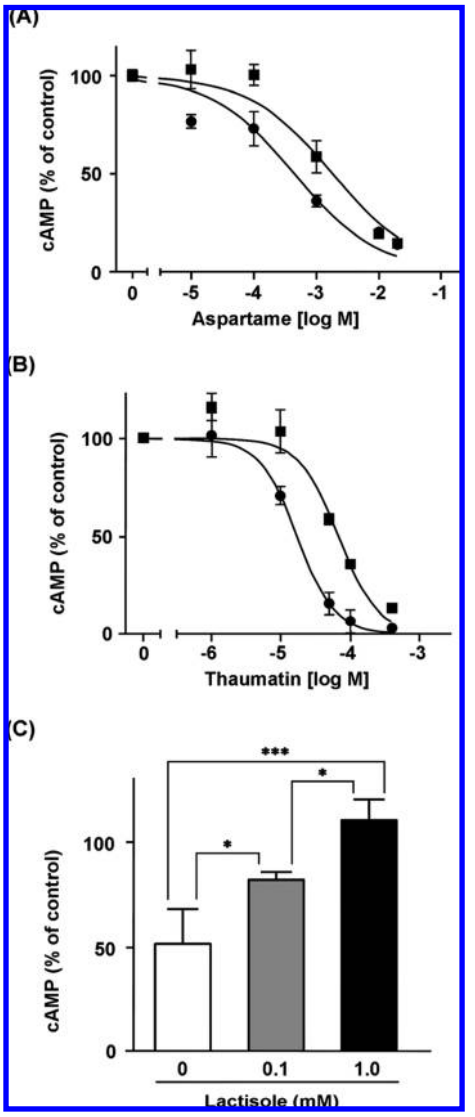

Figure 6. Lactisole suppresses sweetness responses in T1R2+T1R3 receptor-expressing cells: (**A**) concentration−response relationship of aspartame in the presence of 0.1 mM (●) or 1.0 mM (■) lactisole. Hek293-T1R2/T1R3 cells were incubated with 0.5 mM IBMX, 50 μM forskolin, and increasing concentrations of aspartame (10 μM−20 mM) in the presence of 0.1 or 1.0 mM lactisole. (**B**) Concentration−response relationship of thaumatin in the presence of 0.1 mM (●) or 1.0 mM (■) lactisole. Hek293-T1R2/T1R3 cells were incubated with 0.5 mM IBMX, 50 μM forskolin, and increasing concentrations of thaumatin (1−400 μM) in the presence of 0.1 or 1.0 mM lactisole. (**C**) Suppressive effect of lactisole on sweetness responses to 500 μM lysozyme. Hek293-T1R2/T1R3 cells were incubated with 0.5 mM IBMX, 50 μM forskolin, and 500 μM lysozyme in the presence of 0, 0.1, or 1.0 mM lactisole. Each control response was determined in the presence of 0, 0.1, or 1.0 mM lactisole without lysozyme, respectively. Statistical significance was determined by one-way ANOVA with Bonferroni's post hoc test; *, $P < 0.05$; ***, $P < 0.001$.

DISCUSSION

In this study, we cloned the T1R2 and T1R3 cDNAs from the mRNA of human tongue, and then we constructed Hek293 cells that stably expressed the human T1R2+T1R3 sweet-taste receptor. We monitored the levels of intracellular cAMP in one of these cell lines to investigate the interaction between sweet-tasting proteins and the T1R2+T1R3 sweet-taste receptor. The cAMP second messenger system has been proposed as an essential element for transduction cascades in response to sweet,

umami, and bitter stimuli (*22*). We observed that the sweet-tasting proteins thaumatin and lysozyme as well as aspartame decreased the intracellular cAMP levels in cells expressing the T1R2+T1R3 receptor. Furthermore, the sweetness inhibitor lactisole, which functions as an inverse agonist for the T1R2+T1R3 receptor, induced an increase in the intracellular cAMP accumulation of Hek293-T1R2/T1R3 cells in a concentration-dependent manner. It is assumed that the T1R2+T1R3 receptor might couple to the $G\alpha_{i/o}$ family rather than the $G\alpha_s$ family of G-proteins in Hek293 cells. In agreement with our results, in vitro experiments have shown that the T1R2+T1R3 sweet-taste receptor, the T1R1+T1R3 umami-taste receptor, and the T2Rs bitter-taste receptor all effectively trigger the $G\alpha_{i/o}$-signaling pathway in the heterologously expressed cells (*15*, *20*).

Six sweet-tasting proteins, thaumatin, monellin, brazzein, mabinlin, curculin (also termed neoculin), and lysozyme, elicit a sweet-taste response in humans, and these threshold values have a wide range from approximately 50 nM (thaumatin and monellin) to 7 μM (lysozyme) (*1*). Although it has been suggested that a single receptor (T1R2+T1R3) is involved in the perception of sweetness of sweet-tasting proteins as well as low molecular mass sweeteners, little is known of whether the responses of sweet-tasting proteins to the sweet-taste receptor reflect a difference in threshold values of sweet-tasting proteins determined by sensory analysis in humans. Furthermore, it has not been elucidated whether lysozyme, a low-affinity sweet-tasting protein, activates the T1R2+T1R3 sweet-taste receptor. In the present study, we confirmed that thaumatin and lysozyme activate the T1R2+T1R3 receptor in a concentration-dependent manner, and EC_{50} values reflect the sweetness threshold values determined by sensory analysis in the presence of 140 mM NaCl. This receptor activation by thaumatin was not observed with R82A-thaumtin. In a previous study, we demonstrated that most of the basic amino acid residues in a cleft region are required for thaumatin sweetness and that Arg82 is particularly important for the elicitation of the sweetness response to thaumatin by sensory analysis in humans (*17*). These results strongly suggest that the Arg82 residue itself or its neighboring residue(s) of the thaumatin molecule would bind to a critical site in the T1R2+T1R3 sweet-taste receptor with high affinity. Although lysozyme indeed activated the T1R2+T1R3 receptor in a concentration-dependent manner (**Figure 4A**), a high concentration of lysozyme inhibited cAMP accumulation through a T1R2+T1R3-independent mechanism (**Figure 4B**). This mechanism might be due to nonspecific binding to some GPCRs endogenously expressed in parental Hek293 cells and/or the direct activation of membrane-bound $G\alpha_{i/o}$ proteins. Naim et al. reported that $G\alpha_{i/o}$ proteins purified from bovine brain membrane were directly activated by some amphiphilic tastants including the sweet-tasting protein monellin (*23*). Furthermore, some studies indicated that the intact form of lysozyme was able to penetrate the membrane and was absorbed from the intestine in rats (*24*, *25*). These results lead to the hypothesis that a high concentration of lysozyme penetrates the cell membrane and directly activates $G\alpha_{i/o}$ proteins in taste bud cells. The T1R2+T1R3-independent mechanism might be contributing to the differences of the sweet-taste quality of thaumatin and lysozyme. Further studies are necessary to clarify this mechanism.

In agreement with our data, most in vitro experiments with heterologous expression cells indicate that low molecular mass sweeteners, such as aspartame and saccharin, activated the T1R2+T1R3 receptor at the physiologically relevant concentration, whereas in the case of sweet-tasting proteins, a considerably higher protein concentration than the physiological threshold was needed to activate the T1R2+T1R3 receptor (*10*−*12*, *15*). This low sensitivity of sweet-tasting proteins for the T1R2+T1R3 receptor expressed in culture cells would be due to the strong influence of NaCl on the interaction between sweet-tasting proteins and the sweet-taste receptor. We observed that NaCl significantly suppressed the sweetness of thaumatin and lysozyme by sensory analysis. In contrast to the sweet-tasting proteins, the sweetness threshold values of aspartame and sucrose were not affected by NaCl. The importance of charged residues on the surface of sweet-tasting proteins has been widely suggested. Temussi proposed the mechanism of interaction of sweet-tasting proteins to the T1R2+T1R3 receptor, which showed that thaumatin, monellin, and brazzein fit a large cavity of the receptor with the wedge-shaped surfaces of their structures (*26*). The in silico docking study suggested that charge complementation is responsible for efficient binding, because the cavity of the T1R2+T1R3 receptor is predominately negative and the interacting surfaces of sweet-tasting proteins are mainly positive (*26*−*28*). Indeed, mutagenesis studies of monellin (*29*), brazzein (*30*), curculin (*31*), lysozyme (*7*), and thaumatin (*17*) indicate that the electrostatic potential of the protein molecule is important for the elicitation of sweetness. Thus, some electrostatic interactions are important for the interaction of sweet-tasting proteins with the T1R2+T1R3 receptor, and NaCl inhibits this interaction. Our preliminary cell-based assay with NaCl substitutes including KCl, NH_4Cl, and *N*-methyl-D-glucamine chloride in the isotonic assay buffer suggests that the suppression of the sweetness of sweet-tasting proteins is not specific to NaCl and that the degree of suppression may be due to the ionic strength of the medium (unpublished observation).

Lactisole inhibits sweetness elicitation by many sweeteners for humans, whereas such an effect is not observed for rodents. Lactisole interacts with the transmembrane domain of human T1R3 that participates in both sweet taste and umami taste perception (*14*, *32*, *33*). Therefore, it has been demonstrated that lactisole inhibits umami taste as well as sweet taste (*32*, *34*). Schiffman et al. reported that lactisole at both 1.25 and 2.5 mM significantly suppressed the sweetness of several low molecular mass sweeteners by sensory analysis in humans (*13*). Meanwhile, the sweetness perception of thaumatin was lactisole-insensitive (*13*). In the present study, however, the results from both the in vitro cell-based assay and the in vivo sensory analysis clearly indicate that the sweetness of thaumatin is significantly suppressed by 1 mM lactisole. Lactisole also suppressed the sweetness of another sweet-tasting protein, lysozyme, showing that the perception of sweetness by sweet-tasting proteins is lactisole-sensitive. Thus, our results are inconsistent with previously reported results. This difference might be due to the individual variability of human subjects and/or experimental methodology. In the case of umami taste, 1 mM lactisole significantly suppresses the umami taste elicited by a mixture of monosodium glutamate (MSG) and inosine monophosphate (IMP) by sensory analysis (*32*). However, Galindo-Cuspinera et al. reported that lactisole has no inhibitory effect on mixtures of MSG with IMP, whereas lactisole inhibits the umami taste elicited by MSG alone (*34*). The sensitivity of lactisole for sweetness perception of sweet-tasting proteins and umaminess perception may be due to the variation by SNPs of a taste-receptor gene and/or taste-related gene. Further studies are needed to clarify the factor responsible for the diversity of lactisole sensitivity.

So far, the discrepancy between the results of in vivo and in vitro experiments has suggested that lactisole-insensitive sweet-taste receptors other than the T1R2+T1R3 receptor greatly participate in the sweetness perception of sweet-tasting proteins. In the present study, however, the results from in vivo and in vitro experiments show that the sweetness of sweet-tasting proteins is mainly perceived through the T1R2+T1R3 receptor.

Supporting Information Available: Sample concentrations used in sensory analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Masuda, T.; Kitabatake, N. Developments in biotechnological production of sweet proteins. *J. Biosci. Bioeng*. **2006**, *102*, 375–389.

(2) Ming, D.; Hellekant, G. Brazzein, a new high-potency thermostable sweet protein from *Pentadiplandra brazzeana* B. *FEBS Lett*. **1994**, *355*, 106–108.

(3) van der Wel, H.; Loeve, K. Isolation and characterization of thaumatin I and II, the sweet-tasting proteins from *Thaumatococcus daniellii* Benth. *Eur. J. Biochem*. **1972**, *31*, 221–225.

(4) Ide, N.; Kaneko, R.; Wada, R.; Mehta, A.; Tamaki, S.; Tsuruta, T.; Fujita, Y.; Masuda, T.; Kitabatake, N. Cloning of the thaumatin I cDNA and characterization of recombinant thaumatin I secreted by *Pichia pastoris*. *Biotechnol. Prog*. **2007**, *23*, 1023–1030.

(5) Maehashi, K.; Udaka, S. Sweetness of lysozymes. *Biosci., Biotechnol., Biochem*. **1998**, *62*, 605–606.

(6) Masuda, T.; Ueno, Y.; Kitabatake, N. Sweetness and enzymatic activity of lysozyme. *J. Agric. Food Chem*. **2001**, *49*, 4937–4941.

(7) Masuda, T.; Ide, N.; Kitabatake, N. Structure−sweetness relationship in egg white lysozyme: role of lysine and arginine residues on the elicitation of lysozyme sweetness. *Chem. Senses* **2005**, *30*, 667–681.

(8) Chandrashekar, J.; Hoon, M. A.; Ryba, N. J.; Zuker, C. S. The receptors and cells for mammalian taste. *Nature (London)* **2006**, *444*, 288–294.

(9) Nelson, G.; Hoon, M. A.; Chandrashekar, J.; Zhang, Y.; Ryba, N. J.; Zuker, C. S. Mammalian sweet taste receptors. *Cell* **2001**, *106*, 381–390.

(10) Li, X.; Staszewski, L.; Xu, H.; Durick, K.; Zoller, M.; Adler, E. Human receptors for sweet and umami taste. *Proc. Natl. Acad. Sci. U.S.A*. **2002**, *99*, 4692–4696.

(11) Jiang, P.; Ji, Q.; Liu, Z.; Snyder, L. A.; Benard, L. M.; Margolskee, R. F.; Max, M. The cysteine-rich region of T1R3 determines responses to intensely sweet proteins. *J. Biol. Chem*. **2004**, *279*, 45068–45075.

(12) Nakajima, K.; Asakura, T.; Oike, H.; Morita, Y.; Shimizu-Ibuka, A.; Misaka, T.; Sorimachi, H.; Arai, S.; Abe, K. Neoculin, a taste-modifying protein, is recognized by human sweet taste receptor. *Neuroreport* **2006**, *17*, 1241–1244.

(13) Schiffman, S. S.; Booth, B. J.; Sattely-Miller, E. A.; Graham, B. G.; Gibes, K. M. Selective inhibition of sweetness by the sodium salt of ±2-(4-methoxyphenoxy)propanoic acid. *Chem. Senses* **1999**, *24*, 439–447.

(14) Jiang, P.; Cui, M.; Zhao, B.; Liu, Z.; Snyder, L. A.; Benard, L. M.; Osman, R.; Margolskee, R. F.; Max, M. Lactisole interacts with the transmembrane domains of human T1R3 to inhibit sweet taste *J. Biol. Chem*. **2005**, *280*, 15238–15246.

(15) Ozeck, M.; Brust, P.; Xu, H.; Servant, G. Receptors for bitter, sweet and umami taste couple to inhibitory G protein signaling pathways. *Eur. J. Pharmacol*. **2004**, *489*, 139–149.

(16) Kaneko, R.; Kitabatake, N. Structure−sweetness relationship study of sweet protein thaumatin: Importance of lysine residues. *Chem. Senses* **2001**, *26*, 167–177.

(17) Ohta, K.; Masuda, T.; Ide, N.; Kitabatake, N. Critical molecular regions for elicitation of the sweetness of the sweet-tasting protein, thaumatin I. *FEBS J*. **2008**, *275*, 3644–3652.

(18) Masuda, T.; Ide, N.; Kitabatake, N. Effects of chemical modification of lysine residues on the sweetness of lysozyme. *Chem. Senses* **2005**, *30*, 253–264.

(19) Kim, U. K.; Wooding, S.; Riaz, N.; Jorde, L. B.; Drayna, D. Variation in the human TAS1R taste receptor genes. *Chem. Senses* **2006**, *31*, 599–611.

(20) Sainz, E.; Cavenagh, M. M.; LopezJimenez, N. D.; Gutierrez, J. C.; Battey, J. F.; Northup, J. K.; Sullivan, S. L. The G-protein coupling properties of the human sweet and amino acid taste receptors *Dev. Neurobiol*. **2007**, *67*, 948–959.

(21) Galindo-Cuspinera, V.; Winnig, M.; Bufe, B.; Meyerhof, W.; Breslin P. A. A TAS1R receptor-based explanation of sweet 'water-taste'. *Nature (London)* **2006**, *441*, 354–357.

(22) Roper, S. D. Signal transduction and information processing in mammalian taste buds. *Pfluegers Arch*. **2007**, *454*, 759–776.

(23) Naim, M.; Seifert, R.; Nürnberg, B.; Grünbaum, L.; Schultz, G. Some taste substances are direct activators of G-proteins. *Biochem. J*. **1994**, *297*, 451–454.

(24) Nishikawa, M.; Hasegawa, S.; Yamashita, F.; Takakura, Y. Hashida, M. Electrical charge on protein regulates its absorption from the rat small intestine. *Am. J. Physiol.: Gastrointest. Liver Physiol*. **2002**, *282*, G711–G719.

(25) Takano, M.; Koyama, Y.; Nishikawa, H.; Murakami, T.; Yumoto, R. Segment-selective absorption of lysozyme in the intestine. *Eur. J. Pharmacol*. **2004**, *502*, 149–155.

(26) Temussi, P. A. Why are sweet proteins sweet? Interaction of brazzein, monellin and thaumatin with the T1R2-T1R3 receptor. *FEBS Lett*. **2002**, *526*, 1–4.

(27) Morini, G.; Bassoli, A.; Temussi, P. A. From small sweeteners to sweet proteins: anatomy of the binding sites of the human T1R2_T1R3 receptor. *J. Med. Chem*. **2005**, *48*, 5520–5529.

(28) Walters, D. E.; Hellekant, G. Interactions of the sweet protein brazzein with the sweet taste receptor. *J. Agric. Food Chem*. **2006**, *54*, 10129–10133.

(29) Esposito, V.; Gallucci, R.; Picone, D.; Saviano, G.; Tancredi, T.; Temussi, P. A. The importance of electrostatic potential in the interaction of sweet proteins with the sweet taste receptor. *J. Mol. Biol*. **2006**, *360*, 448–456.

(30) Jin, Z.; Danilova, V.; Assadi-Porter, F. M.; Aceti, D. J.; Markley J. L.; Hellekant, G. Critical regions for the sweetness of brazzein. *FEBS Lett*. **2003**, *544*, 33–37.

(31) Kurimoto, E.; Suzuki, M.; Amemiya, E.; Yamaguchi, Y.; Nirasawa, S.; Shimba, N.; Xu, N.; Kashiwagi, T.; Kawai, M.; Suzuki, E.; Kato, K. Curculin exhibits sweet-tasting and taste-modifying activities through its distinct molecular surfaces. *J. Biol. Chem*. **2007**, *282*, 33252–33256.

(32) Xu, H.; Staszewski, L.; Tang, H.; Adler, E.; Zoller, M.; Li, X. Different functional roles of T1R subunits in the heteromeric taste receptors. *Proc. Natl. Acad. Sci. U.S.A*. **2004**, *101*, 14258–14263.

(33) Winnig, M.; Bufe, B.; Meyerhof, W. Valine 738 and lysine 735 in the fifth transmembrane domain of rTas1r3 mediate insensitivity towards lactisole of the rat sweet taste receptor. *BMC Neurosci*. **2005**, *6*, 22.

(34) Galindo-Cuspinera, V.; Breslin, P. A. The liaison of sweet and savory. *Chem. Senses* **2006**, *31*, 221–225.

Received for Review December 20, 2008. Revised manuscript received April 5, 2009. Accepted April 10, 2009. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (T.M. and N.K.). The equipment used in this research was supported, in part, through a cooperative agreement with the Agricultural Research Service of the U. S. Department of Agriculture, Project 1935-42000-035.