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# Introduction of a negative charge at Arg82 in thaumatin abolished responses to human T1R2–T1R3 sweet receptors

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ABSTRACT

Thaumatin, an intensely sweet-tasting protein, elicits a sweet-taste sensation at a level as low as 50 nM. Although previous sensory analyses have suggested that Lys67 and Arg82 are important to the sweetness of thaumatin, the exact effects of each residue on sweet receptors are still unknown. In the present study, various mutants of thaumatin altered at Arg82 as well as Lys67 were prepared and their sweetness levels were quantitatively evaluated by cell-based assays using HEK293 cells expressing human sweet receptors. Mutations at Arg82 had a more deteriorative effect on sweetness than mutations at Lys67. Particularly, a charge inversion at Arg82 (R82E) resulted in an abolishment of the response to sweet receptors even at a concentration as high as 1 mM. These results indicate that Arg82 plays a central role in determining the sweetness of thaumatin. A strict spatial charge location at residue 82 appears to be required for interaction with sweet receptors.

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## 1. Introduction

There are a wide variety of sweeteners including natural sugars, sugar alcohols, and certain amino acids [1]. Besides them, six proteins, thaumatin [2], monellin [3,4], brazzein [5], mabinlin [6], neoculin (curculin) [7,8] and hen egg lysozyme [9,10], have been identified to elicit a sweet-taste. Threshold values of sweetness for sweet-tasting proteins range from 50 nM to 7 μM, being lower than that of sucrose which elicits a sweet-taste at more than 10 mM [11]. Thaumatin is perhaps the most promising candidate for a low-calorie sugar substitute since it has a threshold value of 50 nM. We expect, therefore, unique structural characteristics of thaumatin to be involved in the interaction with sweet receptors.

Our previous study using human sensory analysis demonstrated that lysine residues located on the cleft-containing side are involved in sweetness [12]. More recently, we have shown that several basic amino acid residues on the cleft-containing side are required for sweetness, particularly Lys67 and Arg82 [13]. As the mutants K67A and R82A exhibited a reduction in sweetness by approximately 20-fold [13], Lys67 and Arg82 might bind strongly to sweet receptors, but the importance of the charge or size of the side chains of these two residues have not been fully investigated. Furthermore, it remains unclear whether the results of human sensory analyses correlate with responses of thaumatin mutants to sweet receptors.

T1Rs are a family of class C G-protein-coupled receptors and heterodimers comprising the T1R2 and T1R3 subunits function as sweet receptors [14–18]. Heterologous expression studies have revealed that all class of sweeteners including sugars, artificial sweeteners, amino acids, and sweet-tasting proteins are perceived by the T1R2–T1R3 heterodimer.

In the present study, to clarify the roles of Lys67 and Arg82 in the interaction with sweet receptors, various mutants of thaumatin were prepared with the *Pichia* expression system [19,20]. The sweetness of each mutant was evaluated by a cell-based assay. Since the introduction of a negative charge at position 82 abolished the response to sweet receptors, Arg82 plays a central role in the sweetness and charge at this position appears critical to the interaction with sweet receptors.

## 2. Materials and methods

### 2.1. Materials

Saccharin and sucralose were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Probenecid was purchased from Sigma–Aldrich Inc. (St. Louis, MO). Thaumatin I was purified from

*Abbreviations:* HEK293, human embryonic kidney 293; CD, circular dichroism; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HBSS, Hank's balanced salt solution; $EC_{50}$, 50% effective concentration.

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crude thaumatin powder (Wako Pure Chemical Industries Ltd.) as described previously [12].

### 2.2. Preparation of thaumatin mutants

Site-directed mutagenesis was performed as described previously [13]. The expression of mutant thaumatin proteins was achieved using a *Pichia pastoris* expression system [13,20]. The culture medium supernatant was dialyzed against 5 mM sodium phosphate buffer, pH 7.0, containing 0.1 mM EDTA at 4 °C. Subsequently the dialysate was purified by SP Sephadex C-25 ion-exchange chromatography (Amersham Biosciences, Piscataway, NJ) followed by gel filtration chromatography with a Toyopearl HW-50F column (Tosoh, Tokyo, Japan). The purity of mutant thaumatin proteins was confirmed by SDS–PAGE and native–PAGE.

### 2.3. Fluorescence spectra and CD spectra

Fluorescence spectra of plant thaumatin and its mutants were recorded in 5 mM sodium phosphate buffer, pH 7.0, at 25 °C with an F-3000 fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan) at wavelengths of 280 nm excitation and 300–450 nm emission. The concentration of each protein was adjusted to 5.7 μM.

Far-UV CD spectra were measured with a J-720 spectropolarimeter (Jasco, Tokyo, Japan) at 20 °C at wavelengths from 250 to 200 nm. The protein solutions were prepared at a concentration of 22.5 μM in 5 mM sodium phosphate buffer, pH 7.0.

### 2.4. Functional expression of human sweet receptors

Human T1R2 cDNA and T1R3 cDNA were cloned as reported [21,22]. HEK293 cells stably expressing $G\alpha_{16gust44}$ were prepared by established procedures [23]. Human T1R2- and T1R3-containing plasmids were transiently transfected into $G\alpha_{16gust44}$-expressing HEK293 cells as described previously [22]. At 6 h after transfection, the DMEM was replaced with low-glucose DMEM supplemented with GlutaMAX and 10% FBS (Invitrogen). After 18 h, $1.5 \times 10^5$ cells were seeded onto each well in polylysine-coated 96-well culture plates (BD Biosciences, Bedford, MA) and incubated a further 24 h. They were then loaded with 50 μL of 3 μM Fluo-8 AM (ABD Bioquest Inc., Sunnyvale, CA) in HBSS containing 20 mM Hepes, pH 7.4 and 1.25 mM probenecid for 30 min at 37 °C. Stimulation was performed by addition of 50 μL of agonist solution dissolved in 20 mM Hepes-HBSS, pH 7.4. Calcium mobilization was detected by fluorescence (excitation at 495 nm and emission at 514 nm) using an Infinite F200 fluorescence reader (Tecan Group Ltd., Männedorf, Switzerland). Calcium mobilization was expressed as the change in peak fluorescence ($\Delta F$) over the base-line level ($F$) from at least three independent experiments. Data were presented as $\Delta F/F$ and plotted using Origin 8 (OriginLab, Northhampton, MA). The 50% effective concentration ($EC_{50}$) for ligand-receptor interactions was also determined and represented as the mean ± SE of value from the dose–response analysis.

### 2.5. Human sensory analysis

The human sensory analysis was performed by means of a triangle test as described previously [24]. Seven subjects aged 22–38 yrs participated, having first given their informed consent. The test protocol was approved by the Graduate School of Agriculture, Kyoto University. Briefly, 5 mL of test solution prepared in a 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. The threshold value of sweetness was evaluated on a scale of 0–5. A score of '0', '1' and '2' meant no taste, some taste, and sweetness, respectively. As the sweetness increased, scores of '3–5' were given. The mean concentration of a protein sample which obtained a score of '2' was defined as the sweetness threshold value. Student's $t$-test was used for calculating $p$-values. A $p$-value < 0.01 was considered significant.

## 3. Results

### 3.1. Characterization of thaumatin mutants

SDS–PAGE and native–PAGE of the purified mutant proteins gave a single band, suggesting that no contaminants were included in the fractions (data not shown). The structure of the plant thaumatin and its mutants was investigated using fluorescence spectra and CD spectra (Fig. 1). The fluorescence spectra of the mutants were identical to that of plant thaumatin but different from that of plant thaumatin denatured by 6 M urea, suggesting the environment around the tryptophan residues of thaumatin to be generally unchanged by mutation (Fig. 1A). In addition, the CD spectra of the mutants also overlapped with that of plant thaumatin, indicating no substantial change in secondary structure (Fig. 1B).

### 3.2. Determination of $EC_{50}$ values of thaumatin mutants for sweet receptors

Transfectants responded to saccharin and sucralose in a dose-dependent manner (Fig. 2A) as reported previously [16,21,22,25],

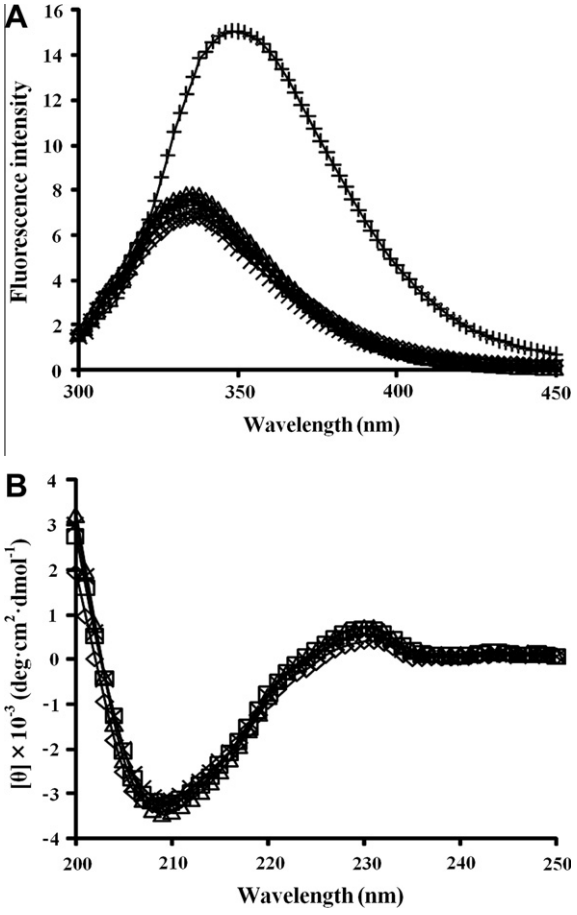


**Fig. 1.** Structural analysis of thaumatin mutants. (A) Fluorescence spectra were recorded in 5 mM sodium phosphate buffer, pH 7.0. Plant thaumatin (□), plant thaumatin denatured by 6 M urea (+), R82Q (×), R82E (Δ), and K67E (◇). (B) Far-UV CD spectra. Plant thaumatin (□), R82Q (×), R82E (Δ), and K67E (◇). The conditions are described in Section 2.

indicating that functional sweet receptors were successfully expressed on HEK293 cells. By using this cell-based assay, we investigated the effects of mutation at Lys67 and Arg82. To precisely evaluate the role of each residue in the interaction with sweet receptors, the dose–response relationship of each mutant was examined (Fig. 2). To date, few investigations have objectively determined $EC_{50}$ values of both sweet-tasting proteins and mutants thereof, except for neoculin [26,27]. Given that the taste-modifying effect and pH-sensitivity of neoculin have been successfully assessed by a comparative analysis of $EC_{50}$ values among neoculin mutants, we reevaluated the contribution of Lys67 and Arg82 in thaumatin to the interaction with sweet receptors by precisely estimating $EC_{50}$ values of thaumatin mutants.

First, in order to estimate the role of Lys67 in thaumatin, dose–response curves for mutants altered at residue 67 were investigated by the cell-based assay. As shown in Fig. 2B, the substitution of lysine with alanine (K67A) resulted in a rightward shift of the dose–response curve, suggesting a reduction in the sweetness level. The $EC_{50}$ value of K67A is 193 μM, approximately 18-fold that of plant thaumatin (10.8 μM) (Table 1). A charge inversion substitution at Lys67 (K67E) resulted in an increase in the $EC_{50}$ to 334 μM, about 31-fold that of plant thaumatin. When compared to K67A, a slight increase in the $EC_{50}$ value was observed (1.7-fold).

We next examined the effect of mutations at residue 82. As shown in Fig. 2C, the dose–response curve of R82A shifted to the right, and the $EC_{50}$ value was 244 μM, 23-fold that of plant thaumatin. In contrast, no cellular response to the charge inversion mutant R82E was observed even at 1 mM, giving an $EC_{50}$ value above 1 mM (Fig. 2C, Table 1). These results suggest that mutations at Arg82 have a greater effect on the interaction with sweet receptors than do mutations at Lys67.

Since the charge inversion mutant of R82E provoked no response to sweet receptors, to further clarify the effect of charge at position 82 of thaumatin, an arginine to glutamine substitution (R82Q) was investigated. The $EC_{50}$ value of R82Q was 298 μM, similar to that of R82A, but distinct from that of R82E (Fig. 2C and Table 1). These results suggest that a strict spatial charge location at residue 82 to be required for interaction with sweet receptors.

Although it had previously been suggested by sensory analysis that substituting arginine for lysine at position 82 (R82K) resulted in a slight increase (5.3-fold) in the threshold value of sweetness (Table 1), we reexamined the effect of the mutation using the cell-based assay. The $EC_{50}$ value of R82K was 54.1 μM, approximately 5-fold that of plant thaumatin (Fig. 2C). These results indicate that the ratios of $EC_{50}$ values of thaumatin mutants against plant thaumatin well correlated with those of threshold values for plant thaumatin calculated by human sensory analysis (Table 1).

### 3.3. Human sensory analysis

Although sweet-tasting proteins activate sweet receptors, their $EC_{50}$ values were much higher than the threshold values of sweetness determined by sensory analysis. This suppression seems attributable to differences in conditions, particularly in the concentration of NaCl, between the cell-based assay and sensory analysis. Approximately 150 mM of NaCl is included in HBSS buffer for cell-based assays, whereas the sensory analysis was done in 5 mM sodium phosphate buffer. Since the positively charged residues in thaumatin are important for sweetness, electrostatic interaction may contribute to the binding between sweet-tasting proteins and sweet receptors. It has been shown that the threshold value of thaumatin in sensory analyses increases to about 2.7 μM in the presence of 140 mM NaCl which is similar to the $EC_{50}$ value obtained from cell-based assays [21]. Given that a human sensory analysis is sensitive to 5 mM sodium phosphate buffer, we could determine the threshold value of R82E whose $EC_{50}$ value could not be determined in the cell-based assay. The threshold value for R82E was 8800 nM, approximately 200-fold that of plant thaumatin (Table 1). The threshold value of R82Q was about 24-fold that of plant thaumatin but significantly lower than that of R82E, suggesting the charge at position 82 to be important for sweetness (Table 1). In contrast, the threshold value for K67E was 1500 nM, 33-fold that of plant thaumatin and only 1.7-fold that of K67A, suggesting that residue 67 is not as restricted as residue 82.

## 4. Discussion

In the present study, we clarified the effects of mutations at Lys67 and Arg82 on the interaction with human sweet receptors using HEK293 cells.

The $EC_{50}$ values of K67A and K67E were 193 μM and 334 μM, approximately 18-fold and 31-fold that of plant thaumatin (10.8 μM) (Table 1), respectively, suggesting that a positively charged environment at residue 67 is involved in the interaction with sweet receptors. In agreement with the results of cell-based assays, the threshold value of K67A and K67E was also increased 19.3-fold and 33.3-fold, respectively. When the $EC_{50}$ values of the thaumatin mutants were plotted against the sweetness threshold values determined by the sensory analysis (Fig. 3), there was a quite a good correlation between the two. The equation of the regression line was $y = 22.1 + 4.08x$ and the correlation coefficient was 0.97. The cell-based assay conducted in this study enabled us to reliably and quantitatively estimate the sweetness of thaumatin mutants ranging from 10 μM to 330 μM. Extrapolation of the regression line gives an $EC_{50}$ value for R82E of 2 mM, as reflected by the finding that the charge inversion mutant, R82E, could not evoke an apparent response to sweet receptors even at 1 mM.

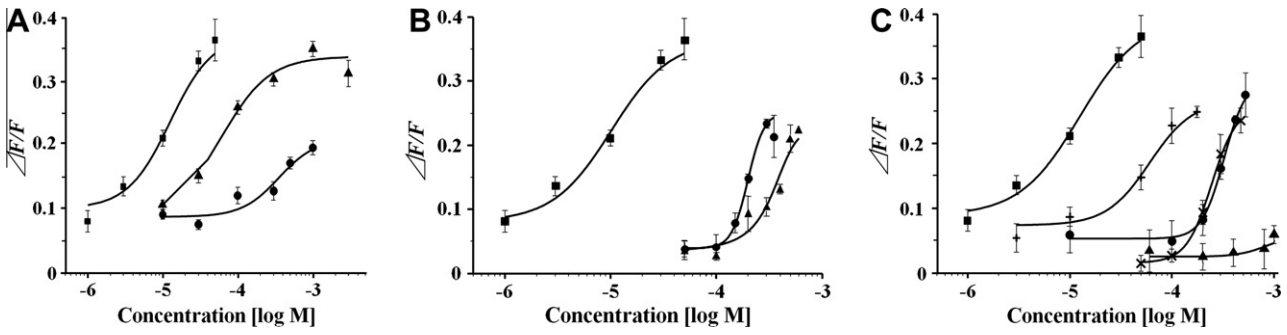


**Fig. 2.** Cell-based assays of various sweeteners. (A) The dose–response analysis of saccharin (●), sucralose (▲), and thaumatin (■). (B) The dose–response analysis of thaumatin (■) and Lys67 mutants, K67A (●) and K67E (▲). (C) The dose–response analysis of thaumatin (■) and Arg82 mutants, R82A (●), R82K (+), R82Q (×), and R82E (▲). The dose response analyses in (A), (B), and (C) were performed at least three times independently.

**Table 1**
$EC_{50}$ values and sweetness threshold values of 1 thaumatin mutants.

| Thaumatin type | $EC_{50}$ (μM) | Ratio (mutants/plant) | Sweetness threshold (nM) | Ratio (mutants/plant) |
|---|---|---|---|---|
| Plant thaumatin | 10.8 ± 1.7 | | 45 ± 9 | |
| K67A | 193 ± 3.0 | 17.9 | 870 ± 92**a | 19.3 |
| K67E | 334 ± 53 | 30.9 | 1500 ± 61** | 33.3 |
| R82A | 244 ± 11 | 22.6 | 1100 ± 58**a | 24.4 |
| R82K | 54.1 ± 8.6 | 5.0 | 240 ± 36*a | 5.3 |
| R82Q | 298 ± 62 | 27.6 | 1010 ± 135** | 22.4 |
| R82E | >1000 | >92.6 | 8800 ± 1600** | 195.6 |

* $p < 0.001$.
** $p < 0.0001$ compared to plant thaumatin.
[a] The sweetness threshold values of K67A, R82A, and R82K were determined in a previous study [13].

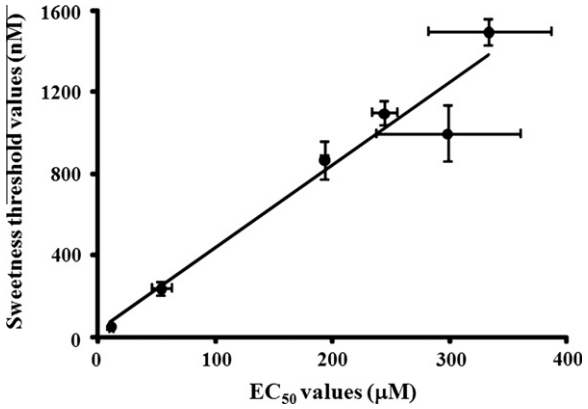


**Fig. 3.** Comparison of $EC_{50}$ values with sweetness threshold values. The linear regression line is shown and its equation is $y = 22.1 + 4.08x$, where $y$ = sweetness threshold values, and $x$ = $EC_{50}$ values. The correlation coefficient is 0.97.

Since sweet-tasting proteins have a high and wide-ranging affinity, from 50 nM (thaumatin and monellin) to 7 μM (lysozyme), they could be useful tools for unveiling how the ligand-binding site of sweet receptors confers a broad and/or specific receptive range.

In lysozyme, the critical residues for sweetness are gathered together in a given area, and no significant change in sweetness was induced by changing lysine to arginine [28]. In contrast, the $EC_{50}$ value of R82K thaumatin is slightly higher than that of plant thaumatin, suggesting that the guanidino group in the arginine residue prefers the ε-amino group in the lysine residue at position 82. These results also indicated that the charge at residue 82 to be important for the interaction with sweet receptors and that mutations at Arg82 have a greater effect on sweetness than mutations at Lys67.

Several examples have been reported in which the introduction of a negative charge leads to a loss of sweetness in protein molecules. For monellin, the mutants M42E, Y63E, and Y65E exhibited a reduction in sweetness of 20- to 100-fold [29], and R72E and R86E, a decrease of 25-fold and approximately two orders of magnitude, 100-fold, respectively [30]. In brazzein, sweetness also decreased by 15-fold or more for the mutants R33A, R43A, and R43E [31–33,25]. However, the introduction of a negative charge at position 82 in thaumatin caused the most serious deteriorative effect on sweetness among sweet-tasting proteins.

It has been suggested that sweet-tasting proteins fit into a large wedge-shaped cavity of sweet receptors [34,35]. Recently, the human sweet receptor subunit T1R3 was implicated in the interaction with sweet-tasting proteins [22,25,36,37]. The extracellular cysteine-rich domain (CRD) of human T1R3 might be required for interaction with thaumatin, however, also CRD seems to play a major role in the conformational change from the ligand-binding domain to the transmembrane domain [38]. It remains to be clarified whether the CRD in T1R3 is directly involved in the interaction with thaumatin and its mutants. Recent high-resolution structural analyses of thaumatin have revealed that the side chain of the residue at 82 has two conformations, suggesting the flexibility and fluctuation of the side chain to be suitable for interaction with sweet receptors [39,40]. We are currently performing an X-ray crystal structural analysis of thaumatin mutants to better clarify the structural requirement for sweetness in thaumatin.

In conclusion, we found mutations at Arg82 to have a greater effect on sweetness than mutations at Lys67. Arg82 in thaumatin plays a central role in determining the sweetness of thaumatin and the charge at Arg82 appears to be very important to the interaction with sweet receptors. Insights into the molecular mechanism concerning residue 82 of thaumatin may help in understanding the interaction with sweet receptors.

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