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Abstract

Thaumatin, a sweet-tasting plant protein, elicits a sweet sensation at 50 nM in humans but not rodents. Although it was shown that the cysteine-rich domain (CRD) of human T1R3 (hT1R3) is important for the response to thaumatin, the amino acid residues within CRD critical for response are still unknown. A comparison of the amino acid sequence (69 amino acid residues) of CRD between hT1R3 and mouse T1R3 (mT1R3) revealed sixteen amino acids that differ.

In the present study, we converted each of these sixteen amino acids in hT1R3 to their mouse counterpart and examined the response to thaumatin and sucralose using a cell-based assay. No significant decrease in the response to sucralose was seen among any of the sixteen mutants. However, five mutants (Q504K, A537T, R556P, S559P, and R560K) exhibited a significantly diminished response to thaumatin. The five critical residues involved in the response to thaumatin were dispersed in the CRD of hT1R3 and widely distributed when compared to brazzein.

The unique intense sweet-taste of thaumatin might be attributed to the different receptor activation mechanism compared to the small molecule sweetener sucralose.

Keywords: Thaumatin; Sweet-tasting protein; Sweet receptor; Cysteine-rich domain
1. Introduction

Thaumatin is one of the sweetest proteins known and used as a low-calorie sugar substitute as well as for medical purposes for lifestyle-related diseases. Thaumatin is 100,000-fold sweeter than sucrose on a molar basis and this intense sweetness makes thaumatin useful for unveiling the interaction between sweeteners and sweet receptors. As sweet-tasting proteins are too large to fit the cavity of the interaction sites for small sweeteners, the activation of sweet receptors by sweet-tasting proteins seems to occur in a different manner compared to other small sweeteners [1-3]. Previous mutational studies of thaumatin suggested that K67 and R82 are important to the sweetness of thaumatin, and mutations at R82 had a more deteriorative effect on sweetness than mutations at K67 [4].

The heterodimers comprising the subunits T1R2 and T1R3, which belong to a family of class C G-protein-coupled receptors, are known to function as sweet receptors [5-8]. Each subunit of sweet receptor possesses a large N-terminal domain (NTD) and a cysteine-rich domain (CRD), and followed by a seven-helix transmembrane domain (TMD). The CRD links the NTD and TMD. Previous studies have shown that sweet-tasting proteins as well as aspartame can be perceived by humans, apes, and Old
World monkeys but not New World monkeys and rodents [9, 10]. Species differences in the response to sweeteners would provide valuable information on the molecular mechanism by which sweet receptors function as well as aid the identification of interaction sites in receptors [10-14]. Recently, we have shown that the CRD within hT1R3 is important for the response toward thaumatin [14]. However, it remains unclear whether two sweet-tasting proteins, thaumatin and brazzein, interact with the same amino acid residues in the CRD of human sweet receptors.

In the present study, to clarify the amino acid residues within the CRD of hT1R3 critical for thaumatin reception and to clarify the mechanisms by which thaumatin activates sweet receptors, we performed site-directed mutagenesis in the CRD of hT1R3 relative to the CRD of mT1R3. The findings should help clarify the activation mechanisms of proteinous sweeteners and might lead to the design of new sweeteners.

2. Materials and methods

2.1. Materials

Thaumatin I was purified from crude thaumatin powder as described previously [15]. Sucralose were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan).
2.2. Site-directed mutagenesis of the CRD of hT1R3

The plasmid pcDNA3.3-hT1R3 was used as the template for mutagenesis [14]. Site-directed mutagenesis was performed using a KOD Plus DNA polymerase (Toyobo Co. Ltd., Osaka, Japan) with two synthetic complementary to opposite strands of oligonucleotide primers containing the desired mutation (Operon Biotechnologies, Tokyo, Japan, Supplement Table 1). The desired mutations were confirmed by DNA sequencing.

2.3. Functional expression of human sweet receptors

Human T1R2- and T1R3- or T1R3 mutant-containing plasmids were transiently transfected into HEK293 cells stably expressing the chimeric G-protein, Gα16gust44 as described previously [14]. After the transfection, cells were seeded onto polylysine-coated 96-well culture plates (1.5 × 10^5 cells/well) (BD Biosciences, Bedford, MA) and incubated for 24 h. They were then loaded with 50 μL of 3 μM fluo-8 AM (ABD Bioquest Inc., Sunnyvale, CA) in Hank’s balanced salt solution (HBSS) containing 20 mM Hepes and 1.25 mM probenecid for 30 min at 37 °C. The cells were incubated with 180 μL of 20 mM Hepes-HBSS containing 0.625 mM probenecid for 10 min at 37 °C. Stimulation was performed by adding 20
μL of agonist solution dissolved in 20 mM Hepes-HBSS. The response to
sucralose (1mM) or thaumatin (50 μM) was detected by measuring
fluorescence (excitation at 495 nm and emission at 514 nm) using an
Infinite F200 (Tecan Group Ltd., Männedorf, Switzerland) as described
previously [14]. The response of hT1R2- wild-type hT1R3 (no mutation)
was defined as 100% and the response of each hT1R3 mutant was
compared to that of hT1R2- wild-type hT1R3.

3. Results and Discussion

Since thaumatin elicits an intense sweet taste compared to other
artificial sweeteners, identifying the amino acid residues required for the
response to thaumatin and the mechanisms by which receptors are activated
by thaumatin would shed light on how to design new intense sweeteners.
Thaumatin is a basic protein (isoelectric point =12) [16], and we reported
previously the importance of the basicity for its sweetness [4, 17]. These
positively-charged residues might affect some charged residues within the
CRD of hT1R3. First, three mutants (Q504K, E525K, and Q531K) were
prepared to examine the response to thaumatin as well as sucralose. No
significant decrease in the response to sucralose was observed among the
three mutants, whereas the Q504K mutant showed a significantly diminished response to thaumatin (Fig. 1). Q504 is located in the N-terminal region in the CRD of hT1R3 and is an essential determinant of the specific response to thaumatin (Fig. 2). Since the N-terminal region of the CRD was found to be important for the response to thaumatin, the effect of the mutation E505D which is adjacent to Q504 was investigated. No significant decrease in the response to either sucralose or thaumatin was observed. These results suggested that only the glutamine residue at 504 is essential to the response to thaumatin. Besides the Q504K mutant, a relative decrease in the response to thaumatin was seen for the E525K mutant (Fig. 1B). These results suggest that strict amino acid positions as well as acidic and/or polar residues are important for the response to thaumatin. A relative increase in the response to thaumatin was seen for the Q531K mutant, and the N532H mutation had no effect on responses to thaumatin and sucralose (Fig. 1). The requirement of the N-terminal region of CRD in hT1R3 for the response to thaumatin may be a novel target for the activation of receptors by signal transduction.

Brazzein is a small (6.5 kDa) sweet-tasting protein [18]. Two amino acid residues, A537 and F540, located in the middle of CRD are important for the response to brazzein (Fig. 2A) [11]. Next we examined the effect of
mutating A537 and F540 on the response to thaumatin. As shown in Fig. 1B, the A537T mutant had a significantly reduced response to thaumatin. Although a relative decrease in the response to thaumatin as well as sucralose was seen in the F540P mutant, the F540P mutant did not significantly affect the response to thaumatin or sucralose in comparison to the A537T mutant (Fig. 1). These results suggested F540 to be involved in the response to brazzein but not thaumatin. The responses of the mutants I536F, A537T, F540P, G542N, and E545Q to brazzein and small sweeteners such as sucrose were previously examined [11]. Our results showed responses of the I536F, G542N, and E545Q to thaumatin and sucralose to be reduced in comparison with those of wild-type hT1R3 but not significantly and were similar to those of Jiang et al [11].

We next converted each of the remaining six amino acids of hT1R3 located in the C-terminal region of the CRD in hT1R3 to their mouse counterpart and examined the response to thaumatin and sucralose. All six mutants (R550K, R553A, F555L, R556P, S559P, and R560K) responded to sucralose, however, three mutants (R556P, S559P, and R560K) showed diminished responses to thaumatin and were located near the transmembrane domain (Fig. 1B, 2B). These mutants might not be directly involved in the interaction with the large sweet protein, thaumatin, because
of steric hindrance between receptors and large protein-ligands.

Interestingly, R556 and S559 resulted in a loss of response with substitution to a proline residue. Previous observations by Jiang et al indicated that the mutation A537P resulted in unresponsive to most sweeteners [11]. They suggested that a change in backbone flexibility might alter the formation of the predicted β-strand and thereby alter the conformation of this region in a way that makes it less able to transmit the signal through the receptor. Our observation is distinct in that the loss of response was only to thaumatin, not to sucralose. Taken together, the substitution of proline residues in the C-terminal region of the CRD in hT1R3 seems to be critical for the response to thaumatin.

Although the three-dimensional structure of sweet receptors has not yet been determined, homology modeling using a metabotropic glutamate receptor as a template has provided various models for the interaction between sweet receptors and sweet-tasting proteins [1-3]. In the wedge model, sweet-tasting proteins fit into a large wedge-shaped cavity and activate sweet receptors by binding to an external site, thus stabilizing an active form of the receptors in the absence of small sweeteners [3]. Recently, the CRD of hT1R3 was implicated in the response to sweet-tasting proteins [8, 11, 14]. However, it was suggested that the CRD
plays a major role in the conformational change from the ligand-binding
domain to the transmembrane domain [19]. Dose response curves for the
five mutations to thaumatin were further investigated (Supplement Fig. 1).
These results showed that no significant increases of responses were
detected for five mutations up to 100 µM, suggesting that five residues in
the CRD of hT1R3 were involved in the response to thaumatin. Recent
high-resolution structural analyses of thaumatin have revealed the
flexibility and fluctuation of the side chains of critical residues to be
suitable for interaction with sweet receptors [15, 20]. Sweet-tasting
proteins would be useful for unveiling how the ligand-binding site of
sweet receptors confers a broad and/or specific receptive range.

In conclusion, we found only five amino acid residues in the CRD of
hT1R3 to be involved in the response to thaumatin and the mechanisms of
receptor activation by thaumatin to be different from that for the small
molecule sweetener sucralose. Furthermore, the residues involved in the
response to thaumatin are dispersed in the CRD of hT1R3.

Insights into the molecular mechanism by which thaumatin activates
sweet receptors may help in understanding the signal transduction by
sweeteners.
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Figures and legends

Figure 1. Five amino acid residues in the CRD of hT1R3 affect the response to thaumatin.

Human T1R2- and T1R3 mutant containing plasmids were transiently transfected into Gα_{16gust44}-expressing HEK cells and responses to sucralose (A) and thaumatin (B) were investigated by cell-based assay. The responses of 16 mutants were averaged and analyzed with a one-way ANOVA (analysis of variance). *P < 0.05, **P < 0.01.

Figure 2. Alignment of amino acid sequences of the cysteine-rich domain (CRD) of T1R3 derived from humans and mice.

(A) Conserved residues are indicated with black letters, and non-conserved residues are in white. The residues important for the thaumatin response are indicated in red circles. Two residues, Ala537 and Phe540, previously
identified as important for the response to brazzein [11] are also shown in blue circles. (B) Schematic representation of the structure of the sweet receptor. CRD of T1R3 is shown in blue and the five amino acid residues involved in the response for thaumatin are shown in red. The figures were prepared using Pymol [21] and Modeller [22].

Supplement Figure 1. Dose-response of five mutants of CRD in hT1R3 to thaumatin.

The dose–response analysis of wild-type (black circle), Q504K (red diamond), A537T (brown square), R556P (green triangle), S559P (cyan circle), and R560K (purple triangle). The response of hT1R2- wild-type hT1R3 (no mutation) to thaumatin (50 µM) was defined as 100%.

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