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Design and Evaluation of New Analogs of the Sweet Protein Brazzein

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Abstract

We have previously modeled the interaction of the sweet protein brazzein with the extracellular domains of the sweet taste receptor. Here, we describe the application of that model to the design of 12 new highly potent analogs of brazzein. Eight of the 12 analogs have higher sweetness potency than wild-type brazzein. Results are consistent with our brazzein–receptor interaction model. The model predicts binding of brazzein to the open form of T1R2 in the T1R2–T1R3 heterodimer.

Key words: brazzein, labeled magnitude scale, *Pentadiplandra brazzeana*, psychophysics, sweetener, taste receptor

Introduction

Brazzein is a small protein (54 amino acids) derived from the African plant *Pentadiplandra brazzeana* (Ming and Hellekant 1994). It is potently sweet (potency = 2000× a 2% sucrose solution on a weight basis, 37 500× a 2% sucrose solution on a molar basis) and is extremely heat stable. A number of point mutations have been made, and these have identified a number of residues in brazzein that are important for interaction with the sweet taste receptor (Assadi-Porter et al. 2000; Jin, Danilova, Assadi-Porter, Aceti, et al. 2003; Jin, Danilova, Assadi-Porter, Markley, and Hellekant 2003). Among these are several charged amino acids on the surface of brazzein, including Glu41 and Arg43. These amino acids were previously suggested to form part of the ‘‘sweet finger'' of brazzein by analogy to structure–function studies of aspartame and other sweeteners (Temussi et al. 1984; Tancredi et al. 2004). But a simple sweet finger–binding affinity determinant may not be a viable model for the sweet proteins. A much larger binding surface, including these amino acids, is required to elicit a response in what Tancredi et al. term the ‘‘wedge model'' of binding. Jiang et al. have shown that brazzein activates the T1R2–T1R3 receptor of humans (Jiang et al. 2004). In their paper, human–mouse chimeric T1R2 + T1R3 receptors showed that, although several human-specific small molecule sweeteners require only human T1R2 for effective response, brazzein was dependent on both human sequences in the heterodimer.

We have previously constructed homology-based models of the ligand-binding domains of the sweet taste receptor, T1R2/T1R3, and have used a combination of docking calculations and structure–taste relationships to identify the likely conformation of the brazzein-sweet receptor complex (Walters and Hellekant 2006). Our model suggests the open conformation of the T1R2 active site is the preferred brazzein-binding site; this model is consistent with 21 of 23 previously designed brazzein mutants. We have now used our proposed model to design new brazzein single and double mutants. The purpose of these new mutants was 2-fold: to improve the sweetness of brazzein and to test the validity of our binding model. Here, we report results for 12 new brazzein single and double mutants.

The model was applied and evaluated using several kinds of mutations. Tyr39 and Arg43 mutations were expected to decrease sweetness, through loss of important receptor interactions in the sweet finger domain/loop (residues 39–45). The double mutant Cys16Ala/Cys37Ala tested the importance of one of the disulfide bonds in brazzein, adjacent to loop 39–45, likely supporting structural integrity and rigidity of this critical domain. Mutation of Asp40 and Lys42 was

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expected to have no effect on sweetness, because these residues do not contact the receptor in the modeled conformation. Mutation of Asp29, Glu41, Asp50, and Tyr54 was expected to increase interaction, either by removing repulsive interactions or, in the case of Tyr54Trp, by increasing a hydrophobic interaction.

Materials and methods

Preparation and isolation of mutants

We produced the mutants in a protein expression system of *Escherichia coli*, as described earlier (Assadi-Porter et al. 2000). In short, brazzein mutants were prepared by site-directed mutagenesis on the template gene encoding WT brazzein (des-pGlu1 brazzein) (Quick Change polymerase chain reaction kit, Stratagene) and expressed as a fusion protein with staphylococcal nuclease (SNase). The fusion protein is found in inclusion bodies and refolded using 6 M guanidinium hydrochloride in the presence of reducing agent dithiothreitol, followed by dialysis in acetic acid. The purity following refolding is consistently greater than 80%. The brazzein molecule was released from the fusion protein by CNBr cleavage and purified by cation exchange chromatography (removing the SNase) and reverse-phase high-performance liquid chromatography (separating unfolded and misfolded brazzein). Protein purity was confirmed using sodium dodecyl sulfate polyacrylamide gel electrophoresis, and concentrations were determined by Pierce bicinchoninic acid protein assay (Thermo Scientific).

Taste evaluation of brazzein mutants

Brazzein mutants and wild-type brazzein were dissolved in deionized water at a concentration of 100 μg/mL and adjusted to pH 7.0 with 0.1 N NaOH or HCl. All stimuli were presented at room temperature. The taste panel was composed of 5 females and 7 males, ages 20–65, (average 30.92 ± 15.59) with normal taste acuity. The subjects tasted 150 μL samples delivered with a 200-μL pipette to the anterior part of the tongue. Each sample was presented 3 times and in quasi-randomized order and on separate occasions. The subjects kept the stimulus in the mouth, moving the sample around the mouth and allowing it to mix well with saliva, to get a ''whole-mouth'' sensation, then expectorated and rinsed their mouth with tap water for 1 min before the presentation of the next stimulus. This same sample size and method of application have been used in our previous studies and it is, in our experience, sufficient for comparison of sweetness of samples (Jin, Danilova, Assadi-Porter, Aceti, et al. 2003; Jin, Danilova, Assadi-Porter, Markley, and Hellekant 2003).

The subjects were asked to score the sweetness of taste stimuli with the Labeled Magnitude Scale, a semantically labeled scale for rating sensation intensity (Green et al. 1996; Bartoshuk et al. 2004). The qualitative scale was later converted to a numerical scale and average sweetness scores were calculated for each stimulus.

Sweetness scores were first evaluated with repeated measurements analysis of variance, ANOVA ($F = 30.023$, df = 14, $P < 0.001$), with a significance level of $P \leq 0.05$ considered significant. This was followed by multiple pairwise comparisons by *t*-test, comparing means of the different mutants to wild-type brazzein. A significance level of $P \leq 0.05$ was considered significant for the pairwise comparisons. No effect of gender was found ($F = 2.834$, df = 1, $P = 0.131$), and the interaction between gender and stimulus effect was not found to be significant ($F = 0.714$, df = 14, $P = 0.576$).

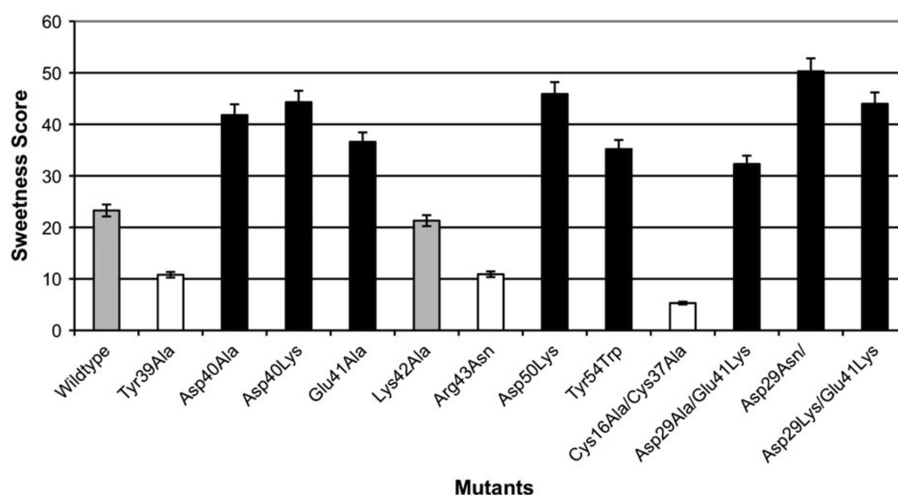

Figure 1 Psychophysical results of pairwise comparison of 12 new brazzein mutants with wild-type brazzein (WT). Data from each panelist were averaged for 3 taste tests and then evaluated by repeated measures with ANOVA. $P < 0.05$ was considered significant. Black bars, significantly higher sweetness than wild type; open bars, significantly lower sweetness than wild type, not different from water; gray bars, comparable sweetness to wild type. Error bar Standard error of the mean.

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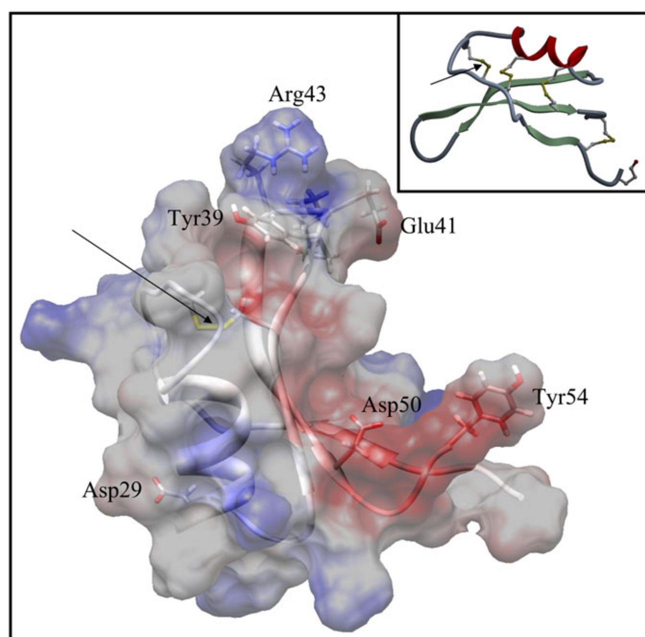

Figure 2 Binding surface and position of mutated amino acids on the brazzein backbone structure. The position of mutated amino acids on the modeled brazzein–T1R2/T1R3 interaction surface is shown, 2 mutated amino acids Asp40 and Lys42 are facing away from this surface. The Coulombic surface was calculated after adding hydrogens and AM1-BCC charges and displayed using Chimera (Pettersen et al. 2004). Inset shows the ribbon backbone structure of brazzein and the position of secondary structures and 4 disulfide bonds. In both structural representations, the arrow indicates the location of the mutated disulfide bond.

Results and discussion

The taste results of the 12 single and double mutants are compared with wild-type brazzein in Figure 1. Location of the mutated amino acids is shown in Figure 2 with the brazzein receptor–binding surface displayed. Three mutants are less potent than wild-type brazzein, 1 is about equal in potency, and 8 are significantly higher in potency.

Table 1 summarizes the results with respect to the model-based rationale for their synthesis. The Cys16Ala/Cys37Ala double mutant demonstrates the importance of the Cys16–Cys37 disulfide bond in brazzein, as it loses almost 80% of its sweetness. This is only 1 of 4 disulfide bonds contributing to the extreme thermal stability of brazzein (Figure 2, inset). It is likely that removal of this disulfide not only dramatically alters the dynamics of the loops between β-sheet 1 and α-helix and between β-sheets 2 and 3, but the effect is also propagated throughout the structure. Single point mutants Arg33Ala, Arg43Ala, and Asp50Ala were previously shown to have dramatically reduced sweetness (Assadi-Porter et al. 2003). Associated with the loss of sweet taste was a significant change in hydrogen bonding and chain mobility that had propagated from the site of mutation throughout the brazzein structure. This was not observed for mutations retaining near wild-type sweetness. These observations show the importance of rigidifying disulfide bonds in brazzein and the critical role of chain dynamics in receptor binding. Mutation of Tyr39 and Arg43 was expected to remove favorable binding interactions, and these mutants were found to be significantly less sweet than brazzein. These residues fall in the loop expected to be stabilized by the Cys16/Cys37 disulfide bond described above, and they make contact, in an extended binding interface, with T1R2 in our current model. Figure 2 shows the face of brazzein suggested to interact with the receptor and the location of these amino acids. Arg43 is near Glu252 of T1R2, whereas Tyr39 is excluded from water in the binding interface with the potential for hydrogen bonding with Ser251 of T1R2.

Table 1 Evaluation of mutants with respect to previously published docking model

Brazzein mutant	Sweetness, relative to wild type	Comments
Tyr39Ala	Less sweet	Consistent with model—lost interaction with T1R2–Ser251
Asp40Ala	Sweeter	Not consistent with model—Asp40 does not contact receptor
Asp40Lys	Sweeter	Not consistent with model—Asp40 does not contact receptor
Glu41Ala	Sweeter	Consistent with model—lost repulsion with T1R2–Glu252
Lys42Ala	Equally sweet	Consistent with model—Lys42 does not contact receptor
Arg43Asn	Less sweet	Consistent with model—lost interaction with T1R2–Glu253
Asp50Lys	Sweeter	Consistent with model—lost repulsion with T1R2–Asp278
Tyr54Trp	Sweeter	Consistent with model—more interaction with hydrophobic pocket formed by T1R2–Leu281, Phe285, Trp304, and Leu323
Cys16Ala/Cys37Ala	Less sweet	Consistent with model—lost a disulfide that stabilizes brazzein conformation
Asp29Ala/Glu41Lys	Sweeter	Consistent with model—lost repulsion with T1R3–Glu178 and gained attractive interaction with T1R2–Glu252
Asp29Asn/Glu41Lys	Sweeter	Consistent with model—lost repulsion with T1R3–Glu178 and gained attractive interaction with T1R2–Glu252
Asp29Lys/Glu41Lys	Sweeter	Consistent with model—gained attractive interactions with T1R3–Glu178 and T1R2–Glu252

Figure 3A shows our previously described model for brazzein–receptor interaction. Most of the binding interactions take place between brazzein and the open ligand-binding region of T1R2. In our model, we observed 3

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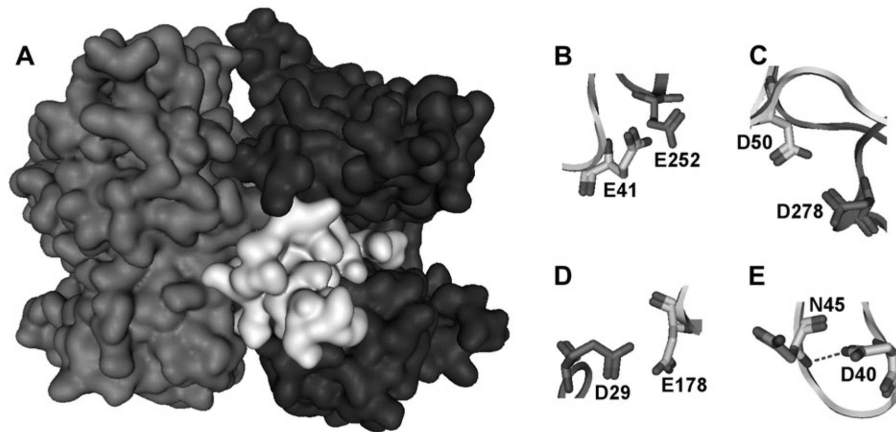

Figure 3 Brazzein–receptor interactions and rationale for brazzein mutants. **(A)** Previously described model (Walters and Hellekant 2006) of the interaction of brazzein (light gray) with the ligand-binding domains of T1R2 (dark gray) and T1R3 (medium gray). **(B)** Close proximity of brazzein–Glu41 to T1R2–Glu252. **(C)** Close proximity of brazzein–Asp50 to T1R2–Asp278. **(D)** Close proximity of brazzein–Asp29 to T1R3-Glu178. **(E)** Stabilization of beta loop of brazzein by side chain of Asp40.

potentially repulsive interactions between charged side chains of brazzein and of the receptor. Glu41 was found to be in close proximity with Glu252 of the T1R2 component of the receptor (Figure 3B). We reasoned that mutation of Glu41 to alanine should remove a repulsive interaction and improve sweetness, and the results confirm this prediction. Similarly, Asp50 of brazzein is close to Asp278 of T1R2 (Figure 3C), and conversion of Asp50 to lysine was expected to improve sweetness; this was also confirmed by experiment. Finally, Asp29 is close to Glu178 of T1R3 (Figure 3D); mutation of Asp29 to alanine, asparagine, or lysine (in double mutants with Glu41Lys) led, in each case, to significantly higher sweetness. Unlike the T1R3-binding model proposed by Temussi, our modeling and mutational analysis indicates brazzein's preference for T1R2 (Temussi 2002; Walters and Hellekant 2006). Like the T1R3 binding site, that of T1R2 is predominantly acidic in nature. The enhanced binding on removal or reversal of negative surface potential on brazzein, above, is consistent with this observation. A semitransparent surface potential of brazzein is shown in Figure 2.

In our docking model, Tyr54 of brazzein lies in a hydrophobic pocket formed by Leu281, Phe285, Trp304, and Leu323 of T1R2. Mutation of Tyr54 to the more hydrophobic Trp was predicted to improve the hydrophobic interaction, and this mutant was also found to be significantly sweeter than brazzein.

Three of our mutants were predicted to have no effect on sweetness, because they are in brazzein residues that do not contact the receptor. We expected that mutation of Asp40 to alanine or lysine, or mutation of Lys42 to alanine, should have no effect. These mutations are positioned away from the binding surface shown in Figure 2. The Lys42 mutant was, in fact, equal in sweetness to brazzein. But the Asp40 mutants were both unexpectedly sweeter than brazzein. We note that, in the wild-type structure, the side chain of Asp40 helps to stabilize a turn between 2 strands of beta sheet (Figure 3E); perhaps, loss of this interaction allows the Asp40 mutants to adopt a conformation even more favorable for interaction with the receptor.

Thus, these experimental results strongly support our previously described model for brazzein–receptor binding to the ligand-binding domains of T1R2 and T1R3. In our previous paper (Walters and Hellekant 2006), we noted that Jiang et al. propose that brazzein interacts instead with the cysteine-rich region of T1R3, based on point mutations they made in this domain (Jiang et al. 2004), we proposed an alternate explanation for their results. We suggest that this region acts to communicate/translate binding to the transmembrane domain through conformational change distant from the site of binding. This is consistent with the observation that even small sweeteners, such as sucrose, are known to have reduced response in the mutant forms. Jiang et al. also point out that binding of brazzein to human T1R2 is enhanced over mouse T1R2, also consistent with our proposed model. Our results continue to be consistent with brazzein interacting primarily with the ligand-binding domain of T1R2, and to a lesser extent with the ligand-binding domain of T1R3 through an extended interaction surface. The extended interaction surface is also consistent with the "wedge model" of sweet protein binding but is not compatible with the sweet finger model described earlier. The sweet finger model is also inconsistent with human T1R2 mutational analysis of the putative sweet finger–binding site (Jiang et al. 2005).

In summary, we have used our previously described model of brazzein–receptor binding to design 12 new single and double mutants. Taste results for 10 of these are fully consistent with our model-based predictions. Eight of the 12 have significantly higher sweetness than wild-type brazzein. This outcome suggests that brazzein's interaction with the T1R2–T1R3 heterodimer occurs predominantly through the T1R2 binding site.

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Finally, although our model is relatively low resolution, it has very promising predictive capability suggesting further improvement to the sweet taste properties of brazzein will be possible.

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