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Monkey Electrophysiological and Human Psychophysical Responses to Mutants of the Sweet Protein Brazzein: Delineating Brazzein Sweetness

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Abstract

Responses to brazzein, 25 brazzein mutants and two forms of monellin were studied in two types of experiments: electrophysiological recordings from chorda tympani S fibers of the rhesus monkey, *Macaca mulatta*, and psychophysical experiments. We found that different mutations at position 29 (changing Asp29 to Ala, Lys or Asn) made the molecule significantly sweeter than brazzein, while mutations at positions 30 or 33 (Lys30Asp or Arg33Ala) removed all sweetness. The same pattern occurred again at the β-turn region, where Glu41Lys gave the highest sweetness score among the mutants tested, whereas a mutation two residues distant (Arg43Ala) abolished the sweetness. The effects of charge and side chain size were examined at two locations, namely positions 29 and 36. The findings indicate that charge is important for eliciting sweetness, whereas the length of the side-chain plays a lesser role. We also found that the N- and C-termini are important for the sweetness of brazzein. The close correlation ($r = 0.78$) between the results of the above two methods corroborates our hypothesis that S fibers convey sweet taste in primates.

Key words: low-caloric natural sweeteners, Magnitude Labeled Scale, rhesus monkey, sweet taste receptor

Introduction

As obesity becomes a major health issue in the USA and other industrialized countries around the world, the demand for low-caloric natural sweeteners increases. Among low-caloric natural sweeteners, three very sweet proteins, brazzein, monellin and thaumatin, can be or have been used as non-sugar alternatives, flavor enhancers and masking agents. Among these, brazzein has the greatest potential because it combines a good sweet taste and thermostability with a long history of human consumption (Hladik *et al.*, 1984). Fruit brazzein consists of two forms, one with pGlu at its N terminus and one without (des-pGlu) which is twice as sweet as the first form (Caldwell *et al.*, 1998a). The latter form is 500 times sweeter than a 10% sucrose solution and up to 2000 times sweeter than a 2% sucrose solution on a weight basis. It has the smallest molecular size among sweet proteins (6.5 kDa) and high solubility in aqueous solution (Ming and Hellekant, 1994). Its exceptional thermostability is probably due to its four intramolecular disulfide bonds and absence of a free sulfhydryl group. This evenly distributed web of disulfide bridges stabilizes brazzein by limiting the conformational entropy of the unfolded molecule (Ishikawa *et al.*, 1996; Kohmura *et al.*, 1996; Caldwell *et al.*, 1998a,b; Gao *et al.*, 1999).

Because of its proteinaceous nature, intense sweetness and stability at extreme pH and temperature, brazzein offers a good system for investigating the chemical and structural determinants for sweet taste. Our earlier studies of brazzein revealed that the sweetness of the molecule could be enhanced, decreased or even abolished by mutations at single sites. As a result of these studies, we suggested that the N- and C-termini and the β-turn around Arg43 are involved in the sweetness of brazzein (Assadi-Porter *et al.*, 2000b).

Sweet taste has recently been linked to the T1R2/T1R3 member of a family of taste receptors discovered in human and mouse (Nelson *et al.*, 2001). It has been shown that HEK-293 cells expressing human T1R2/T1R3 respond to a variety of diverse sweeteners, carbohydrates, high potency artificial sweeteners and large molecules of sweet proteins (Li *et al.*, 2002) (M. Max, personal communication). However, the murine T1R2/T1R3 receptor differs from the human homologue in that HEK-293 cells expressing the murine T1R2/T1R3 failed to be activated by brazzein or any of the other sweet proteins. These findings reinforce the fact that species differences play a decisive role in taste. They also

support our conclusion that the sweetness of the sweet proteins, including brazzein, is limited to catharrhina primates, which include humans, apes, and Old World monkeys, but exclude New World monkeys, such as marmosets (Danilova *et al.*, 2002c; Hellekant *et al.*, 1997a; Hellekant *et al.*, 1997b). In particular, in the rhesus monkey, *Macaca mulatta*, and chimpanzee, *Pan troglodytes*, brazzein stimulated a specific cluster of taste fibers, called S fibers, which respond predominantly to sweeteners. Furthermore, in behavioral experiments these animals preferred brazzein as strongly as they preferred other sweet compounds. In contrast, brazzein did not elicit any taste responses in marmosets, hamsters, pigs, or mice, confirming the above *in vitro* observation on the murine T1R2/T1R3 receptor.

Here we designed a series of brazzein mutants, including multiple mutations at several specific positions, and evaluated their sweetness in both human psychophysical and monkey electrophysiological experiments. Our goal was to determine the critical regions in brazzein molecule that elicit its sweetness in order to elucidate receptor–ligand interaction as well as to design novel natural sweeteners.

Material and methods

Electrophysiological recordings

Animals and surgery

Five *Macaca mulatta* rhesus monkeys, two females and three males, were used. The animals were 3 years old and weighed 3.6–4.8 kg at the time of recording. After initiation of anesthesia with i.m. ketamine, 10 mg/kg body wt, the monkey was intubated and put on 0.7–1.0% halothane mixed with oxygen. During the experiments body temperature, arterial O_2, CO_2 in respired airheart and respiratory rates were continuously monitored, and fluid losses were replaced with i.v. 5% dextrose in lactated Ringer's solution.

The method used to dissect and isolate the right CT nerve has been described earlier (Danilova *et al.*, 2002b). Briefly, the right CT was reached through an incision along the mandibular angle of the mandibular bone and dissected free from the point where it joins the lingual nerve to the tympanic bulla. After the operation the wound was closed and the animal was allowed to recover.

Stimuli and stimulation

We produced the mutants in a protein expression system of *Escherichia coli*, as described earlier (Assadi-Porter *et al.*, 2000a,b). In short, brazzein mutants were prepared by site directed mutagenesis (Quick Change™ PCR kit, Stratagene, La Jolla, CA) on the template gene encoding des-pGlu brazzein (WT) and expressed as a fusion protein. The brazzein molecule was released from the fusion protein by CNBr cleavage and purified by cation exchange chromatography and reverse-phase HPLC.

The tastants were delivered to the anterior part of the tongue with an open flow system, Taste-O-Matic, controlled by a computer. Custom-made software controlled stimulus delivery and stored pulse interval data together with information on the presented stimulus (Hellekant and Roberts, 1995). The system delivered the solutions at given intervals, over a preset time, under conditions of constant flow and temperature (34°C). Stimulation time was 10 s and rinsing time 45 s. Artificial saliva was used for rinsing between stimulations (Hellekant *et al.*, 1997a).

A single taste fiber was first characterized with a concentration series of the four basic taste qualities: 0.07, 0.1, 0.2 M NaCl; 20, 40, 70, 100 mM citric acid; 10, 50, 100 mM ascorbic acid; 1, 5, 15, 30 mM quinine hydrochloride (QHCl); 0.2, 0.5, 1, 2 mM denatonium benzoate; 0.1, 0.3, 0.6, 0.8 M sucrose. In addition, we used 0.01, 0.04, 0.1 and 1 mM SC45647 (high potency artificial sweetener), and 5 mM aspartame. All stimuli were dissolved in artificial saliva. After we had obtained sufficient background information about the response profile, we then stimulated the area of innervation with a few ml of solutions of wild type brazzein (WT), 25 brazzein mutants, monellin and single chain monellin (SCM). All proteins were dissolved in artificial saliva at a concentration of 100 μg/ml.

Recording technique and data analysis

Fine strands of the CT nerve were placed on a silver wire electrode held by a micromanipulator. The indifferent electrode was positioned in nearby tissue. The nerve fiber activities were recorded through an impulse-amplitude analyzer with a window of adjustable upper and lower levels. A pulse was triggered when the nerve impulse exceeded the lower but not the upper level. These pulses were processed by a computer. Custom-made software controlled stimulus delivery and stored pulse interval data, together with information on the presented stimulus (Hellekant and Roberts, 1995). The identity of the stimulus, its order, the level of nerve activity before, during and after each stimulation as well as other parameters of importance, were continuously presented on the computer screen and printed out during the experiment.

The measure of a single fiber response is the mean frequency of impulses during stimulation minus the mean frequency of spontaneous activity. We define here spontaneous activity as the impulse activity preceding the stimulation during rinsing of the tongue with artificial saliva for 5 s.

A fiber was considered to be responsive to a stimulus if the mean activity rate during stimulation was >2 SD of the spontaneous activity of the fiber. The responses to sweet proteins were evaluated with two-way ANOVA. This was followed by pair-wise comparison of stimuli using Fisher's

least significant differences. $P = 0.05$ was considered to be significant.

Psychophysical experiments

Subjects

The human taste panel consisted of 14 healthy volunteers, six females and eight males. All were students or staff at the University of Wisconsin—Madison.

Stimuli

The wild type des-pGlu brazzein (WT), the 25 brazzein mutants and monellin and SCM were dissolved in doubly deionized water, which was also used as a control stimulus. All proteins were dissolved to 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.

Experimental procedure and data analysis

The subjects tasted 150 µl samples delivered with a 200 µl pipette to the anterior part of the tongue. The small size of the samples was due to the limited amounts of mutant proteins. The subjects kept the stimulus in the mouth to get 'whole-mouth' sensation, then expectorated and rinsed their mouth with tap water for one min before the presentation of next stimulus. Each stimulus was tested three times and presented in quasi-randomized order.

The subjects were asked to score the sweetness of taste stimuli with a Labeled Magnitude Scale, a semantically labeled scale for rating sensation intensity (Green *et al.*, 1996). The semantic labels, no sensation, barely detectable, weak, moderate, strong, very strong and strongest imaginable, are unequally, quasi-logarithmically positioned on the scale. The position of the mark on the scale was later coverted to sweetness score, a percentage of a full-scale length. Then average sweetness score was calculated for each stimulus.

Sweetness scores were first evaluated with repeated measurements ANOVA followed by pair-wise comparisons of different mutants' scores using Fisher's least significant differences. A probability level of $P = 0.05$ was considered as significant.

Although subjects were asked to rate the sweetness of the stimuli, they were encouraged to notice and write down any additional taste quality or unusual sensation they experienced during tasting.

To compare the results of psychophysical and electrophysiological experiments we normalized the responses. The average response to WT brazzein in both types of experiments was considered as the standard and assigned the value 100. Then responses to all stimuli (the sweetness scores in psychophysical experiments and single fibers responses in electrophysiological experiments) were expressed in percent of the standard.

Results

Nerve recordings

Figure 1 shows a part of the recordings from the CT filament. For this recording the window discriminator was adjusted to count only action potentials with large amplitude. Based on the responses to the non-protein stimuli this fiber had been classified as an S fiber. The recordings on the left show that sucrose elicited a strong response, while denatonium benzoate and ascorbic acid were not effective stimuli. The top middle recording with WT brazzein shows a slowly growing response with a maximum frequency at the end of stimulation. This slow development is typical for the brazzein response both in non-human primates (Hellekant *et al.*, 1998) and humans (Ming, 1994). Figure 1 also shows responses to five brazzein mutants. Asp29Ala, Asp29Asn, and Glu41Lys elicited strong responses with fast onset and with a temporal profile reminding of sucrose rather than WT brazzein, while neither mutant Glu36Ala nor His31Ala gave a response.

In all, we recorded responses in 11 single S fibers. Their average spontaneous activity during rinsing with artificial saliva was 4.8 ± 0.9 (SE) imp/s. Figure 2 shows the average responses of these S fibers to the non-protein stimuli. All three sweeteners: sucrose, SC-45647 and aspartame, elicited large responses in these fibers (black columns). In contrast, NaCl, ascorbic acid and the bitter compound denatonium benzoate did not stimulate these fibers. Although 40 mM citric acid and 5 and 15 mM QHCl elicited small responses, they were significantly smaller than responses to the sweeteners.

After we ascertained that a fiber belonged to the S cluster we stimulated with the sweet proteins. The averaged results for 11 fibers are displayed in Figure 3. The leftmost column shows that WT brazzein elicited a moderate response. Monellin and SCM, used as controls, gave significantly larger responses than brazzein. The responses to the brazzein mutants ranged from no response to three times that of WT brazzein. Five mutants, Asp2Asn, Asp29Ala, Asp29Lys, Asp29Asn, and Glu41Lys, (black columns) elicited significantly larger responses than WT brazzein. In contrast, the responses of 13 mutants (white columns) did not meet the criterion of a response. The responses to seven mutants (grey columns) did not differ from that to WT brazzein.

Psychophysical results

The average sweetness scores of all stimuli tested are shown in Figure 4. Four mutants, Asp29Ala, Asp29Lys, Asp29Asn, and Glu41Lys, (black columns) were scored significantly sweeter than WT brazzein. In contrast, the sweetness of eight mutants was significantly decreased (hatched). Ten brazzein mutants (white) were scored similar to water. Three

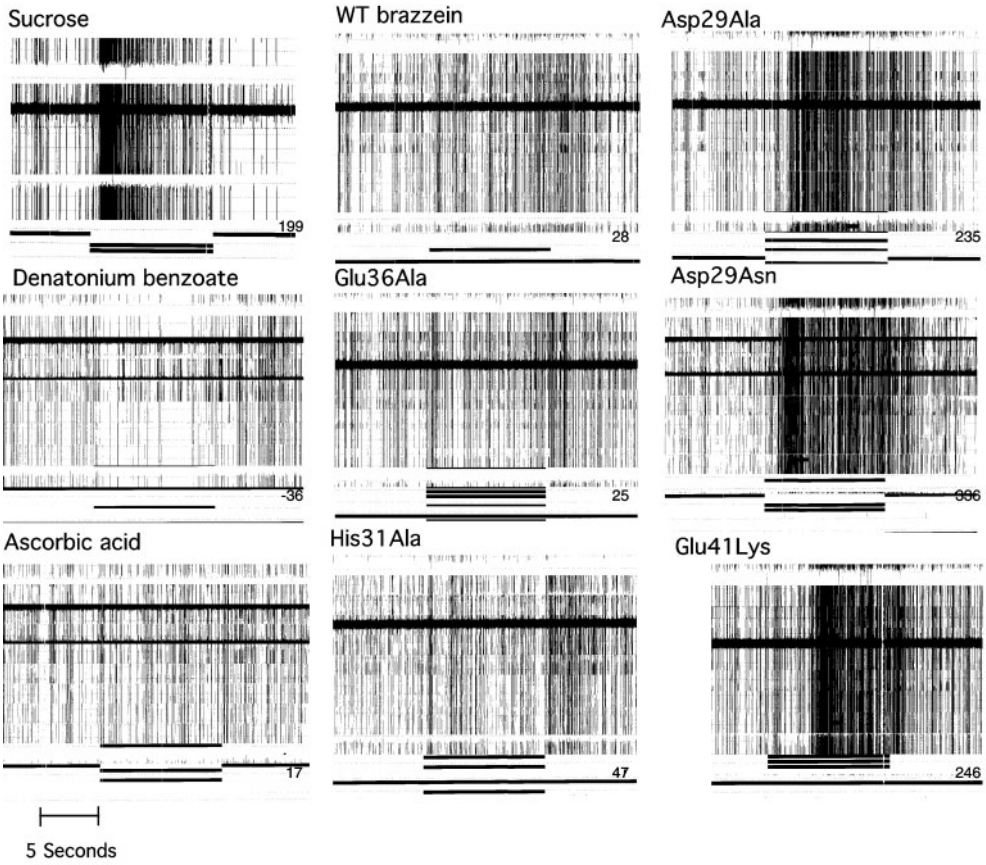

Figure 1 Recordings of a chorda tympani proper filament during stimulation with sucrose, denatonium benzoate (DB), ascorbic acid (Ascorbic), des-pGlu-brazzein (WT) and five brazzein variants. Onset and offset of stimuli are shown as changes in bar code. Stimulation time 10 s. Before and after stimulation tongue was rinsed with artificial saliva.

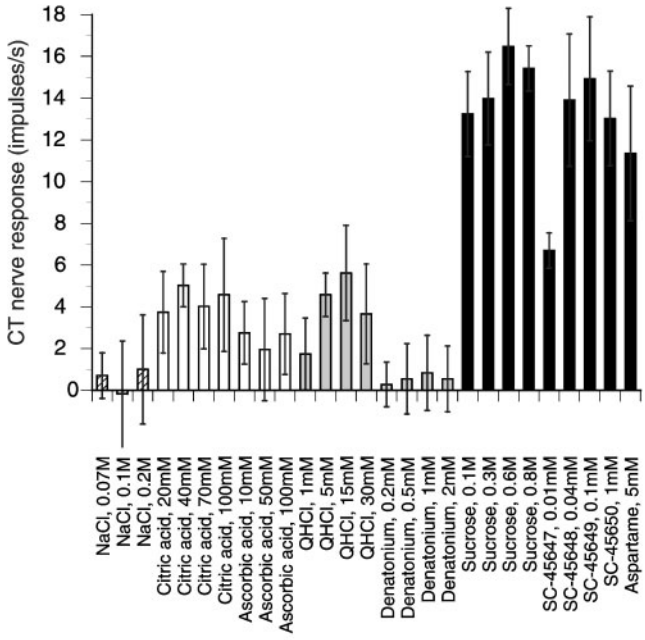

Figure 2 Average response profile of 11 S fibers of rhesus CT nerve. Column patterns indicate different taste qualities: salt hatched, acids white, bitter grey and sweet black. Error bars: SE.

brazzein mutants and both types of monellins (hatched columns) did not differ from the WT.

Some subjects commented that its sweet taste developed slowly. None of the subjects, however, reported any taste qualities besides sweet.

Comparison of psychophysical and electrophysiological results

Figure 5 compares the results obtained from humans and monkeys when sweetness scores and nerve responses are expressed relative to those of WT brazzein. As can be seen the results from the two methods were highly correlated. The correlation coefficient between sweetness scores and average nerve responses was 0.78 ($P < 0.001$). The most notable discrepancy was larger differences between the nerve responses than between sweetness scores in the psychophysical experiments. For example, in humans the sweetness of monellin and WT brazzein did not differ significantly, while in monkeys the average response to the monellins was 4–5 times larger than that to WT brazzein.

Figure 6 summarizes the effects of the different mutations on sweetness of brazzein. It shows the three-dimensional structure of brazzein where red color indicates substitutions

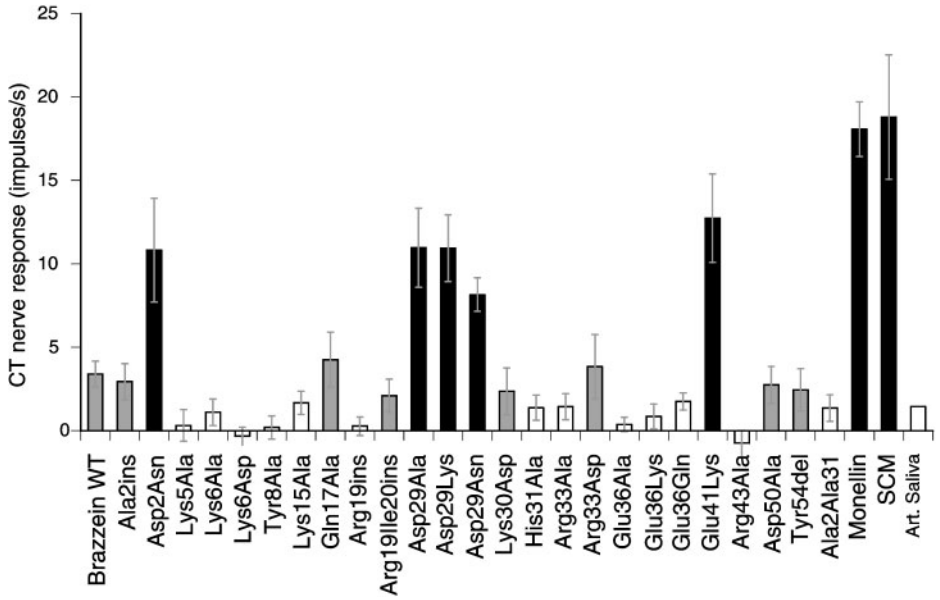

Figure 3 Average responses of 11 S fibers in CT nerve to brazzein variants and monellins (100 μg/ml). Column patterns indicate different levels of response in comparison with response to WT brazzein: black, significantly stronger than WT; grey, no difference; white columns, no response. Error bars: SE.

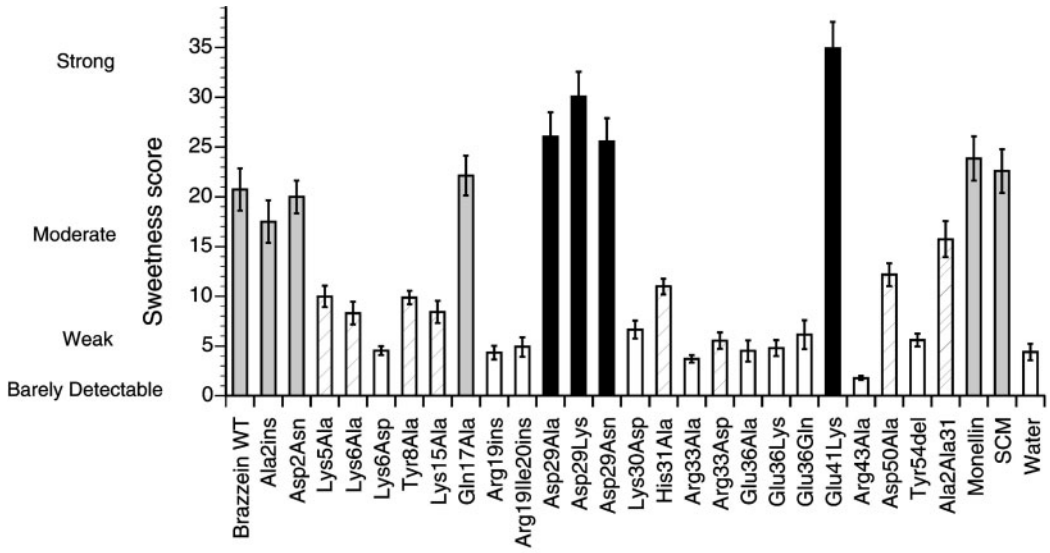

Figure 4 The results of psychophysical experiments with brazzein variants and monellins (100 μg/ml). Data were averaged for 14 subjects. Column patterns indicate different levels of sweetness in comparison with WT brazzein: black, significantly sweeter than WT brazzein; grey, not different from WT brazzein; striped and white, significantly less sweet than WT brazzein. White columns also indicate that the scores were not different from that of water. Error bars: SE.

leading to increased sweetness, black no change from WT, and blue decreased sweetness.

Two features are noteworthy. One is that addition of an N-terminal Ala or mutation of the side chain from Asp to Asn did not change the sweetness. The other is that mutations of residues close to one another in the protein sequence led to remarkable differences in sweetness. For example, mutation at position 29 (changing Asp29 to Ala, Lys or Asn) made the molecule much sweeter than WT, while mutations at positions 30 or 33 (Lys30Asp or Arg33Ala) removed all sweetness. The same pattern occurred again at the β-turn region, where Glu41Lys gave the highest sweetness score among the mutants tested, whereas a mutation two residues distant (Arg43Ala) abolished the sweetness.

To further delineate chemical and structural requirements for the sweetness of brazzein, we examined effects of side chains of different size and charge at two locations, namely

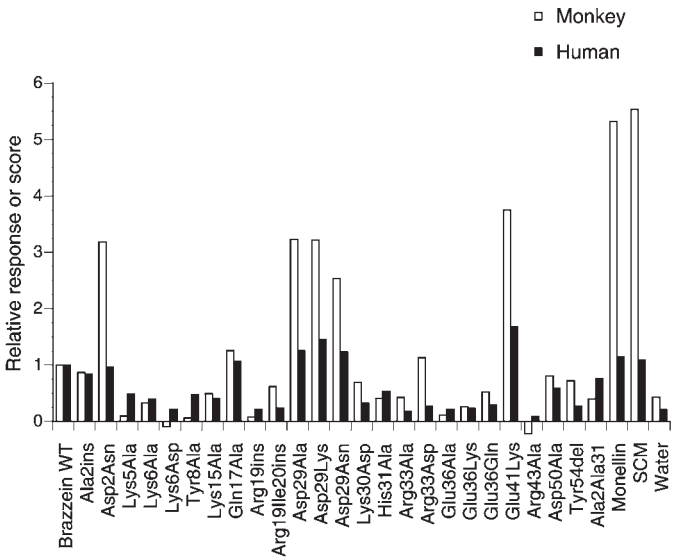

Figure 5 Comparison between human psychophysical and monkey electrophysiological results. The responses to all stimuli are expressed in relation to brazzein response.

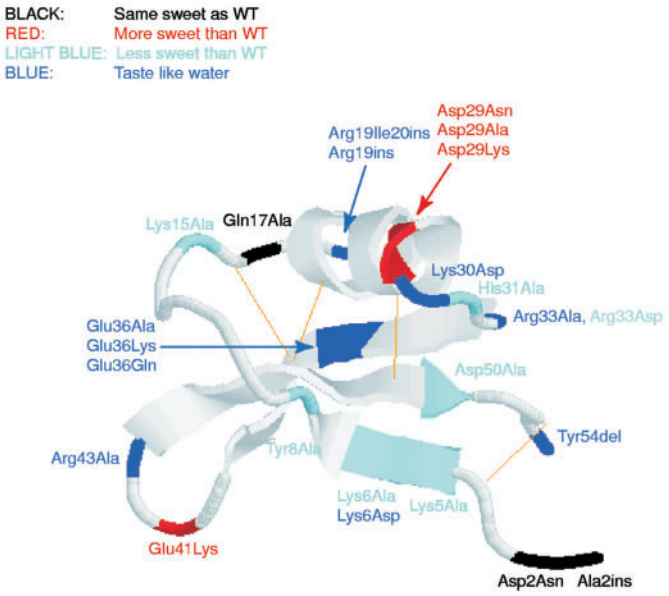

Figure 6 Diagram shows the position of mutations on the brazzein molecule and the corresponding changes in taste (red, increased sweetness; black, the same; light blue, decreased sweetness in comparison with WT brazzein; dark blue, scored as water). Intramolecular disulfide bonds are shown as yellow lines.

positions 29 and 36. It was unexpected to find that mutations of Asp29 to Ala, Lys or Asn each gave increased sweetness, whereas mutations of Glu36 to Ala, Lys, or Gln each abolished the sweetness. This suggests that at these sites, charge is important for eliciting sweetness, whereas the length of the side-chain play a lesser role.

Discussion

This study is a continuation of our efforts to define critical regions of sweetness in the brazzein molecule. It also serves as another test of our hypothesis that in primates the S fibers convey sweet taste. Hence we evaluated sweetness of 25 brazzein mutants in two models, humans and rhesus monkeys, and found a striking correlation between scores of sweet perception in humans and electrophysiological responses in monkey S fibers. The significant positive correlation between monkey and human data with a variety of brazzeins supports the idea that the monkey is a good model for the study of sweet taste. In the following we will discuss the effects of substitutions in two of the three loops as well as in the N and C termini and effects of changes in charge.

Accurate sensory analyses are vital, not only for the directions of future modifications of the brazzein molecule, but also for the development of new sweeteners. To accomplish these we used two methods, human psychophysical measurements and single fiber recordings from monkey S fibers. The advantage of a human sensory panel was that the results could be applied directly to human taste. However, to rely on the use of humans has certain disadvantages, related to regulatory and toxicological concerns that accompany such methods. Experiments involving rinsing of the tongue of an anesthetized animal carry no risk of the compounds being ingested, in contrast to experiments with humans. On the other hand, in animal experiments there is a need to demonstrate that the results apply to human.

WT brazzein has no bitter or any other taste quality. One might expect that the modifications in molecular structure might have in addition to removing sweetness added a bitter taste, as many times is the case when the structure of a sweetener is changed. Therefore it may be of particular interest that the only change that occurred with brazzein was that its sweetness was changed. No traces of bitterness or any other taste quality were reported in the psychophysical experiments.

We previously showed that in higher non-human primates, taste fibers could be grouped on the basis of their predominant response to human taste qualities. We also demonstrated that in monkeys and apes, S fibers are narrowly tuned for sweet stimuli, sufficient for distinguishing sweet tasting compounds from other taste qualities and do not change specificity with increased concentrations (Danilov *et al.*, 2002a; Hellekant *et al.*, 1986, 1997b). Here the high correlation between the electrophysiological and psychophysical responses to brazzein mutants adds another support to our longstanding hypothesis that the sweet taste is linked to S fibers.

The nerve response to a compound differs from that to another compound not only in what taste fibers it stimulates and number of impulses within the response, but also in its temporal profile. Thus for example, aspartame elicits a nerve response with the largest magnitude within the first few seconds of stimulation, while the response to thaumatin

represents the other extreme with a response that slowly grows over >10 s (Hellekant, 1994). This feature is reflected in the taste sensations that increase slowly as is the case for thaumatin and rapidly for aspartame. The same parallel was observed for brazzein because the maximum nerve response to WT brazzein developed by the end of stimulation (Figure 1) and its sweet taste developed relatively slowly compared to that of sucrose. The same type of responses to brazzein was recorded in chimpanzee's CT S fibers (Hellekant *et al.*, 1998) and in humans (A. Nobel, personal communication).

We previously tested 11 of these 25 mutants in psychophysical experiments (Assadi-Porter *et al.*, 2000b). Although we used a different psychophysical method, the results were similar. That study demonstrated the importance of residue 43. Here, in addition to confirming this, we expanded the study to include changes at position 41 and found that this dramatically increased the sweetness. Further substitutions of the residues in this flexible loop may very well cause other changes of sweetness.

We suggested earlier the importance of another region 29–33 (Ming, 1994; Caldwell *et al.*, 1998a). New mutations for this study were made at positions 29, 30 and 33. Changes of sweetness at neighboring 29 and 30 confirmed the importance of this area.

Here we found a decrease of sweetness after mutation of His to Ala at position 31, while in our previous study at the lowest concentration tested this mutation increased sweetness. However, at 100 μg/ml, a concentration used in both studies, the difference was much less between the studies. Furthermore, the fact that here the same results were recorded in both human and monkey, strengthens the present conclusion on a decrease. Our previous study, as well as the present one, indicate strongly a role for the C terminus. First, deletion of Tyr54 abolished sweetness. Second, the NMR solution structure suggested that Arg33 is hydrogen bonded to Asp50, bringing it close to the aromatic ring residues Tyr51 and Tyr54 (Caldwell *et al.*, 1998a; Assadi-Porter *et al.*, 2000b, 2003; DeRider, 2001). This puts the region containing Arg33 in close contact with the C-terminus.

With regard to the N terminus, removal of the pyroglutamate significantly increased its sweetness (Ming, 1994). The pyroglutamyl residue lacks the positive charge of a free amino terminus. Thus the finding here that alanine at the N-terminus or substitution of aspartic acid to aspargine at position 2 had no effect on sweetness may be explained by the fact that these mutations preserve the N-terminus positive charge present in WT. Alternatively, the bulkiness of the cyclic pyroglutamyl residue may account for its lower sweetness in comparison with the WT.

As mentioned above charge is important for the sweetness of brazzein. Mutations of the negatively charged Asp 29 residue, to either a neutral or positively charged residue (Asp29Ala, Asp 29Asn, Asp 29Lys), markedly increased sweetness. Similar types of mutations performed at the Glu36 site (Glu36Ala, Glu36Gln, Glu36Lys) decreased the sweetness to the level of no taste. Thus these results suggest that at these sites, charge is important for eliciting sweetness, whereas the length or composition of the side-chain plays a lesser role. A similar conclusion has been reached in study with thaumatin, namely that elimination of negative charge with the positively charged lysine could increase its sweetness (Kaneko and Kitabatake, 2001).

It has been shown that the recently discovered human heterodimeric T1R2/T1R3 receptor, responds *in vitro* to monellin (Li *et al.*, 2002). It also responds to brazzein (M. Max, personal communication). Thus, human T1R2/T1R3, expressed in HEK-293 cells and monitored with calcium imaging, was activated by brazzein with typical S-shaped dose response relationship, while mouse T1R2/T1R3 did not respond to brazzein at all.

To explain why molecules of such varying size as, for example, brazzein (6.5 kDa) and aspartame (0.4 kDa) are able to interact with the same T1R2/T1R3 heterodimer, Temussi recently hypothesized that the receptor exists as an equilibrium of two conformations (Temussi, 2002). Low molecular weight sweeteners form a complex with the resting state of T1R2/T1R3 and shift the equilibrium to its active state, whereas sweet proteins interact with a secondary binding site, a large cavity of the T1R3 extracellular domain with an average negative charge. The binding then stabilizes the receptor in its active state. Their computer models developed in support of this hypothesis pointed to the importance of electrostatic complementarity between the protein and receptor. Single chain monellin has a positive surface, and other sweet proteins are predominantly positively charged. This prediction is consistent with the present results showing that changing the negative Asp29 to a neutral (Ala, Asn) or positive residue (Lys), increased sweetness, with Asp29Lys exhibiting the largest effect. Introduction of a positive charge at another site in brazzein (Glu41Lys) also greatly increased the sweetness. These results suggest that charge plays an important role in controlling whether brazzein is perceived as sweet or tasteless.

Histochemical data in mice indicate that the T1R2 and T1R3 receptors are co-expressed only on the back of the tongue (Nelson *et al.*, 2001). Here we recorded from the CT, which innervates the anterior part of the tongue, and our human subjects reported sweetness mostly from the anterior area of the tongue. It is interesting that electrophysiological recordings from mice also show a stronger response to sweeteners from the tip of the tongue than from the back (Danilova and Hellekant, 2003). In mice, this discrepancy can be explained by the existence of another sweet receptor on the anterior part of the tongue. It is possible that the T1R3, which is expressed alone in the fungiform papillae (Nelson *et al.*, 2001), might interact with another not yet identified T1R. In human and monkey it is possible that the T1R2/T1R3 complex is present on the anterior part of the

tongue or that there is another sweet receptor yet to be discovered.

In summary, our results with two very different methods to measure sweetness, monkey S fiber response and human magnitude estimation, gave very similar and corroborative results. This further strengthens our hypothesis that S fibers convey sweet taste in primates. Specific mutations of amino acid residues at different location of the brazzein molecule, combined with the above two methods for sweetness measurements, suggest a multipoint interaction between brazzein and its receptor in which charge plays a significant role. Specifically, our findings suggest that residues 29–33 and residues 39–43, plus residue 36 connecting these stretches, as well as the N- and C-termini determine the sweetness of brazzein. As a consequence of these studies, brazzein variants identified to have enhanced sweet qualities could become candidates for a new generation of low-caloric natural sweeteners.

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References

Assadi-Porter, F.M., Aceti, D.J., Cheng, H. and **Markley, J.L.** (2000a) *Efficient production of recombinant brazzein, a small, heat-stable, sweet-tasting protein of plant origin*. Arch. Biochem. Biophys., 376, 252–258.

Assadi-Porter, F.M., Aceti, D.J. and **Markley, J.L.** (2000b) *Sweetness determinant sites of brazzein, a small, heat-stable, sweet-tasting protein*. Arch. Biochem. Biophys., 376, 259–265.

Assadi-Porter, F.M., Abildgaard, F., Blad, H. and **Markley, J.L.** (2003) *Correlation of the sweetness of variants of the protein brazzein with patterns of hydrogen bonds detected by NMR spectroscopy*. J. Biol. Chem., in press.

Caldwell, J.E., Abildgaard, F., Dzakula, Z., Ming, D., Hellekant, G. and Markley, J.L. (1998a) *Solution structure of the thermostable sweet-tasting protein brazzein*. Nat. Struct, Biol., 5, 427–431.

Caldwell, J.E., Abildgaard, F., Ming, D., Hellekant, G. and **Markley, J.L.** (1998b) *Complete 1H and partial 13C resonance assignments at 37 and 22 degrees C for brazzein, an intensely sweet protein*. J. Biomol. NMR, 11, 231–232.

Danilova, V. and **Hellekant, G.** (2003) *Comparison of the responses of the chorda tympani and glossopharyngeal nerves to taste stimuli in C57BL/6J mice*. BMC Neurosci., 4, 5.

Danilov, Y., Danilova, V. and **Hellekant, G.** (2002a) *Specificity of sucrose-best fibers in rhesus monkey chorda tympani does not depend on stimulus concentration*. AChems XXIV, 57:228.

Danilova, V., Danilov, Y., Roberts, T., Elmer, D. and **Hellekant, G.** (2002b) *Electrophysiological recordings of mammalian taste nerves*. In Simon, S.A. and Nicolelis, M.A.L. (eds), Methods and Frontiers in Neuroscience. CRC Press, Boca Raton, FL, pp. 239–264.

Danilova, V., Danilov, Y., Roberts, T., Tinti, J.-M., Nofre, C. and **Hellekant, G.** (2002c) *The sense of taste in a New World monkey, the common marmoset: recordings from the chorda tympani and glossopharyngeal nerves*. J. Neurophysiol., 88, 579–594.

DeRider, M.L. (2001) Conformational Investigations: I. Prolyl Ring Conformations II. Solution Structure Refinement Of Brazzein Chemistry. PhD thesis, University of Wisconsin, Madison, WI.

Gao, G.H., Dai, J.X., Ding, M., Hellekant, G., Wang, J.F. and **Wang, D.C.** (1999) *Solution conformation of brazzein by 1H nuclear magnetic resonance: resonance assignment and secondary structure*. Int. J. Biol. Macromol., 24, 351–359.

Green, B.G., Dalton, P., Cowart, B., Shaffer, G., Rankin, K. and **Higgins, J.** (1996) *Evaluating the 'Labeled Magnitude Scale' for measuring sensations of taste and smell*. Chem. Senses, 21, 323–334.

Hellekant, G. (1994) *The physiology of thaumatin*. In Whitty, M. and Higginbotham, J.D. (eds), Thaumatin. CRC Press, Boca Raton, FL, pp. 99–113.

Hellekant, G. and **Roberts, T.W.** (1995) *Whole nerve and single fiber recordings in non-human primates*. In Spielman, A.I. and Brand, J.G. (eds), Experimental Cell Biology of Taste and Olfaction: Current Techniques and Protocols. CRC Press, Boca Raton, FL, pp. 277–290.

Hellekant, G., van der Wel, H., Brouwer, J.N., Glaser, D., Segerstad, C.H. and **Roberts, T.W.** (1986) *Chorda tympani proper nerve responses to intra-arterial and surface stimulation of the tongue in rhesus monkey and rat*. Chem. Senses, 11, 557–566.

Hellekant, G., Danilova, V. and **Ninomiya, Y.** (1997a) *Primate sense of taste: behavioral and single chorda tympani and glossopharyngeal nerve fiber recordings in the rhesus monkey,* Macaca mulatta. J. Neurophysiol., 77, 978–993.

Hellekant, G., Ninomiya, Y. and **Danilova, V.** (1997b) *Taste in chimpanzees II: single chorda tympani fibers*. Physiol. Behav., 61, 829–841.

Hellekant, G., Ninomiya, Y. and **Danilova, V.** (1998) *Taste in chimpanzees III: Labeled line coding in sweet taste*. Physiol. Behav., 65.

Hladik, A., Bahuchet, S., Ducatillion, C. and **Hladik, E.M.** (1984) *Les plantes a tubercules de la foret dense d'afrique centrale*. Rev. Ecol. (Terre Vie), 39, 249–290.

Ishikawa, K., Ota, M., Ariyoshi, Y., Sasaki, H., Tanokura, M., Ding, M., Caldwell, J. and **Abildgaad, F.** (1996) *Crystallization and preliminary X-ray analysis of brazzein, a new sweet protein*. Acta Crystallogr. D Biol. Crystallogr., 52, 577–578.

Kaneko, R. and **Kitabatake, N.** (2001) *Structure–sweetness relationship in thaumatin: importance of lysine residues*. Chem. Senses, 26, 167–177.

Kohmura, M., Ota, M., Izawa, H., Ming, D., Hellekant, G. and **Ariyoshi, Y.** (1996) *Assignment of the disulfide bonds in the sweet protein brazzein*. Biopolymers, 38, 553–556.

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

Ming, D. (1994) Brazzein, a New Sweet Protein from *Pentadiplandra brazzeana*. PhD thesis, University of Wisconsin at Madison.

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

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

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

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