Critical regions for the sweetness of brazzein[1]

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Abstract **Brazzein is a small, heat-stable, intensely sweet protein consisting of 54 amino acid residues. Based on the wild-type brazzein, 25 brazzein mutants have been produced to identify critical regions important for sweetness. To assess their sweetness, psychophysical experiments were carried out with 14 human subjects. First, the results suggest that residues 29–33 and 39–43, plus residue 36 between these stretches, as well as the C-terminus are involved in the sweetness of brazzein. Second, charge plays an important role in the interaction between brazzein and the sweet taste receptor.**

Key words: Brazzein; Sweet protein; Mutagenesis; Sweetness determinant; Sweetness evaluation; Taste

1. Introduction

Six sweet proteins have been discovered over the last 30 years. The latest discovered is brazzein, isolated from the fruit of *Pentadiplandra brazzeana* Baillon [2]. Brazzein is a single-chain polypeptide of 54 amino acid residues with four intramolecular disulfide bonds, no free sulfhydryl group, and no carbohydrate [3]. It is rich in lysine but contains no methionine, threonine or tryptophan. Brazzein exists in two forms in the ripe fruit. The major form contains pyroglutamate (pGlu) at its N-terminus; the minor form is without the N-terminal pGlu (des-pGlu1). Taste comparisons of chemically synthesized brazzein and des-pGlu1 brazzein revealed that the latter protein has about twice the sweetness of the former [4]. We use 'WT (wild type) brazzein' to denote recombinant des-pGlu1 brazzein.

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[1] For brazzein mutants, the same nomenclature system was followed as in the previous publication [Assadi-Porter et al., Arch. Biochem. Biophys. 376 (2000) 259–265]. For example, Asp29Ala indicates the Asp of residue 29 was replaced by Ala; Arg19Ile20ins is a double insertion with Arg inserted at position 19 and Ile inserted at position 20; mutant Ala2Ala31 indicates the additive mutations of Ala2ins and His31Ala.

Abbreviations: CNBr, cyanogen bromide; ddH_2O, doubly deionized water; MALDI, matrix-assisted laser desorption/ionization; NMR, nuclear magnetic resonance; pGlu, pyroglutamic acid; SCM, single-chain monellin; SNase, staphylococcal nuclease; WT, wild type; WT brazzein, recombinant des-pGlu1 brazzein

Brazzein is very water-soluble. Its isoelectric point (pI = 5.4) is lower than those of other sweet proteins, which all have pI > 7.0 [5]. Brazzein is remarkably heat-stable, and its sweet taste remains after incubation at 80°C for 4 h. Chemical modification studies suggested that the surface charge of the molecule is important and led to the conclusion that Arg, Lys, Tyr, His, Asp, and Glu are important for brazzein's sweetness and should be studied further [5,6]. In addition, studies with other sweet proteins, thaumatin and monellin, have suggested a role for lysine and/or carboxyl groups in the sweet protein–receptor(s) interaction [7–9].

The structure of brazzein was determined by ^{1}H nuclear magnetic resonance (NMR) spectroscopy in solution at pH 5.2 and 22°C [10]. The study revealed that brazzein contains one short α-helix (residues 21–29) and three strands of antiparallel β-sheet (strand I, residues 5–7; strand II, residues 44–50; strand III, residues 34–39) held together by four disulfide bonds. The authors proposed that the small connecting loop containing His31 and the random coil loop around Arg43 were the possible determinants of the molecule's sweetness.

Site-directed mutagenesis was used to change surface residues of WT brazzein at different locations along its sequence [11]; and taste studies of 14 brazzein variants showed that most mutations decreased sweetness but that two increased sweetness. On the basis of these studies, the authors suggested that the N- and C-termini and β-turn around Arg43 are involved in the sweetness of brazzein [1].

Here we present results from a larger set of brazzein variants, which includes multiple mutations at several specific positions, aimed at delineating further how changes of charges and side chains affect the sweetness of brazzein. The ultimate goal, unrealized as yet, is to predict sweetness from the chemical structure of a compound, or from the amino acid sequence of a sweet protein. Because New World primates and other mammals do not perceive brazzein as sweet, investigations of its sweetness must be carried out with humans or Old World primates. This investigation describes and analyzes the effects of site-directed mutations of brazzein on its sweetness as perceived by human subjects.

2. Materials and methods

2.1. Preparation of brazzein variants

A protein expression system for WT brazzein, developed in *Escherichia coli*, allowed brazzein variants to be produced efficiently [1]. Brazzein mutants were prepared by site-directed mutagenesis (Quick Change™ PCR kit, Stratagene) for substitutions of specific amino acids based on the template gene encoding WT and expressed as a fusion protein (staphylococcal nuclease (SNase)–Met–brazzein). The

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brazzein molecule was released from the fusion protein at the unique Met linkage by CNBr (cyanogen bromide) cleavage, then purified by cation exchange chromatography, followed by reverse-phase high-performance liquid chromatography purification to remove all unfolded or mis-folded brazzein proteins [11].

Because brazzein contains no tryptophan, its extinction coefficient was determined at 205 nm instead of 280 nm. The extinction coefficient ε_{205} of each brazzein variant was calculated from measurements of the absorbance of solutions at 280 and 205 nm according to the formula [12]: $\varepsilon_{205}^{1\ mg/ml} = 27.0+120(A_{280}/A_{205})$.

All protein solutions were scanned at 195–290 nm at medium speed with a Cary Win UV Scan spectrophotometer (Varian Analytical Instruments, Walnut Creek, CA, USA). Doubly deionized water (ddH_2O) was used as the blank, and the baseline was subtracted automatically. Samples were diluted by factors that provided absorbance values of about 0.7 at 205 nm; higher absorbances were avoided to minimize effects of stray light. The concentrations of brazzein solutions were determined from the absorbance at 205 nm and the extinction coefficient for the particular variant.

The molecular weights of the brazzein variants were confirmed with matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. Samples were run on a Bruker Biflex III MALDI time-of-flight mass spectrometer in the linear mode using α-cyano-4-hydroxycinnamic acid as the matrix. The external calibration was performed with bovine insulin and ubiquitin as standards.

One-dimensional (1D) ^{1}H NMR spectroscopy was used to check that the mutants were folded correctly. Each brazzein protein (0.5–1.0 mM) was dissolved in 10% ^2H_2O at pH 7.0, and 1D ^{1}H NMR spectra were acquired with a Bruker DMX 500 MHz spectrometer in a 5 mm ^{1}H probe at 298 K.

2.2. Stimuli and sensory analysis

WT brazzein, brazzein variants, monellin, and single-chain monellin (SCM) were dissolved in ddH_2O. The solutions were adjusted to pH 7.0 with 0.1 M NaOH or HCl. The concentrations of monellin and SCM were determined spectrophotometrically using a calculated molar extinction coefficient ε_{280} of 15 930 M^{-1} cm^{-1} [13] and a mass of 11 197 Da for monellin and 11 050 Da for SCM, respectively (SWISS-PROT).

The taste panel consisted of six females and eight males (ages 17–70 years) of reported good health and normal sense of taste. The experimental protocol was approved by the University of Wisconsin–Madison Human Subjects Committee. The subjects tasted 26 brazzeins, two monellins as positive controls, and deionized water as the negative control in double blind experiments. The sample volume was 150 μl. All proteins were used at a concentration of 100 μg ml^{-1}. The solutions were delivered with a micropipet to the anterior part of the subject's tongue. The subject tasted the compound without any time constraints, then expectorated, followed by ad lib. rinsing with tap water within a 1 min interval. Each subject tested each stimulus three times, and the presentations were randomized.

Between each presentation the subjects were asked to score the sweetness of the stimulus on a Labeled Magnitude Scale (LMS) [14]. This scale is a semantically labeled scale, which we used for rating the intensity of a taste sensation. The scale is composed of verbal labels: 'barely detectable', 'weak', 'moderate', 'strong', 'very strong', and 'the strongest imaginable'. The intensity of the sweetness was later converted to a numerical value. The data were averaged between subjects, and standard deviations were calculated. Sweetness scores were first evaluated with repeated measurements ANOVA (analysis of variance) followed by pairwise comparisons of the scores for different variants using Fisher's least significant differences. Probability $P \leq 0.05$ was considered to be significant.

3. Results

3.1. Characterization of brazzein variants

Fig. 1 shows the 1D ^{1}H NMR spectra of two of the most sweet brazzein variants (Asp29Ala, Asp29Lys) and three of the least sweet brazzein variants (Glu36Ala, Glu36Lys, Glu36Gln). Each spectrum exhibits peaks at low frequency (around 0.4 ppm from methyl groups in the protein core) and at high frequency (around 10.1 ppm from the hydroxyl proton of Tyr11) that are characteristic for folded protein. Similar results obtained for the other mutants indicated that they too were correctly folded.

3.2. Human evaluations

Fig. 2 shows the average sweetness scores of all stimuli used. In Fig. 2 we use black columns to indicate significantly increased sweetness, gray columns for no change, striped columns for significantly less sweet than WT brazzein, and white columns for no difference from water. The bars denote S.E.M.

Four brazzein mutants (Asp29Ala, Asp29Lys, Asp29Asn, and Glu41Lys) were scored significantly sweeter than WT brazzein. Three brazzein mutants (Ala2ins, Asp2Asn, and Gln17Ala) were scored as sweet as WT brazzein. In eight mutants the sweetness decreased significantly but the score was different from that of water, and in 10 mutants the sweetness score did not differ significantly from that of water.

To visualize the structural correlations for these results, we have indicated the position of each mutation on the three-dimensional backbone of brazzein (Fig. 3). One dramatic change in sweetness occurred with mutants at or near Asp29: mutation of Asp29 to Ala, Lys or Asn gave significantly increased sweetness, while mutation of His31 to Ala or Arg33 to Asp reduced sweetness; mutating Lys30 to Asp or Arg33 to Ala yielded compounds tasting not significantly different from water. Glu41Lys gave the highest score of sweetness; however, Arg43Ala had no sweetness. Notably, the mu-

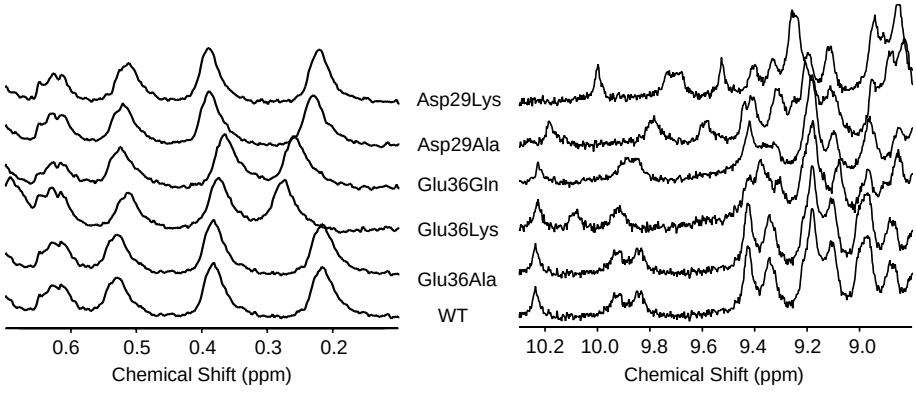

Fig. 1. 1D ^{1}H NMR spectra (500 MHz) of WT brazzein and five representative mutants in 10% ^2H_2O at pH 7.0 and 25°C. All spectra exhibit features indicative of proper protein folding, including the expected chemical shift dispersion with peaks present at high and low frequency.

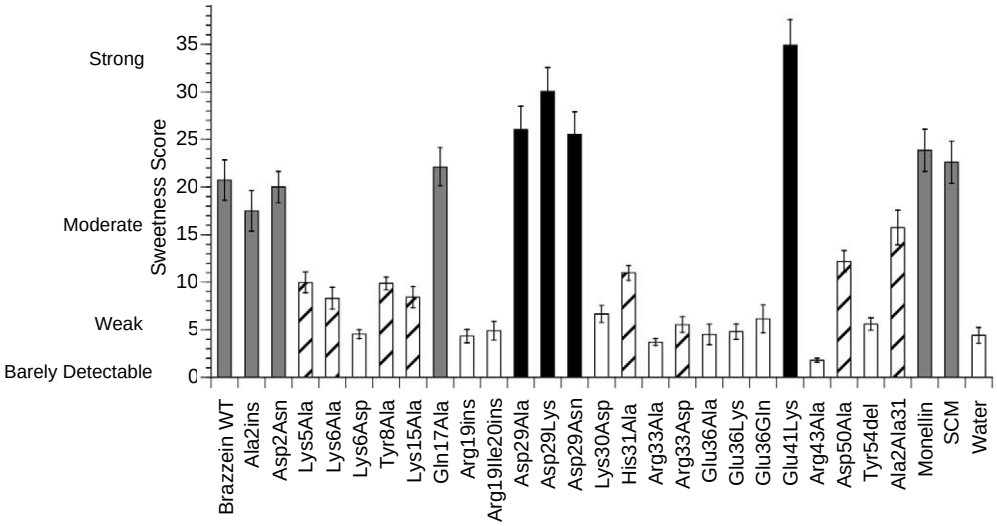

Fig. 2. Results of psychophysical experiments with brazzein mutants and monellins. Data were averaged for 14 subjects. Error bars are S.E.M. Column patterns indicate different levels of sweetness in comparison with WT brazzein: black, significantly sweeter than WT brazzein; gray, not different from WT brazzein; both striped and white, significantly less sweet than WT brazzein. White columns also indicate that the scores were not different from that of water.

tations at the N-terminus of brazzein (Ala2ins, Asp2Asn) brought no changes in sweetness. Furthermore, substitutions of Glu36 to Ala, Lys, or Gln all abolished sweetness. In addition, mutation of Lys6 to Asp, insertion of Arg at position 19, double insertions of Arg and Ile at position 19, and deletion of Tyr at the C-terminus all yielded compounds with taste indistinguishable from water.

4. Discussion

The present study included a considerable number of mutants from our earlier study [1] but used a different method of sensory evaluation. In the following we discuss the possible structure–sweetness relationship and the sensory methods applied in this and the earlier study. The current set of mutants

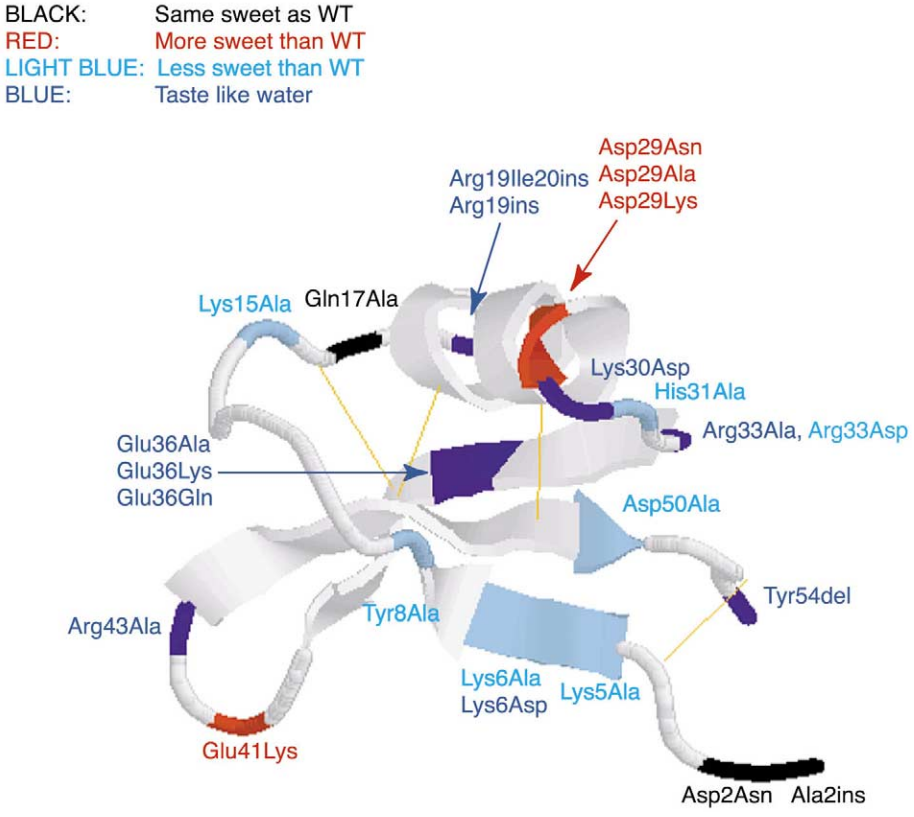

Fig. 3. Diagram showing the three-dimensional backbone of brazzein [10] with the position of mutations studied. The residues are color-coded to indicate the taste properties of mutants at these positions relative to those of WT brazzein: red, increased sweetness; black, the same sweetness; light blue, decreased sweetness; dark blue, taste equivalent to water. Intramolecular disulfide bonds are indicated as yellow lines.

is discussed in the context of the three-dimensional structure of brazzein [10]. Because brazzein's fold is constrained by four disulfide bridges and is so thermostable, it is likely that the structure determined at pH 5.2 and 22°C provides a valid structural model for brazzein at pH 7.0 and 37°C (the conditions of the sensory evaluations).

The two brazzein mutants with the largest increase in sweetness were Glu41Lys and Asp29Lys, while mutations in the immediate vicinity of these regions essentially rendered brazzein tasteless. Thus, the mutations that dramatically changed the sweetness of brazzein are localized within two regions ($AspLysHisAlaArg_{29-33}$ and $TyrAspGluLysArg_{39-43}$). This suggests that these are the critical regions of the molecule for eliciting sweetness. These two regions have been previously suggested to be important for its sweetness as determined from a comparison of the NMR solution structure of brazzein with taste analyses of 14 brazzein variants [1,10]. In the three-dimensional structure of brazzein, the region containing Arg33 is close to residues Tyr54 and Tyr51 (particularly the aromatic ring) and Arg33 itself is hydrogen-bonded to Asp50 [15]. This shows that the region containing Arg33 is in close contact with the C-terminus. Additionally, deletion of Tyr54 abolished the sweetness of brazzein almost completely [1]. Overall, these data indicate that the C-terminus is a necessary component for sweetness in brazzein.

Mutations of the negatively charged Asp29 residue, to either a neutral or positively charged residue (Asp29Ala, Asp29Asn, Asp29Lys), all markedly increased sweetness. Similar types of mutations performed at the Glu36 site (Glu36Ala, Glu36Gln, Glu36Lys) all decreased the sweetness to the level of no taste. This suggests that at these sites, charge is important for eliciting sweetness, whereas the length or orientation of the side chain plays a lesser role.

Research on thaumatin suggested that the positive charge of lysine residues is important in the determination of its sweetness [7]. Furthermore, recent computer modeling investigations of interactions between the sweet taste receptor and sweet proteins point to the importance of electrostatic complementarity between the protein and receptor [16]. The author proposed that sweet proteins activate the T1R2–T1R3 receptor by interacting with the free form II (active state) of the receptor and stabilizing it. Most of the preferred binding solutions for the docking of SCM with the receptor were centered on a large cavity of the T1R3 extracellular domain, which has an average negative charge. Complementary to it, SCM has a positive surface. Similar docking studies were performed on computer models of thaumatin and brazzein, and again it was found that the surface of the sweet protein interacting with the receptor is predominantly positive [16]. This prediction is consistent with the present results, which show that changing the negative Asp29 to a neutral (Ala, Asn) or positive (Lys) residue 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. Because its p*I* is so low (5.4), brazzein may have a higher potential than the other sweet proteins for engineering enhanced sweetness through the introduction of positive charges at critical sites.

To elucidate the effects of mutations on brazzein sweetness, it was important that accurate sensory analysis be carried out in psychophysical experiments. Methods for such evaluations in humans range from simple tasting at the 'lab bench' to quantitative measurements of gustatory sensation described in a number of textbooks and scientific articles [14,17,18]. Two different methods to evaluate sweetness on brazzein mutants were used here and in our previous study. In our earlier study we used a stepwise scale to measure sweetness and drew conclusions from the observed threshold concentrations. Here we used a well-established method of sensory analysis [14] that allowed us to convert semantic expression into a continuous function and worked with above-threshold concentrations. Furthermore, as has been demonstrated with the sweet proteins thaumatin and monellin, sweetness of high-potency compounds does not increase linearly as with sucrose, but rather non-linearly, asymptotically approaching maximal response [19]. This complicates comparison of the current results with those from our earlier study, even though 11 of the variants studied were the same.

In future studies of brazzein, it will be interesting to investigate the effects of changing Lys30, His31, and Lys42 to neutral, negative, or positively charged residues. In fact, brazzein is a sufficiently small protein so that all of its amino acids, except for the cysteines, which are involved in disulfide bridges, have some surface exposure. It may be worthwhile to systematically mutate all residues with surface exposure to thoroughly study the structure–activity relationships. Interesting mutants could be labeled with ^{15}N and/or ^{13}C for detailed NMR analysis of possible structural changes. As a by-product of these studies, brazzein variants identified to have enhanced sweet qualities could become candidates for a new generation of low-caloric natural sweeteners.

In summary, our results suggest a multi-point interaction between brazzein and its receptor in which charge plays a significant role. Our findings also suggest that residues 29–33 and residues 39–43, plus residue 36 in the peptide connecting these stretches, as well as the C-terminus are involved in determining the sweetness of brazzein.

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References

[1] Assadi-Porter, F.M., Aceti, D.J. and Markley, J.L. (2000) Arch. Biochem. Biophys. 376, 259–265.
[2] Ming, D. and Hellekant, G. (1994) FEBS Lett. 355, 106–108.
[3] Ming, D., Markley, J.L. and Hellekant, G. (1995) Pept. Res. 8, 113–114.
[4] Izawa, H., Ota, M., Kohmura, M. and Ariyoshi, Y. (1996) Biopolymers 39, 95–101.
[5] Ming, D., Hellekant, G. and Zhong, H. (1996) Acta Bot. Yunnancia 18, 123–133.
[6] Ming, D. (1994) Ph.D. thesis, 150 pp., University of Wisconsin at Madison.
[7] Kaneko, R. and Kitabatake, N. (2001) Chem. Senses 26, 167–177.
[8] Slootstra, J.W., De Geus, P., Haas, H., Verrips, C.T. and Meloen, R.H. (1995) Chem. Senses 20, 535–543.
[9] Kohmura, M., Nio, N. and Arioshi, Y. (1992) Biosci. Biotech. Biochem. 56, 1937–1942.
[10] Caldwell, J.E., Abildgaard, F., Dzakula, Z., Ming, D., Hellekant, G. and Markley, J.L. (1998) Nat. Struct. Biol. 5, 427–431.
[11] Assadi-Porter, F.M., Aceti, D.J., Cheng, H. and Markley, J.L. (2000) Arch. Biochem. Biophys. 376, 252–258.
[12] Scopes, R.K. (1974) Anal. Biochem. 59, 277–282.

[13] Pace, C.N., Vajdos, F., Fee, L., Grimsley, G. and Gray, T. (1995) Protein Sci. 4, 2411–2423.
[14] Green, B.G., Dalton, P., Cowart, B., Shaffer, G., Rankin, K. and Higgins, J. (1996) Chem. Senses 21, 323–334.
[15] DeRider, M.L. (2001) Ph.D. thesis, 135 pp., University of Wisconsin at Madison.
[16] Temussi, P. (2002) FEBS Lett. 526, 1–4.
[17] DuBois, G.E., Walters, D.E. and Kellogg, M.S. (1991) in: Sweet-taste Chemoreception (Mathlouthi, M., Kanters, J.A. and Birch, G.G., Eds.), pp. 237–267, Elsevier Applied Science, London.
[18] Bartoshuk, L.M. (2000) Chem. Senses 25, 447–460.
[19] DuBois, G.E. et al. (1991) in: Sweeteners: Discovery, Molecular Design, and Chemoreception (Walters, D.E., Orthoefer, F.T. and DuBois, G.E., Eds.), pp. 261–276, American Chemical Society, Washington, DC.