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Probing the sweet determinants of brazzein: Wild-type brazzein and a tasteless variant, brazzein-*ins*(R_{18a}-I_{18b}), exhibit different pH-dependent NMR chemical shifts ☆

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Abstract

Brazzein is a small, intensely sweet protein. As a probe of the functional properties of its solvent-exposed loop, two residues (Arg-Ile) were inserted between Leu_{18} and Ala_{19} of brazzein. Psychophysical testing demonstrated that this mutant is totally tasteless. NMR chemical shift mapping of differences between this mutant and brazzein indicated that residues affected by the insertion are localized to the mutated loop, the region of the single α-helix, and around the Cys_{16}–Cys_{37} disulfide bond. Residues unaffected by this mutation included those near the C-terminus and in the loop connecting the α-helix and the second β-strand. In particular, several residues of brazzein previously shown to be essential for its sweetness (His_{31}, Arg_{33}, Glu_{41}, Arg_{43}, Asp_{50}, and Tyr_{54}) exhibited negligible chemical shift changes. Moreover, the pH dependence of the chemical shifts of His_{31}, Glu_{41}, Asp_{50}, and Tyr_{54} were unaltered by the insertion. The insertion led to large chemical shift and pK_a perturbation of Glu_{36}, a residue shown previously to be important for brazzein's sweetness. These results serve to refine the known sweetness determinants of brazzein and lend further support to the idea that the protein interacts with a sweet-taste receptor through a multi-site interaction mechanism, as has been postulated for brazzein and other sweet proteins (monellin and thaumatin).

Keywords: Brazzein; Sweet-taste determinants; NMR spectroscopy; pK_a; Mutagenesis

Seven proteins from different plants have been found to induce or modify sweet taste in humans and Old World primates. Their primary amino acid sequences and tertiary structures differ from one another ([1,2] and references therein), and all have been found to belong to protein families that include members with other known functions. Thus, the sweet properties of each protein appear to have evolved separately. (1) Monellin from *Dioscoreophyllum cumminsii* (West Africa) is structurally related to cystatin [3–5]. (2) Thaumatin from *Thaumatococcus danielli* (West Africa) resembles plant defense proteins; one of its homologues can bind (1,3)-β-D-glucan [6]. (3) Mabinlin from *Capparis masaikai* (subtropical Yunnan, China) has sequence homology to seed storage proteins from several plants [7,8]. (4) Brazzein from *Pentadiplandra brazzeana* (Africa) has sequence and three-dimensional structural similarity to rapeseed serine proteinase inhibitors and a group of plant defensins [1,2,9]; pentadin, which is isolated from a smoke-dried form of the same fruit, probably is simply a dimeric form of brazzein [1]. (5) Curculin from *Curculigo latifolia* (Malaysia) is also a non-functional

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mannose-binding lectin [10]. (6) Miraculin from *Synsepalum dulcificum* (Africa) has a sequence similar to those of pathogen-related proteins from tomato, potato, and soybeans [11,12]. (7) Gurmarin from *Gymnema sylvestre* (tropical forests of central and southern India), which suppresses sweet taste in animals, although not a K^+ or Ca^{2+} channel blocker, has structural similarity to the spider neurotoxin δ-atracotoxin [13].

The origins of the sweetness of monellin, thaumatin, and brazzein (sweet determinants) have been studied extensively; however, a common mechanism for their action is yet to be discovered. Residues Glu_2, Asp_7, Arg_{39}, Arg_{70}, and Arg_{86} of monellin have been shown to influence its sweetness [14–16]. Mutations of these residues, especially Asp_7 and Arg_{39}, decrease the thermostability of monellin as well as lead to subtle changes in the organization and orientation of the secondary structure. It has been proposed that the orientation of the unique α-helix in monellin is responsible both for its stability and for orienting side-chains responsible for its sweetness [16]. A similar conclusion has been drawn from structural studies of a mutant of monellin (G16A) with reduced sweetness [5]. For thaumatin, the basicity of the protein has been proposed to be important for its sweetness. Modifications of lysine residues (78, 97, 106, 137, and 187), but not acidic residues, led to a substantial reduction in sweetness [17]. In brazzein, mutations of residues Asp_2, Lys_{15}, Gln_{20}, His_{31}, Arg_{33}, Glu_{36}, Arg_{43}, Asp_{50}, and Tyr_{54} have been shown to decrease the sweetness of the protein by differing extents [18–20], while reduction of negative charges at Asp_{29} and Glu_{41} led to sweeter variants [19,20]. Insertion of an arginine residue or an arginine–isoleucine dipeptide between Leu_{18} and Ala_{19} on the surface-exposed loop also significantly decreased the sweetness of the molecule [19,20]. In an early chemical modification study of brazzein, lysine residues were implicated as essential for sweetness [1]. In the above cases, the residues linked to sweetness are distributed over a large region of the surface of each protein [17], and may even include opposite sides of the molecule [18–20]. A conserved "sweet finger" hypothesis is receiving less support, since engineered peptides failed to show sweetness [21]. Therefore, a "multi-point" interaction between these sweet-taste proteins and their target appears more plausible.

Recently, a candidate for the human sweet-taste receptor, T1R3, has been discovered [22,23]. The gene, which encodes a G-protein-coupled receptor, functions as a heterodimer with T1R2 for recognition of diverse sweeteners [22,24,25]. The presence of different binding sites for sweeteners on the sweet-taste receptor has been demonstrated [26]; this result is consistent with the "multi-point" interaction hypothesis mentioned above. A common cysteine-rich region on T1R3 has been discovered to be responsible for sensing the sweet taste of monellin, brazzein, and thaumatin [27].

brazzein	C_{16}-Q_{17}-L_{18}————A_{19}-N_{20}-Q_{21}-C_{22}
brazzein-ins(R_{18a})	C_{16}-Q_{17}-L_{18}-R_{18a}——A_{19}-N_{20}-Q_{21}-C_{22}
brazzein-ins(R_{18a}-I_{18b})	C_{16}-Q_{17}-L_{18}-R_{18a}-I_{18b}-A_{19}-N_{20}-Q_{21}-C_{22}

Fig. 1. Sequence of the solvent-exposed loop of brazzein [1], which is bounded by two cysteines, and the residues inserted in the two variants discussed here: brazzein-*ins*(R_{18a}) and brazzein-*ins*(R_{18a}-I_{18b}). The inserted residues are shown in bold letters.

We describe here the detailed investigation of a fully tasteless variant of recombinant brazzein,[1] brazzein-*ins*(R_{18a}-I_{18b}), produced by inserting two amino acid residues (Arg-Ile) into the solvent-exposed loop between Leu_{18} and Ala_{19} (Fig. 1). In rapeseed-type proteinase inhibitors (29–30% sequence identity with brazzein) this loop is enlarged by three residues (Arg-Ile-Tyr, or Arg-Ile-Phe) and constitutes the reactive site (site of proteinase interaction). Thus, brazzein-*ins*(R_{18a}-I_{18b}) contains the consensus dipeptide of the expanded reactive-site loop of the proteinase inhibitors. CD and NMR analyses of brazzein-*ins*(R_{18a}-I_{18b}) have indicated that the protein is properly folded; however, the protein is not sweet, not even when tasted in powder form [19,20,28]. Experiments described here indicate that the effects of the dipeptide insertion are linked to changes in the region of the Cys_{16}–Cys_{37} disulfide bond. These changes affect the chemical shifts as well as the pK_a value of Glu_{36}, a residue whose mutation to Ala results in a tasteless brazzein variant [19,20].

Materials and methods

Proteins. Synthetic genes coding for brazzein, brazzein-*ins*(R_{18a}), and brazzein-*ins*(R_{18a}-I_{18b}), each with an extension coding for an N-terminal fusion with staphylococcal nuclease linked by an engineered cyanogen bromide cleavage site, were overexpressed in *Escherichia coli* under the control of T7 promoter as described previously [28,29]. The fusion proteins were isolated from inclusion bodies. Following cleavage by cyanogen bromide, isolation, and purification, brazzein, brazzein-*ins*(R_{18a}), and brazzein-*ins*(R_{18a}-I_{18b}) were judged to be fully refolded on the basis of analysis by circular dichroism (CD), reverse-phase HPLC (RP HPLC), and 1D ^{1}H NMR spectroscopy [28,29]. Note that "brazzein" refers here to recombinant wild-type brazzein, which is identical to the minor (sweeter) form of the protein isolated from the fruit of *P. brazzeana* Baillon; the major (less sweet) form of the protein contains an additional N-terminal pyroglutamate residue. The residue numbering scheme used here is that of the longer protein;

[1] *Abbreviations:* ATT, *Arabidopsis thaliana* gene coding for a protein proteinase inhibitor (GenBank Accession No. Z46816); "brazzein" is used here to represent recombinant protein with sequence identical to the minor form of the sweet protein isolated from the fruit of *Pentadiplandra brazzeana* Baillon, which lacks the N-terminal pyroglutamate residue of the major form of the sweet protein (the peptide numbering system used here is that for the longer protein, hence the N-terminal residue of brazzein is designated as residue 2); brazzein-*ins*(R_{18a}-I_{18b}), brazzein variant containing the dipeptide Arg-Ile inserted between residues Leu_{18} and Ala_{19}; brazzein-*ins*(R_{18a}), brazzein variant containing arginine inserted between residues Leu_{18} and Ala_{19}.

thus the N-terminal residue of recombinant wild-type brazzein is residue 2.

NMR spectroscopy. Data were collected at the National Magnetic Resonance Facility at Madison. 2D homonuclear TOCSY data [30] were collected on a Bruker DMX 500 MHz spectrometer equipped with a triple-resonance {^{13}C,^{15}N} ^{1}H probe and triple axis pulse field gradient. The temperature was regulated at 310 K. Samples contained 10 mg/mL protein, 100 mM KCl, and 0.1 mM DSS dissolved in 90% H_2O/10% D_2O. The pH meter (Corning G-P micro combo electrode, Corning, NY) was calibrated with two buffers (at pH 1.0, 4.0, 7.0, or 10.0) spanning the region of interest. The pH was adjusted to the desired value with HCl and KOH, and the pH reading was recorded before and after the experiment. The differences between these values were usually less than 0.04 pH units. Data were processed with XWIN-NMR software version 2.60 (Bruker BioSpin, Billerica, MA). Chemical shifts were referenced to internal DSS as 0 ppm.

Determination of pK_a values. Samples used for pH titration studies contained no buffer and 2 M KCl was added to a final concentration of 100 mM to maintain constant salt content. The pH dependencies of chemical shifts were analyzed by non-linear least-squares curve fitting to a modified form of the Henderson–Hasselbalch equation [31]:

$$\delta_{\text{obs}} = \delta_{\text{B}} + \sum_i \frac{\Delta_i \times 10^{n(\text{pH}-\text{p}K_{\text{a}})}}{1 + 10^{n(\text{pH}-\text{p}K_{\text{a}})}}, \quad (1)$$

where δ_{obs} is the observed chemical shift in ppm, δ_{B} is the chemical shift of the deprotonated form, Δ is the chemical shift difference between deprotonated and protonated forms, i indicates the number of titrating groups involved, and n is the Hill coefficient, which reflects the degree of cooperativity. For titration data that showed a single protonation event ($i = 1$), we used a UNIX shell script to fit the data (generous gift from Dr. Henrik W. Anthonsen); for cases of $i > 1$, the program SigmaPlot was used to fit the data to a non-interaction model.

Results and discussion

Chemical shift assignments and comparisons

^{1}H chemical shift assignments for the major form of brazzein from fruit (the form in which the first residue is pyroglutamate) have been reported [32] and are available (BMRB accession code 4067) for two different pH values (pH 3.7 and 5.2) and two different temperatures (298 and 310 K). Complete assignments for recombinant wild-type brazzein at pH 5.2 and 310 K have been reported by Dr. M.L. DeRider (BMRB accession 5230) [33]. The chemical shift assignments determined here for brazzein and brazzein-*ins*(R_{18a}-I_{18b}) at pH 4.9 and 310 K are included in Appendix A. Assignments for brazzein were achieved by following the previously published data at pH 5.2 and 310 K. Overlapping peaks could be resolved in most cases by examining a series of spectra collected at different pH values. For assignments of brazzein-*ins*(R_{18a}-I_{18b}), we used the minimum perturbation method; in ambiguous situations, residues farthest from the mutation sites were assumed to have the smallest chemical shift differences [34]. In data from both brazzein and brazzein-*ins*(R_{18a}-I_{18b}), no signals could be found corresponding to Pro_{12} (the only proline residue) or Asp_2 or Lys_3 (the two N-terminal residues). In spectra of brazzein-*ins*(R_{18a}-I_{18b}), no signal was found corresponding to Asp_{25}; signals assigned to this residue in brazzein were weak. Stereospecific assignments were inferred from the results of Caldwell et al. ([32], BMRB accession 4067).

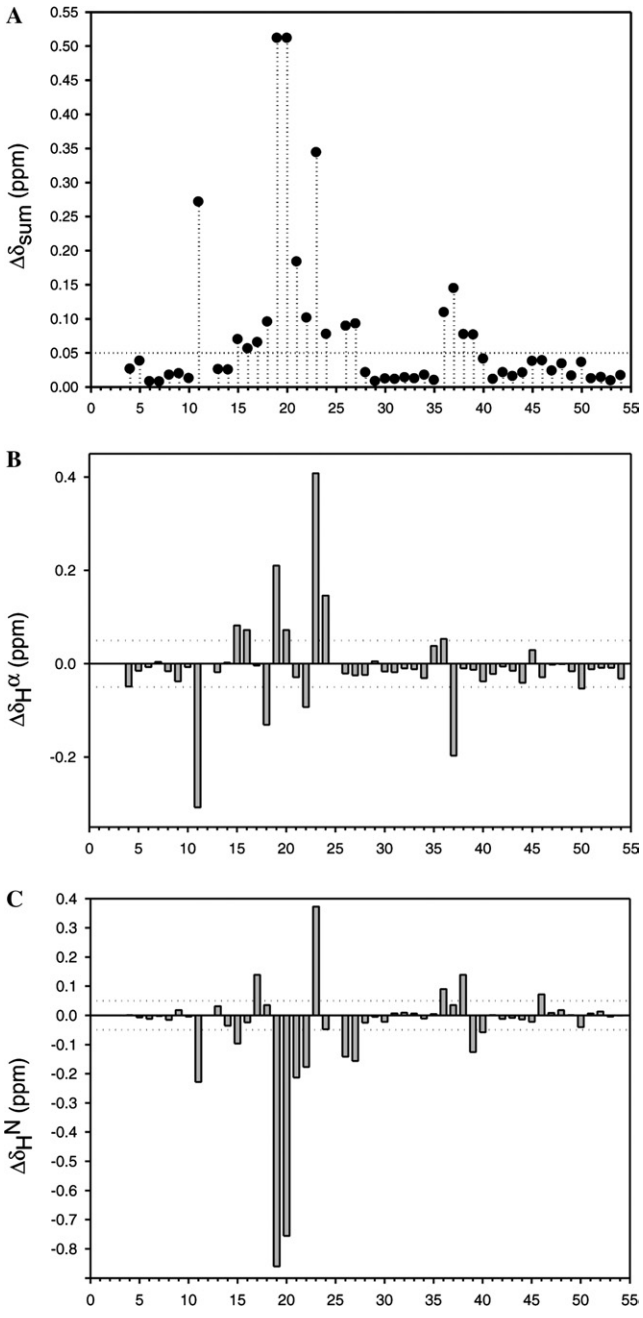

Fig. 2. Differences in chemical shifts for various atoms of brazzein and brazzein-*ins*(R_{18a}-I_{18b}) plotted as a function of the residue number: (A) cumulative chemical shift differences for all observed resonances in the residue (defined in Materials and methods); (B) $^{1}H^{\alpha}$ chemical shifts; and (C) $^{1}H^{N}$ chemical shifts. The dotted lines indicate a chemical shift difference of ±0.05 ppm. (The difference in $^{1}H^{N}$ and $^{1}H^{\alpha}$ is calculated as ($\delta_{\text{ins}} - \delta_{\text{wt}}$), where ins is brazzein-*ins*(R_{18a}-I_{18b}) and wt is recombinant brazzein.)

The aggregate chemical shift difference at residue j ($\Delta\delta_j$) [35] between brazzein and brazzein-*ins*(R_{18a}-I_{18b}) was compared and calculated from Eq. (2):

$$\Delta\delta_j = \sqrt{\frac{\sum_{i=1}^{n}\Delta\delta_{ij}^2}{n}}, \quad (2)$$

where n is the number of chemical shifts used for comparison at residue j (usually those from $^1H^N$, $^1H^\alpha$, and side-chain proton resonances if available) and $\Delta\delta_i$ is the chemical shift difference for a particular atom within residue j; $\Delta\delta_i = \delta_{i\,\mathrm{mut}} - \delta_{i\,\mathrm{wt}}$ (mut represents brazzein-*ins*(R_{18a}-I_{18b}) and wt represents recombinant brazzein). The results are shown in Fig. 2A. Differences in the

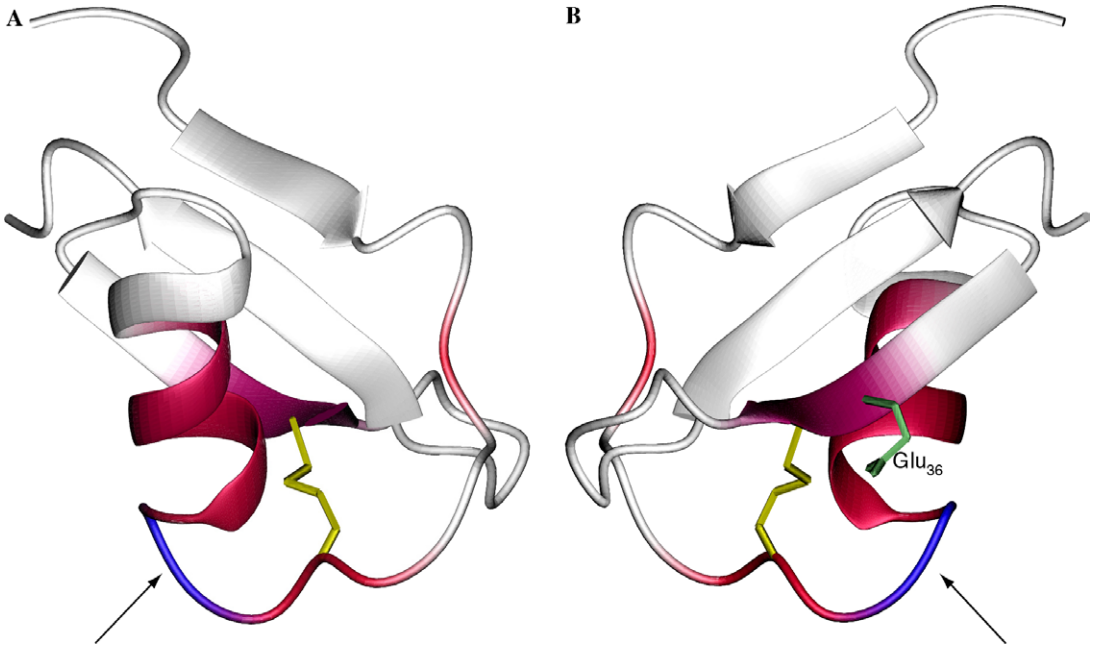

Fig. 3. Residues that are colored red or blue on the ribbon diagram (mapped onto the structure of brazzein [2], PDB code 2BRZ) are those that exhibit chemical shift differences between brazzein and brazzein-*ins*(R_{18a}-I_{18b}) > 0.05 ppm. Residues Leu_{18} and Ala_{19} are colored blue and the arrow indicates the insertion point. The Cys_{16}–Cys_{37} disulfide bond is shown in yellow. The side chain of Glu_{36} is shown in green. The two figures (A,B) are related by 180° rotation about an axis parallel to the paper.

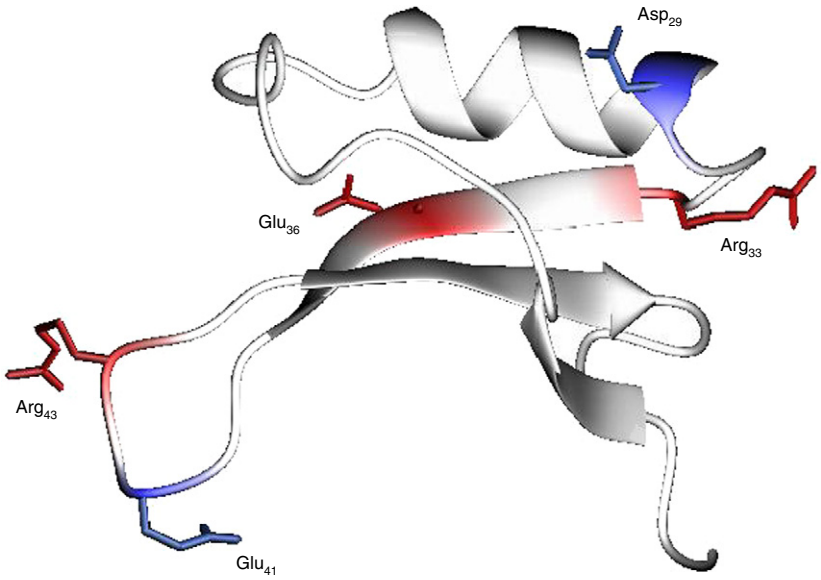

Fig. 4. Residues crucial for the sweet taste of brazzein [2] (PDB code 2BRZ). Substitutions of residues colored in red reduce the sweetness of brazzein. Substitutions of residues colored in blue increase the sweetness of brazzein.

$^1H^N$ and $^1H^\alpha$ chemical shifts are also shown (Figs. 2B and C).

The cumulative chemical shift differences for most residues common to brazzein and brazzein-*ins*(R_{18a}-I_{18b}) were less than 0.05 ppm, which is close to the experimental error. Residues with larger $\Delta\delta_j$ values were 11, 15–27 (excluding 25, which as noted above was unassigned in brazzein-*ins*(R_{18a}-I_{18b})), and 36–39. We have mapped these residues onto the tertiary structure of brazzein (PDB code 2BRZ) (Fig. 3). They can be classified into two categories: (1) residues near the Cys_{16}–Cys_{37} disulfide bond: Tyr_{11}, Lys_{15}, Cys_{16}, Gln_{17}, Leu_{18}, Ala_{19}, Asn_{20}, Glu_{36}, Cys_{37}, Phe_{38}, and Tyr_{39}; and (2) residues in the α-helix: Gln_{21}, Cys_{22}, Asn_{23}, Tyr_{24}, Asp_{25}, Cys_{26}, and Lys_{27}. The chemical shifts of several residues previously found to be implicated in the sweetness of brazzein showed negligible $\Delta\delta_j$ values; these are His_{31}, Arg_{33}, Glu_{41}, Arg_{43}, Asp_{50}, and Tyr_{54}, each of which when mutated to Ala resulted in decreased sweetness, and, Asp_{29} and Glu_{41}, each of which when mutated to Ala, led to increased sweetness [19,20]. Lys_{15}, which was previously found to decrease the sweet taste of brazzein when altered [18], was moderately affected, with $\Delta\delta = 0.069$ ppm (cumulative chemical shift difference based on chemical shifts from $^1H^N$, $^1H^\alpha$, $^1H^\beta$s, $^1H^\gamma$s, $^1H^\delta$s, and $^1H^\varepsilon$s). Asp_2, which when substituted with Asn led to a 10-fold decrease in sweetness [18], was not assigned in this study; therefore, the effect on this residue in brazzein-*ins*(R_{18a}-I_{18b}) could not be assessed.

pK_a values

Groups with unusual pK_a values have been linked to active sites of enzymes [36], ligand-binding sites [37,38], and regions of protein–protein interaction [39]. Abnormal pK_a values may result from one or more of the following factors: burial of ionizable groups, charge–charge interactions, or complex formation [40]. Because four residues with titratable side chains (His_{31}, Glu_{36}, Asp_{50}, and Tyr_{54}) had been implicated previously in the sweetness of brazzein [18], it was of interest to determine whether the pH titration properties of brazzein are altered in the tasteless mutant. The results (Table 1) indicated no significant differences in pK_a values between brazzein and brazzein-*ins*(R_{18a}-I_{18b}) for residues His_{31}, Glu_{41}, Asp_{50}, and Tyr_{54}, but significant changes in the pK_a values of residues Asp_{29} and Glu_{36}.

The pH dependence of the chemical shifts of the $H^{\delta2}$ and $H^{\varepsilon1}$ protons of His_{31} can be fitted to a single protonation step in both proteins. The pK_a of His_{31} derived from these data was the same within experimental error in both brazzein (7.25–7.28) and brazzein-*ins*(R_{18a}-I_{18b}) (7.28); both values were within the normal range for histidine (6.0–7.0) [41]. The structure of brazzein shows that no other ionizable group is within 6 Å of the ring atoms of this histidine.

The pH dependence of the chemical shifts of the two methylene protons of Asp_{50} yielded separate pH_{mid} values (Table 1). However, the values were the same within experimental value for brazzein and brazzein-*ins*(R_{18a}-I_{18b}): 4.7 ± 0.1 from the titration curve of $^1H^{\beta2}$ and 3.6 ± 0.1 from the titration curve of $^1H^{\beta3}$.

The lower value, which is within the normal pK_a range for Asp side chains (3.5–4.3) [42], probably represents the pK_a of Asp_{50}; the higher value likely results from a spectroscopic perturbation from another titrating group.

The amide proton of Tyr_{54}, the C-terminal residue, exhibited two resolvable pH_{mid} values (Table 1). Fitting of these data to a two-proton dissociation model yielded pH_{mid} values of 2.5 and 5.1 for brazzein, and 2.5 and 5.2

Table 1
Summary of pK_a values derived from the pH dependence of the chemical shifts of the atoms indicated in brazzein and brazzein-*ins*(R_{18a}-I_{18b})

Residue	Atom observed	Brazzein			Brazzein-*ins*(R_{18a}-I_{18b})		
		pK_a	$\Delta\delta$ (ppm)	Hill coefficient	pK_a	$\Delta\delta$ (ppm)	Hill coefficient
His_{31}	$H^{\delta2}$	7.29 ± 0.01	0.93	0.87 ± 0.01	7.28 ± 0.01	0.91	0.95 ± 0.02
	$H^{\varepsilon1}$	7.25 ± 0.02	0.38	0.92 ± 0.03	7.28 ± 0.05	0.35	0.96 ± 0.06
Glu_{41}	H^N	4.12 ± 0.01	0.48	1.19 ± 0.03	4.04 ± 0.02	0.58	0.98 ± 0.03
	$H^{\gamma2}$	3.99 ± 0.03	0.24	0.81 ± 0.05	4.12 ± 0.03	0.16	1.60 ± 0.17
Asp_{50}	$H^{\beta2}$	4.61 ± 0.08	0.16	0.66 ± 0.08	4.83 ± 0.06	0.13	0.91 ± 0.10
	$H^{\beta3}$	3.64 ± 0.10	0.47	0.45 ± 0.04	3.72 ± 0.05	0.44	0.52 ± 0.03
Glu_{53}	$H^{\gamma2}$	4.80 ± 0.09	0.13	0.63 ± 0.05	4.68 ± 0.06	0.13	1.06 ± 0.13
	$H^{\gamma3}$	4.86 ± 0.06	0.19	0.87 ± 0.09	4.74 ± 0.05	0.18	1.14 ± 0.12
Glu_{53}	H^N	2.81 ± 0.10	0.17	0.68 ± 0.07	2.90 ± 0.12	0.15	0.59 ± 0.08
	H^N	4.83 ± 0.05	0.16	0.68 ± 0.04	5.07 ± 0.04	0.15	0.64 ± 0.14
Tyr_{54}	H^N	2.55 ± 0.03	0.58	1.13 ± 0.05	2.48 ± 0.03	0.54	1.27 ± 0.08
	H^N	5.13 ± 0.04	0.19	0.91 ± 0.06	5.25 ± 0.06	0.19	0.88 ± 0.10
Asp_{29}	H^N	2.83 ± 0.04	0.33	0.77 ± 0.04	3.34 ± 0.07	0.28	0.82 ± 0.08
	$H^{\beta3}$	3.32 ± 0.03	0.23	0.94 ± 0.06	3.90 ± 0.05	0.15	1.21 ± 0.15
Glu_{36}	$H^{\gamma2}$	4.18 ± 0.03	0.28	0.80 ± 0.04	4.49 ± 0.05	0.20	0.94 ± 0.11

$\Delta\delta$ is the chemical shift difference (in ppm) between the deprotonated (basic) and protonated (acidic) forms.

for brazzein-*ins*(R_{18a}-I_{18b}). These values are identical within experimental error. The lower value for each protein (2.5) is near the normal range for α-carboxyl groups (3.5–4.3, depending on the amino acid type [42]) and is close to that of the carboxylate of free tyrosine (2.2 [41]); thus it is assigned to the carboxyl group of Tyr_{54}. Amide proton chemical shifts are sensitive to changes in the electronic environment [43] and frequently reflect long-range interactions with ionizable groups in proteins [44]. To investigate the origin of the higher pK_a sensed by the Tyr_{54} H^N, we inspected the structure of brazzein (PDB code 2BRZ) for nearby ionizable groups and found that the $O^{\varepsilon 1}$ atom of Glu^{53} is ~3.8 Å from the amide proton of Tyr_{54}. Analysis of the pH dependencies of the amide proton of Glu^{53} in brazzein and brazzein-*ins*(R_{18a}-I_{18b}) (Fig. 4) revealed biphasic titration curves, reciprocal to those of the Tyr_{54} $^1H^N$. Fitting of these curves to two independent dissociations yielded pH_{mid} values of 2.8–2.9 and 4.8–5.0, with very little difference between brazzein and brazzein-*ins*(R_{18a}-I_{18b}). The lower value (2.8–2.9) is assigned to a spectroscopic perturbation of Glu^{53} from the protonation of Tyr^{54} (p$K_a \approx 2.5$), and the higher value (4.8–5.0) is assigned to the actual pK_a of Glu_{53}.

When Asp_{29} was substituted by Asn or Ala, the sweetness of brazzein increased [19,20]. We have found abnormally low pK_a values for the Asp_{29} in wild-type brazzein (Table 1): 3.32 from the pH dependence of the chemical shift of $^1H^{\beta 3}$ and 2.83 from the pH dependence of $^1H^N$. The lower values, which are outside the normal pK_a range for Asp side chains (3.5–4.3) [42], are attributed to the interaction with Tyr_8 as judged by the side-chain proximity of the two residues. In brazzein-*ins*(R_{18a}-I_{18b}), the pH dependence of Asp_{29} signals yielded a more normal pK_a (Table 1). Since changes at Asp_{29} correlate with increased sweetness, rather than decreased sweetness, we conclude that the changes observed here probably do not account for the loss of sweetness of brazzein-*ins*(R_{18a}-I_{18b}).

Substitutions at Glu_{36}, however, significantly decrease the sweetness of brazzein. The aggregate chemical shift difference is 0.10 ppm, which is above experimental error. The pK_a derived from the pH dependence of Glu_{36} $^1H^{\gamma 2}$ is significantly lower in brazzein (4.18) than in brazzein-*ins*(R_{18a}-I_{18b}) (4.49) (Table 1).

Conclusions

In this study, we have used NMR spectroscopy to investigate changes in the structure and chemical properties of brazzein resulting from a two-residue insertion in an exposed loop of the protein. Insertion of a single residue in this loop to produce brazzein-*ins*(R_{18a}) results in a protein with sweetness reduced by a factor of about 2 [18] or more [19,20]. Insertion of two residues to produce brazzein-*ins*(R_{18a}-I_{18b}) results in a totally tasteless protein. Rapeseed proteinase inhibitors contain three residues inserted at this position (Arg-Ile-Tyr or Arg-Ile-Phe). None of these inhibitors has been reported to be sweet, and a recombinant inhibitor identical to ATT from *Arabidopsis thaliana* has been tested and found to be fully tasteless [28].

Residues outside this loop have been shown previously to influence the sweetness of brazzein [18–20]. Thus, it was of interest to investigate whether the loss of sweetness from the two-residue insertion could be explained by an indirect effect on one or more of these residues. The methods used here, chemical shift mapping and analysis of the pH dependence of chemical shifts, showed clearly that the two-residue insertion has affected residues in close spatial proximity to the Cys_{16}–Cys_{37} disulfide bond, especially Glu_{36}, which previously has been shown to be critical to brazzein's sweetness. The other two critical residues, Arg_{33} and Arg_{43}, are unaffected as determined by the parameters measured here (Fig. 4). The results reported here serve to better define the parts of the brazzein molecule that are essential for sweetness and lend further support to the hypothesis that brazzein, like other sweet proteins, is recognized by a sweet-taste receptor through a multi-point interaction [17,18].

Acknowledgments

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Appendix A. Supplementary data

Chemical shift assignments of recombinant wild-type brazzein and variant brazzein-*ins*(R_{18a}-I_{18b}) are described in Tables S1 and S2, respectively. Sample pH values are given in Table S3. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2005.07.049.

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