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Mutagenesis of Critical Amino Acid Residues in α-Helix and β-Sheet Structures of Brazzein

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There is a high demand for non-calorigenic, protein-based sweeteners with favorable taste properties. The optimal design of such sweeteners requires knowledge of structure-function relationships and identification of chemical entities that trigger the sweetness response. Brazzein is an intensely sweet-tasting plant protein with good stability at high temperatures and over a wide pH range. Brazzein, with a molecular mass of 6.5 kDa, is the smallest naturally occurring, sweet-tasting protein described to date.[1,2]

The three-dimensional structures of brazzein have been determined using nuclear magnetic resonance (NMR) spectroscopy, and brazzein contains one short α-helix (residues 21-29) and three strands of an antiparallel β-sheet (strand I, residues 5-7; strand II, residues 44-50; strand III, residues 34-39).[3,4] According to earlier studies, charge and polar residues appear to be critical for the sweetness of thaumatin and monellin.[5-7] A previous chemical modification study suggested that the surface charge of the molecule is important, which led to the conclusion that arginine, lysine, tyrosine, histidine, aspartic acid, and glutamic acid are important for brazzein's sweetness.[8]

A number of site-directed mutagenesis studies have been conducted, and suggest a number of residues in brazzein that may be important for interaction with the sweet taste receptor. Assadi-Porter *et al*.[9] suggested that two surface areas are involved in the sweetness of brazzein. Modeling studies have suggested that brazzein binds to the open form of T1R2 in the sweet taste receptor, T1R2-T1R3 heterodimer.[10,11] Although several investigations of brazzein have been performed, the critical molecular region and sweetness elicitation mechanism of brazzein is still not well understood.

In the present study, to better understand the molecular determinants of brazzein's sweetness, we made four mutations of the three putative interaction sites (at position 6, 29, and 36) in the secondary structures (α-helix and β-sheets) of brazzein using site-directed mutagenesis (Table 1 and Fig. 1). We employed the sequence of the minor form as the basis for designing the brazzein mutants because the minor form, which lacks the *N*-terminal pyroglutamate, possesses twice the sweetness of the major form. The brazzein mutants were expressed in *E. coli* under the control of a T7 promoter and efficiently produced in the soluble, active form into the periplasm in amounts of approximately 80-90% of the total periplasmic proteins. The secretion of brazzein, having four intramolecular disulfide bonds in the periplasmic space in *E. coli*, has a better chance of proper folding due to the increased oxidizing conditions of this extracellular compartment. Moreover, production into the periplasmic space can also facilitate purification. For these reasons, the expressed brazzein mutants were isolated and purified to electro-

Table 1. Residues selected for site-directed mutagenesis and substituted residues

Position	Residue Substitution		Primer	Residue Character	
	Before	After		Before	After
6	Lys	Arg	tgc aaa **cgt** gtt tac	positive	positive
29	Asp	Lys	aag ctt **aa**a aag cat	negative	positive
		Arg	aag ctt **aga** aag cat		positive
36	Glu	Asp	tct gga ga**t** tgc ttt	negative	negative

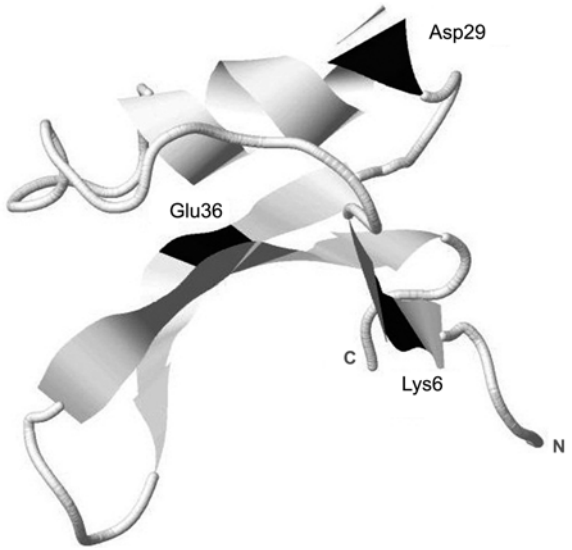

Figure 1. Diagram showing the three-dimensional backbone of brazzein and the position of the mutations studied. The amino acid residues mutated in this study are shown in blue.

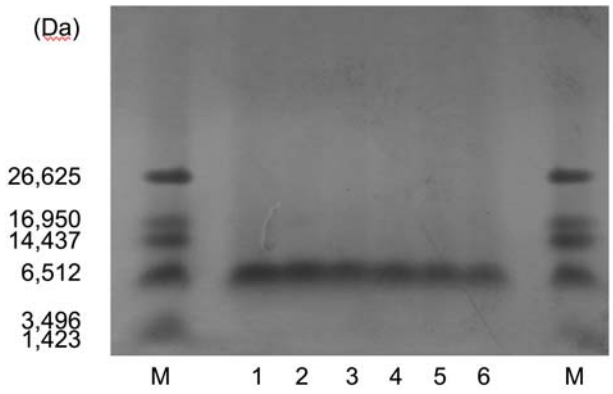

Figure 2. SDS-PAGE analysis. Lane M, molecular weight standard marker; lane 1, purified recombinant wild-type brazzein; lane 2, Brazzein(Met-); lane 3, K6R mutant; lane 4, D29K mutant; lane 5, D29R mutant; lane 6, E36D mutant.

Table 2. Comparison of the sweet taste properties of sucrose, wild type-brazzein and brazzein mutants

Sweet tasting molecule	Molecular mass (Da)	Experimental taste threshold		Sweetness in comparison to sucrose	
		(g(100 mL)$^{-1}$)	(μM)	(g/g)	(molecule)
Sucrose	342.3	2.0	58,000	1	1
WT-Brazzein	6353	0.0025	3.94	800	14,848
Brazzein(Met-)	6204	0.0011	1.77	1840	33,349
Lys6Arg	6383	0.0049	7.64	410	7,645
Asp29Lys	6368	0.0008	1.26	2390	46,032
Asp29Arg	6396	0.0009	1.41	2210	41,135
Glu36Asp	6341	0.0006	0.95	3310	61,053

phoretic homogeneity through extraction of the periplasmic fraction and thermal treatment. The brazzein mutants were purified to yield approximately 3.0-17.0 mg/L of purified brazzein, the purity and conformational state mutants of which were confirmed using SDS-PAGE and HPLC. The purified brazzein mutants appeared as a single band on SDS-PAGE with an apparent M_r of 6,500 Da (Fig. 2); the elution times for the folded brazzein mutants were 11.0 ± 0.5 min, as denoted by RP-HPLC.

The taste results of the four mutants at the putative interaction sites in the secondary structures (α-helix and β-sheets) of brazzein are compared with that of the wild-type in Table 2 and Figure 3. To elucidate the role of Lys6 in β-strand I (residues 5-7) of brazzein, we made Lys6Arg mutants in order to increase the size of the side chain while maintaining the charge. The mutation of Lys6 to Arg caused a notable reduction in sweetness. Jin *et al.*[1] reported that Lys6Ala and Lys6Asp replacements result in decreased sweetness. Based on these results, we suggest that the charge and the length of the side chain at position 6 in brazzein are important for eliciting sweetness, and only the Lys residue at position 6 allows brazzein to maintain full sweetness.

To further study the role of Asp29 in only one α-helix structure (residues 21-29) of brazzein, we also made Asp29Lys and Asp29Arg mutants, changing negatively charged residues

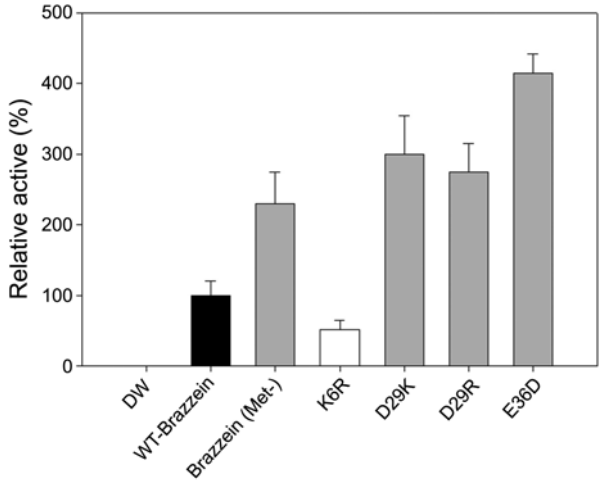

Figure 3. Taste evaluation results of a pair-wise comparison of four new brazzein mutants with wild-type brazzein (WT-Brazzein) and minor-type brazzein (Brazzein(Met-)). Data were averaged for 20 subjects. Error bars represent the standard error of the mean. The black column indicates the wild-type brazzein. The gray columns indicate more sweetness than the wild-type brazzein, whereas the white columns indicate less sweetness.

to positively charged residues. Mutations of Asp29 to Lys or Arg increased sweetness (Table 2 and Fig. 4). Among these mutations, the activity for sweetness of the Asp29Lys mutant was approximately 1.4-fold higher than that of the wild-type brazzein. The three-dimensional structure of brazzein on NMR spectroscopy indicated that is Asp29 located at the C-terminal of one short α-helix structure (residues 21-29) in brazzein, where it may generate a negative electrostatic potential.[3] Jin *et al.*[1] reported that mutations of this residue to a neutral residue (Asp29Ala and Asp29Asn) markedly increase sweetness. A modeling study of the brazzein-receptor interaction indicated that Asp29 of brazzein was found to be in close proximity to Glu178 of the T1R3 component of the receptor, suggesting potentially repulsive interaction between charged side chains of brazzein and the receptor.[10] These results suggest that at this site, charge is important for eliciting sweetness, whereas the length or orientation of the side chain plays a lesser role.

One dramatic change in sweetness occurred with a mutant at the Asp36 residue. The mutation of Glu36 to Asp significantly increased sweetness and this mutant proved to be approximately 3.5-fold sweeter than the wild-type brazzein (Table 2 and Figure 3). In this case, the three-dimensional structure of brazzein according to NMR spectroscopy showed that Glu36 is located in the β-strand III (residues 34 to 39) near the C-terminus, suggesting a direct interaction with the sweet receptor.[9] Jin *et al.*[1] reported that mutation of this residue to a neutral residue (Ala, Gln) or a positively charged residue (Lys) decreases sweetness such that the mutant is rendered tasteless. In addition, significant contributions to brazzein's sweetness were also made by nearby residues 29-33 and 39-43, in addition to residue 36 between these stretches and the *N*- and *C*-terminal regions.[1,2,12] From these results, we surmise that the charge and length of the

side chains at position 36 in brazzein are important for eliciting sweetness.

In summary, to identify critical residues important for sweetness of the sweet protein brazzein, four forms of brazzein with mutations at residues (Lys6, Asp29 and Glu36) in the α-helix and β-sheet structures were constructed by site-directed mutagenesis, and the effects of the mutations were evaluated by a human taste panel. Mutations of Lys6 to Arg in β-strand I (residues 5-7) significantly decreased sweetness. Conversely, the mutations of Asp29 in the C-terminus of one short α-helix (residues 21-29) to Lys or Arg and Glu36 in β-strand III (residues 34-39) to Asp significantly increased sweetness. Particularly, the sweetness of the Glu36Asp mutant was approximately 3.5-fold higher than that of the wild-type brazzein, indicating that the negative charge and the length or orientation of the side chain of the amino acid at position 36 are important for eliciting sweetness. We infer that the Glu36 residue in the β-strand III of brazzein may be an important determinant of the molecule's sweetness. In future studies of brazzein, it would be interesting to investigate the structural differences among brazzein mutants using NMR techniques and structure-activity relationships. Moreover, it would be worthwhile to investigate brazzein mutant binding properties to T1R2-T1R3 receptors using X-ray crystallography.

Experimental Section

Materials. The pET-26b(+) expression vector and *E. coli* strain BL21 Star (DE3) used in this study were supplied by Novagen (Madison, WI, USA). Restriction enzymes, the Mutant^{TM}-Super Express Km Kit, and DNA-modifying enzymes were obtained from Takara Shuzo (Otsu, Shiga, Japan). The IPTG and kanamycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The synthesis of DNA primers for mutagenesis was performed by COSMO Genetech (Seoul, Korea). All chemicals and reagents used were commercially available and of the highest reagent grade.

Preparation of Brazzein Mutants by Site-directed Mutagenesis. Wild-type brazzein and Brazzein(-Met), the latter lacking an *N*-terminal methionine, were obtained by expression of the synthetic gene based on the amino acid sequence of naturally occurring brazzein[13] in *E. coli*, as described in our previous paper.[14] The oligonucleotide primers used for site-directed mutagenesis of brazzein are shown in Table 1. Mutagenesis was performed according to the Mutant^{TM}-Super Express Km Kit protocol (Takara Shuzo). Construction of the DNA template for mutagenesis, confirmation of mutation, and construction of the expression plasmid of the mutants were performed as described in a previous paper.[15] The resulting vectors of the mutant proteins were transformed into *E. coli* strain BL21 Star (DE3).

Overexpression and Purification of Brazzein Mutants. The overexpression and purification of the mutant proteins were performed as previously described.[14] Protein concentrations for particular mutants were determined based on the absorbance at 205 and 280 nm, as brazzein lacks tryptophan. The extinction coefficient (ε_{205}) of each brazzein mutant was calculated from measurements of the absorbance of solutions at 205 and 280 nm, according to the following formula[16]: $\varepsilon_{205}^{1.0\ mg/mL} = 27.0 + 120\ (A_{280}/A_{205})$.

Analysis of the Sweetness Properties of Brazzein Mutants. The sweet-tasting activity of the wild-type and mutant proteins were assayed by sensory analysis using a double-blind taste test with 20 individuals. The purified proteins were lyophilized and dissolved in water (1.0 mg/mL). The brazzein solutions were prepared in concentrations ranging from 0.1 to 50.0 μg/mL. A 1% sucrose solution was prepared for comparison since 1% sucrose is the lowest concentration detectable by humans. The taste panel consisted of ten females and ten males of reported good health and normal sense of taste. Two-hundred-microliter samples were applied to the anterior part of the tongue. The mouth was rinsed with tap water after each test. The panel tasted the samples in order of increasing concentration and each taster indicated the first sample in which they could detect sweetness. Sweetness potencies were reported relative to sucrose on a molar basis.

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