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Design and Evaluation of Synthetic Peptides Corresponding to the Sweetness Loop of the Sweet-Tasting Protein Brazzein

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Humans recognize five tastes, namely, sweet, bitter, sour, salty, and umami, through taste buds on the tongue. These five tastes give organism essential information with which to evaluate the nutritional components of food, as well as prevent the intake of toxic substances. The desirable tastes of umami and sweetness promote consumption of nutritive food. On the other hand, bitterness and sourness alert organisms to toxins and promote rejection of harmful foods. Identifying sweet-tasting foods is particularly important as it provides organisms with a means to seek out carbohydrates with high nutritive value.[1] However, excessive intake of carbohydrate sweeteners causes diabetes and obesity. For these reasons, the demand for non-calorigenic protein-based sweeteners with favorable taste is of high priority.

The optimal design of new sweeteners requires knowledge of the interaction between protein-based sweeteners and the sweet taste receptor T1R2/T1R3.[2] To date, only eight sweet-tasting proteins have been known to elicit sweetness.[3] Among them, brazzein is the smallest sweet-tasting protein, with a molecular mass of 6.5 kDa. This protein was isolated from the fruit of the West African plant *Pentadiplandra brazzeana* Baillon.[4] Brazzein has been most well-known and well-characterized for its relationship between structure and sweetness.[2] NMR studies on the three-dimensional (3-D) structures of brazzein have revealed that brazzein contains one short α-helix (residues 21–29) and three strands of antiparallel β-sheets (strand I, residues 5–7; strand II, residues 44–50; strand III, residues 34–39).[5] Previously, we have constructed 15 mutants of residues in the flexible loops and the α-helix and β-sheet structures of brazzein using site-directed mutagenesis.[6,7] Our studies suggested that the His31 and Glu41 residues in the flexible loops and the Glu36 residue in the β-strand III are critical for eliciting sweetness. In spite of these studies, the mechanism of interaction of brazzein with the sweet taste receptor, T1R2/T1R3, has not yet been elucidated.

To gain insight into the mechanism of interaction between sweet-tasting proteins and the sweet taste receptor, we designed four peptides derived from the important regions of brazzein as follows: BZ1, DKHARSGECFYDEKR corresponding to the loop-β-strand III-loop; BZ2, KKRARSGD CFYDAKR derived from the BZ1 peptide; BZ3, DEKR corresponding to the sweetness loop; and BZ4, DAKR

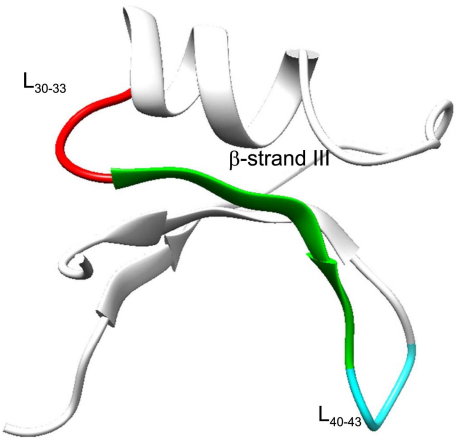

Figure 1. Potential sweet fingers of the sweet-tasting protein brazzein. Loops $L_{30–33}$, $L_{40–43}$, and β-strand III of brazzein. The 3-D models were manipulated and rendered in UCSF Chimera.

derived from the BZ3 peptide (Fig. 1). All designed peptides were synthesized with a peptide synthesizer and purified by HPLC and Sephadex G-10 columns. The purified peptides were lyophilized and subsequently dissolved in water for sensory analysis. Docking tasks were performed between the designed peptides and the predicted model of sweet taste receptor, T1R2.

The structures of peptides were predicted by the peptide tertiary structure prediction server PEP-FOLD (Diderot University, France). The predicted structure of BZ1 corresponding to the two flexible loops and the β-strand of brazzein tended to make a long elliptical form (Fig. 2(a)). The structure of BZ2, derived from BZ1 through mutations in four residues, showed a randomly twisted shape that was different in structure from the elliptical form of BZ1 (Fig. 2(b)). The predicted structures of BZ3 and BZ4 had hairpin loop structures as expected (Fig. 2(c) and 2(d)). The secondary structures of peptides were also analyzed using circular dichroism (CD) spectroscopy (Fig. 3). Contrary to the predicted structures, the CD spectra of BZ1 and BZ2 appeared denatured forms. Particularly, the CD spectra of BZ3 and BZ4 exhibited representative extended disordered conformations, showing a strong negative band at 197 nm and a small positive band at 217 nm. From these results, we suggest that the structures of the four designed peptides did not

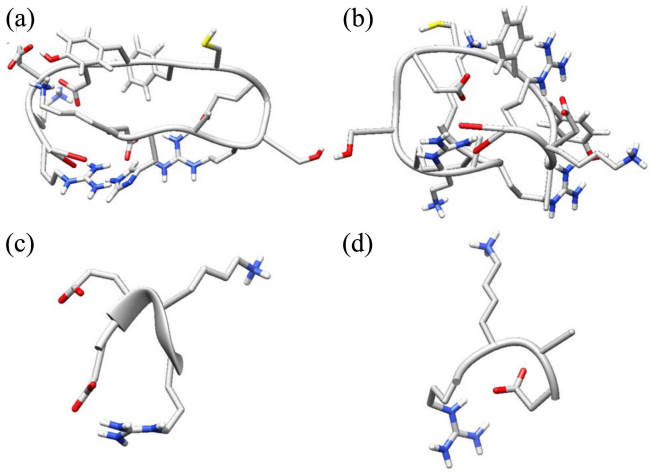

Figure 2. Predicted structures of peptides. (a) BZ1, (b) BZ2, (c) BZ3, and (d) BZ4. The structures of peptides were predicted with the PEP-FOLD tool.

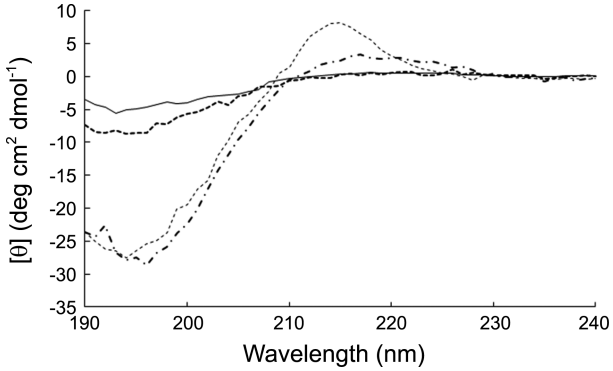

Figure 3. Circular dichroism (CD) spectra of peptides. (a) CD spectra of BZ1 (solid line), (b) BZ2 (dotted line), (c) BZ3 (dashed line), and (d) BZ4 (dot-dashed line).

retain the original conformations of the corresponding residues of the parent protein, although the predicted structures using PEP-Fold were similar to the original conformations.

Results from mutagenesis and chimera studies of the receptor have demonstrated that the Venus flytrap module of T1R2 is important for brazzein agonism.[8] Homology modeling studies using the X-ray structure of the mGlu receptor have also shown that the best docking orientation places brazzein in contact with the T1R2 subunit rather than the T1R3 subunit.[9] Therefore, designed peptides were docked onto T1R2. Receptor-ligand interactions for T1R2 bound to brazzein and peptides are shown in Figure 4, and their binding free energies (ΔGb, kcal/mol) were compared in Table 1. According to the results of the docking task, the predicted docking model of brazzein was bound to the whole region of a Venus Flytrap Domain (VFD), the known binding site of T1R2, with −14.0 kcal/mol of binding free energy (Fig. 4(a)). There were seven possible hydrogen bonds between brazzein and T1R2. PCA1 and Asp2 residues in the N-terminal of brazzein formed two hydrogen bonds with Lys60 in T1R2. Glu36, Try39, and Arg43 residues in the *β*-strand III and the flexible loop ($L_{40–43}$) of brazzein formed hydrogen bonds with Tyr314, Leu313, and Ser458 of T1R2, respectively. Most of peptides tend to bind to the right cleft of the VFD of T1R2 with approximately −7.6 to −12.4 kcal/mol except BZ3 bound to the left side with −8.3 kcal/mol (Fig. 4(b)-(e)). These results indicate that binding sites of the designed peptides to the sweet taste receptor moderately coincide with the binding region of the $L_{40–43}$ in brazzein, not with the binding regions of the N-terminal in brazzein.

The sweet taste receptor T1R2/T1R3 belongs to a family of G-protein-coupled receptors and binds all classes of sweet micro- and macromolecules. Several models predicting the mechanism of interaction between T1R2/T1R3 and sweet materials have been proposed.[10] The AH-B theory, based on the structures of sweeteners, was one of the most widely accepted models for sweetener and sweetener binding site interaction. This model proposed that a sweet-tasting compound must contain a hydrogen bond donor (AH) as well as a hydrogen bond acceptor (B) and was reasonable only for sweet micromolecules. The wedge model has been emphasized for understanding the relationship between sweet proteins and sweet taste receptors.[11] According to the wedge model, the strong binding of protruding structure of the sweet protein and the wedge of the sweet taste receptor leads to the activation of the receptor. The sweet finger mechanism is the most widely regarded model as a protruding structure for activating the sweet taste receptor. In the present study, we designed the sweet finger candidates, *β*-sheet loops in brazzein, based on results of mutagenesis studies and structural similarities. However, the designed peptides were unable to elicit a sweet taste (Table 1). BZ1, BZ2, and BZ4 had no taste. In fact, BZ3 had a weak salty flavor. BZ3 also appeared a different result in the docking task (Fig. 4). The binding regions of BZ1, BZ2, and BZ4 to sweet taste receptor moderately matched up with those of corresponding sequences in brazzein. Otherwise, the binding region of BZ3 was different from those of the flexible loop

Table 1. Sequence, taste, and binding free energy between the designed peptides and sweet taste receptor

Peptide	Sequence	Binding free energy ΔGb (kcal/mol)	Taste	Intensity
BZ1	DKHARSGECFYDEKR	−12.4	None	
BZ2	KKRARSGDCFYDAKR	−11.4	None	
BZ3	DEKR	−8.3	Salty	++
BZ4	DAKR	−7.6	None	
Brazzein		−14.0	Sweet	+++++

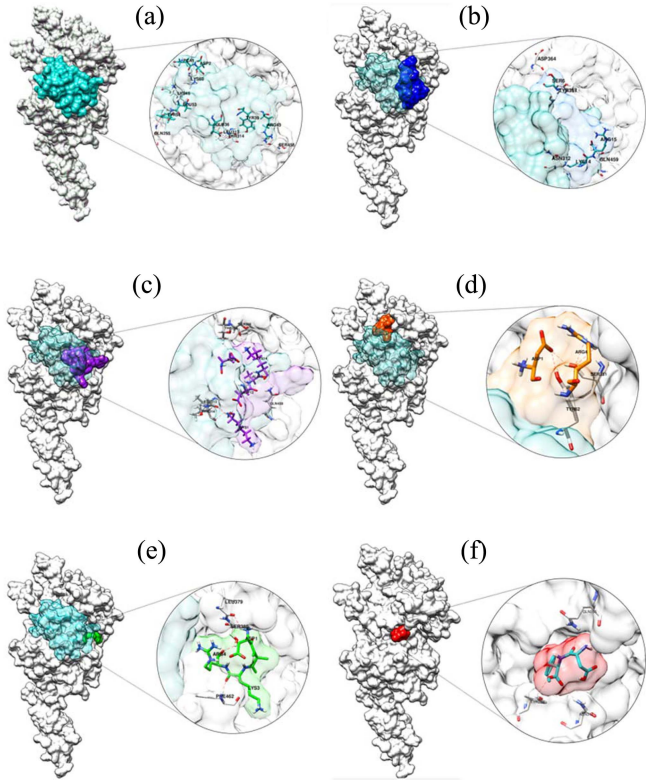

Figure 4. Predicted docking models and interaction of brazzein, designed peptides, and aspartame with sweet taste receptor T1R2. (a) brazzein, (b) BZ1, (c) BZ2, (d) BZ3, (e) BZ4, and (f) aspartame. Model building of the sweet taste receptor T1R2 was performed with the SWISS-MODEL program. The glutamate receptor (2E4U.pdb) was used as a template due to homologous sequences. Designed peptides and brazzein were docked into the ligand-binding cleft of the T1R2 using AutoDock Vina.

($L_{40–43}$) of brazzein and other peptides. The weak salty taste of BZ3 may be due to the different binding mode.

Above all, the lack of sweet taste of the peptides can be attributed to the lack of ordered structure correspond to the structures present in the 3-D structure of brazzein. Unexpectedly, the structures of the synthesized peptides were different from the original conformations of the parent protein judging from CD analysis, although the designed peptides exhibited similar conformations by computer modeling (Figs. 2 and 3). The CD spectra of peptides appeared not loop structure as a sweet finger candidate but denatured form and extended disordered conformation. Therefore, the lack of sweet taste of the peptides may be due in part to these structural differences. The results of docking tasks also indicated that the binding sites that bind all designed peptides to the sweet taste receptor were different from the one that binds aspartame, a representative low molecular weight sweetener. Aspartame was bound to the center region of the VFD of T1R2, forming 3 hydrogen bonds as follows: two hydrogen bonds between methyl ester which is a functional group near the phenylalanine residue of aspartame and Tyr282 and Asn312 residues of T1R2, and one hydrogen bond between aspartic acid of aspartame and Gln355 residue of T1R2 (Fig. 4(f)). These results suggest that sweet proteins recognize distinct binding site different from that of low molecular weight sweeteners.

The lack of sweet taste also can be attributed to insufficient binding sites necessary to trigger response. The results of docking tasks indicated that multiple sites including the N-terminal and the loop $L_{40–43}$ of brazzein were bound to sweet taste receptor T1R2 (Fig. 4). Similar results were observed with studies on peptides corresponding to the best potential "sweet fingers" of sweet-tasting proteins.[11,12] Tancredi *et al.* designed several cyclic peptides corresponding to the β-sheet loop structure of sweet-tasting proteins, but none had a sweet taste.[11] Designed β-hairpin peptides derived from the N- and C-termini of brazzein were also unable to elicit a sweet taste.[12] These results indicate that the interaction of the sweet-tasting protein with the sweet taste receptor may involve multiple binding sites. Although the peptides may be tightly bound to the sweet taste receptor, binding of only one part of the multiple binding sites is insufficient to elicit a sweet taste by itself. Recently, studies focused on mutagenesis and chimeras of the sweet receptor have led to the development of a multi-point interaction model to describe the interaction between the sweet taste receptor and sweet material.[8] This model was supported by our previous study using multiple mutations of the critical amino acid residues of brazzein.[13] The saturation transfer difference NMR spectroscopy study also suggested a multi-point interaction between brazzein and the sweet receptor.[8] Taken together, we suggest that the interaction of sweet protein with the sweet taste receptor mainly relies on the structural integrity of the binding sites in sweet proteins and/or may involve a much larger surface area of sweet proteins, supporting the multi-point interaction model that sweet proteins bind to multiple sites of the sweet taste receptor.

Experimental

Design of Peptides. To find the critical structure for the sweetness of sweet-tasting proteins, we compared the relationship between the structure and function of sweet proteins. Although homology was not found among their amino acid sequences, there were some similarities among the tertiary folds, such as β-sheet loops (Fig. 1). The 30-KHAR-33 and 40-DEKR-43 loops of brazzein have been found to contain important sweet taste determinants.[6,7,13] Site-directed mutagenesis studies suggested that two residues (H31 and E41) in the flexible loops of brazzein are the critical residues of the molecule for eliciting sweetness.[6,7] Our studies also suggested that the Glu36 residue in the β-strand III (residues 34-SGECFY-39) is a critical residue of the molecule for eliciting sweetness.[6] More recently, multiple mutations of the critical amino acid residues in brazzein suggested that it binds to the multisite surface of the sweet taste receptor.[13] According to these results, we have designed BZ1 peptide (29-DKHARSGECFYDEKR-43) containing two flexible loops, the β-strand III, and the BZ3 peptide (40-DEKR-43) corresponding to sweetness loops of brazzein

(Fig. 1). Our studies also suggested that mutations reducing the overall negative charge and/or increasing the positive charge favor sweet tasting protein potency.[6,7,13] Based on these results, we also designed the BZ2 peptide (KKRARS GDCFYDAKR) derived from the BZ1 peptide, the BZ4 peptide (DAKR), and the BZ3 peptide.

Synthesis of Peptides. All peptides were synthesized by the Fmoc-based solid-phase method with a C-terminal amide using a Pioneer Peptide Synthesizer (Applied Biosystems, USA) and purified at Peptron (Deajeon, Korea). The purity of all peptides was determined by HPLC to be more than 95% pure. Synthesized peptides were dissolved in water to a concentration of 3 mg/mL and purified again on Sephadex G-10 columns (GE Healthcare, USA) to remove residual trifluoroacetic acid. Purified peptides were dried with a vacuum freeze dryer (Martin Christ, Germany).

Model Building. Model building of the sweet taste receptor T1R2 was performed with the SWISS-MODEL program (The Swiss Institute of Bioinformatics and the Biozentrum of the University of Basel, Basel, Switzerland). The glutamate receptor (2E4U.pdb) was used as a template due to its homologous sequences.[14] The structures of peptides were predicted with the PEP-FOLD tools (Diderot University, France). PEP-FOLD is based on the concept of Hidden Markov Model Derived Structural Alphabet (HMM-SA) which is an ensemble of elementary prototype conformation to describe the whole diversity of protein structures.[15] HMM discretizes protein backbone conformation as series of overlapping states of four residues.[16] To build 3D structures from HMM-SA profile, PEP-FOLD uses a zip operator which can start building process at any position, and OPEP v3.1 (optimized potential for efficient structure prediction version 3.1), in the case of a coarse grained force field, is used for protein folding and aggregation. The accuracy of PEP-FOLD was verificated comparing the prediction results of 52 peptides as a benchmark with the NMR results of benchmark peptides.[15] The structures of BZ3 and BZ4 could not be predicted by PEP-FOLD because of the short length of their amino acids. Therefore, five alanine sequences were added to the C-terminal sequences of BZ3 and BZ4, and the added five alanine sequences were manually deleted with UCSF Chimera. Among all five suggested peptide models, the model with the lowest binding free energy was chosen for docking tasks. All preparation (removing water molecules, adding polar hydrogen, and allowing torsions) of materials was performed using AutoDock Tools (ADT, The Scripps Research Institute, California, USA). Prepared peptides were docked into the ligand-binding cleft of the T1R2 using AutoDock Vina.[17] Models calculated as having the lowest binding energy were regarded as the binding form between the sweet taste receptor T1R2 and the designed peptides. Molecular graphics and analyses were performed with the UCSF Chimera package (University of California, San Francisco, California, USA).

Circular Dichroism (CD) Analysis. CD spectra were recorded at 25 °C on a Jasco 815 spectropolarimeter (Jasco, Tokyo, Japan) using a 0.1-cm path-length quartz cell. The CD spectra were measured for the 500 μM of peptides in the wavelength range from 190 nm to 240 nm. A reference spectrum of triple distilled water as solvent was also recorded. The CD spectra of the peptides were obtained after subtracting the reference spectrum.

Sensory Analysis. Freeze-dried peptides were dissolved in distilled water to 3.0 mg/mL. The taste panel consisted of healthy, non-smoking individuals who were well trained through previous studies on sweet-tasting proteins.[7] In total, there were ten female subjects and ten male subjects, aged 24–55 years. Taste tests were conducted before mealtime or at least 1 h after the last meal. Before the sample was tasted, the mouth was thoroughly rinsed with distilled water. The sample solution was dropped on the tongue, held in the mouth for approximately 10 s, and then spat out. Taste tests were performed using a double blind model. The panel was allowed to freely express their sense.

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