Chem. Senses 30 (suppl 1): i90–i91, 2005 doi:10.1093/chemse/bjh128

Brazzein, a Small, Sweet Protein: Effects of Mutations on its Structure, Dynamics and Functional Properties

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Key words: brazzein, conformation, hydrogen bond, mutagenesis, structure–function, sweet taste

Introduction

The demand for non-calorigenic protein-based sweeteners with favorable taste properties is high. The optimal design of such sweeteners requires knowledge about structure–function relationships and the identification of chemical entities that trigger the sweetness response. Among the known, naturally occurring, sweet-tasting proteins, brazzein has properties that make it particularly attractive as a potential economic sweetener. It is highly stable over wide temperature and pH ranges and has taste properties that resemble those of carbohydrate sweeteners. Brazzein is a single polypeptide of 54 standard amino acids and contains no carbohydrate. The brazzein protein originally was purified from the fruit of *Pentadiplandra brazzeana*, a climbing vine that grows in Gabon, Zaire and Cameroon (Ming and Hellekant, 1994). The fruit is consumed by the local population and is prized for its sweetness. Pure brazzein has been shown to elicit sweetness responses in humans by taste trials and in a non-human primate (rhesus monkey) as determined from electrophysiological recordings of signals from the chorda tympani (van der Wel *et al.*, 1989).

Recently receptor proteins have been discovered that are responsive to sweet ligands. The human receptor appears to be a heterodimer of two conventional seven-transmembrane-helix G-coupled type receptors (T1R2/T1R3) but with unusually large ectodomains ((Nelson *et al.*, 2001; Li *et al.*, 2002); P. Jiang and M. Max, personal communication). Different sweet proteins interact with and activate the same heterodimeric receptor in somewhat different ways and tentative models for such interactions have been elaborated ((Temussi, 2002); M. Max, personal communication). The discovery of the sweet taste heteroreceptor opens up exciting new avenues for research on the mechanism of action of sweet substances. Brazzein is an excellent candidate for experimental investigations of the chemical and structural requirements for extracellular triggering of a sweet response in humans and for understanding the mechanism of the signal transduction.

Early investigations of brazzein

The amino acid sequence of brazzein was determined by peptide sequencing (Ming and Hellekant, 1994) and the three-dimensional structure of brazzein was solved by homonuclear ^{1}H NMR spectroscopy (Caldwell *et al.*, 1998a). The protein has a highly compact structure consisting of one short α-helix and three anti-parallel β-strands held together by four disulfide bridges. No significant sequence or structural similarity was found between brazzein and the two other sweet-tasting proteins of known three-dimensional structure: monellin (Somoza *et al.*, 1993) and thaumatin (Ogata *et al.*, 1992; Ko *et al.*, 1994). Early variable temperature NMR studies of brazzein showed very little change in its ^{1}H NMR spectrum over a wide range of temperatures (32–82°C; Caldwell *et al.*, 1998b) and the sweetness profile was shown to be undiminished after incubation at 100°C for 4 h (Ming *et al.*, 1996).

The original NMR studies of fruit brazzein indicated that the protein adopts a cysteine-stabilized αβ (CSαβ) fold in which the α-helix and β-strands are stabilized by the presence of four disulfide bridges (Caldwell *et al.*, 1998a). Other proteins with this fold include members of the rapeseed family of serine proteinase inhibitors, scorpion toxins, insect defensins and plant-derived γ-thionins. Apart from the conserved cysteines, little sequence identity is found between members of the different families. Brazzein is the only CSαβ protein known to be sweet.

Production of recombinant brazzein, stable isotope labeled brazzein and brazzein mutants

We developed an efficient bacterial production system for brazzein (Assadi-Porter *et al.*, 2000a). The recombinant protein produced has a sequence identical to the minor form of brazzein isolated from fruit, the form that lacks the N-terminal pyro-glutamate (pGlu) residue (des-pGlu1-brazzein) and that has been shown to have about twice the sweetness of the pGlu containing variant. The fusion protein is expressed as an insoluble product, which we solubilize and fold. The conditions we developed for folding and oxidation of the disulfides lead to a product with native structure (as determined by NMR spectroscopy) and with full activity as a sweetener (as determined by taste tests) (Assadi-Porter *et al.*, 2000a).

This methodology, along with quick-change mutagenesis, has allowed us to make a variety of brazzein mutants. We discovered mutants with sweet-taste properties that appear to be superior to those of the wild-type protein (Assadi-Porter *et al.*, 2000b). Studies have indicated that the presence of positive charges on the surface of brazzein enhances sweetness: mutating some of these positive charges to neutral or negative charge significantly decreases the sweetness (Jin *et al.*, 2003a,b).

Production of brazzein from *Escherichia coli* has also enabled us to make samples labeled with stable isotopes (^{15}N or ^{13}C and ^{15}N) for NMR investigations of the structure and dynamics of the protein. For our detailed structural and dynamic analyses of brazzein variants, we chose wild-type brazzein (des-pGlu1-brazzein) and five mutants (two with increased sweetness and three with decreased sweetness). The ribbon-diagram in Figure 1 shows the backbone of wild-type brazzein and the positions of the five mutations (Assadi-Porter *et al.*, 2003). Four of the sites of mutation (Ala2 insertion, His31Ala, Arg33Ala and Asp50Ala) are spatially close to one another. Two of the mutants (Ala2 insertion and His31Ala) have about twice the sweetness of wild-type brazzein; the other three mutants (Arg33Ala, Arg43Ala and Asp50Ala) have greatly reduced sweetness (Assadi-Porter *et al.*, 2000b). Arg43Ala is tasteless.

NMR studies

In only one of the five mutants (Arg43Ala) were the chemical shifts changes resulting from the mutation propagated to other parts of the

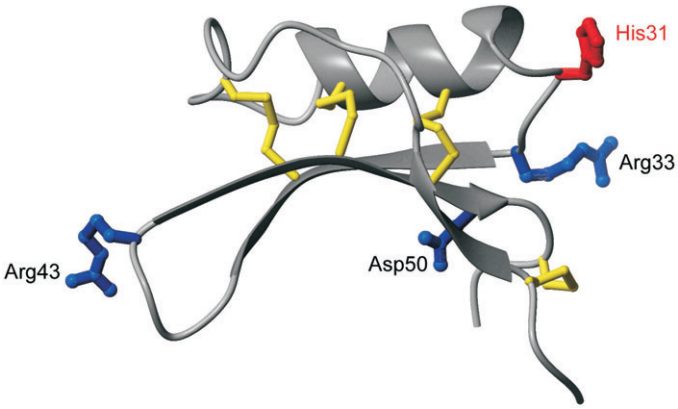

Figure 1 Backbone ribbon diagram of wild-type brazzein (Caldwell *et al.*, 1998a). Residues found to be critical for the sweetness of the protein are indicated in colour: red, enhanced sweetness; blue, decreased sweetness.

molecule. In this mutant, changes in chemical shifts were observed in the N- and C-terminal regions.

Analysis of the H-bonds in the six brazzein variants through measurements of trans-H-bond couplings has shown that single-site mutations can give rise to subtle structural changes (Assadi-Porter *et al.*, 2003). Wild-type brazzein and the two variants with sweetness equal to or greater than wild-type brazzein had similar patterns of H-bonds, whereas all three variants with reduced sweetness exhibited changes in H-bonding (Assadi-Porter *et al.*, 2003).

As determined by NMR relaxation measurements, the mutations that decrease sweetness were found to decrease the flexibility of the protein. The results suggested, in addition, that loop 9–19 of brazzein exists as two or more sub-structures. We measured residual dipolar couplings (RDCs) as a means for determining whether loop regions are disordered. The RDCs from the sweeter brazzein analog (Ala2insertion) were similar to those from wild-type brazzein; this confirmed that the two proteins have similar structures. Furthermore, the RDC results indicated that residues 11–18 in the loop between the first β-strand and α-helix are disordered in both proteins (F.M. Assadi-Porter and C.C. Cornilescu, unpublished results).

Investigation of model peptides

On the basis of our model for multi-site brazzein:receptor interactions, we designed a small cyclic peptide corresponding to regions of the N- and C- termini connected by a tri-peptide linker (PGN) at one end and a disulfide bond at the other end. The resulting cyclic peptide, c[(D^{2}KCKKV^{7})-PGN-(D^{50}YCEY^{54})], was designed to contain a proper β-turn (type I or II) motif. This conformation was confirmed by homonuclear ^{1}H-^{1}H 2D TOCSY and NOESY NMR data (F.M. Assadi-Porter, unpublished results). In a taste test, however, the peptide was found to be tasteless. Similar results were obtained in the Temussi laboratory on a different cyclic peptide. That study examined the cyclic peptide c[C^{37}FYDEKRNLQC^{47}], which proved to be tasteless (Tancredi *et al.*, 2004). Both investigations of model peptides suggest that a more extensive structure is required for sweetness.

Relationship between brazzein and proteinase inhibitors

The sequence of brazzein is very similar to that of the rapeseed-type proteinase inhibitors from plants and the structures are also similar (Caldwell *et al.*, 1998a; Zhao *et al.*, 2002). The reason why brazzein has no activity as a proteinase inhibitor is explained by its lack of the reactive site dipeptide (Zhao *et al.*, 2002). Attempts to convert brazzein into a trypsin inhibitor by inserting the missing residues or even by introducing the entire reactive site loop of the *Arabidopsis thaliana* inhibitor proved unsuccessful; these insertions or modifications either destabilized the mutated protein or prevented it from folding (Zhao, 2001; F.M. Assadi-Porter and J.L. Markley, unpublished data).

Acknowledgements

We thank Dr Marianna Max and P. Jiang for sharing information about their research prior to publication. This work was supported by grants RR02301 (JLM) and DC006016 (Göran Hellekant, P.I.).

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