Crystal structure of Mabinlin II: A novel structural type of sweet proteins and the main structural basis for its sweetness

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Abstract

The crystal structure of a sweet protein Mabinlin II (Mab II) isolated from the mature seeds of \textit{Capparis masaikai} Lev., grown in Southern China has been determined at 1.7 A˚ resolution by the SIRAS method. The Mab II 3D structure features in an “all alpha” fold mode consisting of A- and B-chains crosslinked by four disulfide bridges, which is distinct from all known sweet protein structures. The Mabinlin II molecule shows an amphiphilic surface, a cationic face (Face A) and a neutral face (Face B). A unique structural motif consisting of B54–B64 was found in Face B, which adopts a special sequence, NL–P–NI–C–NI–P–NI, featuring four [Asn-Leu/Ile] units connected by three conformational-constrained residues, thus is called the [NL/I] tetralet motif. The experiments for testing the possible interactions of separated A-chain and B-chain and the native Mabinlin II to the sweet-taste receptor were performed through the calcium imaging experiments with the HEK293E cells coexpressed hT1R2/T1R3. The result shows that hT1R2/T1R3 responds to both the integrated Mabinlin II and the individual B-chain in the same scale, but not to A-chain. The sweetness evaluation further identified that the separated B-chain can elicit the sweetness alone, but A-chain does not. All data in combination revealed that the sweet protein Mabinlin II can interact with the sweet-taste receptor hT1R2/T1R3 to elicit its sweet taste, and the B-chain with a unique [NL/I] tetralet motif is the essential structural element for the interaction with sweet-taste receptor to elicit the sweetness, while the A-chain may play a role in gaining a long aftertaste for the integrate Mabinlin II. The findings reported in this paper will be advantage for understanding the diversity of sweet proteins and engineering research for development of a unique sweetener for the food and agriculture based on the Mabinlin II structure as a native model.

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Keywords: Sweet protein; Mabinlin; Crystal structure; Sweet receptor; hT1R2/T1R3-Mab II interaction; Structural basis of sweet taste

1. Introduction

Sweet proteins are low-calorie sweetener (\textit{Kant, }2005). In recent years, the prevalence of the diseases related to the consumption of sugar, such as obesity, diabetes, hyperglycemia, and caries, has increased dramatically. For example, the number of diabetes mellitus patients in China has been estimated at 50 million in 2004 and it is forecast to increase by 1.5–2 million per year (\textit{Zhang, }2004). A series of artificial sweeteners like Saccharin, Aspartame, and Cyclamate are used world wide as low calorie replacements for these patients. So far the most popular sweeteners are small molecule compounds, which may incur some site effects such as psychological problems, mental disorders, bladder cancer, and heart failure (\textit{Weihrauch et al., }2002; \textit{Kanarek, }1994; \textit{Cohe, }2001; \textit{Nabors, }1988; \textit{Hagiwara et al., }1984). Instead, sweet proteins can be used as natural, low calorie sweeteners without triggering a demand for insulin in these patients whereas sucrose does. Therefore, they are potential replacements for artificial sweeteners
and sugars and may find applications in food and beverage industries.

Until now, five sweet proteins have been identified, namely thaumatin (Van der Weld and Loewe, 1972), monellin (Morris and Cagan, 1972), brazzein (Ding and Hellekant, 1994), neoculin (Shirasuka et al., 2004; Suzuki et al., 2004) (formerly known as curculin (Yamashita et al., 1990)), and mabinlin (Hu and He, 1983). Among them, the 3D structures of thaumatin (Ogata et al., 1992), monellin (Somoza et al., 1993), neoculin (Shimizu-Ibuka et al., 2006), and brazzein (Caldwell et al., 1998) have been determined. Besides, by using the site-directed mutagenesis analysis a series of residues related to the sweet activity have been identified for specific protein sweeteners, including monellin (Mizukoshi et al., 1997; Somoza et al., 1995), thaumatin (Kaneko and Kitabatake, 2001), and brazzein (Assadi-Porter et al., 2000; Jin et al., 2003a,b).

On the other hand, the human receptors for sweet taste, hT1R2/T1R3, have been identified recently (Li et al., 2002). Meanwhile Temussi proposed a “wedge model” (Temussi, 2002) for the interactions between sweeteners and T1R2–T1R3 receptor. More recently, Temussi and coworkers built all four possible forms of the extracellular heterodimeric sweet receptor by homology modeling to perform docking with sweet proteins, including brazzein, monellin, and thaumatin and described full account of the “wedge model” for sweet proteins (Morini et al., 2005). Some recent reports on sweet proteins, such as neoculin (Shimizu-Ibuka et al., 2006), brazzein (Walters and Hellekant, 2006) also demonstrated the validity of the “wedge model”. However, there are no significant similarities in either amino acid sequences or three-dimensional structures in all sweet proteins reported. Moreover, no common structural basis for sweetness has been identified from these proteins. Therefore it is significant to find new type of sweet protein for understanding their structural diversities. Here we report the X-ray crystal structure of a highly thermo-stable sweet protein Mabinlin II (Mab II), which shows a novel, all alpha fold with two polypeptide chains crosslinked by four disulfide bridges. A docking study of Mab II onto the model of sweet receptor hT1R2/hT1R3 revealed the main structural basis for the Mab II–hT1R2/T1R3 interaction to elicit its sweet taste. Besides, an interesting functional ramification of A- and B-chains in eliciting the sweet taste of Mab II was also observed. The results provided another example of the validity of the “wedge model”.

Mabinlin II is isolated from the seeds of mabinlang, the plant of Capparis masaikai Lev. growing in the subtropical region of the Yunnan province of China and bearing fruits in a unique condition of high temperature at 310 K. The native data were collected with a crystal-to-detector distance of 100 mm, Δθ = 1° and 300 s exposure time. A total of 180 frames were collected. MOSFLM (V6.2.3), TRUNCATE, and SCALA from CCP4 program.

2. Experimental procedures

2.1. Purification

The Mab II protein used in crystallization was purified from the mature seeds of C. masaikai plants grown in the method described previously (Hu and He, 1983).

2.2. Crystallization

All crystallization experiments were performed with the hanging-drop vapor-diffusion method. Mab II was dissolved in pure water at a concentration of 10 mg/ml for crystallization. The crystallization was achieved in a rather unique condition of high temperature at 310 K. The drops were formed by mixing 2 μl of protein solution with 2 μl of reservoir solution and equilibrated against 400 μl reservoir solution in each well. The best crystals were obtained with a reservoir solution (400 μl) containing 0.1 M sodium acetate trihydrate buffer (pH 4.6) and 1.4 M sodium malonate (Li et al., 2006).

2.3. Data collection and processing

All diffraction data were collected on a Rigaku R-Axis IV++ image plate using CuKα radiation (λ = 1.5418 Å) from a rotating anode operating at 40 kV and 20 mA with 0.1 mm confocus incident beam diameter. The crystals were briefly soaked in paraffin oil (Hampton Research) after being mounted in nylon cryoloops (Hampton Research) and then flash-cooled in a nitrogen-gas stream at 85 K. The native data were collected with a crystal-to-detector distance of 100 mm, Δθ = 1° and 300 s exposure time. A total of 180 frames were collected. MOSFLM (V6.2.3), TRUNCATE, and SCALA from CCP4 program.
suit V.4.2.2 (CCP4, 1994) were used for processing, reducing, and scaling of the diffraction data.

2.4. Structure determination and refinement

The structure of Mab II was determined by the SIRAS method using iodine derivative prepared through the cryo-soaking approach (Dauter et al., 2000). To obtain suitable heavy atom derivatives, the native crystals were soaked for 24 h in the mother liquor containing KI reagent at 0.1 M concentration. The heavy atom derivative data were collected with a crystal-to-detector distance of 150 mm, $\Delta \phi = 1^\circ$ and 300 s exposure time. A total of 360 frames were collected. Eighteen possible iodine sites were found by ShelxD (Schneider and Sheldrick, 2002) using the SIRAS method. The program SHARP (De La Fortelle and Bricogne, 1997) was used for phase calculation and density modification. The model was manually built using the program O (Jones et al., 1991). The structure was refined to a resolution of 1.7 Å with R-factor of 0.221 and Rfree of 0.254 using CNS version 1.1 (Brunger et al., 1998). There are two Mab II molecules in an asymmetric unit. The final model contains 189 ordered residues (A4–A28, B4–B69, C2–C29, and D4–D71), one acetate molecule and 125 water molecules in the asymmetric unit. The stereochemical assessments of the structure were performed by PROCHECK (Laskowski, 2001). The statistics data are listed in Table 1.

<table>
<thead>
<tr>
<th>Data collection, phasing, and refinement statistics of Mab II</th>
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<tr>
<td><strong>A. Data collection</strong></td>
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<td>Observation ($I/\sigma(I) &gt; 0$)</td>
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<td><strong>B. Phasing</strong></td>
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<td>Numbers of solvent molecules</td>
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<td>Average B value (Å²)</td>
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2.5. 3D-docking with sweet receptor

To explore the possible interaction between Mab II and hT1R2/T1R3, a 3D-docking computation was performed. Prior to calculation of the docking model, a structural model of the sweet-taste receptor hT1R2/T1R3 was built by homology modeling based on the crystal structure of the extracellular region of the metabotropic glutamate receptor 1 (mGlur), also called ligand-binding region of mGlur1 (m1-LBR) (Kunishima et al., 2000). At present, the crystal structures of m1-LBR in two unliganded forms, called free forms I (PDB entry: 1EWT) and II (PDB entry: 1EWK), and in a complex form with glutamate (PDB entry: 1EWV), and in a complex form with glutamate and so is considered to be similar with the active conformation of sweet protein receptor (Temussi, 2002). Considered that T1R2/T1R3 is a heterodimer while mGlur is a homodimer, as Morini et al. described there are total of four T1R2/T1R3 models should be modeled, (Morini et al., 2005), including two models for the inactive open–open form, henceforth dubbed Roo_AB (where AB means T1R2 modeled on chain A and T1R3 modeled on chain B of the 1ewt template) and Roo_BA (T1R3 modeled on chain A and T1R2 modeled on chain B of the 1ewt template), and two models for the complexed active closed–open form, henceforth dubbed Aoc_AB (T1R2 modeled on chain A and T1R3 modeled on chain B of 1ewk) and Aoc_BA (T1R3 modeled on chain A and T1R2 modeled on chain B of 1ewk).

First, the Aoc_AB model was modeled. The free form II structure of extracellular domain of mGlur (m1-LBR, PDB entry: 1EWV, not 1EWK) was used as a template. The primary sequences of T1R2 and T1R3 were obtained from the Swiss-Prot database (entry codes: TS1R2_HUMAN and TS1R3_HUMAN). Sequences of T1R2, T1R3, and m1-LBR were aligned by CLUSTAL (Thompson et al., 1994). The two chains of the initial m1-LBR molecular model were then replaced by T1R2 and T1R3, respectively, and the resultant complex was refined by Modeller 8V2 (Kelley et al., 2000). The other three models of T1R2/T1R3 (Roo_AB, Roo_BA, Aoc_BA) were built up with the same procedure. The four models were then used in 3D-docking of Mab II molecule.

The docking of Mab II with the hT1R2/T1R3 complex was performed using the program GRAMM v.1.0.3 (Vak-
ser, 1995; Vakser et al., 1999; Tovchigrechko and Vakser, 2005). The representative structure at each protonation state was docked into the hT1R2/T1R3 receptor model. First, docking was evaluated only through energy scores. Then, the models taking the highest score with the lowest energy of Mab II-hT1R2/T1R3 were built and evaluated manually.

2.6. Separation of two chains of Mab II

To verify the main structural basis of Mab II for the sweet taste, which was proposed from the docking test, and distinguish the functional role of A-chain from that of B-chain, the two polypeptide chains of Mab II were separated. Mab II protein powder was dissolved in 6 M guanidine hydrochloride with 5 mM β-mercaptoethanol at a concentration of 5 mg/ml and incubated for 4 h at 310 K. Two peaks were collected after elution from a C18-reverse-phase column (5 μm, 4.6 × 250 mm) on an AKTA Puriﬁer system (Amersham Pharmacia Biotech) and then lyophilized. These two peaks were veriﬁed as the A- and B-chains of Mab II by MALDI-TOF mass spectra.

2.7. Fluorescence emission spectra of Mab II and Mab-B

To compare the conformational state of the separated B-chain (Mab-B) with that in the native Mab II, Mab-B, and Mab II were subjected to fluorescence emission spectrometric analysis. Because there is only one tryptophan residue located at B-chain (TrpB72) in Mab II, the fluorescence emission spectra of this residue can be used to monitor the folding status of the B-chain in comparison with that of the native Mab II (Hu et al., 1985a,b). For the experiment, 1 mg/ml Mab II and Mab-B solutions were prepared. Their intrinsic ﬂuorescence were measured with excitation at 280 nm and slits of 5 nm. The results were recorded at room temperature using a Shimadzu RF5301PC spectroﬂuorometer.

2.8. 1D 1H NMR spectrum

The 1D 1H NMR experiment was taken further to check the fold state of the separated B-chain. The NMR sample used in the experiment contains 0.5 mM Mab-B protein, and 10% (v/v) D2O in 50 mM phosphate buffer, pH 7.0. 1D 1H NMR experiments were performed at 310 K on a Bruker DMX 600 MHz spectrometer equipped with a cryo-probe.

2.9. Test of interactions between Mab molecules and human sweet receptor

Heterologously expressed hT1R2 + hT1R3 has been shown to respond to many sweeteners, including monellin, thaumatin, brazzein, and other sweet compounds (Jiang et al., 2004). The response of the human sweet receptor to Mab II, Mab-A, and Mab-B was tested through the calcium imaging experiments with the expressed hT1R2/hT1R3 cell model (Jiang et al., 2004, 2005). For the experiments, cDNAs encoding hT1R2, hT1R3, and Gz16gust44 (Jiang et al., 2004, 2005) were transiently transfected into HEK293E cells using lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. After 4 h, cells were trypsinized and seeded onto poly-lysine coated 96-well plates at 40,000 cells/well and cultured in low-glucose DMEM supplemented with GlutaMAX-I and 10% dialyzed FBS (Invitrogen). After an additional 40 h, cells were loaded with the calcium dye Fluo-4 (Invitrogen), dissolved in Dulbecco’s PBS buffer (DPBS, Invitrogen) at a concentration of 3 μM, for 1 h at room temperature. After three washes with DPBS, stimulation was performed with DPBS supplemented with tastants. Calcium mobilization was recorded using an Olympus Fluvview confocal microscope. The HEK293E cells coexpressed hT1R2/T1R3 were stimulated with the purified Mab II (0.5%), Mab-A (0.05%), Mab-B (0.5%), and D-tryptophan (10 mM) as a control. Data acquisition and analysis of time series images were performed using the software Fluvview version 2.1. Every experiment was repeated for at least three times.

2.10. Sweet activity test

The sweetness of Mab II, Mab-A, and Mab-B, were evaluated by a regular procedure (Hu and He, 1983). Five milligrams per milliliter solutions of Mab II, Mab-A, Mab-B, and a blank solution (pure water) were prepared. The samples were blindly taken into the test. Each time 10 μl of the solution was dropped on the tongues of the testers (Hu and He, 1983) have been trained by 0.3 M sucrose solution. After about 10 s, the testers identiﬁed whether the solutions were sweet and the results were recorded to identify which solutions were sweet. Total three people were chosen as the tester and every experiment was repeated for at least three times.

3. Result and discussion

3.1. Overall structure of Mab II in crystals

The final Mab II model contains two Mab II molecules in an asymmetric unit (Fig. 1), which are related by a non-crystallographic (NCS) twofold rotation axis nearly parallel with the b axis of the unit cell (Fig. 1a). The general folds of the two Mab II molecules are similar to each other with an rmsd of 0.464 Å for main chain atoms. These two NCS-related molecules contact each other by their respective residues B34–B40 in an antiparallel arrangement through a well-ordered hydrogen-bond network (Fig. 1b). Their surfaces in this contact region display a high degree of shape complementary, exhibiting a shape coefﬁcient (Lawrence and Colman, 1993) of 0.757 (Sc = 1.0 for interface with geometrically perfect ﬁts), and a buried surface area of 435 Å2 as calculated by SURFACE (Chothia,
1975) (the total surface area of a Mab II molecule is 5506 Å²).

3.2. A novel structure type of sweet protein

The full sequence of the monomeric Mab II molecule contains 105 residues (Fig. 2a), which shows no significant homology with that of all the other sweet proteins reported so far. The double chain structure of Mab II belongs to “all alpha protein” in SCOP classification (Murzin et al., 1995), which is distinct from all known structures of the sweet proteins featuring beta or alpha/beta type. Thus, the 3D structure of Mab II presents a novel structure type of the sweet proteins (Table 2). The molecule Mab II is the only sweet protein that consists of two covalent-linked polypeptide chains. These two chains are crosslinked by two inter-
Fig. 2. Unique structure of Mab II molecule. (a) Sequence and secondary structure distribution of Mab II. (b) The stereo drawing of the general fold of Mab II molecule. The unique segment B54–B64 is highlighted by a circle. (c) Topology diagram of the unique [NL/I] tetralet motif. (d) Space-filling model of Mab II. The side chains of Asn residues from B54, 57, 60, and 63 of the unique motif are highlighted, which are rooted in a hydrophobic cluster and presented on the ligand-accessible surface. (e) Structure of the [NL/I] tetralet motif in the orientation same as (d). The side chains only involved in the motif are shown. All side chains of hydrophobic residues are shown in green stick. All O and N atoms in drawings are colored in red and blue, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)
chain disulfide bridges (CysA5-CysB21 and CysA18-CysB10). In addition, the B-chain has two intrachain disulfide bridges (CysB11-CysB59 and CysB23-CysB67) (Fig. 2a and b). The A-chain consists of two α-helices (h1, A4–A12; h2, A15–28) connected with a hydrogen-bonded turn (t1, A13–A14) to form a helix–turn–helix motif (Fig. 2a and b). The B-chain has three α-helices (h3, B2–B15; h4, B21–B37; h5, B41–B59) (Fig. 2a and b). Helices h3 and h4 are connected by a short 3_10 helix (g1, B16–B20) to form a helix–turn–helix motif. Helices h4 and h5 are connected with a short loop (I1, B38–B40). The C-terminus of B-chain is a coil (c1, B65–B71) and connected to h4 through a β-turn (t2, B59–B64). Helix h3, which contains the Cys-Cys motif, is linked to h2 and to the C-terminal end of h5 by disulfide bridges. The N-terminus of h4, which contains Cys-X-Cys motif, connects to h1 and to the C-terminus of the protein (c1) (Fig. 2a and b). The overall structure of Mab II molecule is rather compact: the volume of the protein as calculated with the program VADRA (Wishart et al., 2003) is 12900 Å^3, 12% lower than expected from its molecular weight (about 14500 Å^3). This compactness, together with the existence of four disulfide bridges, may contribute to its high thermostability.

### 3.3. A unique structural motif

Detailed inspection of sequence found that the segment B54–B64 takes a unique residue arrangement, **NLPNIC-NIPN**I, which features four successive (Asn-Leu/Ile) dipolar units connected by three conformation-constrained residues, two prolines and a cystein involved in disulfide bridge, in between (Fig. 2c). We call this special local structure as [NL or NI] tetralet motif. On the tertiary structure this motif is located in the C-terminal part of h5 with the β-turn t2 (Fig. 2a and b). The motif adopts a subtle structural unity. In fact, the side chains of AsnB54, AsnB57, AsnB60, and AsnB63 are all protruded from the specific surface and ligand-accessible. It is plausible to infer that this unique structural motif may possess certain critical structure-function roles, which was generally identified by 3D-docking and experiments as shown below.

### 3.4. An amphiphilic surface

Mab II molecule has five negatively (three Asp and two Glu) and 18 positively (all Arg) charged residues. Among them the negative charged residues are scattered on the molecular surface, including two Glu (A1/C1 and B1/D1) and one Asp (A33/C33) which are disorder and located at the N- and C-terminus. Most of the positively charged residues are distributed on one side of the exterior surface to form a strongly cationic face, Face A (Fig. 3a). While, most of polar residues (Asn, Glu) with certain hydrophobic residues are gathered onto another side of the molecular surface to create a neutral face, Face B (Fig. 3b). It is noted that the four characteristic Asn presented by the unique [NL/NI] tetralet motif described above are just protruded from the neutral Face B (Fig. 3b). This unique “amphiphatic design” of the molecular surface may be related to the primary interaction of Mab II molecule with its receptor.

### 3.5. Mab II-hT1R2/T1R3 complex model from 3D-docking

Following the account of the “wedge model” for sweet proteins by Temussi and coworkers (Temussi, 2002; Morini et al., 2005), the 3D-dockings of Mab II interacting with all four possible forms of the extracellular heterodimeric sweet receptor hT1R2/T1R3 were performed. The refined Mab II structure was first docked into the hT1R2/T1R3 Aoc_AB receptor. A global exhaustive six-dimensional grid search through the relative translations and rotations of the molecules was performed. The energy scores of all possible complex models were sorted, and 10 candidates with the highest score and the lowest energy were built up for the further evaluation. Each generated model was then taken into a manual inspection on its reasonableness from the structural aspect. The detailed stereochemical analysis showed that, among 10 candidates, five models displayed serious close contacts between Mab II and the receptor in some areas (Table 3) showing the spatial hindrance, which therefore were discarded. The remaining five candidates were sorted according to their folding energy and contact areas (Table 3) and the best one in the list, Candidate 1, was chosen as the representive model of Mab II-hT1R2/T1R3 complex.

In this complex model, the Mab II molecule contacts only with protomer T1R3 of the receptor and fits to T1R3 structure well with a good stereochemical complimentary as shown in Fig. 4. There is an area as large as
1532 Å² to be buried at the interface and the extensive intermolecular interactions being found between Mab II and the receptor. These indicate that the model is generally reasonable. Besides, it is noticed that the docking areas of Mab II with the receptor are roughly in the same site of the “wedge model” as described by Temussi and coworkers (Temussi, 2002; Morini et al., 2005).

Overviewing the representative complex model, the B-chain of Mab II is mainly involved in the interaction with T1R3 (Fig. 4). Looking at the interface of the generated complex three essential surface regions of Mab II, designated I1, I2, and I3, respectively, are involved in the direct contacts with target structure T1R3. I1 mainly consists of B-chain segment B54–B69 including the unique [NL/NI] tetralet motif (B54–B64), which is located at the neutral Face B of the molecule. It is interesting to note that the side chains of the specific four Asn in this motif are just protruded from this surface to touch with the target T1R3, which implied that the unique [NL/NI] tetralet motif may play certain important roles in the interaction of Mab II with its receptor and elicitation of the sweetness of Mab II. I2 and I3 consist the segments from B-chain’s N-terminus and C-terminus, respectively, which are distributed in the cationic zone and contact to the two partner surface of T1R3 in charge complimentary (Fig. 4c).

The docking tests of Mab II with the other three models of T1R2/T1R3, Roo_AB, Roo_BA, and Aoc_BA were also performed to search for the possible interactions in between. In the docking of Mab II to the inactive forms of T1R2/T1R3 (Roo_AB and Roo_BA), a series of docking models were built, but they all did not show the good charge complementary and the distinct difference in energy, contact area of surface and molecular orientation. We considered that a specific binding of Mab to this form of the receptor seems unlikely as the same as that of MNEI described by Morini et al. (2005). Furthermore, some models of Mab II and Aoc_BA T1R2/T1R3 were also built up from the docking. Among them, a few models showed Mab II can bind to Aoc_BA T1R2 in the way similar to that of Mab II with Aoc_AB T1R3. However, the detailed evaluation showed that the contact surfaces in both Mab II and Aoc_BA T1R2 are highly positively charged (Fig. 4d), with which the reasonable complex model possessing a suitable stereochemistry could not be constructed. Therefore, the 3D-docking experiment for the whole models of the receptor hT1R2/T1R3 interacting with the whole Mabinlin showed that Mab II seems mainly to interact with the T1R3 promoter of Aoc_AB T1R2/T1R3.

Combined the observations of the unique structure of Mab II and the docking test of Mab II to hT1R2/T1R3, it is plausible to predict that the main structural elements of Mab II for the interaction between Mab II and hT1R2/T1R3, in turn, for eliciting the sweet taste of Mab II are in B-chain of Mab II molecule. And the unique motif B54–B64 of Mab II might be one of the critical sites for the sweetness of Mab II. These predictions were generally identified by the following experimental results.

3.6. Separation of A- and B-chains

To gain the individual A- and B-chains, the denatured Mab II protein by 6 M guanidine hydrochloride and
Fig. 4. Mab II-hT1R2/T1R3 complex model constructed by 3D-docking. (a) Overview of the complex structure of Mab II and Aoc_AB T1R2/T1R3 model drawing with α-carbon traces showing that only B-chain of Mab II and the protomer T1R3 of the receptor are involved in contacts. The A- and B-chains of Mab II and T1R2 and T1R3 of the receptor are colored in blue, red, yellow, and green, respectively. (b) Space-filling drawing of Mab II-T1R3(Aoc_AB) complex showing that the B-chain of Mab II is main structural element involved in contacts with T1R2/T1R3. The A- and B-chains of Mab II and the protomer T1R3 are colored in blue, red, and gray, respectively. (c) Separately drawings of the electrostatic potential surfaces of Mab II (right) and T1R3 (left) in the complex of Mab II and Aoc_AB T1R2/T1R3 model. The three areas involved in the interactions between Mab II and T1R2/ T1R3 are highlighted with circles. The contact site I1 containing the B54–B64 motif of Mab II is located on the neutral Face B showing in Fig. 3. (d) Drawings of the electrostatic potential surface of T1R2 from Aoc_BA T1R2/T1R3 model with the same orientation as in (c), showing the positively charged surface should repel the Mab II’s binding due to Mab II is mainly positively charged. The figures were prepared by Molscript and PyMol.
5 mM β-mercaptoethanol was isolated by an AKTA purifier system on a C18-reverse-phase column. There were two sharp peaks appeared in elution (Fig. 5a). The two lyophilized samples collected from the elution were used in MALDI-TOF mass spectrum analysis. The resulted molecular mass of Peak 1 and Peak 2 are 4146.50 and 8298.84 Da, which are, respectively, coincident with the calculated masses of A-chain (4168.6 Da) and B-chain (8268.7 Da). The result confirmed that A-chain and B-chain of Mab II have well been separated and purified. Then the fluorescent emission of TrpB72, the only tryptophan residue in the B-chain, was employed to probe the conformational state of the separated B-chain. The wavelength of B-chain’s fluorescence was 352.2 nm which generally coincident with that of the native Mab II (355.6 nm) (Fig. 5b). It has reported that the wavelength of the fluorescence emission from the residue TrpB72 has a significant red shift, from 352 to 370 nm, when Mab II was denatured (Hu et al., 1985a,b). At the same time, 1D 1H NMR spectrum of Mab-B has well dispersed peaks indicating that Mab-B was well refolded and has secondary and tertiary structures (Fig. 5c). These results indicate that the separated Mab-B should have a definite fold and may be adopted a conformation similar to that in the native Mab II.

3.7. Experimental test of interactions between hT1R2/T1R3 and Mab II, Mab-A, Mab-B

To determine whether Mab II, Mab-A, and Mab-B can interact with the human sweet receptor hT1R2/T1R3, the human sweet receptor was expressed by transient transfection in HEK293E cells along with G16-gust44 and then monitored activation (calcium mobilization) by indicator dye using D-tryptophan as a positive control. The stimulation experiments showed that the human sweet receptor responds to both Mab II (0.5%) and Mab-B (0.5%) and D-tryptophan (10 mM), but essentially not to Mab-A (0.05%), as shown in Fig. 6. The results indicate that both the integrated Mab II and the individual Mab-B can interact with the sweet receptor hT1R2/T1R3 almost in the same scale. It is identified that Mab-B is the principal subunit of Mab II for its interaction with the sweet receptor hT1R2/T1R3.

3.8. Sweetness evaluation of separated A- and B-chains of Mab II

Regular sweetness test was performed by trained people with the regular procedure (Hu and He, 1983). With the sample solutions at the same concentration both the native Mab II and separated Mab-B elicited strong sweetness, but Mab-A did not induce evident sweet feeling. Interestingly, the sweet feelings elicited by Mab II and Mab-B are rather different. The sweetness produced from Mab-B is immediate and disappears in a short time when Mab-B solution is dropped to the tongue of people, whereas the sweet taste from the native Mab II will be lasted for half an hour. These results are coincident.

Fig. 5. Identification of A- and B-chains’ separation. (a) The elution profile from a C18 reverse phase column (5 µm, 4.6 x 250 mm, Buffer A is 0.1% TFA, Buffer B is 0.1% TFA and 70% acetonitrile) on an AKTA Purifier system (Amersham Pharmacia Biotech). The molecular masses of Peaks 1 and 2 are determined by MALDI-TOF mass spectrum analysis, which are well coincident with the theoretical values of the A-chain (4168.6 Da) and B-chain (8299.7 Da) considering the reasonable error from the facility. (b) The fluorescence emission of Mab II and Mab-B. Compared with the native Mab II the spectrum from the separated B-chain has a small red shift (from 352.2 to 355.6 nm), which indicates that Mab-B is properly refolded as in the native. (c) 1D 1H NMR spectrum of Mab-B. The well-dispersed peaks indicate that Mab-B was well refolded and has secondary and tertiary structures.
with the observations from the binding test in the calcium imaging experiments described above in which hT1R2/T1R3 responded to Mab-B more quickly than it does to Mab II (data not shown). These tests identified that the B-chain of Mab II can elicit the sweet taste alone and should be the main structural element of the sweetness elicited by Mab II, while the A-chain may play a role in gaining the long aftertaste for the native Mab II. The dissection of the main structural basis of the sweet properties eliciting by Mab II will be of benefit to develop a unique sweetener from Mab II.

Fig. 6. Response test of the sweet receptor hT1R2/T1R3 to Mab II, separated A-chain (Mab-A) and B-chain (Mab-B). HEK-293E cells coexpressing human T1R2, human T1R3 along with Gα16-gust44 were loaded with calcium indicator dye Fluo-4, and stimulated with purified Mab II (0.5%), Mab-A (0.05%), Mab-B (0.5%), and D-tryptophan (10 mM). Left panels show the fluorescence images of cells before the addition of the stimuli, and right panels show the fluorescence images 25–35 s after addition of stimuli. The evident responses to Mab II, Mab-B, and D-tryptophan, a positive control, were detected, but no response to Mab-A was detected. The mock transfected cells show no response to any of them (data not shown).

4. Conclusion remark

The sweet protein Mab II isolated from the plant of C. masaikai Levl. (mabinlang) consists of two polypeptide chains. The crystal structure of Mab II has been determined at 1.7 Å resolution, which is unique in an “all alpha” fold type to be distinct from all known sweet protein structures. The Mab II shows an amphiphilic molecular surface, in which most of 18 positively charged residue (all Arg) are distributed on the same side of the exterior surface to form a cationic face (Face A), and some polar residue (mainly Asn and Gln) with certain hydrophobic residues are gathered onto another side to create a neutral face (Face B). This unique “amphipathic design” of the molecule may relate to the primary interactions between Mab II and its receptor. A unique structural motif consisting of the B-chain segment B54–B64 with a special sequence NL–P–NI–C–NI–P–NI is found on the neutral Face B. This motif features four [Asn-Leu/Ile] units connected by three conformation-constrained residues (two Pro and one Cys involved in disulfide bridge), thus called the [NL/I] tetralet motif. In the refined structure, four Asn residues of the motif are rooted in the (Leu/Ile) hydrophobic cluster to be well stabilized and their orientation are directed by Pro or Cys in the disulfide bridge to form a tertiary structural unity on the surface. This special structural organization implies the certain structure–function significance. Following the account of the “wedge model” for sweet proteins interacting with the sweet-taste receptor (Temussi, 2002; Morini et al., 2005), the 3D-docking between Mab II and the sweet receptor hT1R2/T1R3 model were performed, which suggest that the B-chain of Mab II is the main structural element for the interactions with the receptor and the unique tetralet motif consisting of residues B54–B64 may be one of the essential binding sites. To experimentally verify the prediction from the 3D-docking, the two polypeptide chains, A and B, are separated and identified to possess the fold state similar to that in the native Mab II. By using the calcium imaging with the HEK293E cells coexpressed hT1R2/T1R3, the experiments for testing the possible interactions of A-chain, B-chain and the native Mab II, show that hT1R2/T1R3 responds to both the integrated Mab II and the individual B-chain in the same scale, but not to A-chain. The sweetness evaluation further identified that the separated B-chain can elicit the sweetness alone, but A-chain does not. However, compared with the native Mab II, the special aftertaste is lost for the individual B-chain. In combination of all data we conclude that the sweet protein Mab II can interact with the human sweet-taste receptor hT1R2/T1R3 to elicit its sweet taste. The B-chain with a unique [NL/I] tetralet motif is the essential structural element for the interaction with the sweet receptor to elicit the sweetness, and the A-chain may play a role in assisting the integrate Mab II to gain a long aftertaste. The findings reported in this paper will be advantage for understanding the diversity
of sweet proteins and engineering research for development of a unique sweetener based on Mab II structure as a native model.

Protein data bank accession code

Coordinates and structure factors for the structure of Mab II have been deposited at the Protein Data Bank with accession code 2DS2.

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