Summary on Monellin **

Comparative Summary of Sweet protein - Monellin

Rank	Solution	Solution proposed by	Non caloric or very low calorie	Stable at low pH (~pH 3.0)	Sensory profile closer to sugar	No licorice taste or Aftertaste	Thermal stability	Stable to UV exposure	Low cost	Robust supply chain	Solubility
1	Fusing two chains of Monellin protein into one through G6-I46 binding	University of California-Berkeley, **Lucky Biotech Corp**	Yes	**pH stability at 2-6**	Sweetness	**Improved lingering time**	**Renatures easily even after heating to 100°C at low pH**			Mass production	
11	GlyPhe variant of Monellin	University of Naples Federico II, Italy	Yes	**pH stability (sample of 3.2 pH was evaluated)**	Sweetness	**No off-flavour i.e. non-sweet taste like bitterness (aftertaste)**	Heat stability				
17	Mut9 Monellin variant	University of Naples & **Consorzio Sannio Tech**	Yes	**2.5 and 5.1**	Sweetness		85			Cheap and scalable production	
20	New Monellin sweetener	**iSWEETCH**	Yes		3000 times sweeter than sugar	**No aftertaste**				No by-products, green production	Water soluble
30	E23Q Monellin Variant	University of Naples Federico II	Yes	**3.5-6**	Sweetness		**90°**			Mass production	
31	Y65R Monellin variant	Università di Napoli Federico II, University of Sannio	Yes	**3.3–7.0**	0.665 µg/l		80.13 degree			Mass production	Increased solubility at low pH
35	High temperature stable E24Q/Y80R H-Monellin	China Pharmaceutical University	Yes		Sweetness		**Tm: 96.1℃. When treated at 90℃ , it was retained 80% in 1 hour**				
36	High temperature stable E23A H-Monellin	Qilu University of Technology	Yes		Sweetness		**No detectable change after incubation at 80°C for 10 h, and it was totally denatured after heat treatment at 85°C for 2 h**				

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2. Solutions on Monellin Modification

Monellin (1/3)

Introduction

- **Monellin is intensely sweet protein extracted from African berries, the serendipity berry**.
- **The protein is ~ 100 000 times sweeter than sugar** on a molar basis and several thousand times sweeter on a weight basis. Monellin consists of two nonidentical subunits, formed by 45 and 50 amino acid residues, called A and B chains, respectively. **Monellin consists of two peptides, the A-chain of 45 residues and the B-chain of 50 residues.**
- The crystal structures of monellin and thaumatin proteins have been determined at 3 A resolution and there appear to be no significant gross structural similarities except for /J-sheet structural motifs which are generally quite common among protein structures.
- **Monellin is low in calories due to their high potency**.
- **Monellin is safe, natural and neither introduce non-natural metabolites into the body nor perturb the balance of the amino acid pool**. This is not the case with aspartame or with other amino acid sweeteners, these elevate specific amino acid pools and could cause adverse effects after degradation.
- The quaternary structure is kept by the secondary binding forces and the crystal structure was determined. This structure makes native monellin quite unstable.
- **It is relatively simple to clone the genes for the proteins and mass produce the protein for practical use.**
- It is relatively easy to generate a large number of variants with different amino acid substitutions, deletions and insertions at various positions in order to search for more desirable taste as well as physical properties.
- The proteins or their variants can be used as reagents for isolating sweet taste receptors or receptor genes.
- **The protein loses sweetness when heated above 50°C under acidic pH**.
- **Monellin has no disulfide bond and is thermally less stable than thaumatin**.
- **Protein sweeteners with high renaturability are more useful in practice, because people prefer to drink or eat foods which are at a moderate temperature even though they may have been heated to a higher temperature previously**.

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Monellin (1/3)

Introduction

- Monellin is a sweet protein extracted from the West African plant **Dioscoreophyllum cumminsii**.
- **Natural monellin consists of 94 amino acids with a molecular weight of 10.7 kDa. It consists of two chains A and B which are held together by noncovalent interactions**.
- The A and B chains of the protein contain 44 and 50 amino acids, respectively. **Monellin has a secondary structure consisting of five β-strands that form an antiparallel β-sheet and a 17-residue α-helix, as revealed in the resolved crystal structures of this protein and some of its variants (e.g., PDB: 1MOL, 2O9U).**
- Monellin is proposed to be a promising sweetener. However, it is unstable at high temperatures or extremes of pH, which limits its extensive applications in food industry.
- Single-chain monellin protein was created in which the two natural chains are joined via a Gly-Phe dipeptide linker, and site-directed mutagenesis has been extensively used to modify the functional properties of the protein.
- Artificial single-chain monellin is as potently sweet as the wild type and is more stable upon temperature. However, few studies have reported the direct improvement of sweetness and thermostability of the protein by the gene mutation and protein modification techniques until now.
- **Mutations of residues G1M, E2M, and E2N have been shown to result in an obvious improvement of the sweetness**. Rega et al. reported a mutant **Y65R with significant increase of sweetness and solubility in acidic conditions** and compared the structure and function between the single-chain monellin and this mutant.
- Lee et al. studied the thermostability of various E23A variants with the circular dichroism analysis and succeed to transform these variants into tobacco chloroplasts. However, detailed heat resistance and thermal denaturation as well as sensory evaluation of these mutants have not been investigated.
- The sweet taste of monellin can only be detected by human and old-world monkeys in primates. Until now, the mechanism by which the sweet proteins interact with and activate the sweet taste receptor-heterodimeric T1R2/T1R3 remains elusive.
- A wedge model suggests that sweet proteins interact with the large external cavity in the receptor and then induce the receptor activation. **It has been reported that either T1R2 or T1R3 is responsible for the sweet taste difference towards monellin between human and squirrel monkey**, and the electrostatic properties of the receptors probably mediate the species-dependent response to sweet-tasting proteins.

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Monellin (1/3)

Introduction

- **Hydrophobic interactions, conformational entropy, and hydrogen bonding are believed to contribute most to monellin protein stability.**
- The functional analysis of wild type MNEI (single-chain monellin) and the G16A and V37A mutants showed an order of the thermal stability, WT (wild type)>G16A>V37A>G16A/V37A, and an order of sweetness threshold value, G16A>G16A/V37A>V37A>WT.
- The lowest threshold value indicated the protein with most sweetness.
- The least sweet mutant, **G16A-MNEI, was not the least stable protein**.
- The study indicates that there is no correlation between the stability and sweetness of the sweet protein monellin another study pointed out that coulombic interactions are of primary importance for the function of monellin.
- **Charge-charge interactions play a less prominent role in protein assembly and stability compared to interactions involving hydrophobic core residues.** However, it is suggested that the net charge of the protein surface can also affect the protein stability as well as its sweetness.
- Sweet protein monellin is approximately **3000 times sweeter than sugar**. However, the protein has a slow onset of sweetness and a lingering aftertaste. On the other hand, **sweetness of monellin is pH and temperature dependent**.
- **The protein is of taste at pH 2–9, and heat treatment over 50°C at low pHs denatures monellin protein with a loss of the sweetness**.
- The reference here discloses about **methods to increase the thermal stability and quality of sweetness of the sweet-tasting protein by site-directed gene mutagenesis and protein modification.**
- Mutants were obtained by alanine substitution of amino acids at different sites in monellin protein, and their changes of thermal stability and sweetness threshold were evaluated.
- **Monellin is approved as a food additive in Japan.**

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2.1 Fusing two chains of Monellin protein into one through G6-I46 binding

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Fusing two chains of Monellin protein into one through G6-I46 binding (1/3)

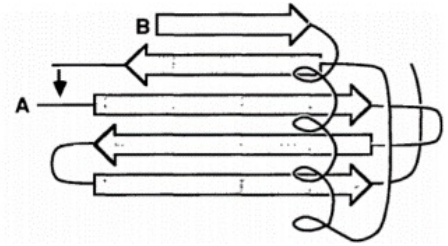
Solution

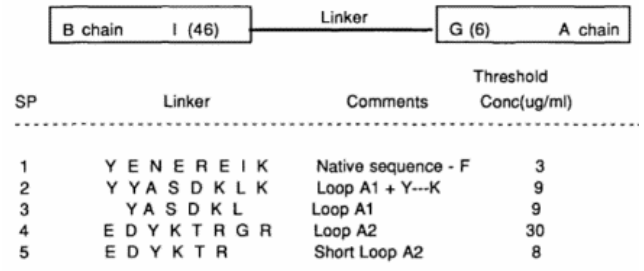
The solution to the problem is proposed by Lawrence Berkeley Laboratory, University of California-Berkeley, Lucky Biotech Corp

Non-patent Published in 1989

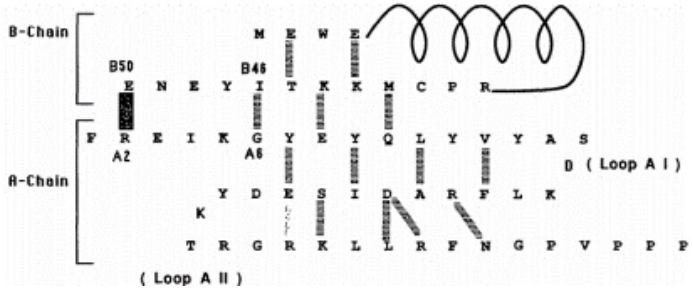
- Based on the crystal structure of monellin, researchers from university of California **fused the two chains into a single chain using several different linkers copied** and 'transplanted' from the same molecule. One of the **newly designed proteins is as potently sweet as the natural one, is more stable upon temperature or pH changes, and renatures easily even after heating to 100°C at low pH**.
- The researchers have **redesigned the molecule from two peptides into a single chain protein which shows substantially increased thermal stability and excellent renaturation properties after heat denaturation**.
- The structure shows that the carboxy-terminal B-strand of the B-chain forms an antiparallel B-sheet with the amino-terminal B-strand of the A-chain. The last three residues of the B-chain and the first four residues of the A-chain are partially disordered. However, we can clearly see that **isoleucine-46 of the B-chain is in register and hydrogen-bonded to glycine-6 of the A-chain**. In designing the junction between these two residues, we considered two main points: (i) the size and sequence of the **connecting peptide should be compatible to maintain the registration of hydrogen bonding in the B-sheet and should preserve the conformation identical to that in native monellin**; and (ii) since it is not known which part of the molecule is the recognition site for the sweet receptor, the **linker region should contain sequences copied and 'transplanted' from other parts of the same molecule, and should not introduce any foreign sequences**.
- **The crystal structure of monellin suggested a very natural and obvious way of fusing the two peptides of monellin together**.
- The structure shows that the **carboxy-terminal /3-strand of the B-chain forms an antiparallel /3-sheet with the amino-terminal /3-strand of the A-chain.**
- **At the current resolution, the last three residues of the B-chain and the first four residues of the A-chain are partially disordered.**
- Isoleucine-46 of the B-chain is in register and hydrogen-bonded to glycine-6 of the A-chain.

Application in beverages.





SP	Linker	Comments	Threshold Conc(ug/ml)
1	Y E N E R E I K	Native sequence - F	3
2	Y Y A S D K L K	Loop A1 + Y---K	9
3	Y A S D K L	Loop A1	9
4	E D Y K T R G R	Loop A2	30
5	E D Y K T R	Short Loop A2	8



Source

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Fusing two chains of Monellin protein into one through G6-I46 binding (2/3)

Tests

Sweetness Assessment Test:

- **Sweetness was assessed by putting on the tongue 50 µl of aqueous solution of the highly purified protein at 80 µg/ml concentration**.
- After a few seconds the tongue was washed with large quantities of water several times.
- Two independent assessments were made for each sample. The temperatures listed in the Table are those of solutions before the taste assay (A) or before cooling the samples to room temperature (B). Actual temperature of the drop on the tongue at the time of response is difficult to measure and may not be relevant from a practical point of view.

Results

- The results show that the overall structure of the single chain monellin does not change very much when the linkers are copied and 'transplanted' from the known loop regions of the same molecule, **suggesting that this approach may serve as a general rule of practical use in protein designing**.
- Several redesigned single chain monellins with different linker sequences taste sweet implies that the **sweet determinant is not located in the linker region of the molecule**.
- The **redesigning of natural two-peptide sweet protein into a single chain protein has been done which has substantially improved thermal and pH stability, excellent renaturability, and is easier to mass produce by currently available technology**. Circular dichroism spectra at room temperature of the fused monellin SP1 are practically identical to that of natural monellin at pH 7, and **stable throughout the entire pH range between 2 and 10**.
- One of the newly designed proteins is as **potently sweet as the natural one**, is **more stable upon temperature or pH changes**, and **renatures easily even after heating to 100°C at low pH**.

Table I. Sweetness after heating

Temp (°C)	pH 2		pH 4		pH 6	
	M	SP1	M	SP1	M	SP1
	(A) Taste response at given temperature					
40	+	+	+	+	+	+
50	−	+	+	+	+	+
60	−	−	+	+	+	+
70	−	−	−	+	+	+
80	nd	nd	−	+	+	+
90	nd	nd	nd	nd	nd	nd
100	nd	nd	nd	nd	nd	nd
	(B) Taste response at room temperature following heating					
40	+	+	+	+	+	+
50	−	+	+	+	+	+
60	−	+	+	+	+	+
70	−	+	+	+	+	+
80	nd	+	+	+	+	+
90	nd	+	+	+	+	+
100	nd	+	+	+	+	+

+, Sweet; −, not sweet; nd, not determined; M, natural two chain monellin; SP1, single chain monellin.

Source

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Fusing two chains of Monellin protein into one through G6-I46 binding (3/3)

Application

- The method is applicable for redesigning a sweet protein which is potently sweet then the natural one and is more stable upon temperature or pH changes, and **renatures easily even after heating to 100°C at low pH**.
- By using this method it becomes relatively easy to generate a large number of variants with different amino acid substitutions, deletions and insertions at various positions in order to search for more desirable taste as well as physical properties.

Conventional Solutions

The conventional monellin protein losses its sweetness above 50°C under acidic pH.

The conventional monellin has a long-lasting lingering effect.

Advantages

The monellin produced by this method renatures easily even after heating to 100°C at low pH.
Newly designed proteins is as **potently sweet as the natural one**, is more **stable upon temperature or pH changes**, and **renatures easily even after heating to 100 degrees C at low pH**.

The monellin produced by this method has an **improved lingering time**.

Other Advantages

- They are low in calories due to their high potency.
- It is relatively simple to clone the genes for the proteins and **mass produce** the protein for practical use.
- The proteins or their variants can be used as reagents for isolating sweet taste receptors or receptor genes.

Comment

- Redesigning the Monellin molecule from two peptides into a single chain protein which shows substantially increased thermal stability and excellent renaturation properties after heat denaturation has been done.
- The newly designed proteins is as potently sweet as the natural one, is more stable upon temperature or pH changes, and renatures easily even after heating to 100 degrees C at low pH.

Source

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2.2 GlyPhe variant of Monellin

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GlyPhe variant of Monellin (1/7)

Solution

The solution to the problem is invented by University of Naples Federico II, Italy

Non-patent Published in 2014

- Different factors, such as cost, availability, legislation, safety, exports or reduced energy intake, often drive food companies to consider the substitution of one sweetener with another appropriate, or even necessary, sweetener. **For these reasons, continuous research is focused on sugar substitutes in order to find one that is noncaloric, safe, economic, without aftertaste and, at the same time, is able to elicit the best sensory performance**.
- Sweet proteins are an attractive alternative to some of the most traditional sweeteners because they respond to the need of diabetic patients and consumers for low-calorie products.
- MNEI, the protein studied in this work, is a single chain derivative of monellin, a **sweet protein extracted from the plant Dioscoreophyllum cumminsii**.
- **The modifications are based on the insertion of two amino acids, i.e., a GlyPhe dipeptide**, **to covalently link the A and B chains** constituting the original molecules, and thus the composition of the single chain MNEI contains only natural building blocks; thus, it is completely compatible with that of natural proteins but **retains its sweetness in the wider range of temperature and pH**.
- For a full sensory characterization of a new sweetener, it could be useful to find its detection and recognition thresholds and to compare them with those of common sweeteners.
- Previously, it was found that **taste detection threshold (DT) and recognition threshold (RT) of MNEI were 3,000 times lower than those of sucrose determined by the same assessors**.
- Considering that the sensory performance of a stimulus can be affected by contour conditions, the effect of different experimental conditions on DT and RT of MNEI in order to assess its stability in a model industrial process was also evaluated.
- **Mineral content and serving temperature affected MNEI DT, whereas pH and thermal treatment did not affect the perceived intensity of sweetness**.

Application in beverages.

Source1, Source2

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GlyPhe variant of Monellin (2/7)

Tests

- **None of the current low-calorie sweeteners match sucrose in terms of sweet taste quality or temporal characteristics**.
- The **time–intensity (t-I) method is used to obtain the temporal profile of a product**, by monitoring, measuring and recording the perceived intensity of each attribute over time. By considering that t-I records the evolution of one specific attribute over time and that data analysis is quite difficult, some scientists have proposed alternative methods to time–intensity, such as the dual-attribute time–intensity, the temporal dominance of sensations and, more recently, the multiple t-I.
- The temporal dominance of sensation (TDS) method determines the dominant sensation or prevalence of one over the others during product evaluation.
- Time–intensity and TDS are both dynamic sensory methods but provide different and complementary information; in fact, **TDS is a descriptive multi-attribute methodology that focuses on the concept of sensory dominance and deals with the interactions among attributes, whereas t-I focuses on the evolution of the intensity of one attribute at a time**.
- Many t-I studies were focused on taste sensation evolution, such as sweetness, whereas TDS was used to evaluate the sensory performance of a new sweetener in coffee with good results.
- The objective of this study was to compare the sensory performance of a sweet protein, MNEI, with aspartame, saccharin and sucrose.
- First, equi-sweet concentrations were found and then the sweetness temporal profiles of the four sweeteners were determined. Finally, the dominant attributes over time in model beverages were monitored.

Source1, Source2

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GlyPhe variant of Monellin (3/7)

Tests

Equi-Sweetness Determination:

- Aspartame, saccharin and MNEI have been found to be 125–238 times, 300–500 times and 3,000 times sweeter than sucrose, respectively, on weight basis. Thus, guided by literature results, different solutions of sucrose (30 g/L; 40 g/L), aspartame (0.18 g/L; 0.21 g/L), saccharin (0.08 g/L) and MNEI (0.013 g/L; 0.0098 g/L) were prepared and tested using a sip-and-spit method. During each session, assessors received a reference sample (R = 40 g/L sucrose) and five samples. Each sample (10 mL) was served, at room temperature (20 ± 3C), in a plastic cup and coded with three-digit random numbers. The assessors were asked to taste the reference first and then each sample. For each sample, they were required to indicate if the sweetness intensity was less, more or equal to that of the reference, using a 10-cm linear scale, anchored from 0 (much less sweet than reference) to 10 (much sweeter than reference) and 5 in the middle (as sweet as R). A complete block design was used. The assessors were asked to rinse their mouths well with water between samples and wait at least 30 s after each evaluation. Three replicates were carried out.
- The scores achieved by all the sweeteners during the difference from control test were submitted to one sample t-test ($P \leq 0.05$) in order to evaluate whether they were significantly different from the reference (sucrose 40 g/L, corresponding to a score of 5 on the scale). The concentration of each sweetener that was not significantly different from a score of 5 was considered as an iso-sweet concentration when compared with the reference.

Time–Intensity:

- Six trained assessors evaluated the sweetness temporal profiles of aspartame, saccharin, sucrose and MNEI in aqueous solution at equi-sweetness concentrations. Ten milliliters of each sample was served in a plastic cup and coded with three-digit random codes. **First assessors sipped an aliquot of sample and started to score the intensity of sweetness perceived, then, they swirled it around their mouths and spat it out after 5 s and continued to score the sweetness intensity, for a total of 60 s**. To record responses, the assessors moved the mouse cursor along a vertical linear anchored scale from 0 (no perceived) to 10 (highest intensity). A period of 90 s between two sample evaluations allowed the assessors to rinse their mouths with water. At each session, assessors evaluated all the samples presented in a randomized order. Five replicates of t-I test were carried out.

Data Analysis:

- Data were collected by FIZZ software. The SPSS v.17 was used to determine the equisweetness concentrations, obtained by the difference from control test, and to analyze the parameters extracted from the t-I curves. The dominance rates of the sensory attributes for each evaluated sweetener were calculated by means of FIZZ Calculation software.

Source1, Source2

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GlyPhe variant of Monellin (4/7)

Tests

TDS:.

- Four model beverages containing equi-sweetness concentration of sweeteners, 0.09 g/L of citric acid and strawberry flavor (50 µL/L) were prepared in order to evaluate the dominant attributes over time. **The pH of samples was 3.2. Samples (10 mL) were presented in a balanced and randomized order to the assessors**; coded plastic cups, covered with plastic lids, were used to serve them. The first session was dedicated to attribute generation; the four samples were simultaneously presented to the assessors, and the attributes cited by at least 50% of the assessors, in at least one sample, were selected. **During the evaluation time (100 s), the assessors tasted the product and immediately chose the attribute considered as dominant from the attributes list provided; when the dominant perception changed, the assessors had to select the new dominant sensation.** The order of attributes on the screen was randomized among the assessors to minimize the effect due to their sequence. There was a break of 90 s between evaluations. At each session, assessors evaluated all the samples, presented in a randomized order. **Five replicates of TDS analysis were made**.

Temporal Dominance of Sensations

- For each product, the dominance rate, defined as the proportion of runs, "n" (subject × replication) for which a given attribute was assessed as dominant, was computed. These proportions, transformed through a smoothing B Spline (Fizz Calculator), were plotted against time and called TDS curves. To assist the interpretation of TDS curves, the chance level and significance level were plotted on the graphs.

TABLE 1. EXPERIMENTAL CONDITIONS OF MNEI SOLUTION EVALUATION

Experimental condition	Water	pH	Thermal treatment	Serving temperature
E_1	A	6.8	held at 20C	20C
E_2	B	7.0	held at 20C	20C
E_3	A	6.8	held at 10C	10C
E_4	A	4.3	held at 20C	20C
E_5	A	6.8	heated from 20 to 90C – held at 90C for 15 min – cooled from 90 to 20C	20C
E_6	A	3.8	heated from 20 to 90C – held at 90C for 15 min – cooled from 90 to 20C	20C

Thermal Treatment and Combined pH Effects:

- In order to study the thermal treatment effect on MNEI sensory perception, conditions E1 and E5 were compared. The effect of thermal treatment on DT of MNEI is illustrated in Fig. 4a. When the assessors evaluated the sweetener after it was submitted to thermal treatment (E5), the 0.84 mg/L was the first concentration of MNEI that significantly differed from the reference ($P < 0.05$), corresponding to a DT of 0.64 mg/L, a concentration lower than that corresponding to condition E1 (1.10 mg/L). Samples submitted to thermal treatment, already at low MNEI concentration, presented greater differences from the reference than untreated samples. Hence, the percentage increase of the samples submitted to thermal treatment was higher than the other one, in the same range of MNEI concentration. The RT of MNEI in condition E5 was 0.84 mg/L corresponding to 71% of corrected answers (Table 2).

Source1, Source2

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GlyPhe variant of Monellin (5/7)

Result

Equi-Sweetness Concentrations:

- The results obtained in the first session showed that saccharin (0.08 g/ L), **MNEI 2 (0.013 g/L)** and aspartame 2 (0.21 g/L) **did not differ significantly from reference**; instead, sucrose (30 g/L) and aspartame 1 (0.18 g/L) were significantly less sweet than reference ($P \leq 0.05$). In the second session, a lower concentration of MNEI (MNEI 1 = 0.0098 g/L) was used in order to verify if a reduction of its concentration would be perceived; furthermore, a hidden reference and two concentrations of aspartame were tested, to confirm the results of the previous session. Sodium saccharin was not tested again since the iso-sweet concentration was lower than others found in the literature. The same results were found for aspartame in both sessions, i.e., the higher concentration was equal to the reference whereas the lower concentration was less sweet than the reference. These results validate the sensory procedure employed. **The higher MNEI concentration tested was found to be equal to the reference in both evaluation sessions**.

TDS:

- **The attributes selected during the first phase of the test implementation were bitterness, metallic taste, saltiness, sourness, strawberry flavor and sweetness**, as they were perceived at least by 50% of the assessors, for at least one sample. Thus, for the number of chosen attributes (P = 6), chance level (P0) and significance level (Ps), were 16 and 25, respectively. The **TDS graphs for the four model beverages; each curve represents the evolution of the dominance rate of each attribute over time**. The panel dominance rate represents the percentage of assessors who recognized an attribute as dominant at a given time. **The only dominant attributes in all of the curves were sweetness and strawberry flavor**, except for saccharin and aspartame, in which a sour taste was slightly dominant. **The other attributes were never dominant and always lower than chance level (P0)**. **The sweetness dominance attributed by assessors to aspartame was much higher (67%) than the significance level**, meaning that a high consensus characterized the panel according to the sweet taste for this sweetener. The other dominant attribute for the beverage prepared with aspartame was the strawberry flavor, from the beginning for 10 s, with a maximum dominance rate at 3 s; sour was a dominant attribute for just 0.5 s. **For the beverage sweetened with MNEI, sweetness and strawberry flavor were both the dominant attributes, but sweetness was mostly dominant at the beginning and lasted from 5 to 60 s**. At 3 s, strawberry flavor was the most dominant but was never the most dominant during the remaining part of the evaluation. The first dominant attribute **for saccharin-sweetened beverage** was strawberry flavor, which lasted from the beginning for 16 s, with a **maximum panel dominance rate of 47% at 3.5 s, after which sweetness became dominant, from 12 to 60 s, and sourness, which was briefly dominant (11–13.5 s)**. **For sucrose-sweetened beverage**, the first dominant attribute was strawberry flavor, from the beginning for 18 s and from 21.5 to 31.5 s with a maximum dominance rate at the beginning, after which **sweetness was dominant, from 2 to 54 s with a maximum dominance rate at 42 s**. The other attributes were never dominant.

Source1, Source2

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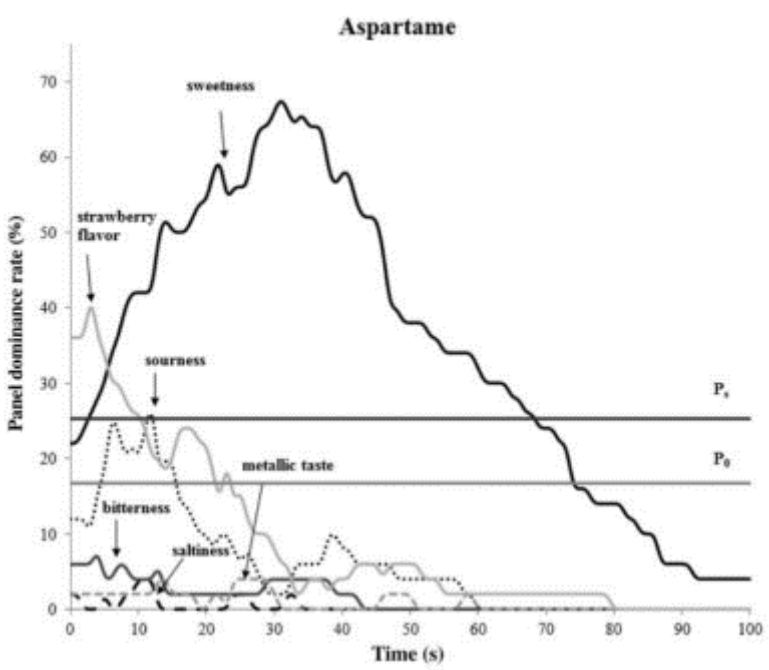
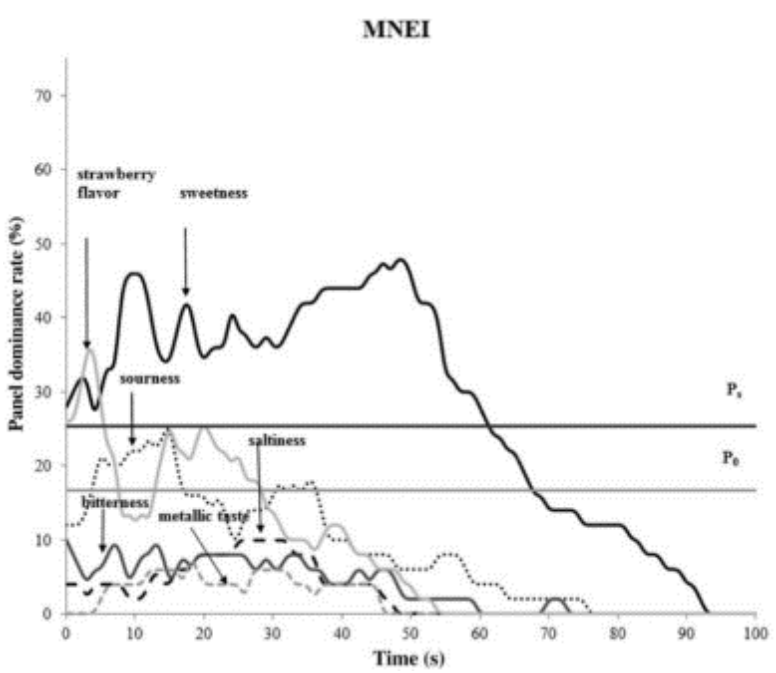
GlyPhe variant of Monellin (6/7)

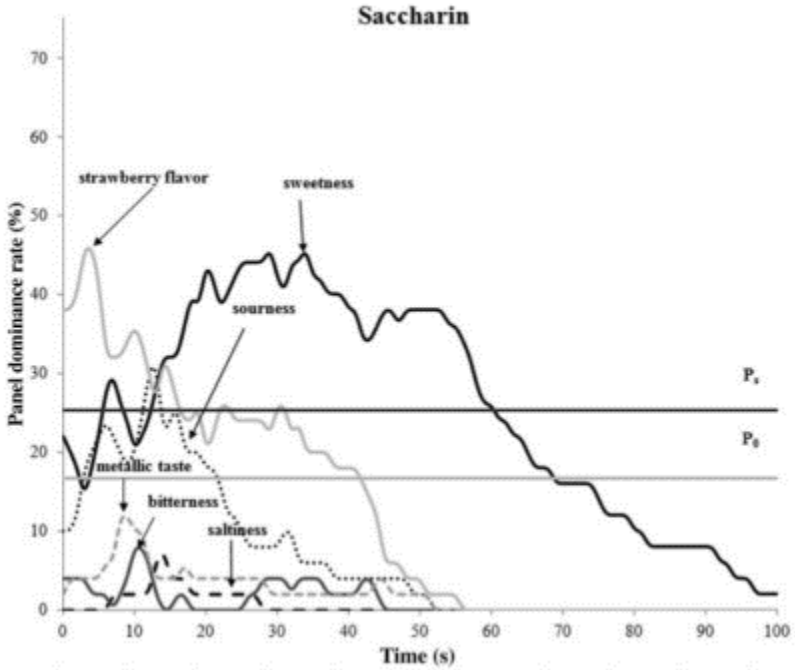
Result

Time–Intensity:

- **Paired t-test between MNEI and aspartame results showed they differed in the plateau end time (tEPI),** the first parameter related to the decreasing phase of the sensation, **indicating that the sweetness imparted by MNEI started to decrease almost 6 s later than that imparted by aspartame.** Indeed, even if the sweetness imparted by MNEI was perceived later than the sweetness provided by sucrose, in the presence of other stimuli, no significant differences were detected.







Source1, Source2

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GlyPhe variant of Monellin (7/7)

Application

- The method is applicable for comparing the sweetness temporal profile of MNEI with commercial sweeteners such as aspartame.

Conventional Solutions

The conventional MNEI had an off- flavour.

Advantages

The MNEI here does not have any off flavour.

Comment

- The MNEI used here don not have any off flavour and also has greater stability at high temperature with low calorie content.
- These properties could make MNEI a good choice as high intensity sweeteners (HIS) for low-calorie beverages and dairy products.
- It is also stable at different experimental conditions tested, even though other conditions, such as specific salt concentrations, more acidic pH or basic pH, should be explored before the application of this HPS in beverages. For a more complete understanding of MNEI sensory properties, other studies are in progress, in particular, to understand MNEI sensory behavior in more complex solutions that imitate beverage models.

Source1, Source2

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2.3 Mut9 Monellin variant **

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Mut9 Monellin variant (1/6)

Solution

The solution to the problem is invented by University of Naples & Consorzio Sannio Tech

Non-patent Published in 2021

Application in food & beverages.

- **To resolve this inconvenience, MNEI was designed by joining both subunits of the protein through a Gly Phe dipeptide linker to enhance its thermal stability. Indeed, MNEI has a melting temperature of over 70 °C and, in certain conditions, can be heated without losing its sweetness. Because of its distinct features, MNEI could be better than monellin as a substitute for commonly used sweeteners in industrial applications. In this reference, the researchers aimed at further improving MNEI features, in particular thermal and chemical stability and sweetness, by applying targeted point mutations.**
- The researchers reviewed the most promising results obtained with MNEI mutants and carefully selected some of the best performing constructs in order to combine them, yielding a "super mutant". **The mutations E23A, C41A, Y65R, and S76Y were selected so that they could be as widely and homogeneously spread on the protein surface as possible**, thus producing the maximum gain in function while not interfering with each other and not affecting the overall structure.
- **The results confirmed that Mut9 further improves the properties of MNEI**, retaining most of the beneficial features previously reported for the individual point mutations.
- **Single-chain Monellin, MNEI**, is among the sweetest proteins known and it could replace sugar in many food and beverage recipes. Nonetheless, its use is limited by low stability and high aggregation propensity at neutral pH. **To solve this inconvenience, a new construct of MNEI have been constructed, dubbed Mut9, which led to gains in both sweetness and stability.** Single-chain monellin derivatives have additional advantages, such as easy, **cheap and scalable production**, absence of insulin release, high sweetness intensity, and low environmental impact. **Mut9 showed an extraordinary stability in acidic and neutral environments, where a melting temperature over 20 °C higher was observed than that of MNEI. In addition, Mut9 resulted twice as sweet than MNEI.**

Cloning, Expression and Purification of the Mutant

- The synthetic full-length gene encoding for the sequence of Mut9 was purchased from Eurofins Genomics. **The gene was cloned into the expression vector pET22b(+) (Novagen) between the NdeI and BamHI restriction sites. The recombinant protein was expressed in Escherichia coli BL21(DE3) and purified from the cell lysate by ion-exchange chromatography followed by size-exclusion chromatography for salt removal.** Protein identity and purity were confirmed by SDS-PAGE and circular dichroism spectroscopy. Protein concentration was measured using UV-Vis spectrophotometer.

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Mut9 Monellin variant (2/6)

Tests

Differential Scanning Calorimetry (DSC)

- **Calorimetric measurements were performed using a Nano-DSC 6300.**
- **Protein samples were prepared in the appropriate buffer solutions with a concentration of 1 mg/mL and ran with a scanning speed of 1 °C/min and in a temperature range of 20–110 °C for Mut9 and 20–100 °C for MNEI.**
- During the temperature scans a total pressure of 3.0 atm was applied to both cells using nitrogen gas. Buffer scans were recorded separately under the same conditions and subtracted from sample scans to obtain the excess molar heat capacity function.
- A second run heating of the protein samples under identical conditions, after cooling down from the first run heating was also performed to verify the reversibility of the process.
- The denaturation temperature, Tm and enthalpy ΔdH were obtained by the maximum of the DSC peak and the integrated area under the peak, respectively.
- All DSC data analysis were performed using the Nano-Analyze software supplied with the instrument.

Sensory Analysis

- Sweetness intensity was evaluated by triangle test. **A team of five panelists participated in the sensory analysis. MNEI solutions and mineral water were used as positive and negative controls, respectively.**
- **Three paper cups, one containing 5 mL of protein sample and two cups containing 5 mL of mineral water were provided for the panelists to taste the samples and record their evaluation from 0 (no taste) to 5**.
- A value of 1 indicated the perception of a taste, 2 meant the taste was recognized as sweet. The sample solutions were provided from the lowest (35 nM) to the highest (220 nM) concentration. Sweetness threshold was the concentration at which the protein scored 2 on average.
- To assess taste performance upon thermal treatment, **a blind sensory analysis was performed on 20 mg/L Mut9 and MNEI sample solutions before and after boiling for 2, 5, and 10 min.**
- The boiled samples were tasted, and the assessments were made on a table with three tasting rates: same sweetness, decreased sweetness, and loss of sweetness. In both experiments, the subjects tested the sample solutions without any time constraints, then spat it out and rinsed their mouth thoroughly with mineral water within 1 min.s

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Mut9 Monellin variant (3/6)

Tests

Circular Dichroism Spectroscopy (CD Spectroscopy)

- **CD measurements were performed on a Jasco J-715 spectropolarimeter**, equipped with a Peltier temperature control system, using a 0.1 cm quartz cell.
- **The CD curves of Mut9 were obtained in 0.020 M sodium phosphate buffer at pH 2.5, 5.1, and 6.8.**
- To assess the effect of temperature, **spectra of Mut9 and MNEI in 0.020 M sodium phosphate buffer at pH 2.5 and 6.8 were measured at 10 °C intervals in the range 25–95 °C and back to 25 °C.**
- In another experiment, **CD spectra were acquired upon boiling Mut9 and MNEI dissolved in the same buffers for 2, 5, and 10 min, and cooling back the protein solutions to room temperature.**
- The spectra were taken in the far UV-range (195–250 nm) with a scan speed of 50 nm/min and each experiment was performed with 3 accumulations.

Shelf-Life Studies

- **The stability of Mut9 and MNEI was evaluated upon extended storage: samples of Mut9 and MNEI at the concentrations of 0.5 and 5.0 mg/mL were prepared at pH 2.5, 5.1, and 6.8 in 0.020 M phosphate buffers.**
- **The samples were stored for 6 months at 4 °C to simulate fridge storage, or at 37 °C for an accelerated shelf-life assessment.**
- The protein concentration of the samples was measured using a UV-Vis spectrophotometer (Thermo GENESYSTM 10UV, USA).
- Prior to each measurement, the samples were diluted 10 times by deionized water and the protein concentration was calculated using the UV absorbance at 280 nm. Protein content (%) = (measured protein concentration/Initial protein concentration) × 100.

Source

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Mut9 Monellin variant (4/6)

Results

Thermal Stability Assessment

- The melting temperatures of Mut9 at the explored pHs were first evaluated by CD thermal denaturation experiments.
- The melting temperatures were found to be near or over the instrumental maximum limit temperature (95 °C) in all conditions except at pH 2.5.
- Therefore, Differential Scanning Calorimetry (DSC) experiments were carried out to assess and compare the Tm of Mut9 and MNEI.
- **Both MNEI and Mut9 thermal stability increased going from pH 2.5 to pH 6.8, with the Tm and the ΔH reaching a maximum at pH 5.1, indicating that both the parent protein and the new mutant favor slightly acidic pH over neutral and strongly acidic environments.**
- The reversibility of the thermal denaturation process by performing a reheating run of the samples after cooling down was also observed.
- **The unfolding of Mut9 showed a very good reversibility at the acidic pHs, 2.5 and 5.1, whereas, at neutral pH, the denaturation process appeared irreversible. On the other hand, the denaturation of MNEI was reversible only at pH 2.5.**
- The stability and reversibility of the unfolding of Mut9 and MNEI were also evaluated by recording a series of CD spectra at acidic and neutral pH at increasing temperatures.
- **At pH 2.5, the spectra of Mut9 remained unchanged until near the protein's Tm (75 °C), whereas, at 85 °C, the line-shape of the spectra dramatically changed due to unfolding.**
- **MNEI was much less stable than Mut9: its unfolding process started above 75 °C and, at this pH, the protein did not refold after cooling down from 95 °C to 25 °C.**
- **Consistently with CD data, both proteins at acidic pH preserved their sweetness upon 10 min of boiling, whereas at neutral pH, the sweetness of MNEI was completely lost after only 2 min of boiling; Mut9, instead, retained its sweetness intensity even after 10 min boiling.**

Secondary Structure Assessment

- The folding and secondary structure content of Mut9 and MNEI from strongly acidic to almost neutral pH (i.e., 2.5, 5.1, and 6.8) was assessed by circular dichroism spectroscopy (CD).
- **At all examined pHs, the spectra of Mut9 were characterized by two minima, located at 201 and 213 nm.**
- **The β-sheet and α-helix content showed minor changes from pH 2.5 to 6.8, confirming the stability of Mut9 fold in a wide pH range.**

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Mut9 Monellin variant (5/6)

Results

Shelf-Life Assessment

- **Mut9 displayed higher stability in all the conditions tested**.
- Samples of Mut9 incubated at 4 °C lost 10% of their initial protein content after the first 8 weeks, at all examined pHs; for the following 16 weeks, the protein under strong or mild acidic conditions remained totally stable, whereas, at pH 6.8 an additional 5% loss could be observed.
- MNEI incubated at the same temperature lost approximately 45% (average of all conditions) of its initial amount after 6 months of incubation, showing the lowest stability upon 6 months incubation at pH 6.8.
- **In the accelerated shelf-life assessment, Mut9 behavior paralleled that observed at 4 °C, while MNEI lost over 60% of its content upon 6 months incubation (average of all conditions).**

Sensory Analysis

- The sweetness threshold of Mut9 was evaluated by a panel of five tasters using the triangle test technique.
- **The sweetness threshold of Mut9 was 0.8 mg/L (71 nM).**
- **The same panel tasted MNEI as a positive control, and the sweetness threshold resulted in 1.48 mg/L (132 nM), which is in good agreement with literature data.**
- **Based on these results, the point mutations included in Mut9 were able to increase the parent protein's sweetness by almost 2 folds, in line with what observed for Y65R-MNEI.**

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Mut9 Monellin variant (6/6)

Application

- A 6-month shelf-life assessment was performed, and the data confirmed the greater stability of the new construct in a wide range of conditions.

  Mut9 has an even greater potential for food and beverage applications than MNEI.

- **Deep structural studies of receptor–protein complexes in parallel with protein engineering techniques are the key for building new, enhanced constructs that could find wide use in food and beverage products.**

Conventional Solutions	Advantages
Use of Single-chain Monellin, MNEI is limited by low stability and high aggregation propensity at neutral pH.	We have designed a new construct of MNEI, dubbed Mut9, which led to gains in both sweetness and stability.
MNEI lost its structure and function.	Mut9 preserved its structure and function even after 10 min boiling, with the greatest differences being observed at pH 6.8, where it remained folded and sweet

Comment

- Mut9 contained four point mutations compared to the parent protein MNEI: E23A, C41A, Y65R and S76Y. We characterized Mut9 and MNEI using sensory and biophysical techniques, confirming the additivity of the features associated with the mutation introduced.
- The high stability gain in the case of Mut9 can be explained by the effect of the point mutations E23A and C41A that introduce apolar side chains into two distinct hydrophobic pockets that, instead, host ionizable/polar residues in the parent protein MNEI.

Source

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2.4 New Monellin sweetener **

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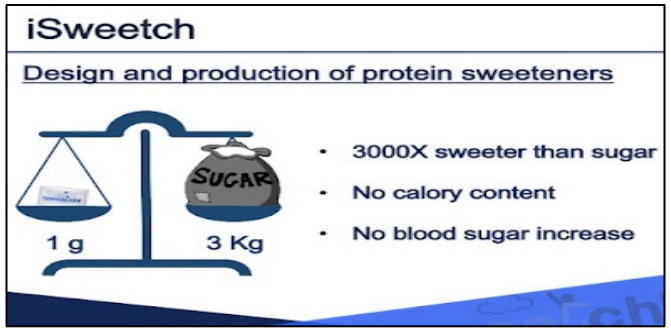
New Monellin sweetener (1/3)

Introduction

The solution to the problem is invented by iSweetch

- iSweetch is a start-up based in Naples, Italy. It was incorporated in 2015 as a spin-off of the University of Naples Federico II. Its field of activity is the design and production of protein-based High-Intensity Sweeteners to be used as sugar substitutes and as alternatives to the most common sweeteners whose safety has always been controversial. Delia Picone is Chief Executive Officer of iSWEETCH.



Application in food & beverages.

- Italian scientists almost ready to launch a NEW TYPE OF SWEETENER which is: **3000 times sweeter than sugar, has NO by-products, has NO aftertaste, green production, No calorie content, NO blood sugar increase.**
- **The sweetener is made form monellin** which is a sweet protein discovered in 1969 in the fruit of the West African shrub known as serendipity berry (Dioscoreophyllum cumminsii). Monellin is perceived as sweet by humans and some Old World primates, but is not preferred by other mammals. The relative sweetness of monellin varies from 800 to 2000 times sweeter than sucrose. **Monellin can be useful for sweetening some foods and drinks**, as it is a protein **readily soluble in water** due to its hydrophilic properties. **However, it may have limited application because it denatures under high temperature conditions, which makes it unsuitable for processed food.** It may be relevant as non-carbohydrate tabletop sweetener, especially for individuals such as diabetics who must control their sugar intake. In addition, monellin is costly to extract from the fruit and the plant is difficult to grow. Alternative production such as chemical synthesis and expression in micro-organisms are being investigated. **Legal issues are the main barrier in its widespread use as a sweetener, as monellin has no legal status in the European Union or the United States.** However, it is **approved in Japan as a harmless additive**, according to the List of Existing Food Additives issued by the Ministry of Health and Welfare (published in English by JETRO).

Source1 Source2

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2.5 E23Q Monellin Variant **

Back to Ranking

E23Q Monellin Variant (1/4)

Solution

The solution to the problem is invented by University of Naples Federico II

Non-patent Published in 2016

Application in food & beverages.

- **Native monellin loses its activity when heated above 50°C, due to disruption of the heterodimeric structure. To increase thermal stability, single chain derivatives have been designed, joining the two subunits directly together or through a dipeptide Gly-Phe linker. These proteins are more stable than the parent protein and, at acidic pH, they regain sweetness even after few minutes boiling in water solution**.
- In addition to temperature, **single and double chain monellins also show a marked stability dependence from pH**. Analyses of the unfolding kinetics have proven that both variants are strongly destabilized by the increase of pH from 4.0 to 10.0. **Aghera et al. demonstrated that this effect is due to a single glutamic residue buried at the C-terminal region of the helix.**
- **As a consequence of its position in a hydrophobic pocket, the side chain of E23 exhibits a high pKa,** a phenomenon often observed when ionizable residues are located in the interior of the protein fold, which often leads to a marked pH-dependent stability. **From the analysis of the folding kinetics, Aghera et al. estimated that the pKa of the side chain of E23 in the native state is approximatively 7.5.** The abrupt change in pKa (to ~4.5 for the exposed glutamic side chain) that occurs with unfolding is the cause of the observed destabilization of MNEI at neutral to alkaline pHs. Destabilization of the native state of globular proteins can also lead to other unfavorable or uncontrollable phenomena, among which aggregation and former studies have indeed pointed out the tendency of MNEI to form fibrillar aggregates, a tendency accentuated by increasing pHs or temperatures. All these phenomena reduce the potential of MNEI for industrial applications, and should therefore be resolved before the protein can be actually employed in large scale processes. The researchers have evaluated the theoretical pKa of E23 side chain using Multi Conformation Continuum Electrostatics calculations on various available experimental structures, and performed molecular dynamics simulations at different pHs and temperatures, in order to define the contribution of E23 to the fold stability.
- **Researchers then designed a stabilized mutant, MNEI-E23Q, in which replacement of the glutamic residue with a glutamine allows the preservation of the network of interactions of the native state in a pH-independent manner. Increased stability of such mutant, as predicted by MD simulations, has been then confirmed by thermal unfolding studies using CD spectroscopy.**

Proteins expression and purification:

- The **synthetic gene encoding for MNEI-E23Q was purchased from Eurofins Genomic and cloned in the pET22b+ vector (Novagen) within the Nde I and BamH I restriction sites.** Vector **pET22b+ carrying the gene encoding for MNEI**. To express the recombinant proteins, **cells of Escherichia coli BL21(DE3) were transformed with said plasmids, cells were cultured in 1L of LB medium containing 100 mg/L ampicillin. Protein expression was induced at 0.6 OD with 5 mM lactose and cells were harvested by centrifugation (4°C, 3000 x g, 20 min) after 20 h induction at 25°C**, washed with cold PBS and stored frozen until extraction. **Purification was achieved in a one-step procedure.** Cell lysates in 50 mM sodium acetate at pH 5.5 were applied to a DEAE-Sepharose (20 mL) connected in series to a Macro-Prep High S (15 mL, Bio-Rad). **Proteins were then eluted from the Macro-Prep High S with 2 CV of 100 mM NaCl in 50 mM Sodium Acetate, pH 5.5.** The protein containing fractions were desalted by Size Exclusion Chromatography on a Sephadex G-25 column (GE Lifesciences, 2.5 x 26.5 cm, 130 mL) in 75 mM AcOH at 5 mL/min and freeze-dried, **purity was assessed by SDS-PAGE**. **Protein yield was estimated by UV absorbance and was on average 50 mg for both proteins per liter of culture.**

Source

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E23Q Monellin Variant (2/4)

Tests

CD spectroscopy:

- **Protein fold integrity was assessed by circular dichroism (CD) spectra** recorded on a Jasco J715 spectropolarimeter equipped with a Peltier temperature control system (PTC-348WI). Molar ellipticity per mean residue [θ] in deg cm2 dmol−1 was calculated from the equation: [θ] = [θ]obs mrw/(10 × l × C), where [θ]obs is the ellipticity measured in degrees, mrw is the mean residue molecular weight of the protein (Da), C is the protein concentration in g/mL and l is the optical path length of the cell in cm. **Cells of 0.1 cm path length were used**. **CD spectra were recorded with a time constant of 4s, a 2 nm band width and a scan rate of 20 nm/min, and the signal was averaged over three scans and baseline corrected by subtracting a buffer spectrum. Spectra were recorded in 20 mM phosphate buffer at pH 3.5, 5.1 and 6.8 and in 20 mM TrisHCl at pH 8.0.**
- A concentration of 0.25 mg/mL protein was used for each sample, as determined by UV absorbance at 280 nm prior to CD measurement. **Thermal denaturation experiments were recorded following the signal at 215 nm while varying the temperature from 30 to 95°C at a rate of 1°C/min.** For each condition**, three independent measures were performed.** Experimental points were fitted to a Boltzmann curve, and fraction of unfolded protein (fu) was calculated.

MD simulations:

- Molecular dynamics were performed on model 1 of 1FA3 as the starting structure. **Dynamics and trajectory analysis were performed with the software package GROMACS 4.6.4 using the AMBER ff03 force field.** Structures with protonated and ionized side chain for E23 and for mutant MNEI-E23Q were generated and immersed in a rhombic dodecahedron box with periodic boundary conditions and solvated with TIP3P water molecules. The appropriate number of Clions was added to neutralize the system. **Long-range electrostatic interactions were treated with the particle-mesh Ewald method with a grid spacing of 0.12 nm, and a long range cutoff of 8 Å was used. The LINCS algorithm was used to constrain bond lengths and a 2 fs time step was used.** For the simulations at room temperature, **the molecules were submitted to initial energy minimization with 5000 steps of steepest descent, followed by 100 ps NVT and 300 ps NPT equilibration at 300 K with position restraints.** For the high temperature simulations, after solvent relaxation, initial velocity distributions were generated at 300 K, followed by 50 ps equilibration at this temperature. Temperature was then increased step-wise over 40 ps to 473 K and the system temperature was further equilibrated for an additional 10 ps. Production runs were performed for 10 ns with a 2 fs step. Temperature and pressure coupling were obtained with the v-rescale and the Parrinello-Rahman algorithms, respectively. Surface accessibility of the residues was calculated with the program g_sas from the GROMACS package was used. Relative surface accessibility was estimated by normalizing the values obtained over time to the maximum surface accessibility as calculated in the tripeptide Gly-X-Gly.

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E23Q Monellin Variant (3/4)

Result

Continuum Electrostatic pKa determination:

- **A comparison of region 21–33 in the two models, corresponding to the C-terminus of the helix and to the facing portion of loop Lα2, shows a RMSD between heavy atoms of only 1.7 Å**. In the crystal structure**, the region appears slightly wider than in the NMR ensemble as indicated by the distances between the carboxyl oxygens of E23 and the carbonyl oxygen of Y29 (5.7 Å and 6.2 Å in 1FA3.15 and 2O9U, respectively) and E23(O) and Q28(N) (2.9 and 4.7 Å).** The crystal structure also shows that a **molecule of water (W17 in the PDB) is stably bound and buried in correspondence of the loop, but this water molecule was not observed in solution NMR studies with paramagnetic probes of the hydration of MNEI surface**. NMR and crystallization experiments were performed at different pHs (2.9 and 5.6, respectively). **The occurrence of water in the crystal structure might be a consequence of forced packing interactions, leading to the underestimation of E23 pKa.** Taken together, **the results suggest that loop Lα2 is provided with a certain flexibility, and that slight displacements, even thermal motions, can produce significant changes in the accessibility of the protein interior to water, with consequent changes in the polarizability experienced by E23 side chain, abrupt changes of its pKa and significant variations of MNEI stability.**

MD simulations of different protonation states:

- The simulations at room temperature confirmed the structure stability at every pH, in accordance with experimental data. **The Cα-RMSD plot for each simulation reaches a plateau within 2 Å from the NMR structure, whereas the plot of the RMS fluctuation for each residue shows that the regions of higher flexibility are localized, as expected, at the N- and C-termini and at the loops between the strands. The helix appears stably positioned in the β-grasp and the mobility of its C-terminus, where E23 is located, is substantially unaffected by its protonation state. Upon increase of the simulation temperature to 473 K, the structure of MNEI-GLH is substantially unaffected: the RMSD remains within 3 Å of the NMR structure for the first 7 ns of the simulation and unfolding begins only at the end of the MD run.** This is consistent with the experimentally observed thermostability at acidic pH of MNEI. **When E23 is deprotonated, the RMSD diverges, reaching values above 5 Å after only 4 ns, and the protein proceeds toward fast unfolding.** A plot of the RMSF shows that N-terminal of the protein, up to residue 40, becomes increasingly mobile, with the helix being displaced from its original position up to 7 Å. The remaining portion of the protein is more stable, but either the loops and the β-strands have higher mobility compared to MNEI-GLH, suggesting that the destabilization is conveyed through the entire protein structure.

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E23Q Monellin Variant (4/4)

Result

Thermal stability of MNEI-E23Q:

- In order to experimentally validate these theoretical results, we expressed and purified MNEI and MNEI-E23Q. **The mutant shared the fold of the parent protein, as confirmed by Circular Dichroism spectroscopy.** CD spectroscopy was also used to monitor thermal denaturation and compare the proteins stability. Buffers at four pH values were used, namely 3.5, 5.1, 6.8 and 8.0, and unfolding was monitored through the signal at 215 nm. **Despite being very resistant to thermal denaturation in acidic conditions, at neutral to alkaline pH MNEI melting temperature decreases about 15°C compared to the maximum of 90° at pH 3.5. MNEI-E23Q, which exhibits comparable stability at acidic pH, preserves this characteristic also at pH 6 and above. Preliminary sensory evaluation showed that the new construct has comparable sweetness with MNEI, indicating that the biological activity is not affected by the point mutation**.
- The University had developed mass production of monellin variant in 2018. The mass production approach has been developed in collaboration with Institute of Biosciences and BioResources, University of Naples Federico II. **No significant difference was evident between the thermal treatment at 60 or 70 °C for both Mut3 and E23Q, the thermal treatment at 60 °C for 20 min was done for subsequent downstream processes and biochemical characterization. The product has purity of at least 99%. All plant-derived proteins revealed a sweetness threshold of 1.08±0.04 mg/l, 1.64±0.04 mg/l and 0.40±0.02 mg/l for Y65R-MNEI, E23Q-MNEI and Mut3, respectively**. Source

Application

❑ The method is applicable for production of MNEI with increased pH stability.

Conventional Solutions

The conventional MNEI had less stability at acidic pH

Advantages

The method is capable in producing MNEI variant with greater acidic pH stability.

Source

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2.6 Y65R Monellin variant **

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Y65R Monellin variant (1/7)

The solution to the problem is invented by Università di Napoli Federico II, University of Sannio

Solution

Non-patent Published in 2014

Application in food & beverages.

- In this reference, **researchers have focused on a comparison of structural and functional properties of two single chain derivatives of monellin**, one of the best characterised sweet proteins isolated so far, **i.e. MNEI and its Y65R mutant, in which the tyrosine residue in position 65 is replaced by an arginine residue**.
- **Natural monellin consists of two separate polypeptide chains, A and B, made of 44 and 50 amino acids, respectively, forming a single domain through non-covalent interactions.**
- Its three dimensional structure has an a-b architecture with a single helix held in a coiled antiparallel b-sheet, Based on structural similarity, monellin has been classified as a member of the cystatin superfamily.
- Being many thousands of times sweeter than sucrose on a molar basis, monellin has significant potential as an alternative to carbohydrate or artificial sweeteners because it combines a marked persistence of taste sensation with a very low caloric power.
- **Thermal denaturation of monellin, which occurs when the protein is heated above 50 C, leads to a loss of sweetening power, clearly indicating that a correctly folded three-dimensional structure is a prerequisite for sweetness.**
- **To enhance the thermal stability of monellin, recombinant single chain constructs, obtained by joining the two chains either by a direct amide bond (SCM) or by the insertion of a dipeptide linker (MNEI) were generated, and found to be as sweet as native monellin, but endowed with increased thermal stability.**
- **Both single chain constructs refold easily even after boiling at low pH and remain potently sweet as wild type monellin.**
- In silico studies of the interaction between MNEI and the sweet taste receptor lead to the design of charged mutants of MNEI which were successfully produced in Escherichia coli. Among them, **the mutant Y65R was proven particularly promising for its high sweetness.**
- Here, researchers provide a thorough physico-chemical comparison of Y65R and MNEI, together with an extensive evaluation of their sweetness in different experimental conditions.
- These conditions have been chosen to reproduce those of some common sweet drinks, which we believe could constitute the first applicative target of new sweeteners structurally derived from monellin.

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Y65R Monellin variant (2/7)

Tests

Circular dichroism:

- **Circular dichroism (CD) spectra were recorded with a Jasco J-715 spectropolarimeter equipped with a Peltier type temperature control system (Model PTC-348WI).** Molar ellipticity per mean residue, [h] in deg cm2 dmol1 , was calculated from the equation: [h]=[h]obs mrw/(10 l C), where [h]obs is the ellipticity measured in degrees, mrw is the mean residue molecular weight of the protein (Da), C is the protein concentration in g/ml and l is the optical path length of the cell in cm. Cells of 0.1 cm path length were used. **CD spectra were recorded with a time constant of 4 s, a 2 nm band width and a scan rate of 20 nm/min**, and the signal was averaged over at least three scans and baseline corrected by subtracting a buffer spectrum. **The thermal unfolding curves were recorded in the temperature mode, by following the change of the CD signal at 215 nm with a scan rate of 1.0 C/min. Protein concentration was kept constant at about 0.3 mg/ml**. For each sample, three repetitive scans were obtained and averaged.

NMR spectroscopy:

- **Nuclear magnetic resonance (NMR) measurements on unlabeled samples were performed at pH 3.3 and 5.1, using 2 mM protein solutions in 20 mM sodium phosphate buffer containing 10% of 2 H2O**. **NMR experiments were carried out at 500 MHz**, on a Varian Unity 500 spectrometer using standard acquisition programs. Mono-(1D) and two-dimensional (2D) spectra were accumulated with a spectral width of 6493 Hz and were acquired at 35 C. 2D TOCSY experiments were recorded with a mixing time of 70 ms and the water suppression was achieved by DPFGSE sequence. **Typically, 65 transients of 2 K data points were collected for each of the 256 increments; the data were zero filled to 1 K in x1.** Data were processed and analysed using NMRpipe and Sparky software.

Aggregation propensity:

- **The aggregation propensity of MNEI and Y65R was assessed by incubating protein aliquots in 100 mM ammonium acetate buffer, pH 5.1 or in 100 mM phosphate buffer at pH 6.8.**
- The protein samples, all at a concentration of 0.5 mg ml1 , were incubated at different temperatures for times ranging from 2 to 24 h.
- **At the time indicated aliquots of 20 ll were withdrawn for the control, while the remaining part of the sample was centrifuged at 13.000 rpm for 3 min, to separate the supernatant from the pellet, which was suspended in 100 ll of loading buffer.**
- Identical volumes (20 ll) of supernatant and precipitate were then loaded on the same gel, to evaluate protein solubility and tendency to form sodium dodecyl sulphate (SDS) resistant aggregates.
- **SDS polyacrylamide gel electrophoresis (PAGE) was performed according to the Laemmli protocol using 15% polyacrylamide gels.**
- Gels were stained with Coomassie Brilliant Blue R-250.

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Y65R Monellin variant (3/7)

Tests

Molecular dynamics:

- The starting structure for molecular dynamics for MNEI was extracted from the protein data bank (PDB) file 1FA3, using the first model of the NMR ensemble. Based on the evidence of comparable fold from the NMR and circular dichroism (CD) analysis, the model for Y65R was built on the same structure by manual amino acid replacement. **Continuum Electrostatic calculations were realised with the H++ server, with an external dielectric of 80, an internal dielectric of 10 and choosing a reference pH of 5.1 and a salt concentration of 20 mM. Molecular dynamics (MD) and trajectory analysis were performed with the software package GROMACS 4.6.4 using the AMBER ff03 force field. The structures were protonated to reproduce pH 5.1,** according to the results of electrostatic calculations, **and the appropriate number of Na+ and Cl ions was added to neutralise the system and reproduce a 20 mM salt concentration.** The structures were put in a rhombic dodecahedron box with periodic boundary conditions and solvated with TIP3P water molecules. **Long-range electrostatic interactions were treated with the particle-mesh Ewald method with a grid spacing of 0.12 nm and cutoff of 8 Å.** The molecules were submitted to initial energy minimisation with the steepest descent for 5000 steps followed by 100 ns NVT and 300 ps NPT equilibration with position restraints. **Production runs were performed for 10 ns with a 2 fs step.** Temperature and pressure coupling were obtained with the v-rescale and the Parrinello–Rahman algorithms, respectively. Visual inspection of the trajectories and model manipulations were performed with the UCSF Chimera package (version 1.9).

Sensory evaluation:

- **Stock solutions of 1 mg/ml of both MNEI and Y65R were prepared by dissolving each protein in mineral water, pH 6.8.** In order to determine the detection threshold (DT) and recognition threshold (RT), nine concentrations of each protein obtained from stock solution**, going from 0.49 mg/l to 5.32 mg/l, were prepared**. The range of concentrations was chosen on the basis of the results from previous research. **Two different pH conditions were investigated: 6.8 and 5.1**. **Citric acid powder was used as an acidifying agent in both protein solutions at a concentration of 19 mg/l to reach a pH equal to 5.1**. A modified version of the ‘‘difference from the reference’’ method was used to determine DT and RT of the proteins. Thirteen assessors participated in the study; they were selected for having the same DT and RT for the basic tastes in order to avoid errors due to different thresholds. For each pH condition, the presentation of the samples was carried out according to an incomplete block design: during each session, assessors compared a reference sample of pure water (R), with a hidden reference and four samples, two of MNEI and two of Y65R, containing different concentrations of each stimulus.

Source

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Y65R Monellin variant (4/7)

Result

Structural comparison:

- Analysis of the spectra revealed that under all the conditions explored both proteins retain very similar secondary structure populations, characterised by a high b-sheet and low a-helix content, coherently with the structure determined by NMR studies at acidic and neutral pH for MNEI. **Comparison of the CD curves intensity, evidences a similar solubility of the proteins at pH 5.1 reported for MNEI. Both proteins reveal a lower solubility at neutral pH. Interestingly, in the case of Y65R the CD signal intensity increases by lowering the pH from 5.1 to 3.3, although the curve shape is identical, indicating that the protein structure does not change. Deeper structural comparison was carried out by acquiring 1D and 2D NMR spectra of both proteins at pH values of 3.3 and 5.1, in which the proteins display higher solubility.** An overlay of the 1D NMR spectra of the two proteins at the two different pH is reported in the supplemental material for MNEI and for Y65R. The overlay of the 2D spectra allows a better comparison among the tertiary structures of the proteins compared to the 1D spectra: proton chemical shifts are indeed significantly affected by the chemical environment, so that they represent extremely sensitive tools to monitor even small changes in the protein folds. **The spectra reported in indicate convincingly that at pH 3.3 the 3D structures of the two proteins are very similar to each other, and only minor local differences are evident from a few slightly shifted signals.** Based on the available resonance assignments for MNEI at pH 2.9, the researchers have been able to identify the most shifted residues between MNEI and Y65R at pH 3.3, which are mostly located in proximity of the mutation (for instance residues 64 and 66). The same considerations apply for the spectra recorded at pH 5.1.

Thermal stability:

- Y65R and MNEI progressively unfold into a random coil conformation, as indicated by the disappearance of the negative band at 215 nm and by the appearance of a strong negative band with a maximum just below 200 nm. **At pH 3.3 the parent protein resulted more stable in solution, as Tm values are about 81.63 C and 75.99 C for MNEI and Y65R, respectively**. **Upon increasing the pH by about two units, from 3.3 to 5.1, the researchers found again the wild-type protein to be more stable than the mutant, because the Tm values are 82.78 C and 80.13 C for MNEI and Y65R, respectively. Heat denaturation curves registered at pH 7.0 gave Tm values of 72.28 C and 66.36 C for MNEI and Y65R, respectively, confirming that the higher thermal stability of MNEI also occurs at neutral pH.** Analysis of these data indicates that the parent protein **MNEI preserves its superior thermal stability within the pH range 3.3–7.0 and that at pH 5.1 both proteins possess the maximum relative value of Tm**, also showing the lowest difference in thermal stability between them.

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Y65R Monellin variant (5/7)

Result

Thermal treatment and aggregation propensity:

- **At pH 5.1 both proteins were quite stable in solution**, as they remain soluble and mainly in monomeric form upon incubation up to 24 h at 4 C. **Only a small amount of SDS resistant dimer was formed which, in the case of MNEI, precipitated, whilst remained soluble in the case of Y65R. However, at the same pH and at 60 C, the aggregation propensity of both proteins was increased,** although some differences among their solubility persisted. Upon incubation for 24 h at 60 C both proteins were partially converted into SDS-resistant dimers and for MNEI, (panel C), into even higher molecular weight insoluble aggregates. Moreover, **even in dimeric state Y65R is largely found in the soluble fraction (panel D). At pH 3.3 the parent protein resulted more stable in solution, as Tm values are about 81.63 C and 75.99 C for MNEI and Y65R, respectively.** At pH 6.8 (panels E–H), the differences between the two proteins are reduced, as they displayed a very similar behaviour, characterised by lower solubility, as indicated by the presence of insoluble aggregates already at 4 C (panel E-F). As the incubation temperatures increased from 4 to 60 C the solubility also decreased, and significant amounts of dimers and high molecular weight forms were found in the precipitated fractions of both protein samples (panel G-H). **The parent protein MNEI preserves its superior thermal stability within the pH range 3.3–7.0 and that at pH 5.1 both proteins possess the maximum relative value of Tm,** also showing the lowest difference in thermal stability between them

Molecular dynamics:

- **The net charge of the two proteins at this pH is +3 for MNEI and +4 for Y65R.** After the 10 ns simulation in explicit solvent, the experimental similarities between the two proteins are also evident in the dynamic behaviour of the structures. **The analysis of the Root Mean Square Fluctuation (RMSF) per residue of the protein backbones exhibits similar patterns, in accordance with what was previously observed for MNEI and the G16A mutant with the highest flexibility in correspondence of the loops L23 (residues 49–53), and L45 (79–82).** The replacement of Y65 in the original structure with an arginine residue has only a moderate effect on the local motions.This residue is in fact located at the C-terminal portion of the b3 strand, flanking the loop L34, and does not appear to be vital for the conservation of the protein fold. **In the mutant, the side chain of R65 can in fact dynamically interact with the carboxylic oxygen from the backbone of D68 through a hydrogen bond with a 64.6% occupancy, but this only leads to a slight difference over time of the position of L34 from the experimental data collected on MNEI, with a minimal impact on the motion of the area, as observed in the RMSF plot.**

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Y65R Monellin variant (6/7)

Result

Sensory evaluation:

- Data show that by increasing concentrations of both proteins, the differences from the reference increased too, **Y65R being sweeter than MNEI in all the investigated concentrations, with the exception of the highest one, where no differences are found among sweetness intensities**.
- **The first significantly ($p < 0.05$) different concentration of Y65R from the reference was 0.84 mg/l, whereas the first such difference was observed at a MNEI concentration of 1.86 mg/l ($p < 0.05$).**
- **The DT, i.e. the geometric mean between the highest undetected and lowest detected concentration, were 0.665 mg/l for Y65R and 1.645 mg/l for MNEI.**
- Furthermore, **the recognition threshold (RT) of Y65R was 1.43 mg/l, a concentration at which 62% of assessors correctly recognised the sweet taste; whereas for MNEI the RT was 1.86 mg/l, a concentration correctly recognised as sweet by the 55% of assessors**.
- The first significantly ($p < 0.05$) different concentration of Y65R from the reference is 1.43 mg/l, whereas the first such difference is observed for the concentration of MNEI equal to 1.86 mg/l ($p < 0.05$).
- Then, in this pH conditions the DT of Y65R was 1.265 mg/l and that of MNEI 1.645 mg/l, whilst the RT of Y65R was 1.43 mg/l and RT of MNEI was 1.86 mg/l, with the percentage of corrected responses of 70% and 65%, respectively.
- The University had developed mass production of monellin variant in 2018. The mass production approach has been developed in collaboration with Institute of Biosciences and BioResources, University of Naples Federico II. **No significant difference was evident between the thermal treatment at 60 or 70 °C for both Mut3 and E23Q, the thermal treatment at 60 °C for 20 min was done for subsequent downstream processes and biochemical characterization. The product has purity of at least 99%. All plant-derived proteins revealed a sweetness threshold of 1.08±0.04 mg/l, 1.64±0.04 mg/l and 0.40±0.02 mg/l for Y65R-MNEI, E23Q-MNEI and Mut3, respectively**. Source

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Y65R Monellin variant (7/7)

Application

- The method is applicable for production of a sweeter mutant of monellin with increased stability at high temperatures and low pH and also increased solubility at low pH.

Conventional Solutions

The conventional monellin had low stability at high temperature and intense pH.

Advantages

The monellin mutans produced by this method has enhanced temperature and pH stability. The solubility is also better at low pH.

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2.6 High temperature stable E24Q/Y80R H-Monellin

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High temperature stable E24Q/Y80R H-Monellin (1/5)

Solution

The solution to the problem is invented by China Pharmaceutical University

Non-patent Published in 2019

Application in food industry.

- The sweetness level would decrease when the temperature reaches 50℃, **to increase the thermostability**, **many protein engineering strategies were used. For example, single-chain monellin (SCM) was generated by linking C terminus of the B chain sequenced by A-chain N-terminus13**.
- Another mostly studied **single-chain monellin (MNEI) was developed by connecting two natural chains sequential by a Gly-Phe dipeptide linker** and have been successfully expression in varies organism such as bacteria, yeast, tobacco.
- The **Tm（denaturation midpoint**; assuming two-state protein folding), for folded and unfolded states are equally populated at equilibrium of MNEI when the temperature rising from 55℃ to 65℃.
- Recent studies also show that when treating monellin under high temperature, irreversible protein aggregations with some hetero protein aggregations will form.
- According to the folding mechanism research, there are at least four distinct conformations, including unfolded and native conformations.
- The chain–chain association step occurs after the initial folding of the two chains, so **heat resistance of monellin is depended on its structural and refolding ability**.
- **Protein circulation was the best choice because it facilitates refolding after thermal stress**.
- Many circular proteins such as β-lactamase and dihydrofolate reductase could refold correctly after boiling. **The university had tried the circular monellin, which was stable after heating, but totally lost the sweetness after masking its critical active sites at the chain A C- terminal domain**.
- **Inspired by the high Tm value in RNA hairpin containing structure, the university proposed that introduced the beta-hairpin structure in some sites of protein would delay the collapse when heating.**
- **The hairpin structure would first form to minimize potential aggregation of the free chains and increase the correct protein refolding when annealing.**
- **The beta-hairpin structure was** usually used for FRET linker or anti-enzyme hydrolysis linker but little **used for increase the Tm of proteins**. The university introduced three kinds of **beta hairpin analogues (TWLRWXXAKXP, XKWTWNPATGKWTWQE, XCTWEPDGKLTCXDNA) between the two polypeptide chains of monellin**.
- After selection, the **sweetness and thermostability of three mutants were tested with Tm 79.5℃, 89.1℃, 89.4℃. and combined with E24Q/Y80R mutation, the mutated E24Q/Y80R hairpin monellin Tm was as high as 96.1℃.** Overall, this **hairpin structure encoded significantly increased the thermostability of monellin** and may be applied to other proteins.

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High temperature stable E24Q/Y80R H-Monellin (2/5)

Solution

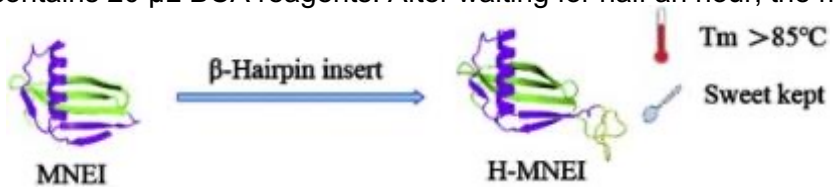
The solution to the problem is invented by China Pharmaceutical University

Non-patent Published in 2019

Homology modelling of hairpin contained monellin: The structural homology modelling of hairpin contained monellin was done with Swiss Model and Discovery Studio based on PDB code 1FA3 as a template. Visualization and manipulations of the structures were performed with PyMOL.

Plasmid and Library construction: DNA manipulation, ligation, transformation and plasmid preparation were performed following standard techniques. All DNA constructs were sequenced. The plasmid pET28a-MNEI was generated by inserting MNEI gene between NdeI and HindIII endonucleases in pET28a, and it contains His-tag at the N-terminal. The TWLRWXXAKXP, XKWTWNPATGKWTWQE, XCTWEPDGKLTCXDNA libraries were prepared by overlapping PCR and mutation using oligonucleotides containing random sites. Then, the library was digested with DpnI endonucleases and transformed into E. coli strain BL21-AI cells by electroporation. The size of the library was determined by plating on LB-Kan agar plate incubated at 37℃ . The mixture plasmids of the plates were extracted and sequenced. For each beta hairpin, fifteen clones were sequenced to characterize the library quality.

Heat-stain selection method: The Hairpin monellin library was picked in 96 Deep Well Plate. After it grew at 37℃ overnight, transformed 100uL culture into another 96 Deep Well Plate. Then it grew at 37℃ for another 3 h, library was induced with 1% arabinose and 1mM IPTG 3 h. Next, Induced E. coli cells in 96 Deep Well Plate were harvested by centrifugation (4000 g, 3 min) and the supernatants were discarded before adding lysis buffer (1 mL 20 mM Tris PH8.0, 100 μL lysozyme 200 μg/mL, 150 μL 1% Triton X-100). The deep well plates were shank in 37℃ for half an hour and sequenced settled in the 80℃ -water bath for 1 hour. By centrifugation (4500 g, 15 min), the supernatants of 96 deep well plate were separated, and 20 μL supernatants each hole was transformed to a 96 well plate contains 20 μL BCA reagents. After waiting for half an hour, the most-deep-purple-hole was chosen for another round test.



Purification of sweet protein: The pET28a-MNEI and selected sweet proteins were expressed in E. coli BL21 (DE3) after 6 hours of 1 mM IPTG induction and identified by SDS-PAGE. Then, the protein was purified by Ni-chelating affinity chromatography. After collection of the protein elution, they were dialyzed with distilled water to remove the imidazole and then freeze-dried for the activity test. The SDS-PAGE was performed according to the Laemmli protocol using 15% polyacrylamide gels, which were stained by Coomassie Brilliant Blue R-250. HPLC assay were performed by Agilent 1260 Infinity. HPLC conditions: ZORBAX 300SB-C18 column as stationary phase, H2O- acetonitrile as mobile phase (95:5) 5min; (35:65) 25min; (5:95) 10min with the flow rate of 1mL/min. The temperature of column was at 20℃ , the wavelength of detection was 215 nm.

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High temperature stable E24Q/Y80R H-Monellin (3/5)

Tests

Sweet activity analysis:

- The freeze-dried protein was dissolved in distilled water, then centrifuged to remove the little precipitate and quantified with BCA method to get the stock solution (about 250 μg/mL). A series of concentrations ranging from 1 to 40 μg/mL were diluted with corresponding buffers, ex. PH7.0 (distilled water); PH5.1 (citric acid, 19 mg/L). Sucrose solutions ranging from 1 to 40 μg/mL were used as the positive control. **Ten healthy volunteers, five males and five females, age from 18 to 22, joined the test** (single-blind test, the volunteers did not know what tasted it is, but the researchers known). **The sucrose or proteins were tasted by each volunteer from the lowest to the highest concentration**. Before each assay, rinsed with water at least twice to avoid the residual taste remained. Subsequently, **3 mL of the sample solution was taken into the mouth**. **The solution was held in the mouth for at least 10s before spitting out**. The volunteer should grade the sample as follows: 0 for non-sweet or uncertainty at this level; 1 for faintly sweet; 2 for sweet; 3 for very sweet; 4 for intense sweet. Each volunteer did repeat another independent test to confirm the grade values were correct. Temperature-dependent sweet activity assay and the reversion test were similar as above.

Thermal treatment and aggregation test:

- **The aggregation propensity of sweet proteins were tested by assessing 100 μL of the protein (250 μg/mL) incubated at different temperatures (70℃ , 80℃ and 90℃) at the time of 15 min, 30 min, 1 h and 2 h**. All the treated samples were centrifuged at 12000 rpm for 10 min to separate the supernatant from the pellet. Then, 20 μL loading buffer (with beta-mercaptoethanol) was added to the supernatant. 100 μL distilled water and 20 μL loading buffer (with beta-mercaptoethanol) were added to the pellet. Identical volumes (20 μL) of **supernatant and precipitate were then loaded on the same SDS-PAGE, to evaluate the protein thermal resistant and aggregative properties**.

Circular dichroism detection:

- Circular dichroism (CD) spectra were recorded on Chirascan plus CD spectrometer equipped with a thermostatically controlled quartz cell with a 0.1 cm path length. Protein concentration in the solution was 100 μg/mL. The signals were recorded at 215 nm in the thermal denaturation experiments with the temperature varying from 25℃ to 90℃ at a rate of 1 ℃ /min. For each condition, three independent signals were detected, and the baseline was corrected by subtracting the buffer spectrum. Then, experimental results were fitted to a Boltzmann curve.

Differential scanning calorimetry detection:

- The proteins were sent to Shanghai Jiang Tong University and tested by Nano DSC. The concentration of protein samples was diluted to 0.1 mg/mL and centrifuged at 12 000 rpm for 20 minutes. Then the supernatant was applied as Nano DSC sample. The temperature range of scanning is 20-100 ℃. The scanning results are analysis by two-state scaled model.

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High temperature stable E24Q/Y80R H-Monellin (4/5)

Results

Sensory Evaluation:

- There was **no significant (p<0.05) difference in the concentration** of MNEI and H-Monellin from 5μg to 40 mg/L.
- There is **no significant difference between the sweetness** of MNEI and H-Monellin in each concentration.

Thermal treatment and aggregation test:

- MNEI was totally disappeared in the supernatant in 1 hour, with all of that was transferred in precipitated fraction**.**
- Some aggregation could be found in the precipitated fraction, and there might exist some dimers, trimers or tetramers according to the molecular weight.
- The **H-Monellin was different with MNEI**, **since it could always be found in the supernatant, and it will retain 80% after being treated for 1 hour and 50% for 2 hours**.

CD detection:

- Compared with MNEI, the **H-Monellin showed an identical fold**, **and a typical β-sheet rich structure**.
- The MNEI exhibited a property of Tm (75℃), whereas the **H-Monellin showed a higher stability above 85℃** .
- The **CD detection temperature (90℃) did not reach the plateau of H-Monellin**.
- **Combined with thermal aggregation tests, when treated at 90℃ , it was retained 80% in 1 hour.** Therefore, it was about at least 10℃ higher than MNEI.

Differential scanning calorimetry detection:

- The **Tm of** MNEI is 54.7℃, H-MNEI is 79.5℃; **E24Q/Y80R H-Monellin is 96.1℃**.
- The Tm of another two selected hairpin contain mutants also tested by Nano DSC around 90℃.

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High temperature stable E24Q/Y80R H-Monellin (5/5)

Application

- The method is applicable for producing H-Monellin which could tolerate boiling water for 10 mins without getting degraded.

Conventional Solutions	Advantages
The conventional Monellin gets degraded at high temperatures.	The monellin produced by this method do not degrades at high temperature.
The conventional monellin had low potential for sweeteners	The monellin produced by this method showed remarkable potential for further sweeteners.

Comment

- The monellin produced by this method showed remarkable stability at very high temperatures.

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2.7 High temperature stable E23A H-Monellin **

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High temperature stable E23A H-Monellin (1/5)

Solution

The solution to the problem is invented by Qilu University of Technology

Non-patent Published in 2016

Application in beverages.

- **Cloning, Expression, and Purification of the Monellin Protein:**
- The **monellin gene of full-length 294 bp was synthesized** with the optimized codon usage.
- The gene was then **cloned into the plasmid pET15b with two restriction enzyme sites NdeI and BamHI at the N and C terminals**, respectively.
- The recombinant plasmid was designated as **pET15b-MNEI** (single-chain monellin) and verified by DNA sequencing.
- The monellin variants were constructed on the basis of the recombinant plasmid.
- Primers of the **MNEI mutants were designed to amplify the gene by PCR.**
- The **PCR product was digested by DpnI enzyme** and then **transformed and cultured in E. coli DH5α cells**.
- The plasmid of the mutated gene was purified and verified by DNA sequencing.
- To overexpress the recombinant wild type and mutated proteins, **the plasmids were transformed into E. coli BL21-CodonPlus(DE3)-RIL.**
- **An overnight culture in LB medium at 37°C was diluted 1 : 100 and grown until the OD600 reached 0.6 and then induced with 0.4 mM IPTG at 37°C for 4 h.**
- Harvested cells were **resuspended in lysis buffer** (20 mM sodium phosphate buffer, 20 mM imidazole, and 500 mM NaCl, pH 7.4) and **disrupted by 20 min sonication**.
- The **cell debris was removed by centrifugation at 10,000 rpm for 30 min**.
- The proteins in the supernatants were **purified by Ni Sepharose High Performance (nickel column affinity chromatography).**
- The purified proteins were dialysed into **MilliQ water and analyzed by SDS-PAGE.**
- **Another method by Nagoya University, Kirin Brewery Co., Ltd, Northwest University increases yield production of monellin by using modified fed batch culture.** Source

Escherichia coli strains DH5α and BL21-CodonPlus(DE3)-RIL and plasmid pET15b were from Novagen.
Easy Pfu DNA polymerase and restriction enzyme FastDigest DpnI were from Beijing TransGen Biotech Co. and Thermo Scientific, respectively.
All other molecular manipulation enzymes were from Takara Bio (Dalian, China). Ni Sepharose High Performance was from GE Healthcare.
All other chemicals were of analytical grade and obtained from Sangon Biotech (Shanghai, China).

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High temperature stable E23A H-Monellin (2/5)

Tests

Concentration Measurement and Sweetness Threshold Assay of the Wild Type and Variants Proteins:

- The **concentration of proteins was measured by the Bradford method.**
- **Double-blind taste assays were performed by a panel of ten healthy volunteer tasters, five males and five females, 20–60 years old.**
- The compounds tested were the MNEI protein, MNEI variants proteins, sucrose, and MilliQ water. Stock solutions of proteins were diluted by MilliQ water immediately prior to the taste assay.
- An initial series of **protein samples with concentrations 0.25, 0.5, 0.75, 0.8, 0.9, 1.0, 1.5, 2.0, 2.5, 3.0, 5.0, 10, 15, 20, 45, 90, 100, and 150 μg/mL was tested by one taster.**
- To further determine the accurate threshold values of each sample, **after the initial evaluation, 0.05 μg/mL was selected as the concentration interval (0.25, 0.3,0.35, 0.4,0.45,0.5,…1 μg/mL) for the proteins with sweetness threshold 0-1 μg/mL and 0.1 μg/mL as the concentration interval (1, 1.1, 1.2, 1.3, 1.4, 1.5,…10 μg/mL) for the proteins with sweetness threshold 1–10 μg/mL, respectively.**
- Samples were tasted in order starting from the lowest concentration until at least two consecutive concentrations were judged as sweet. Before each sample, **tasters rinsed the mouth with tap water at least twice until no residual taste remained**. Then, 1-2 mL of sample was taken into the mouth.
- The **solution was held in the mouth for at least 10 s and then spit out**.
- The tasters then graded the sample using the following notation to score their response (numbers indicate how these responses were scored): nonsweet and uncertainty at the threshold level of detection: 0; faintly sweet: 1.0; sweet: 2.0; very sweet: 3.0; intensely sweet: 4.0.
- Numerical scores for all tasters were combined to yield averages. The detection threshold was taken as the lowest concentration at which the taster recognized the sweetness as perceptible.

Thermostability Assay of the Sweet-Tasting Protein Monellin and Its Variants:

- **100 μL stock solutions of the dialyzed proteins were incubated in water bath at different temperatures for up to 10 h**, respectively.
- A series of temperatures **40°C, 45°C, 50°C, 55°C, 60°C, 65°C, 70°C, 75°C, 80°C, 85°C, and 90°C were used and the proteins were taken at various times (2 h once).**
- The proteins with water bath treatment were **centrifuged to remove the sediment** and **the supernatants were analyzed by SDS-PAGE**.
- Each **experiment was carried out in three parallel experiments and the results were averaged.**

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High temperature stable E23A H-Monellin (3/5)

Tests

Comparison of the His-Tagged and No-Tagged MNEI and Its Mutants:

- **To investigate the probable effect of His-tag sequence on the properties of the sweet protein, The enzyme thrombin was used to cleave and remove the His-tag sequence of the MNEI and its mutants E23A and C41A.**
- The **overexpressed proteins in the supernatants obtained as described above were loaded on the nickel column** (Ni Sepharose High Performance).
- The column was washed with distilled water, binding buffer (50 mM Tris-Hcl, 20 mM imidazole, and 150 mM NaCl, pH 7.4), and washing buffer (50 mM Tris-Hcl, 40 mM imidazole, and 150 mM NaCl, pH 7.4).
- **The bottom of the column was plugged with a plug, followed by addition of 10 mL cleavage buffer (50 mM Tris-Hcl, 150 mM NaCl, and 2.5 mM CaCl2, pH 7.4) containing thrombin (100 U/mL, 0.5 mL).**
- After **incubation at 25°C for 16 h**, the plug was removed and the no-tagged proteins were collected. **This procedure ensured that the residual His-tagged proteins in the thrombin digested reaction mixture were removed.**
- The **resultant no-tagged proteins were then purified with an anion exchange column to remove thrombin.**
- The sweetness threshold and thermostability of His-tag removed MNEI and its mutants were investigated with the same experimental procedures described above as the His-tagged proteins.

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High temperature stable E23A H-Monellin (4/5)

Result

- The result indicated that **six variants (N14A, F18A, D21A, E23A, N24A, and I26A) were consistent with the wild type single-chain monellin protein in sweetness.**
- The **sweetness threshold of C41A was lower than that of the wild type MNEI, demonstrating an improved sweetness of this mutant.**
- The sweetness thresholds of other mutants was increased, **and the K17A mutant was the highest. Indeed, the K17A mutant tasted almost no sweet, and its thermostability was obviously reduced**.
- It is worth noting that the **A19E mutant was not successfully expressed, suggesting that this alanine residue plays a vital role in the correct folding of the protein.**
- Most mutants displayed the remaining thermostability as the wild type MNEI.
- **The E23A mutant showed remarkable improvement of thermostability that the protein was stable and had no detectable change after incubation at 80°C for 10 h, and it was totally denatured after heat treatment at 85°C for 2 h.**
- **The mutants P10A, F11A, N14A, N24A, and I26A resulted in slight increase of thermostability (about 5°C).**
- These amino acids are located at the two ends of the α-helix, and their distribution is symmetrical.
- The results conclude that manipulation of gene expression and modification of the original protein are effective for improvement and optimization of the properties of sweet-tasting proteins.
- It was found that there was **no difference of the sweetness threshold and thermostability between the tagged and no-tagged proteins**, indicating that the His-tag sequence has no influence on the native protein.
- The study of the thermal stability and the sweet threshold of the mutants of single-chain monellin (MNEI), **It was found that two mutants C41A and E23A exhibited improved sweetness or thermal stability**.

Name	Sweetness threshold (μg/mL)	Maximum heat resistance temperature (°C)
Sucrose	10000 ± 150	—
MNEI	0.8 ± 0.05	65
P10A	3.0 ± 0.4	70
F11A	2.0 ± 0.25	70
T12A	10 ± 1.1	55
N14A	0.9 ± 0.1	70
L15A	2.0 ± 0.2	45 ± 5
K17A	>450	<40
F18A	0.9 ± 0.05	55 (50)
A19E (insoluble)	—	—
V20A	2.5 ± 0.5	45
D21A	1.0 ± 0.2	65 (45)
E22A	2.0 ± 0.3	60
E23A	0.8 ± 0.1	85
N24A	0.9 ± 0.15	75 (70)
I26A	0.8 ± 0.05	70 ± 5
C41A	0.5 ± 0.05	70

Source

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High temperature stable E23A H-Monellin (5/5)

Application

❑ The method is applicable for producing novel monellin with improved thermostability and sweetness.

Conventional Solutions

Advantages

The conventional monellin had less sweetness.	→ The method is capable of producing monellin with higher sweetness.
The conventional monellin had limited application because of its instability at high temperatures.	→ The monellin produced by this method had higher stability at high temperature.

Comment

- The method produces monellin with higher sweetness content and higher thermostability.
- MNEI, expressed in E. coli, denatured after heating at 65 C for 6 h. However, **monellin variant production in P. pastoris retained solubility under the same conditions. Also, proteins expressed in P. pastoris had better stability than those expressed in E. coli.**
- Another research by the university disclose that E2N/E23A variant is stable at 84.9 degree. Source

Table 3
Sweetness and thermostability of the wild-type MNEI and its mutants.

Samples	Sweetness threshold (μg/ml)[a]	Times than sucrose	Thermostability (T_m, °C)[a]
Sucrose	4000	1	
Wild-type	1.1 ± 0.09	3636	74.2 ± 0.2
E2N[b]	0.33 ± 0.02	12121	68.4 ± 0.1
E2N/E23A[b]	0.38 ± 0.02	10526	84.9 ± 0.1
E2M	0.35 ± 0.02	11429	70.4 ± 0.3
E2K	0.31 ± 0.03	12903	72.5 ± 0.4
E23K	1.1 ± 0.06	3636	74.2 ± 0.1
E23R	1.2 ± 0.05	3333	74.1 ± 0.5

[a] Standard deviation was calculated from the results of three paralleled experiments.
[b] Reference13.

Source

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