Comparative Summary of Sweet protein - Brazzein

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
4	Cweet by Natur Research Ingredients Inc	**Natur Research Ingredients Inc**	40% fewer calories than sugar, no calorie		2000x times sweeter than cane sugar	**Without any aftertaste**	Heat stable		Low cost		Highly soluble
6	Mass production of brazzein by Magellan Life Sciences	**Magellan Life Sciences**	Yes	**pH stable**	1200 times sweeter than sugar	**No bitter or metallic aftertaste**	**90°C for 1 hour**		Low cost		
7	Mass production of Brazzein by Novo holdings	**Novo Holdings**	Yes	**2-9**	510 times sweeter than sucrose	**Sweet taste delay of the brazzein was estimated to be 1.5 sec±1.2 sec (SD)**				Mass production with high yield and purity	
8	AmideBio Brazzein	**AmideBio**	Yes	**2.5 to 8**	2000 to 4000 times that of sucrose		**98°C for 2 hours**		Low cost		
14	Mass production of brazzein by Roquette + BRAIN + AnalytiCon	**Roquette + BRAIN + AnalytiCon**	Yes		Sweetness	**No bitter or metallic after taste**	Stable under extreme thermal and chemical conditions			Scale-up production	
15	Heat stable Brazzein mutant	University of Wisconsin	Yes	**Acid resistance**	Sweetness		**100°C for 4 h**				
18	Efficient Production of Recombinant Heat-Stable Brazzein	University of Wisconsin	Yes		Sweetness	**Not exhibit aftertaste for longer durations**	Heat stable at 85				
19	Heat treated H30R, E35D, E40A, E40D, E40K, E40H, E40R brazzein variants	**Biosweet**	Yes	**Acid resistance**	2-20 times than native form		**Thermal stability (70 to 90° C. for 15 to 60 minutes)**			Large scale production by using E.coli, Kluyveromyces lactis	Water stability
21	Brazzein variants of Ala29, Ala40, Ala50, Ala9, Ala41, Ala53	**Ajinomoto**	Yes		Sweetness	**Low lingering effect at sweetness level of variants**					
33	His31, Glu36 and Glu41 amino acids modification in brazzein	Chung Ang University	Yes	**5.4**	7-12-fold of des-pE1M-brazzein						
38	Mass production of Brazzein by FRI srl	**Food Res and Innovation SRL**	Yes		Sweetness					Mass production with high yield and purity	
40	E9K and V7R brazzein variants	Qilu University of Technology	Yes		Sweetness threshold of 1.5 µg/ml						

Back to Contents

1. Solutions on Brazzein Modification

Back to Ranking

Brazzein (1/4)

Introduction

- Ira Remsen (USA) and Constantin Fahlberg (Germany) discovered **saccharin, which is considered to be approximately 500 times sweeter than sugar.**
- Saccharin has an advantage in that it is **not digested in the human body but excreted from the human body**. However, there is controversy over whether saccharin is a carcinogenic substance. Finally, although saccharin was proven to be **harmless to the human body**,
- it is still **hardly used due to its bitter aftertaste**. T2R bitter sensing cells accounting for the bitter aftertaste of saccharin.
- In 1937, the University of Illinois (USA) found that **sodium cyclohexylsulfamate has a sweet taste**. **With the trade name cyclamate**, it was first used in the beginning of 1950, and swept through the global sweetener market in the 1960s.
- However, as the **sodium cyclohexylsulfamate was proven to be a carcinogenic substance**, it has been completely prohibited since the 1970s in Korea.
- An **artificial sweetener most widely used in recent years is aspartame** that was discovered in 1965 by James Schlatter. **Aspartame has a sweetness content approximately 180 to 200 times that of sugar**.
- **Aspartame is included in a majority of currently commercially available diet drinks**, and thus is subjected to a **metabolic pathway to generate phenylalanine** when it is taken up into the human body. Therefore, **because of a congenital deficiency in the enzyme that serves to break down phenylalanine, i.e., phenylalanine hydroxylase, phenylketonuric patients cannot use the enzyme**.
- Continuous research conducted to develop not only artificial sweeteners but also **natural sweeteners**.
- As a result, a compound referred to as **stevioside was found to be present in the leaves of a perennial plant (i.e., Stevia rebaudiana)** in the aster family, which is classified as an herb.
- The natives living in the border between Paraguay and Brazil have used stevioside as a sweetener for over 400 years.
- In Korea, stevioside is sometimes added to a traditional distilled liquor called “soju” and is 200 times as sweet as sugar.
- Increasing attention has been paid to a sweetener protein extracted from a tropical fruit.

Back to Contents

Brazzein (2/4)

Introduction

- The demand for non-calorigenic protein-based sweeteners with favourable taste properties is high.
- To date, only eight sweet-tasting proteins have been known to elicit sweetness. Among them, brazzein (molecular mass of 6.5 kDa) is the smallest sweet-tasting protein isolated from the fruit of the West African plant Pentadiplandra brazzeana Baillon.
- **Brazzein is attractive as a candidate for sweeteners for the control of obesity, oral health and diabetic management, because of its potential sweetness, sugar-like taste and good stability at high temperature and wide pH ranges.**
- The optimal design of new sweeteners requires knowledge about the structure–function relationships of sweet proteins and the interaction mechanisms with the sweet taste receptor.
- **The three-dimensional structures of brazzein have been determined by nuclear magnetic resonance (NMR) spectroscopy. Such NMR studies have revealed that brazzein contains one short a-helix (residues 21–29) and three strands of antiparallel b-sheets (strand I, residues 5–7; strand II, residues 44–50; strand III, residues 34–39).**
- To date, no tertiary structure has been reported for the sweet taste receptor, T1R2–T1R3. The three-dimensional structures of human T1R2–T1R3 by homology modelling have suggested that **brazzein binds to the open form of T1R2 in the sweet taste receptor**, T1R2–T1R3 heterodimer.
- However, results from modelling must be considered carefully because the glutamate receptor from which the 3D structures of human T1R2–T1R3 heterodimers is a homodimer and the sequence identity between these receptors is approximately 23%.
- Brazzein is the only CSαβ protein known to be sweet.

Back to Contents

Brazzein (3/4)

Introduction

- Brazzein is a sweetener protein extracted from the fruit of **Pentadiplandra brazzeana** (Baillon) growing in West Africa.
- Brazzein shows sweetness approximately **500 to 2,000 times that of sucrose**, and is divided into two types: a major type and a minor type.
- The major type accounting for a majority of **brazzein extracted from the plant has 54 amino acids including a pyroglutamic acid residue bound to an amino-terminal region.**
- **The minor type of brazzein has 53 amino acid residues without a pyroglutamic acid residue** bound to an amino-terminal region, and shows stronger sweetness, approximately twice that of the major type of brazzein.
- Brazzein has a **molecular weight of approximately 6.5 kDa**, which is the smallest among the sweetener proteins, and is a monomer composed of one kind of subunit. Also, brazzein consists of a single polypeptide and has one **α-helix and two β-pleated sheets**.
- **Brazzein has very high thermal stability since it has 8 cysteine residues to form 4 disulfide bonds in the molecule**. Also, brazzein shows **very high solubility and pH stability in water**.
- In order to search for a natural sweetener showing high thermal stability and **excellent sweetness**, the prior art here have prepared **variants and multi-variants by mutating wild-type brazzein** through substitution of amino acids at certain positions which are expected not to affect a structure in an amino acid sequence of brazzein, and screening a brazzein variant or multi-variant **having equivalent properties such as thermal stability, pH stability and high water solubility and showing higher sweetness compared to the conventional brazzein**.
- The prior art here is directed to providing a novel brazzein variant or multi-variant having **equivalent properties such as high thermal and pH stabilities and high water solubility** compared to a minor-type protein of the conventional brazzein and showing stronger **sweetness at least 2 times and up to 20 times that of the minor-type protein**.
- **Among all the known sweet-tasting proteins, brazzein is the most stable with the lowest lingering off-taste**, making it one of the most interesting if we also consider its intense sweetness. **Low association and dissociation rate constant of the brazzein can be a reason for its delayed taste and aftertaste**. Brazzein has a delayed sweetness onset, a licorice off-note, and shows concise sweetness lingering.

Source

Back to Contents

Brazzein (4/4)

Introduction

- Brazzein, a sweet protein extracted from the ripe fruit of the west African plant Pentadiplandra brazzeana Baillon, is the smallest (based on molecular weight) naturally occurring sweet-tasting protein discovered up to now.
- The protein consists of a **single chain of 54 amino acids and displays in two forms: the major form, which contains a pyroglutamic acid (pGlu) at its N-terminus**, is about 9,500 times sweeter than sucrose on a molar basis, and the **minor form, in the absence of pGlu (des-pGlu), is two times sweeter than the form with pGlu**.
- Due to its sugar-like taste, high sweetness, and **good stability at high temperatures and wide pH ranges**, brazzein is proposed as a potential replacer of sugars and artificial sweeteners for the control of the lifestyle-related diseases such as obesity, oral health, and diabetes.
- **Purification of the natural brazzein protein has the shortcomings of low yield, time-consuming, and high cost**.
- **To facilitate its production** in food and beverages, the **recombinant des-pGlu brazzein has been heterologously expressed in E. coli, Lactococcus lactis, Kluyveromyces lactis, and P. pastoris**.
- The des-pGlu brazzein expressed in different construct/host E. coli systems exhibited different sweet taste thresholds, while **brazzein expressed in P. pastoris showed a sweet taste threshold about 3 μg/ml**.
- The recombinant brazzein secreted from K. lactis was 2,130 times sweeter than sucrose on a weight basis, whereas the sweetness of recombinant brazzein expressed in L. lactis was not precisely evaluated.
- The **variance of the sweetness thresholds should be due to the different construct/ host systems or applied evaluation methodologies**.
- To further improve the sweet properties of sweet protein brazzein, we expressed the wild-type protein and its three mutants with optimized codon usage and **modified N-terminus**. As a result, the recombinant brazzein displayed significant improvement of sweetness than those in previous reports.

Back to Contents

1.1 Cweet by Natur Research Ingredients Inc. **

Back to Ranking

Solution

Cweet by Natur Research Ingredients Inc. (1/2)

The solution to the problem is invented by Natur Research Ingredients

Cweet™

- **Natur Research Ingredients Inc is a sister company to Natur Research Foods Inc, which has developed low-GI sweetening products including Natur Baker's Blend Natural Sweetener, which has 40% fewer calories than sugar.**
- Los angeles based Natur Research Ingredients **obtained a license in 2007 from the Wisconsin Alumni Research Foundation (WARF) to manufacture and distribute Cweet Natural Intense Sweetener. Cweet is not currently approved for distribution in the U.S., but the company anticipates being available in the next one to two years, reports by FoodNavigator.com.**
- The product has the potential to become highly competitive in the plant-based low-glycemic sweetener category.
- Loren Miles is The CEO of Natur Research Ingredients.Natur Research Ingredients, Inc. is also **in discussions with global food and beverage brands for the exclusive rights to use Cweet within their product category for the first 2-years from availability**. "Manufacturers can lock in their exclusive use now, thus having a market advantage over competing brands in their category, for the first 2-years upon regulatory approval," said Miles.Loren Miles was seeking a strategic partner - ideally with expertise in the sweeteners business and global reach - to help him get it to market. He has been trying to get brazzein to market for some years and admits it's taken longer than he originally envisaged, in part because industry attention has been on stevia.
- Brazzein can be used very cost-effectively to "do the heavy lifting" in many formulations, said Miles.
- **Extracting brazzein from the berries is expensive and undesirable from a sustainability perspective, Miles has acquired the license to produce it from food grade bacteria using a patented process developed by scientists at the University of Wisconsin.** The brazzein this produces can still be listed as brazzein on the ingredients label and is just as 'natural' as the original, Miles claimed.
- **Cweet is a natural intense sweetener and 2000x times sweeter than cane sugar.** Cweet natural intense sweetener is a natural proprietary sweetening agent that is a **highly soluble product with excellent organoleptic properties. Cweet is derived from brazzein,** a protein extracted from the West African fruit of the climbing plant Oubli (Pentadiplandra brazzeana Baillon).**Cweet Natural Intense Sweetener is specifically formulated to be a user friendly sweetening system without any aftertaste, zero calories (per serving) and naturally has a low glycemic index value.** Cweet is an extremely versatile sweetening system that can be used in nearly any application of food, beverage, confection, condiment and pharmaceutical uses. **The sweetener is diabetic friendly, dissolves clear, and heat stable.** The brazzein plant creates a protein that is 2,500 to 4,000 times sweeter than sucrose by weight, which makes it **more affordable than stevia and monk fruit. Production costs are also lower in converting brazzein into a sugar substitute through a unique patented process called "bio-fermentation".** "Bio-fermentation" technology makes it possible, using food-grade bacteria commonly used today for many foods and beverages, to grow a large quantity of brazzein in large tank vessels in a matter of days. According to FoodNavigator.com, Cweet is not extracted from the actual brazzein berries, but created from food grade bacteria in a process that was developed at the University of Wisconsin. The technology involves "growing" large quantities of the plant protein in tank vessels within several days. The company suggests this bacteria method is more sustainable than stevia or monk fruits because the process involves no tree orchards that require the use of natural resources including land and water.

Application in food & beverages.

Source1 Source2 Source3 Source4 Source5

Back to Contents

Cweet by Natur Research Ingredients Inc. (2/2)

Application

- The protein sweetener is suitable for both diabetics and pre-diabetics, and may stimulate new product development tailored for individuals with these concerns. With its mild, sweet taste, it can function as a non-cariogenic sucrose replacer in a number of foods and drinks, including energy drinks, sports and isotonic drinks, fitness waters, candy and chocolate bars, milk drinks, yogurts, soft drinks, and cereals. The ingredient has GRAS approval in the United States.

Conventional Solutions

Conventional protein sweeteners lacks one or the other qualities to be an ideal solution for food and beverage manufacturers.

Earlier, no natural sweetener was able to replace all the artificial sweeteners in a product.

Advantages

Protein-based sweetener is safe for diabetics, very soluble in water, and heat stable—characteristics that are highly desirable to food and beverage manufacturers. In addition, its sweetness profile is very similar to sucrose with no lingering aftertaste.

Cweet can used to replace all artificial sweeteners in a product.

Comments

- Global companies are testing samples of Brazzein and their response has been very positive.
- The results have proven to be highly cost-effective, maintain a high level of standardization that meets or exceeds current quality control standards, and as compared to conventional farming [the process] has a fraction of the carbon footprint.

Source1 Source2 Source3
Source4

Back to Contents

1.2 Mass production of brazzein by Magellan Life Sciences **

Back to Ranking

Mass production of brazzein by Magellan Life Sciences (1/4)

Solution

The solution to the problem is invented by Magellan Life Sciences

Patent Published in 2019

Application in food & beverages.

- **Magellan Life Sciences is an innovation driven synthetic biotechnology company that discovers and develops plant inspired proteins for commercial applications in Food and Beverage industries. It is addressing the important issue of sugar reduction by providing alternatives to carbohydrates with new protein sweeteners.**
- The company was founded on October 22, 2013 and the headquarters regions is Asia-Pacific (APAC). It is classified as Non-govt company and is registered at Registrar of Companies, Hyderabad. Magellan's proprietary protein production platform XSeed® bridges the gap from R&D to consumer markets by allowing for economically viable industrial scale production of unique natural proteins. **The sweet tasting protein Brazzein is the leading product from the company's pipeline. Brazzein holds significant commercial potential as the next-gen protein sweetener that is 1200 times sweeter than sugar, zero caloric, pH and heat stable. Unlike all its competitors, Brazzein has a sugar-like taste with no bitter or metallic aftertaste, thus requiring zero masking agents, thereby reducing formulation and product costs; a high value proposition for today's F&B industry.**
- **Using XSeed®, the company have developed a patented commercially viable production process for Brazzein.** XSeed® Platform Technology – The proprietary synthetic biology-based platform is built upon a vast library of proprietary synthetic secretion signal molecules. **Magellan's new protein sweeteners are produced using its proprietary expression and fermentation platform XSeed, and is nearly indistinguishable in terms of taste to sucrose but with a far higher sweetening power and no undesirable metallic aftertaste.**
- **Engineering of product-specific, production strains with the appropriate combinations of these secretion signals allows for extremely high titers of secreted product. Proprietary, effective and simplified Down-Stream Processing protocols complement XSeed® enabling Magellan to reach unrivaled lower production costs.**
- Abhiram Dukkipati is the founder and CEO of the company and Laxmi Wagle is the CSO. Roquette Ventures, SOSV and the angel investors invest in Magellan. The funding allowed to expand the R&D team and scale the proprietary manufacturing process. Magellan has bagged seed investment led by the French ingredients major's Roquette Ventures, joined by U.S. tech startup venture capital SOSV and three European business angel investors. **The capital will be used to scale the British startup's plant protein sweeteners produced using fermentation, and will accelerate its go-to-market plans. The company has filed 1 patent titled, "Process for extracellular secretion of brazzein" on 27 January, 2017. US2019153455A1**
- **Stable under thermal and chemical condition changes, the sweeteners are ideal for F&B applications to replace the sugar content in products and for producers to develop healthier formulations without having to add another masking agent to hide potential aftertastes associated with existing sweeteners on the market.**
- The company says that Brazzein will also help alleviate rising health epidemics such as diabetes and obesity.
- **Fermentation technology produces novel ingredients that are better from a health or environmental standpoint has been gaining momentum. The company developed fermentation process using an FDA-approved microorganism strain and food-grade chemicals to provide carbon and nitrogen sources for the fermentation process. Although the microorganism is genetically modified (GM), the brazzein does not contain this and the final product therefore be labelled as GM-free. The platform's industry leading productivity level combined with simplified down-stream processing makes it the first commercially viable and scalable production process for brazzein.**

Source1 Source2 Source3 Source4

Back to Contents

Mass production of brazzein by Magellan Life Sciences (2/4)

Test

- **Thermal Stability and Protein Estimation: Cell free supernatant was heated to 90°C for 1 hour in a water bath and spun at 17,500 g for 30 mins.** The supernatant was analyzed by SDS - PAGE . Protein quantitation was done using BCA assay kit . As a result of heating most of the endogenous E . coli proteins present in the cell free supernatant precipitated, **leaving > 90 % pure Brazzein in solution**. Protein estimation of the supernatant from the heated and spun sample demonstrated a yield of 0 . 56 g / L of Brazzein.
- **Sensory Analysis of Brazzein**: A portion of the purified Brazzein (A28D) was lyophilized and re - dissolved in deionized water to 1 . 0 mg / mL . From this , Brazzein (A28D) solutions with following concentrations were made : 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10 . 0 ug / mL . A 1 % (w / v) sucrose solution , the lowest concentration of sucrose detectable by humans , was used as a reference . The taste panel consisted of fifteen females and fifteen males with normal health and normal sense of taste . Two - hundred - microliter samples were applied to the anterior region of the tongue . After each test , the mouth was rinsed with tap water . The tasters sampled from the lower concentration samples to higher . Each taster chose the first sample that could be sensed for sweetness . Sweetness potencies were reported relative to sucrose on a weight basis . **The purified Brazzein (A28D) was found to be 1660 times sweeter than sucrose on a weight basis**.

Sensory Analysis of Purified Brazzzein

Molecule	Experimental Threshold % [g/100 mL]	Relative Sweetness (by weight)
Sucrose	1	1
Brazzein(A28D)	0.0006	1660

- Speaking with Food Navigator, Nuttin (GM of Roquette Ventures) explained that Magellan's proprietary platform X-Seed enables high levels of protein secretion into the extracellular fermentation media, which enables 'very efficient downstream purification of the desired protein'.

Back to Contents

Mass production of brazzein by Magellan Life Sciences (3/4)

Applications

- Global health challenges such as the rise of sugar-related diseases like diabetes or obesity, Magellan's protein sweeteners have a tremendous potential to meet the increasing demand of consumers for healthier alternatives to sugar.
- Biospringer has created a new yeast protein using the company's designed patented fermentation technology. Biospringer says the product was specifically for plant-based cheese and meat applications, as it combats the off-notes that some consumers report in existing plant-based alternatives on the market.
- Magellan's solution provides a solution to health and nutrition challenges, especially as more F&B businesses begin to come under pressure to produce healthier products amid the clean label movement.

Conventional Solutions	Advantages
Conventional sweet proteins lacks the potential to withstand extreme temperature and pH conditions.	Water-soluble brazzein withstands typical cooking processes such as high temperatures and freezing and has a range of pH values, making it interesting for food manufacturers.
Conventional protein sweeteners requires masking agents for unpleasant aftertastes.	Brazzein is 1200 times sweeter than sugar, zero caloric, has a sugar-like taste with no bitter or metallic aftertaste, thus requiring zero masking agents, thereby reducing formulation and product costs.

Comments

- The sweet tasting protein Brazzein is the leading product from the company's pipeline.
- The company developed fermentation process using an FDA-approved microorganism strain and food-grade chemicals to provide carbon and nitrogen sources for the fermentation process. Although the microorganism is genetically modified (GM), the brazzein does not contain this and the final product therefore be labelled as GM-free.
- The platform's industry leading productivity level combined with simplified down-stream processing makes it the first commercially viable and scalable production process for brazzein.
- Roquette Ventures has invested in Magellan Life Sciences Ltd., that has developed a proprietary expression and fermentation platform to produce new generations of plant-inspired protein sweeteners.

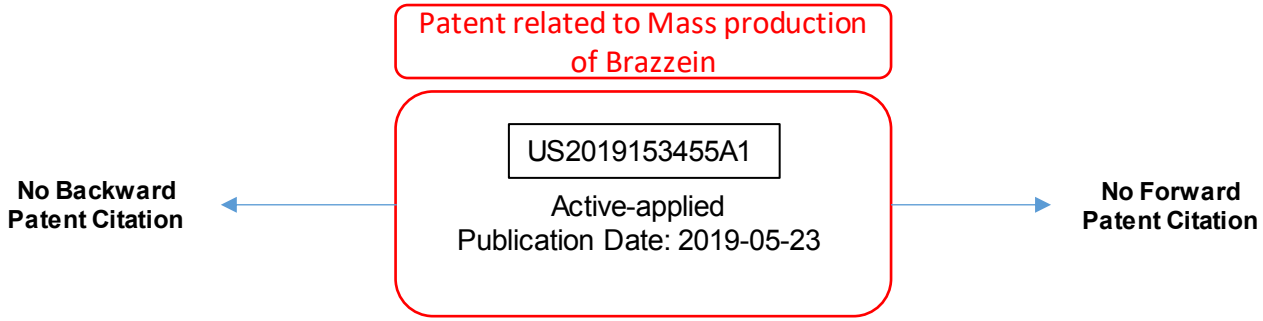
Source1 Source2
Source3

Back to Contents

Mass production of brazzein by Magellan Life Sciences (4/4)

Citation Tree

❑ **Citation tree helps to understand the strength of the technology based on count of companies or universities taking help form the inventive technology.**



Backward Citations are earlier published documents that are publicly available before the filing date of the new patent application, also called "prior art". These can be cited by an applicant, third party or a patent office examiner because of its content relates to the new patent application.

Forward citations are more recently published documents which has gone on to cite the new patent application. These can be cited by an applicant, third party or a patent office examiner because of its content relates to the new patent application.

Note that citation tree has been provided for top ten ranked solutions only.

Back to Contents

1.3 Mass production of Brazzein by Novo holdings **

Back to Ranking

Mass production of Brazzein by Novo holdings (1/4)

The solution to the problem is invented by Novo Holdings

Solution

Patent Published in 2016

Application in food & beverages.

- **The reference discloses about a method of producing a recombinant sweet protein comprising an amino acid sequence having at least 90% identity to amino acids 2 to 54 of SEQ ID NO: 2, which comprises**:
- **a) Cultivating an <u>Aspergillus host cell</u>** comprising a nucleic acid construct comprising a polynucleotide encoding the sweet protein under conditions conducive for production of the sweet protein.
- **B) Recovering the sweet protein in a functional form as secreted from the host cell.**
- **C) Applying the functional sweet protein as active ingredient in the sweetening agent.**
- **The sweet protein is secreted from the host cell in a functional form.**

- **The sweet protein is brazzein or a variant of brazzein.**

- **The nucleic acid construct comprises the polynucleotide encoding the sweet protein operably linked to a first nucleotide sequence encoding a signal peptide comprising or consisting of an amino acid sequence having at least 90% identity to amino acids −55 to −33 of SEQ ID NO: 2, and a second nucleotide sequence encoding a propeptide comprising or consisting of an amino acid sequence having at least 90% identity to amino acids −32 to −1 of SEQ ID NO: 2.**

Source

Back to Contents

Mass production of Brazzein by Novo holdings (2/4)

Tests

Sensory Evaluation of the Sweetness of Brazzein Produced in A. oryzae:

- **A test taste panel of six persons was used to evaluate the brazzein sweetness compared to sucrose.**
- The brazzein batch was diluted 30 fold to a final conc. of 50 mg/l with 20% skim milk in order to avoid adsorption on surfaces.
- **The sweetness was estimated using a standard of 0%, 0.68%, 1.37%, 2.74%, 5.48%, 11.0%, 21.9% (w/v) sucrose diluted in 20% (v/v) skim milk.**

pH Profile of the Sweet Taste of Brazzein Produced in A. oryzae:

- **A taste panel of three persons estimated the pH profile of the sweet taste of brazzein compared to sucrose. 5 g/l whey protein (Lacprodan DI-9224, Arla Foods Ingredients) with 0%, 2%, 4%, 6%, 8%, 10%, 12%, 14% and 16% sucrose or 75 mg/l brazzein was adjusted with H3PO4, Na2HPO4 or NaOH to a final pH of 2.0, 3.9, 4.0, 5.0, 7.0 and 9.0.**

Results

Sensory Evaluation of the Sweetness of Brazzein Produced in A. oryzae:

- The sweetness of this 50 mg/l brazzein dilution corresponded to an average sweetness of 2.57% (w/v) sucrose±0.55% sucrose (SD).
- **The brazzein batch is 510 times sweeter than sucrose on weight basis at the present condition. The sweet taste delay of the brazzein was estimated to be 1.5 sec±1.2 sec (SD).**

pH Profile of the Sweet Taste of Brazzein Produced in A. oryzae:

pH	% sucrose	SD
2.0	6.0	3.5
3.0	6.0	0.0
4.0	4.3	0.6
5.0	6.3	0.6
7.0	5.0	1.0
9.0	2.7	1.2

Source

Back to Contents

Mass production of Brazzein by Novo holdings (3/4)

Application

- The method is applicable for producing a protein sweetener brazzein with high purity and the yield production is also more than conventional methods.

Conventional Solutions	Advantages
The conventional methods of brazzein production produce less pure brazzein and the production capacity is also less.	The method produces brazzein with high purity and greater yield.

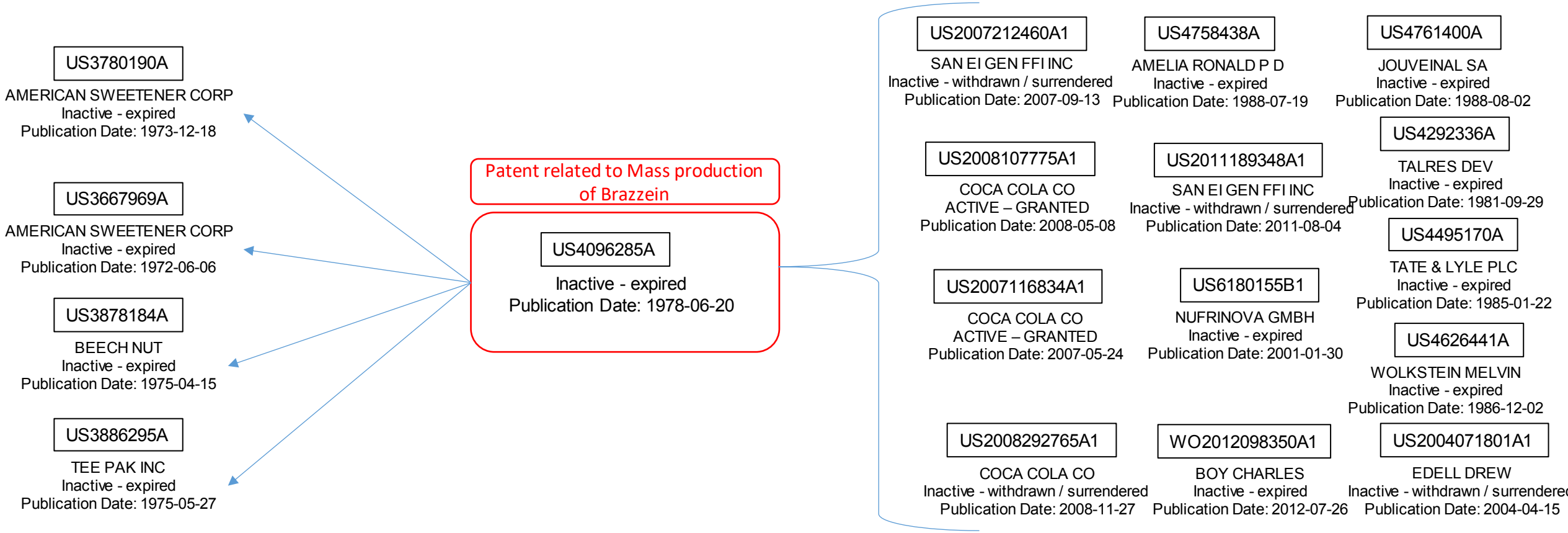
Source

Back to Contents

Mass production of Brazzein by Novo holdings (4/4)

Citation Tree

❑ **Citation tree helps to understand the strength of the technology based on count of companies or universities taking help form the inventive technology.**



Backward Citations are earlier published documents that are publicly available before the filing date of the new patent application, also called "prior art". These can be cited by an applicant, third party or a patent office examiner because of its content relates to the new patent application.

Forward citations are more recently published documents which has gone on to cite the new patent application. These can be cited by an applicant, third party or a patent office examiner because of its content relates to the new patent application.

Note that citation tree has been provided for top ten ranked solutions only.

Back to Contents

1.4 AmideBio Brazzein **

Back to Ranking

AmideBio Brazzein

Solution

The solution to the problem is invented by AmideBio LLC

- **AmideBio develops and commercializes peptide research reagents and clinical products for clinical and therapeutic marketplaces**. Its Headquarter is located in Greater Denver Area, Western US. The company was founded in 2009. Pawel Fludzinski is The CEO and President of AmideBio; and Michael H. B Stowell is CSO and Co-founder.
- **AmideBio can provide innovative biotech service manufacturing for peptides and proteins using a novel recombinant-chemical hybrid process**. From bench scale mg to multi-gram scale, **AmideBio can meet industrial needs by producing higher quality peptides and proteins at lower cost.**
- Brazzein and pentadin are a sweet-tasting protein produced in the fruit of the West African climbing plant Oubli (Pentadiplandra brazzeana Baillon) and found in the pulp tissue surrounding the seeds. Similar to other sweet proteins discovered in plants, such as monellin and thaumatin, it is extremely sweet compared to sucrose with sweetness 2000 to 4000 times that of sucrose. The monomer protein, consisting of 53 or 54 amino acid residues, is the smallest of the sweet proteins with a molecular weigh of 6.5 kDa. The structure of brazzein was determined by proton nuclear magnetic resonance (NMR) and shown to have four evenly spaced disulfide bonds, an alpha-helix and three beta strands forming an anti-parallel beta sheet. **Brazzein is stable over a broad pH range from 2.5 to 8 and heat stable at 98°C for 2 hours**.
- AmideBio provides two different types of Brazzein – 1-54 and 2-54.

Application in food & beverages.

Parameters	Brazzein, 1-54	Brazzein, 2-54
Description	Full length Brazzein	**Truncated Brazzein**
Molecular weight	6.5 kDa	6.5 kDa
Sweetness Index	2000 times sweeter than sucrose on weight basis	4000 times sweeter than sucrose
Stability	stable over a broad pH range and is heat stable at 98°C for several hours.	stable over a broad pH range and is heat stable at 98°C for several hours.
Sequence	QDKCKKVYENYPVSKCQLANQCNYDCKLDKHA RSGECFYDEKRNLQCICDYCEY	DKCKKVYEN YPVSKCQLAN QCNYDCKLDK HARSGECFYD EKRNLQCICD YCEY
Purity	>99%	>99%
Other Characteristics	Compared to sucrose and its sweet perception is more similar to sucrose than the other sweeteners such as stevia, thaumatin, or aspartame	Compared to sucrose and its sweet perception is more similar to sucrose than other sweeteners such as stevia, thaumatin, or aspartame.

Source1 Source2 Source3

Back to Contents

1.5 Mass production of brazzein by Roquette + BRAIN + AnalytiCon **

Back to Ranking

Mass production of brazzein by Roquette + BRAIN + AnalytiCon (1/2)

Solution

The solution to the problem is invented by Roquette+BRAIN+AnalytiCon

- **Roquette is a global leader in plant-based ingredients, a pioneer of plant proteins and a leading provider of pharmaceutical excipients.** Roquette was founded in 1933. Its Headquarters (HQ) is located in Lestrem, France (FR). Pierre Courduroux is The Chief Executive Officer (CEO) of the company. **Roquette Ventures (part of ingredients company Roquette Group), which supports start-ups in the food, nutrition, and health markets, was looking to scale up production of plant protein sweeteners made using precision fermentation. Roquette Ventures has invested in Magellan Life Sciences Ltd., that has developed a proprietary expression and fermentation platform to produce new generations of plant-inspired protein sweeteners. Roquette, biotech company BRAIN AG, and BRAIN Group company AnalytiCon Discovery have completed the R&D phase for the development of protein sweetener brazzein.**
- Roquette and BRAIN will now work on the approval and industrial scale-up of the protein sweetener in the food and beverage sector. Roquette and BRAIN, came together during the DOLCE research program and subsequently decided to further develop the sweetener by fermentation, and said they see good commercial opportunities for the compound, primarily in the beverage industry. Roquette Freres SA filed a patent titled, “Novel sweetening composition” on 2015-07-01. The patent is focused on a sweetening composition that contains 80% to 95% by weight of crystalline pulverulent sorbitol and preparing chewing gums and tablets. **Roquette and its biotech partner Brain say they have wrapped up R&D on a fermentation route to brazzein**. They plan to seek regulatory approval for the sweetener and to scale up output. The Union of European Soft Drinks Associations (UNESDA) has revealed its enhanced health and nutrition targets to help Europeans manage their intake of added sugars from soft drinks with a pledge to reduce sugar by a further 10 percent by 2025. Key sugar reduction players, including SweeGen, Kerry, Roquette and Sensient, also share their insights following the latest moves for sugar reduction.
- **Edouard Nuttin, said that Roquette seeks to help accelerate the development of Magellan’s protein sweeteners to an industrial scale as they contribute negligible calories at the intended levels of usage while still maintaining the sweetness profile required by the applications and formulations.** This is going to be a huge leverage for the food ingredient players seeking to find alternatives to sugar with these plant inspired, protein sweeteners which not only have an overwhelmingly **close taste to sugar with negligible calories, like an intense sweetener with a protein structure, and have absolutely no bitter or metallic after taste. Furthermore, being natural protein sweeteners they do not trigger an insulin response from the body thus making them diabetic friendly and safe. Stable under extreme thermal and chemical conditions, protein sweeteners are known to be ideal candidates for food and beverage applications/formulations.** Roquette has developed solutions to meet the increasing consumers’ demand for healthy and tasty food products. Among these plant-based solutions, Roquette offers Food formulators Nutriose, a soluble fiber for sugar reduction, and SweetPearl maltitol to be used as sugar alternative in sugar-free products.

Application in food & beverages.

Source1 Source2 Source3 Source4 Source5

Back to Contents

Mass production of brazzein by Roquette + BRAIN + AnalytiCon (2/2)

Applications

- The protein sweeteners are promising proteins for the F&B industries. They taste almost identical to sucrose with a much higher sweetening power.
- In the upcoming times, Fermentation can be a powerful technology to manufacture and scale up the production of plant protein sweeteners.

Conventional Solutions	Advantages
Conventional sweeteners have an unpleasant aftertaste and also lacks the property of thermal stability and pH resistance.	Protein sweeteners have no undesirable aftertaste and are stable under various thermal and chemical conditions.
Conventional protein sweeteners were not able to maintain the sweetness profile in formulations.	Protein sweeteners contribute negligible calories at the intended usage levels while still maintaining the sweetness profile required by the applications and formulations.

Comments

- The company supports and invests in new generation sweeteners.
- Roquette, biotech company BRAIN AG, and BRAIN Group company AnalytiCon Discovery have completed the R&D phase for the development of protein sweetener brazzein.
- Brazzein is a low-calorie sweetener and maintaining the sweetness profile required by the applications and formulations.
- Furthermore, it is diabetic friendly, stable under extreme thermal and chemical conditions, have no bitter or metallic after taste.

Source1

Back to Contents

1.6 Heat stable Brazzein mutant **

Back to Ranking

Heat stable Brazzein mutant (1/3)

Solution

The solution to the problem is invented by University of Wisconsin

Non-patent Published in 2005

Application in food & beverages.

- **Production of recombinant brazzein, stable isotope labeled brazzein and brazzein mutants:**
- An efficient **bacterial production system for brazzein** is developed.
- The recombinant protein produced has a sequence identical to the minor form of brazzein isolated from fruit, the form that lacks the N-terminal pyro-glutamate (pGlu) residue (des-pGlu1-brazzein) and that has been shown to have about twice the sweetness of the pGlu containing variant.
- The fusion protein is expressed as an insoluble product, which we solubilize and fold.
- The conditions we developed for folding and oxidation of the disulfides lead to a product with native structure (as determined by NMR spectroscopy) and with full activity as a sweetener (as determined by taste tests). Early variable temperature NMR studies of brazzein showed very little change in its 1H NMR spectrum over a wide range of temperatures (32–82°C) and the **sweetness profile was shown to be undiminished after incubation at 100°C for 4 h**.
- **This methodology, along with quick-change mutagenesis, has allowed us to make a variety of brazzein mutants**.
- **We discovered mutants with sweet-taste properties that appear to be superior to those of the wild-type protein**.

For detailed structural and dynamic analyses of brazzein variants, we chose wild-type brazzein (des-pGlu1-brazzein) and five mutants (two with increased sweetness and three with decreased sweetness).

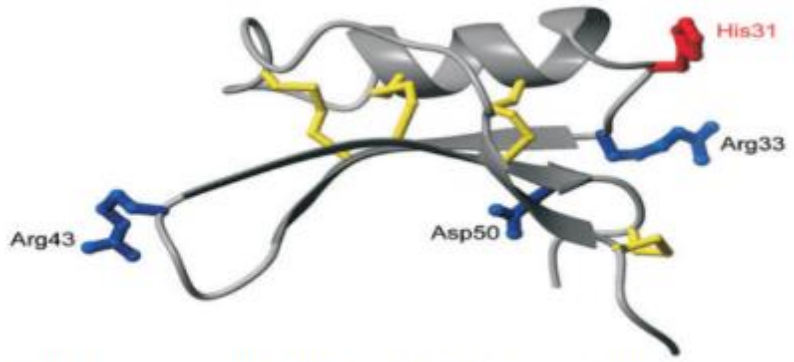


Figure 1 Backbone ribbon diagram of wild-type brazzein (Caldwell *et al.*, 1998a). Residues found to be critical for the sweetness of the protein are indicated in colour: red, enhanced sweetness; blue, decreased sweetness.

The ribbon-diagram in Figure 1 shows the backbone of wild-type brazzein and the positions of the five mutations.

Four of the sites of mutation (Ala2 insertion, His31Ala, Arg33Ala and Asp50Ala) are spatially close to one another.

Arg43Ala is tasteless.

Two of the mutants (Ala2 insertion and His31Ala) have about twice the sweetness of wild-type brazzein; the other three mutants (Arg33Ala, Arg43Ala and Asp50Ala) have greatly reduced sweetness.

Source

Back to Contents

Heat stable Brazzein mutant (2/3)

- **NMR study:** In only one of the five mutants **(Arg43Ala)** were the chemical shifts changes resulting from the mutation propagated to other parts of the molecule. **In this mutant, changes in chemical shifts were observed in the N- and C-terminal regions.** Analysis of the H-bonds in the six brazzein variants through measurements of trans-H-bond couplings has shown that **single-site mutations can give rise to subtle structural changes. Wild-type brazzein and the two variants with sweetness equal to or greater than wild-type brazzein had similar patterns of H-bonds, whereas all three variants with reduced sweetness exhibited changes in H-bonding. As determined by NMR relaxation measurements, the mutations that decrease sweetness were found to decrease the flexibility of the protein.** The results suggested, in addition, that loop 9–19 of brazzein exists as two or more sub-structures. We measured residual dipolar couplings (RDCs) as a means for determining whether loop regions are disordered. The RDCs from the sweeter brazzein analog (Ala2insertion) were similar to those from wild-type brazzein; this confirmed that the two proteins have similar structures. Furthermore, the RDC results indicated that residues 11–18 in the loop between the first β-strand and α-helix are disordered in both proteins

- **Investigation of model peptides**
  - On the basis of our model for **multi-site brazzein:receptor interactions**, we designed a **small cyclic peptide corresponding to regions of the N- and C- termini connected by a tri-peptide linker (PGN) at one end and a disulfide bond at the other end.**
  - The resulting cyclic peptide, c[(D2KCKKV7)-PGN-(D50YCEY54)], was designed to contain a proper β-turn (type I or II) motif.
  - This conformation was confirmed by homonuclear 1H-1H 2D TOCSY and NOESY NMR data.
  - **In a taste test, however, the peptide was found to be tasteless.**
  - Similar results were obtained in the Temussi laboratory on a different cyclic peptide.
  - That study examined the cyclic peptide c[C37FYDEKRNLQC47], which proved to be tasteless.
  - Both investigations of model peptides suggest that a more extensive structure is required for sweetness.
- **Relationship between brazzein and proteinase inhibitors**
  - The sequence of brazzein is very similar to that of the rapeseed-type proteinase inhibitors from plants and the structures are also similar.
  - **The reason why brazzein has no activity as a proteinase inhibitor is explained by its lack of the reactive site dipeptide.**
  - Attempts to convert brazzein into a trypsin inhibitor by inserting the missing residues or even by introducing the entire reactive site loop of the Arabidopsis thaliana inhibitor proved unsuccessful; these insertions or modifications either destabilized the mutated protein or prevented it from folding.

Back to Contents

Heat stable Brazzein mutant (3/3)

Application

- ❑ The discovery of the sweet taste heteroreceptor opens up exciting new avenues for research on the mechanism of action of sweet substances.
- ❑ Brazzein is an excellent candidate for experimental investigations of the chemical and structural requirements for extracellular triggering of a sweet response in humans and for understanding the mechanism of the signal transduction.
- ❑ Production of brazzein from Escherichia coli has also enabled us to make samples labeled with stable isotopes (15N or 13C and 15N) for NMR investigations of the structure and dynamics of the protein.

Advantages

Brazzein has properties that make it particularly attractive as a potential economic sweetener.

Brazzein is highly stable over wide temperature and pH ranges and has taste properties that resemble those of carbohydrate sweeteners.

Brazzein is a single polypeptide of 54 standard amino acids and contains no carbohydrate.

Comment

- ➢ Studies have indicated that the presence of positive charges on the surface of brazzein enhances sweetness: mutating some of these positive charges to neutral or negative charge significantly decreases the sweetness.
- ➢ **Sweetness profile was shown to be undiminished after incubation at 100°C for 4 h**

Source

Back to Contents

1.7 Efficient Production of Recombinant Brazzein, a Small, Heat-Stable, Sweet-Tasting Protein of Plant Origin **

Back to Ranking

Efficient Production of Recombinant Heat-Stable Brazzein (2/7)

Solution

The solution to the problem is invented by University of Wisconsin

Non-patent Published in 2000

Design and synthesis of the brazzein gene:

- **The Genetics Computer Group (GCG) program was used in designing the synthetic gene**, which was based on the amino acid sequence of the naturally occurring protein and was optimized to achieve a stable predicted RNA secondary structure. **NdeI and BamHI sites were designed into the 59 and 39 ends, respectively**, to permit cloning into any of the pET system plasmids (a variety of plasmids characterized by a T7 expression system with an optional fusion to a poly-histidine linker). **A starting codon (Met) was introduced just before the first codon of the synthetic gene, since the amino acid sequence of natural brazzein lacked an N-terminal methionine**. The **brazzein gene was synthesized by ligating eight oligonucleotides per strand. The NdeI/BamHI fragment of the resulting DNA, which codes for the entire sequence of brazzein, was isolated and cloned into the T7 expression vector, pET-16b.** The sequence of the final, **ligated expression vector was confirmed by automated DNA sequencing.** All **mismatches due to errors during synthesis of the original oligonucleotides were corrected by site-directed mutagenesis using PCR.**

Construction of the brazzein expression vectors:

- **The synthetic des-pGlu1-brazzein gene was cut with restriction enzymes and cloned into T7 expression vectors pET-3a, pET-9a, and pET-11a, which contain NdeI and BamHI sites.** The fusion construct was made with a modification of the original nuclease–ovomucoid fusion gene. The four Met codons in the nuclease gene (SNase) were replaced with Ala codons by quick-change site-directed mutagenesis. **The DNA fragment coding for brazzein was excised and cloned between NdeI and BamHI sites at the C-terminus of the modified SNase gene in the pET-3a expression system.** The resulting plasmid, **named pET-3a/SNase-SW, was transformed into the E. coli strain BL21(DE3)/pLysS for protein expression**. The use of pLysS in this strain permits high-level expression of the nuclease–brazzein fusion protein without the deleterious effect of nuclease prior to induction.

Application in food & beverages.

Enzymes, chemicals, bacterial strains, and plasmids. Restriction enzymes and T4 DNA ligase were purchased from Promega (Madison, WI).
E. coli strains, HMS174(DE3, recA2) and BL21(DE3)/ pLysS were purchased from Novagen.
All plasmids were stored in a nonexpression host strain HMS174 and expressed in BL21(DE3)/ pLysS.
Protein expression vectors pET-3a, pET-9a, pET-11a, and pET-16b were purchased from Novagen.
Chemicals, such as GdmCl, Tris–HCl, ME, and DTT were purchased from Sigma (St. Louis, MO).

Source

Back to Contents

Efficient Production of Recombinant Heat-Stable Brazzein (3/7)

Solution

Production of nuclease–brazzein:

- **A single colony of E. coli strain BL21(DE3)/pLysS, containing the plasmid pET-3a/ SNase-SW, was selected and grown overnight at 37°C in 5 mL of Luria broth (LB) medium with 100 mg ampicillin/mL and 34 mg of chloramphenicol/ mL.** The **starting culture was used to inoculate 1 L of LB medium with chloramphenicol (34 mg/mL)/ampicillin (100 mg/mL) at 37°C until an A600 nm of 0.8–1.0 was attained.** Cells were induced for 3 h by addition of IPTG to a final concentration of 0.1 mM. Cells were harvested and rapidly frozen in liquid nitrogen and stored at 270°C. After freeze/thawing once, 4–5 g cells was resuspended in 50 mL lysis buffer (50 mM Tris–HCl, pH 8.0, containing 2 mM EDTA and 10 mM PMSF). The **lysed cells were treated with 10 mM CaCl2 for a period of 15** min and passed through a French press three times to disrupt the cells. The fully **broken cells were centrifuged for 15 min at 12,000g**. The **supernatant and the pellet were analyzed on 16% Tricine gels**. Because more than 70% of the fusion protein was insoluble, the soluble component was discarded.

Purification of brazzein from inclusion bodies:

- The **cell pellet after the French press steps was washed once with 9 vol of wash buffer (50 mM Tris–HCl, pH 8.0, containing 2 mM EDTA), once with 9 vol of the wash buffer containing 0.5% (v/v) Triton X-100 and 100 mM NaCl, and a third time with 9 vol of the wash buffer**. After each addition, the slurry was stirred gently for 5 min and then centrifuged at 12,000g for 10 min at 4°C. The **final pellet was resuspended in 50 mL 6 M GdmCl containing 10 mM EDTA and 100 mM DTT and stirred for 2–3 h at room temperature**. The clear resuspension was dialyzed overnight at 4°C against 4 L deionized water (dH2O) containing 3.5 mL acetic acid (pH 3.8–4.0) to ensure full protonation of the cysteine side chains. The **precipitant was removed by centrifugation at 12,000g.** The **clear supernatant was dialyzed three more times against dH2O and acetic acid for a total period of 36–48 h to completely remove the reducing agent**. At this stage, **more than 60–70% of the fusion protein was refolded, and the purity, as judged by gel electrophoresis, was greater than 80%.**

Source

Back to Contents

Efficient Production of Recombinant Heat-Stable Brazzein (4/7)

Solution

- The typical yield of the fusion protein, after the refolding step, was 130–150 mg/L culture.
- The reduced sulfhydryl groups in the brazzein domain were oxidized by rapidly diluting the dialysate with 4–5 vol of 200 mM Tris–acetic acid, pH 8.0, to a final concentration of 0.3–0.7 mg/mL, and this solution was stirred at room temperature for 24 h.
- Following the oxidization step, the solution was concentrated with an Amicon ultrafiltration apparatus to a final volume of 20–50 mL. When successfully folded and oxidized, the product was a clear solution.
- The concentrated fusion protein was dialyzed once against 10 L of 0.3 M NaCl to remove bound Tris and twice against 10 L of dH2O to remove residual salt; the final lyophilized product was a white powder.

Cleavage of the fusion protein and purification of brazzein:

- **Lyophilized fusion protein (130–150 mg) was dissolved in 65–75 mL water to a final concentration of 2 mg/mL.**
- The pH of the sample was adjusted slowly to 1.5–1.7 by adding 1 M HCl.
- Approximately 70–100 mg of CNBr was added to this solution, which was then stirred in the dark at room temperature for 24 h.
- After lyophilization, the cleaved product was resuspended in 150 mL of dH2O and lyophilized.
- This procedure of resuspension and lyophilization was repeated four times to ensure the complete removal of CNBr.
- The white powder was dissolved in 30 mL 50 mM Tris–HCl, pH 7.6, and applied to a CM–cellulose column (2.5 3 12 cm) preequilibrated with the same buffer.
- **Pure brazzein eluted in the first column volume**.
- **Nuclease and uncleaved fusion protein were eluted with 50 mM Tris–HCl, pH 7.6, containing 0.6 M NaCl.**
- **Brazzein-containing fractions were combined and desalted by dialysis against five changes of dH2O containing 0.1% acetic acid and lyophilized.**

Source

Back to Contents

Efficient Production of Recombinant Heat-Stable Brazzein (5/7)

Tests

Characterization of brazzein:

- **SDS–PAGE was performed with 16% Tricine gels.**
- Gels were stained directly with 0.05% Coomassie brilliant blue R-250 (BioRad) in 50% methanol, 10% acetic acid and destained with a 5% methanol/7% acetic acid destaining solution.
- **Broad range molecular weight markers were used to calibrate the gels.**
- Pure recombinant **brazzein samples at various dilutions were evaluated in double-blind studies by a taste panel in comparison to water, 2% sucrose, and fruit brazzein**.
- The threshold concentrations were the minimum concentrations that were perceived as being sweet.
- The relative sweetness was calculated from the threshold values.
- **NMR spectra were obtained with Bruker DMX600 spectrometer in a 5 mm 1 H probe**.
- **NMR samples contained 4 mM recombinant brazzein (or 8 mM fruit brazzein) in 10% 2 H2O at pH 5.2–5.4. 1 H NMR spectra with water solvent presaturation were obtained at 22°C**; each represented an average of 64 transients.

Source

Back to Contents

Efficient Production of Recombinant Heat-Stable Brazzein (6/7)

Result

- Brazzein was found to **remain folded at temperatures as high as 85°C**, and its sweetness remained undiminished after heating to 80°C for a period of 4 h.
- A **majority of the fusion protein was refolded, and the purity at this stage was greater than 80%.**
- Treatment with **cyanogen bromide led to a cleavage of only 50–70% of the molecules**, suggesting that the **cleavage site might not be fully accessible.**
- The **use of higher concentrations of CNBr in either HCl or formic acid resulted in nonspecific cleavage and final products devoid of sweetness**. Because brazzein is highly acidic, with a pI of about 5.4, whereas nuclease is highly basic, with a pI of 9.4, the two cleavage products were easily separated from one another by a single cation exchange chromatography step.
- The purified recombinant **brazzein had high purity and the correct molecular weight (6.4 kDa)** as judged by gel electrophoresis, MALDI-TOF mass spectrometry, reverse-phase HPLC, and 1 H NMR spectroscopy.
- **Purified recombinant des-Glu1-brazzein was twice as sweet as brazzein isolated from the fruit of P. brazzeana Baillon**, and equivalent to that of des-Glu1- brazzein isolated from the fruit.
- **The studies show that the protein is sweet only when folded correctly**; this was demonstrated earlier for chemically synthesized brazzein and for other sweet proteins.
- **Both unfolded/misfolded des-pGlu1-brazzein and the SNasedes-pGlu1-brazzein fusion were found to have no taste**. Interestingly, **the anti-brazzein antibody was found to be reactive both to the SNase-des-Glu1-brazzein fusion and to des-Glu1-brazzein (isolated following CNBr cleavage) even though the fusion construct is devoid of sweetness.**

Source

Back to Contents

Efficient Production of Recombinant Heat-Stable Brazzein (7/7)

Application

- The method can be used to chemically produce brazzein from the chemically synthesized gene resulted in recombinant protein with sweetness similar to that of brazzein isolated from the original source but with greater production efficiency..

Conventional Solutions

The conventional brazzein production method had less efficiency as brazzein was isolated from plants only.

The conventional brazzein exhibits aftertaste for long duration of time.

Advantages

The method can produce brazzein from the chemically synthesized gene resulted in recombinant protein with sweetness like that of brazzein isolated from the original source but with greater production efficiency.

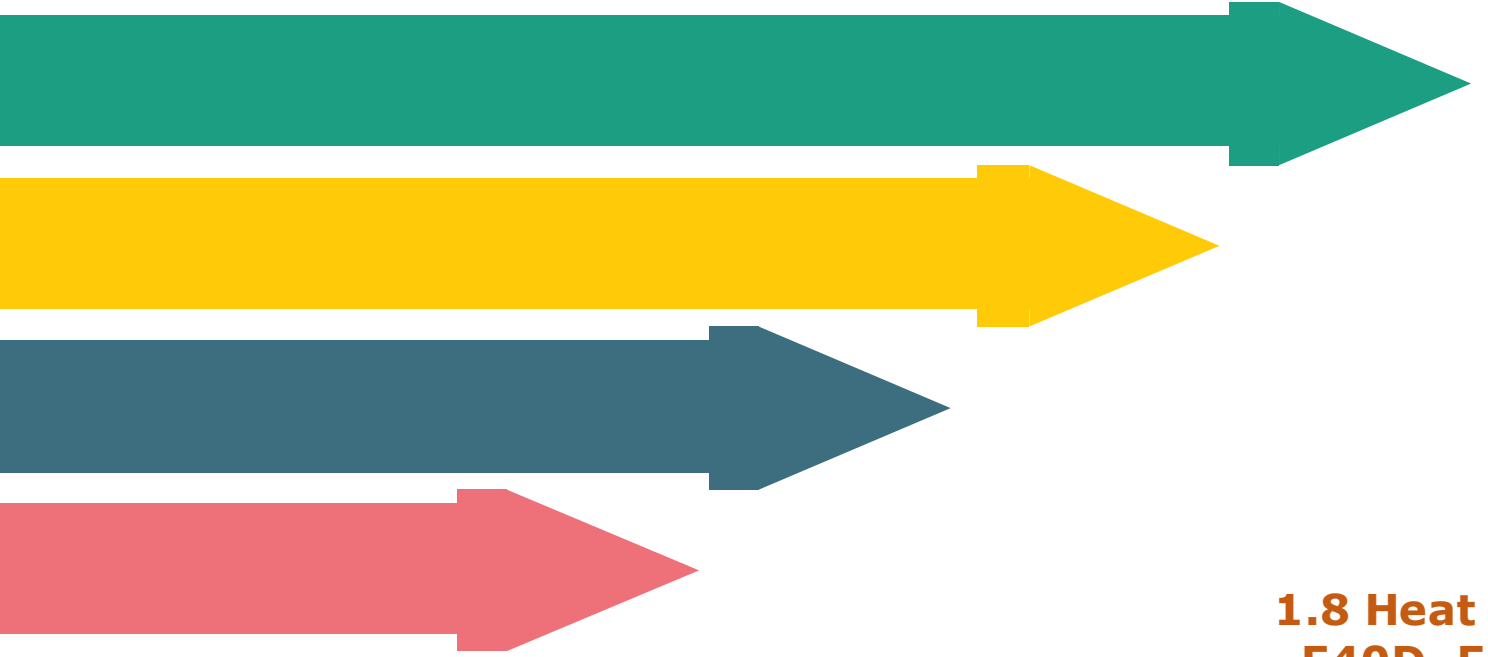
The sweet taste profile of brazzein do not exhibit aftertaste for longer durations.

Comment

- The method produces brazzein from chemically developed gene which produces recombinant proteins with sweetness similar to brazzein isolated from plants.
- Heat stable at 85 degree.

Source

Back to Contents



1.8 Heat treated H30R, E35D, E40A, E40D, E40K, E40H, E40R brazzein variants by Biosweet

Back to Ranking

Heat treated H30R, E35D, E40A, E40D, E40K, E40H, E40R brazzein variants by Biosweet (1/4)

Solution

The solution to the problem is invented by Biosweet

Patent published in 2013

Application in beverages (including alcoholic beverages), fruit juices, various drinks

Biosweet

- The solution relates to a **Method for preparing a polypeptide comprising a brazzein variant.**
- The method comprises **three steps.**
- ***1st step:* Culturing Escherichia coli** (E. coli) transformed with a polynucleotide encoding a **polypeptide comprising an E. coli pelB signal sequence and a brazzein variant amino acid sequence** selected from the group consisting of SEQ ID NOs: 142, 151, 152, 153, 154, 155, 156, 157 and 158;
- ***2nd step:* Isolating the polypeptide from the periplasm of the cultured E. coli**.
- The **isolation method** was performed by collecting the cultured *E. coli* strain, **suspending the collected *E. coli* strain in a 30 mM Tri-HCl (pH 8) solution supplemented with 20% sucrose and eluting an *E. coli* periplasmic protein using an EDTA (pH 8) solution and $MgSO_4$**.
- ***3rd Step: Heat-treating the isolated polypeptide*.**
- ***Thermal treatment*** *to denature proteins other than brazzein indicates that the brazzein variant would be stable at high temperature.*
- The food composition includes all kinds of a functional food, a nutritional supplement, a health food and a food additive. These kinds of food compositions may be prepared into **various formulations such as beverages (including alcoholic beverages),** fruits and their processed foods, fishes, meats, **fruit juices, various drinks**, dairy products (for example, butter, cheese, etc.).

As the transformed E. coli strain is cultured, a brazzein protein containing a pelB signal sequence is expressed under control of an expression control sequence in the expression vector. **Such expression of the brazzein is performed without using a compound**, such as isopropyl-beta-D-thiogalactopyranoside (IPTG), which facilitates the expression of a conventional inducible promoter. The expressed brazzein containing the pelB signal sequence is translocated into the E. coli periplasm by the action of the signal sequence, and the signal sequence is removed by an E. coli signal peptidase to synthesize brazzein.

The **transformation may be performed using a suitable standard technique** Such a standard technique includes electroporation, calcium phosphate (CaPO4) precipitation, calcium chloride (CaCl2) precipitation, microprojectile bombardment, PEG-mediated fusion, microinjection, and a liposome-mediated method.

The brazzein according to the present invention may be **isolated using techniques such as salting out (ammonium sulfate precipitation and sodium phosphate precipitation),** solvent precipitation (precipitation of a protein fraction using acetone or ethanol), dialysis, gel filtration, ion exchange chromatography, reverse phase column chromatography and affinity chromatography, **which may be used alone or in combination**.

The **heat treatment may be preferably performed by heating a cell homogenate at 70 to 90° C. for 15 to 60 minutes to thermally denature proteins other than the brazzein** and centrifuging the cell homogenate at 4° C. and 18,000 g for 30 minutes to isolate the thermally denatured proteins and the brazzein

Vector according to the present invention includes a plasmid vector, a cosmid vector, a bacteriophage vector and a viral vector.

The E. Coli culturing was done on a LB-agar medium.

Source

Back to Contents

Heat treated H30R, E35D, E40A, E40D, E40K, E40H, E40R brazzein variants by Biosweet (2/4)

Tests

Measurement of Sweetness of Primary Brazzein Variants:

- The activity of the recombinant brazzein was measured using the human sense of taste.
- Sugar content measurement was performed on 20 subjects who were trained to feel substantially the same minimum concentration of sucrose in which they could sense sweetness using a sucrose solution.
- A concentration of each brazzein variant in which the subjects could sense sweetness for the first time was measured.
- A **sweetness ratio of the sucrose solution to the wild-type brazzein was 1 g/100 ml**, **which was a minimum stimulation level in which the subjects could sense sweetness.**
- Also, a **sweetness ratio of the minor-type brazzein protein to the wild-type brazzein was 500 µg/100 ml**, which was a minimum stimulation level in which the subjects could sense sweetness.
- **The sweetness was calculated using the sweetness ratios (That is, 1/0.0005=2000 for the minor-type brazzein)**.

Measurement of Thermal Stabilities of Primary Brazzein Variants:

- **100 mg of each of the brazzein variants having high sweetness, that is, brazzein(H30K)** set forth in SEQ ID NO: 99, **brazzein(H30R)** set forth in SEQ ID NO: 100, **brazzein(E35D)** set forth in SEQ ID NO: 109, **brazzein(E40A)** set forth in SEQ ID NO: 113, **brazzein(E40A)** set forth in SEQ ID NO: 113, **brazzein(E40D)** set forth in SEQ ID NO: 114, **brazzein(E40K)** set forth in SEQ ID NO: 115, **brazzein(E40H)** set forth in SEQ ID NO: 116 and **brazzein(E40R)** set forth in SEQ ID NO: 117, **was dissolved in a 50 mM Tris-HCl (pH 8.0) solution, and heated at 80° C. for 4 hours**.
- Based on the sweetness measured before the heat treatment of the respective primary brazzein variants, a sweetness change level of each primary brazzein variant was then measured by the 20 subjects in the same manner. The sweetness change level was calculated as relative activity.

Source

Back to Contents

Heat treated H30R, E35D, E40A, E40D, E40K, E40H, E40R brazzein variants by Biosweet (3/4)

Brazzein variants (H30R, E35D, E40A, E40D, E40K, E40H, E40R) are 2 to 3.3 times sweet than minor type brazzein, and maintain their thermal stability

Results

- It was confirmed that the brazzein variants, that is, brazzein(H30K) set forth in SEQ ID NO: 99, **brazzein(H30R)** set forth in SEQ ID NO: 100, **brazzein(E35D)** set forth in SEQ ID NO: 109, **brazzein(E40A)** set forth in SEQ ID NO: 113, brazzein(E40A) set forth in SEQ ID NO: 113, **brazzein(E40D)** set forth in SEQ ID NO: 114, **brazzein(E40K)** set forth in SEQ ID NO: 115, **brazzein(E40H)** set forth in SEQ ID NO: 116 and **brazzein(E40R)** set forth in SEQ ID NO: 117, **had higher sweetness at least 2 times and up to 3.3 times (at least approximately 4,000 times and up to approximately 6,600 times that of 1 g/100 ml sucrose) that of the minor-type brazzein protein**.
- The **brazzein variant (E40D) showed the highest increase in sweetness**.
- It was confirmed that the brazzein variants such as **brazzein(H30R)** set forth in SEQ ID NO: 100, **brazzein(E35D)** set forth in SEQ ID NO: 109, **brazzein(E40A)** set forth in SEQ ID NO: 113, **brazzein(E40A)** set forth in SEQ ID NO: 113, **brazzein(E40D)** set forth in SEQ ID NO: 114, **brazzein(E40K)** set forth in SEQ ID NO: 115 and **brazzein(E40R)** set forth in SEQ ID NO: 117 **maintained their thermal stabilities**.

Sweetness test results of respective primary brazzein variants

Positions of amino acids in primary brazzein variants	Sequence Nos.	Minimum stimulation level in which one senses sweetness for first time (μg/100 ml)	Sweetness ratios of sucrose (1 g/100 ml) to primary brazzein variants (minor-type brazzein: 2000)	Multiples of increased sweetness to minor-type brazzein
K5A	SEQ ID NO: 83	6,000	167	0.08
K5D	SEQ ID NO: 84	6,000	167	0.08
K5E	SEQ ID NO: 85	6,000	167	0.08
K5H	SEQ ID NO: 86	10,000	100	0.05
K5R	SEQ ID NO: 87	10,000	100	0.05
D28A	SEQ ID NO: 88	10,000	100	0.05
D28H	SEQ ID NO: 89	6,000	167	0.08
D28K	SEQ ID NO: 90	6,000	167	0.08
D28R	SEQ ID NO: 91	6,000	167	0.08
D28E	SEQ ID NO: 92	2,000	500	0.25
K29A	SEQ ID NO: 93	10,000	100	0.05
K29R	SEQ ID NO: 94	10,000	100	0.05
K29H	SEQ ID NO: 95	10,000	100	0.05
K29D	SEQ ID NO: 96	10,000	100	0.05
K29E	SEQ ID NO: 97	10,000	100	0.05
H30A	SEQ ID NO: 98	6,000	167	0.08
H30K	SEQ ID NO: 99	250	4,000	2
H30R	SEQ ID NO: 100	150	6,600	3.3
H30D	SEQ ID NO: 101	3,000	334	0.16
H30E	SEQ ID NO: 102	3,000	334	0.16
R32A	SEQ ID NO: 103	6,000	167	0.08
R32K	SEQ ID NO: 104	3,000	334	0.16
R32H	SEQ ID NO: 105	3,000	334	0.16
R32D	SEQ ID NO: 106	10,000	100	0.05
R32E	SEQ ID NO: 107	10,000	100	0.05
E35A	SEQ ID NO: 108	10,000	100	0.05
E35D	SEQ ID NO: 109	150	6,600	3.3
E35K	SEQ ID NO: 110	6,000	167	0.08
E35H	SEQ ID NO: 111	6,000	167	0.08
E35R	SEQ ID NO: 112	6,000	167	0.08
E40A	SEQ ID NO: 113	150	6,600	3.3
E40D	SEQ ID NO: 114	150	6,600	3.3
E40K	SEQ ID NO: 115	150	6,600	3.3
E40H	SEQ ID NO: 116	250	4,000	2
E40R	SEQ ID NO: 117	250	4,000	2
R42A	SEQ ID NO: 118	10,000	100	0.05
R42K	SEQ ID NO: 119	3,000	334	0.16
R42H	SEQ ID NO: 120	3,000	334	0.16
R42D	SEQ ID NO: 121	10,000	100	0.05
R42E	SEQ ID NO: 122	10,000	100	0.05

Kind of multi-variants Positions of amino acid in variants (Sequence Nos.)	Minimum stimulation level in which one senses sweetness for first time (μg/ml)	Sweetness ratios of sucrose (1 g/100 ml) to brazzein multi-variants	Multiples of increased sweetness to minor-type brazzein
Secondary variants			
H30R_E35D (SEQ ID NO: 142)	1,250	8,000	4
H30R_E40A (SEQ ID NO: 143)	1,250	8,000	4
H30R_E40D (SEQ ID NO: 144)	1,250	8,000	4
H30R_E40K (SEQ ID NO: 145)	1,000	10,000	5
H30R_E40R (SEQ ID NO: 146)	1,000	10,000	5
E35D_E40A (SEQ ID NO: 147)	1,000	10,000	5
E35D_E40D (SEQ ID NO: 148)	1,000	10,000	5
E35D_E40K (SEQ ID NO: 149)	1,250	8,000	4
E35D_E40R (SEQ ID NO: 150)	850	12,000	6
Tertiary variant			
H30R_E35D_E40A (SEQ ID NO: 151)	650	15,000	7.5
H30R_E35D_E40D (SEQ ID NO: 152)	500	20,000	10
H30R_E35D_E40K (SEQ ID NO: 153)	500	20,000	10
H30R_E35D_E40R (SEQ ID NO: 154)	450	22,000	11
Quaternary variant			
29ins30 Lys_H30R_E35D_E40A (SEQ ID NO: 155)	400	25,000	12.5
29ins30 Lys_H30R_E35D_E40D (SEQ ID NO: 156)	350	28,000	14
29ins30 Lys_H30R_E35D_E40K (SEQ ID NO: 157)	350	28,000	14
29ins30 Lys_H30R_E35D_E40R (SEQ ID NO: 158)	250	40,000	20

Source

Back to Contents

Heat treated H30R, E35D, E40A, E40D, E40K, E40H, E40R brazzein variants by Biosweet (4/4)

Application

- The method is applicable for producing novel brazzein variant having higher sweetness at least twice that of a conventional brazzein protein.
- The method can also be used to produce brazzein variant with thermal and pH stability and higher water solubility as compared to the conventional brazzein protein.
- The method can be used to produce brazzein which may be widely used as a sweetener in food compositions since a greater amount of sugar (sucrose) may be replaced with a smaller amount of the brazzein variant.

Conventional Solutions	Advantages
The conventional brazzein had poor thermal stability, acid resistance and water solubility.	The method is capable of producing brazzein with excellent thermal stability, acid resistance and water stability.
The conventional brazzein had less sweetness.	The brazzein produced by this method had at least 2 times and up to 3.3 times higher sweetness as compared to conventional brazzein.

Comment

- The method is suitable for producing brazzein with excellent thermal stability, acid resistance and water solubility compared to a conventional brazzein
- The method also produces brazzein with higher sweetness at least 2 times and up to 3.3 times that of the conventional brazzein.

Source

Back to Contents

Biosweet's work on protein sweetener

Chung Ang University

Chung Ang University

- **US Patent US8592181B2 was first filed by Chung Ang University Industry Academic Cooperation Foundation in 2009.**
- **The US patent was assigned to Biosweet Co. Ltd. In 2016.** However, the US patent is expired on 11/26/2017 due to non-payment of maintenance fee.
- This patent application is also filed in Japan and the still active.

Biosweet

Biosweet

- **Biosweet Co. Ltd. is a Korean company.**
- The company has filed 13 patent applications (belonging to unique 8 INPADOC patent families) worldwide.
- Biosweet Co Ltd has filed a few other patents on brazzein protein:
  - WO2018056747A1 - **Method for mass production of brazzein.**
  - WO2018056746A1 - **Sweet-tasting protein brazzein variant having antiallergic activity**
  - KR102216399B1, KR101795573B1, KR101795569B1 - **Method for estimating sweet-tasting of sweet-tasting proteins**

Kwang-Hoon Kong is the inventor of the US patent. He has approx. 61 research works with 507 citations and 1792 reads. Click here for full read of his work.

The professor has also worked on different brazzein variants. Some interesting work of Prof. Kwang-Hoon Kong has been presented in next slides.

Source

Backward patent citation ← US8592181B2 → **Forward patent citation**

Biosweet

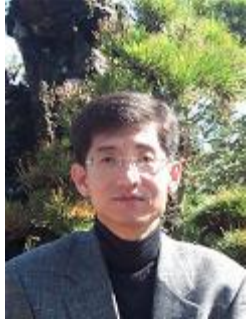
US7153535B2,
Wisconsin Alumini Research Foundation
Replacement of a particular amino acid in the naturally occurring Brazzein sequence with Lys or Asn, the taste profile and sweetness strength are improved.

KR100981087B1, KR100990667B1, KR100809100B1
Chung Ang University Industry Academic Cooperation Foundation
The brazzein variant and the multivariate have at least two times sweeter than the conventional brazzein while having properties such as thermal stability, pH stability and high water solubility compared to the conventional brazzein.

US9826773B2
Owned by Biosweet **Biosweet**
Filed in US, CA, AU, KR
Brazzein multiple variants of increased sweetness, and production method for same

Back to Contents

Chung Ang University's Kwang-Hoon Kong's Work on Brazzein



- Professor **Kwang-Hoon Kong** of Department of Chemistry at Chung-Ang University (CAU) has been named as one of the top 100 scientists by the International Biographical Centre.
- Mr. Kong is most well-known for his research and patent in structure and function of protein.
- **Kwang-Hoon Kong's work includes**:

1. Efficient brazzein production in yeast (Kluyveromyces lactis) using a chemically defined medium with **applications in mass production.** Compositions of defined media were investigated for two phases of fermentation: the first phase for cell growth, and the second for maximum brazzein secretory production. **Secretory brazzein expressed in the optimized defined medium exhibited higher purity than in the complex medium**; purification was by ultrafiltration using a molecular weight cutoff, yielding approximately 107 mg L−1. Moreover, **the total media cost in this defined medium system was approximately 11% of that in the optimized complex medium to generate equal amounts of brazzein**. Therefore, the **K. lactis expression system is useful for mass-producing recombinant brazzein with high purity and yield at low production cost and indicates a promising potential for applications in the food industry**. Source
2. Optimized production and quantification of the **tryptophan-deficient sweet-tasting protein brazzein** in Kluyveromyces lactis. To commercialize brazzein as a sweetener, optimization of fermentation and purification procedure is necessary. Transformed K. lactis was cultured in YPGlu (pH 7.0) at 25 °C and induced by adding glucose:galactose at a weight ratio of 1:2 (%/%) during the stationary phase, which in**creased brazzein expression 2.5 fold compared to the previous conditions**. Cultures were subjected to **heat treatment at 80 °C for 1 h, and brazzein containing supernatant was purified using carboxymethyl-sepharose cation exchange chromatography** using 50 mM NaCl in 50 mM sodium acetate buffer (pH 4.0) as a wash buffer and 400 mM NaCl (pH 7.0) for elution. The **yield of purified brazzein under these conditions was 2.0-fold higher than that from previous purification methods**. NanoOrange assay was a suitable method for quantifying tryptophan-deficient brazzein. Source
3. Multiple mutations of the critical amino acid residues for the sweetness of the sweet-tasting protein, brazzein. we made multiple mutations of three residues (His31 in loop 30-33, Glu36 in β-strand III, and Glu41 in loop 40-43). We found that all double mutations (H31R/E36D, H31R/E41A and E36D/E41A) made the molecules sweeter than des-pE1M-brazzein and three single mutants. Moreover, **the triple mutation (H31R/E36D/E41A) made the molecule significantly sweeter than three double mutants**. Mutations reducing the overall negative charge and/or increasing the positive charge favor sweet-tasting protein potency. Source

Molecule Type	S. No.	Modification	Solution proposed by	Non caloric or very low calorie	Sensory profile closer to sugar
Brazzein	1	Triple mutant brazzein (H31R/E36D/E41A)	Kwang-Hoon Kong	Yes	Higher than above two
	2	Mutant of Lys5 residue to Arg in N-terminal β-strand I of brazzein	Kwang-Hoon Kong	Yes	Moderate sweetness
	3	Mutation of Glu53 to Arg	Kwang-Hoon Kong	Yes	Higher than des-pE1M-brazzein
	4	Mutation of Glu41 to Ala, Lys, or Arg at position 41 in loop 40–43	Kwang-Hoon Kong	Yes	Sweeter than brazzein
	5	Mutation of His31 to Arg	Kwang-Hoon Kong	Yes	Sweeter than brazzein

Source

Back to Contents

Chung Ang University's Kwang-Hoon Kong's Work on Brazzein

4. **Antioxidant, anti-inflammatory, and anti-allergic activities of the sweet-tasting protein brazzein**. Sweet-tasting proteins may be useful as low-calorie sugar substitutes in foods, beverages, and medicines. Brazzein is an attractive sweetener because of its high sweetness, sugar-like taste, and good stability at high temperature and wide pH ranges. To investigate the bioactivities of brazzein, the antibacterial, antifungal, antioxidant, anti-inflammatory, and anti-allergic activities were determined in vitro. Brazzein showed no antibacterial and antifungal activities, although it showed approximately 45% or greater similarity to defensin, which has antimicrobial effects, and drosomycin, which is used as an antifungal agent. However, brazzein exhibited strong antioxidant effects, showing ABTS radical scavenging activity (IC50 = 12.55 μM) and DPPH activity (IC50 > 30 μM). Brazzein also showed anti-inflammatory activity and anti-allergic activity in a β-hexosaminidase assay (IC50 > 15 μM) and cyclooxygenase-2 inhibition assay (IC50 = 12.62 μM), respectively. These results suggest that brazzein has antioxidant, anti-inflammatory, and anti-allergic activities and considerable potential as a functional sweetener. Source

5. **Role of Lys5 Residue in β-Strand I of the Sweet-Tasting Protein Brazzein**. To identify critical residues responsible for sweetness in brazzein and elucidate the interaction mechanisms of brazzein with the sweet taste receptor, three mutants of Lys5 residue in N-terminal β-strand I of brazzein were constructed by site-directed mutagenesis. Mutations of Lys to Asp or Glu at position 5 of brazzein significantly decreased its sweetness, while **mutation of Lys5 to Arg resulted in a molecule with slightly decreased sweetness to des-pE1M-brazzein**. From these results, **it is suggested that the positive charge of Lys5 in β-strand I of brazzein is essential for its function and necessary for structural integrity**. Source

6. Importance of Glu53 in the C-terminal region of brazzein, a sweet-tasting protein. To identify important residues responsible for the sweetness of the protein brazzein, four mutants of the Glu53 residue in the C-terminal region of des-pE1M-brazzein, which lacks the N-terminal pyroglutamate were constructed using site-directed mutagenesis. Mutations of Glu53 substitution to Ala or Asp significantly decreased the sweetness. **Mutation of Glu53 to Arg resulted in a molecule significantly sweeter than des-pE1M-brazzein**, which agrees with previous findings that showed that mutation with positively charged residues results in a sweeter protein. Residue at position 53 is crucial for the sweetness of brazzein, which may be interacting with the sweet-taste receptor. Source

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

8. **Residue mutations in the sweetness loops for the sweet-tasting protein brazzein. mutations of Glu41 to Ala, Lys, or Arg at position 41 in loop 40–43 made the molecules significantly sweeter than brazzein**, while mutations at two distant residues (changing Arg43 to Lys or Glu) decreased sweetness. A similar pattern occurred at loop 30–33, where **mutation of the His31 to Arg significantly increased sweetness**, while mutations at positions 30 or 33 in the immediate vicinity of this region significantly decreased sweetness. In addition, a **Gln17 residue in the loop 9–19 was necessary for structural integrity**. From these results, we suggest that the loops containing His31 and Glu41 are critical regions of the molecule for elicitingsweetness. Source

Source

Back to Contents

1.9 Brazzein variants of Ala29, Ala40, Ala50, Ala9, Ala41, Ala53

Reduction of lingering/ aftertaste of protein sweetener

Back to Ranking

Brazzein variants of Ala29, Ala40, Ala50, Ala9, Ala41, Ala53 (1/3)

The solution to the problem is invented by Ajinomoto Co. Inc

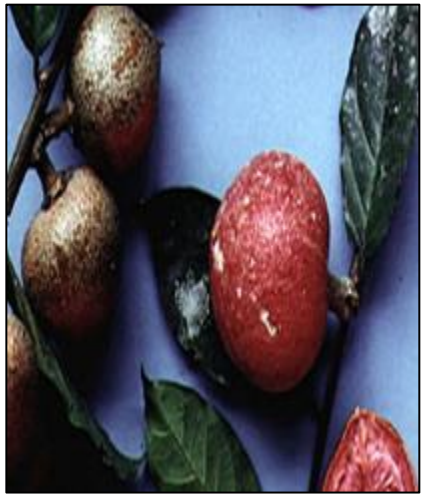
Non-patent Published in 1999

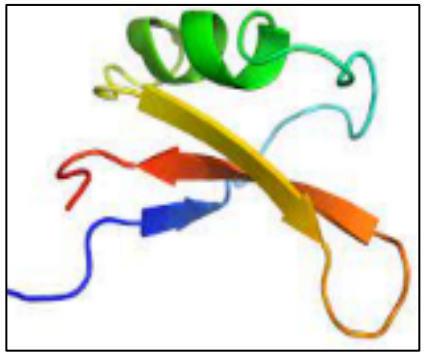
Solution

Application in food & beverages.

❑ METHODOLOGY

- **Nine brazzein analogues were synthesized by the same Fmoc solid-phase method** as used for the synthesis of brazzein.
- **The peptides were synthesized by single coupling of the 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/1-hydroxybenzotriazole (HBTU/HOBt) method,** except for the His and Arg residues which were incorporated by double coupling of the HBTU/HOBt method.
- The peptide-resins were deprotected and cleaved from the resin support.
- **The resulting peptides were treated with dithiothreitol (DTT) in 0.2 M ammonium acetate buffer (pH 8.0) containing 6 M guanidine hydrochloride to prevent polymer formation.**
- **The peptides were purified by preparative reversed phase HPLC (RP-HPLC) and characterized by analytical HPLC and electrospray ionization mass spectrometry (ESIMS), which gave satisfactory results.**
- **The reduced analogues thus obtained were folded simply by keeping them at 25°C in aqueous solutions**, without any redox systems such as cysteine/cystine or glutathione/oxdized glutathione (GSH/GSSG).
- The folding process of each reduced analogue proceeded readily similarly to the process of reduced brazzein.
- **The peptides were purified by RP-HPLC and characterized by analytical HPLC, ESIMS, amino acid analysis and CD spectrometry with satisfactory results.**





Source

Back to Contents

Brazzein variants of Ala29, Ala40, Ala50, Ala9, Ala41, Ala53 (2/3)

Test & Results

- Sweetness was evaluated by matching a threshold concentration of each analogue with that (0.6%w/v) of sucrose. **The threshold concentration was chosen to avoid confusion arising from the persistent lingering sweet taste of the analogues**. The previously synthesized brazzein was 500 times sweeter than sucrose on a weight basis under the same conditions. The **sweetness of six analogues ([Ala 29]-, [Ala 40]-, [Ala 50]-, [Ala9]-, [Ala 41]-, and [Ala 53] brazzeins) was increased**. The hydrophobic groups are known to be as important as the carboxyl groups of sweet compounds for binding with the receptor. The **substitution of Ala for Asp or Glu might affect the hydrophobicity of brazzein**. The low potency of [Ala 36]brazzein suggests that the Glu 36 residue is involved in binding with the receptor. Source

Table 1. Sweetness of brazzein analogues in comparison to sucrose on a weight basis

Brazzein analogues in which an Asp residue was replaced	Sweetness in comparison to sucrose	Brazein analogues in which a Glu residue was replaced	Sweetness in comparison to sucrose
[Ala^{2}] brazzein	500 times	[Ala^{9}] brazzein	1000 times
[Ala^{25}] brazzein	250 times	[Ala^{36}] brazzein	20 times
[Ala^{29}] brazzein	1000 times	[Ala^{41}] brazzein	1000 times
[Ala^{40}] brazzein	1250 times	[Ala^{53}] brazzein	1250 times
[Ala^{50}] brazzein	750 times	Synthetic brazzein	500 times

Source

Back to Contents

Brazzein variants of Ala29, Ala40, Ala50, Ala9, Ala41, Ala53 (3/3)

Application

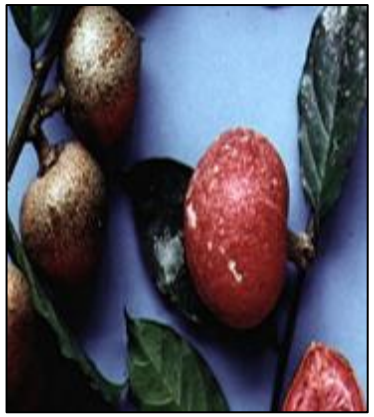
- In future, studies and experiments can be conducted on other amino acid substitutions to look for their role in sweetness potency or other structure-activity relationships of protein sweetener.

Conventional Solutions

- No study has been investigated on the role of acidic amino acids on the sweetness potency before.

Advantages

- Brazzein is a thermostable, sweet-tasting protein.
- The roles of acidic amino acids were investigated by replacing each with an Ala residue by the fluoren-9-yl-methoxycarbonyl (Fmoc) solid-phase synthesis.



Comment

- Brazzein contains five Asp residues and four Glu residues. In this paper, the roles of these acidic amino acids were investigated by replacing each with an Ala residue by the fluoren-9-yl-methoxycarbonyl (Fmoc) solid-phase synthesis.
- The hydrophobic groups are known to be as important as the carboxyl groups of sweet compounds for binding with the receptor.
- The substitution of Ala for Asp or Glu might affect the hydrophobicity of brazzein.

Source

Back to Contents

1.10 His31, Glu36 and Glu41 amino acids modification in brazzein

Back to Ranking

His31, Glu36 and Glu41 amino acids modification in brazzein (1/4)

Solution

The solution to the problem is invented by Chung-Ang University

Non-patent published in 2013

Application in food & beverages.

- The studies suggest that **His31 and Glu41 residues in the flexible loops and Glu36 residue in the b-strand III of the brazzein are the critical residues of the molecule for eliciting sweetness**, and charge and/or structure of the side chain of these residues plays an important role in the interaction between brazzein and the sweet taste receptor.
  - In the present study, **three double mutations and one triple mutation of three putative interaction sites (at positions 31, 36, and 41) in brazzein by site-directed mutagenesis**.
  - **The soluble recombinant mutants were produced by their periplasmic secretion in Escherichia coli and simply purified by the extraction of periplasmic fraction and a thermal treatment**. The effects of mutations on brazzein were evaluated by a human taste panel.
  - This study offers information on the precise interaction mechanism of brazzein with human sweet taste receptor responsible for the sweetness of brazzein, and will be of great value in future design of sweeter brazzein variants.

Preparation of brazzein mutants by site-directed mutagenesis:

- pE1M-brazzein, which has a methionine instead of a pyroglutamate at its N-terminus, and des-pE1M-brazzein, which lacks an N-terminal methionine in its pE1M-brazzein form, were obtained by expression of the synthetic gene that was based on the amino acid sequence of the naturally occurring brazzein in E. coli. Mutagenesis was performed according to the Mutant™-Super Express Km kit protocol (Takara Shuzo). Construction of the DNA template for mutagenesis, confirmation of mutation, and construction of the expression plasmid of the mutants were performed. The resulting vectors of the mutant proteins were transformed into E. coli strain BL21 Star (DE3).

Overexpression and purification of brazzein mutants:

- The overexpression and purification of the mutant proteins were performed. Protein concentration for the particular mutant was determined from the absorbances at 205 and 280 nm, as brazzein lacks tryptophan. The extinction coefficient (e205) of each brazzein mutant was calculated from measurements of the absorbances of solutions at 205 and 280 nm, according to the following formula: 1:0 mg=ml 205 ¼ 27:0 þ 120ðA280=A205Þ.

The pET-26b(+) expression vector and E. coli strain BL21 Star (DE3) used in this study were supplied by Novagen (Madison, WI).
Restriction enzymes, Mutant™-Super Express Km kit, and DNA-modifying enzymes were obtained from Takara Shuzo (Otsu, Shiga, Japan). Synthesis of the DNA primers for mutagenesis was performed by COSMO Genetech (Seoul, Korea).
All chemicals and reagents used were commercially available and of the highest reagent grade.

Source

Back to Contents

His31, Glu36 and Glu41 amino acids modification in brazzein (2/4)

Production Technique

- The multiple mutants were expressed in E. coli under the control of a T7 promoter and efficiently produced in the soluble, active form into the periplasm at an amount approximately 80–90% of the total periplasmic proteins.
- The secretion of multiple mutants, having four intramolecular disulphide bonds into the periplasmic space in E. coli, gives a better chance of proper folding due to the increased oxidising conditions in this extracellular compartment. Moreover, production into the periplasmic space can also facilitate purification.
- For these reasons, the expressed multiple mutants were isolated and purified to electrophoretic homogeneity by extraction of periplasmic fraction and thermal treatment.
- The multiple mutants were purified to approximately 1.0–6.0 mg/L. The purity and conformational state of the multiple mutants were confirmed by SDS–PAGE, HPLC, and CD spectroscopy. The purified multiple mutants appeared as a single band on SDS–PAGE with an apparent Mr of 6500 Da ; the elution times for the folded multiple mutants were 11.0 ± 0.5 min, as denoted by RP-HPLC.
- The structures of the des-pE1-brazzein and the multiple mutants were investigated using CD spectroscopy. No gross change in secondary structure was suggested from comparison of CD spectra of the des-pE1-brazzein and the multiple mutants, although the contribution of a minor conformational change to the increase in sweet taste cannot be ruled out.

Tests

Analysis of the sweet-taste properties of brazzein mutants:

- The sweet-tasting activities of the des-pE1M-brazzein and mutant proteins were assayed by sensory analysis using a doubleblind taste test of 20 individuals.
- Sweetness potencies were reported relative to sucrose on a molar basis.

Circular dichroism analysis of brazzein mutants.

- Circular dichroism (CD) spectra of the des-pE1M-brazzein and mutant proteins were recorded with a J-815 spectropolarimeter at 25 C.
- Far-UV CD spectra were obtained at a protein concentration of 19.7 IM with a 1-mm cell at wavelengths from 250 to 190 nm.
- The measurements are expressed as mean residue ellipticity, [h]Mr, with a mean residue weight (Mr) of 120 for brazzein.
- The data were collected four times and are given as the average mean residue ellipticity.

Source

Back to Contents

His31, Glu36 and Glu41 amino acids modification in brazzein (3/4)

Result

Effects of multiple mutations on sweetness activity of brazzein:

- The taste results on the multiple mutants of these residues are compared with the des-pE1M-brazzein.
- All **double mutants significantly increased the sweetness (H31R/E36D, 12,600 times sweeter than sucrose on a weight basis; H31R/ E41A, 13,200 times; E36D/E41A, 5000 times).** Among these mutations, **mutation (E36D/E41A) that reduced the negative charge and mutation (H31R/E36D) that increased the positive charge had significantly increased the sweetness**. The **sweetness of H31R/E36D was approximately 2.5-fold higher than that of E36D/E41A**, suggesting that the effect of mutation that increased the positive charge is larger than that of mutation that reduced the negative charge. **Mutations (H31R/E41A and H31R/E36D/E41A) that increased the positive charge and reduced the negative charge largely increased the sweetness**, showing approximately 7- and 12-fold higher activity than des-pE1M-brazzein, respectively.
- **These results suggest that alkalinity of brazzein is important for sweetness**, **although brazzein with pI 5.4 is not alkaline protein**. Rather, brazzein with low pI value has a higher potential than other sweet proteins for engineering enhanced sweetness by the introduction of positive charges or the reduction of negative charges at important residues.
- It was reported that the activity for sweetness of H31R mutant was approximately 5.8-fold higher than that of H31A mutant, indicating that the positive charge of the side chain of the amino acid at position 31 influences the sweetness of brazzein.
- Docking studies were performed on computer models of thaumatin and brazzein, and again it was found that the surface of the sweet protein interacting with the receptor is predominantly positive. It conclude that the alkalinity of brazzein is important for the elicitation of sweetness and the positive potential of brazzein plays an important role in the interaction between brazzein and the sweet taste receptor.
- The results presented herein demonstrate that the introduction of multiple mutations of three residues (His31 in loop 30–33, Glu36 in b-strand III, and Glu41 in loop 40–43) leads to stronger sweetness, which, listed in increasing order of sweetness, are: triple mutation (H31R/E36D/E41A) > double mutations (H31R/E36D, H31R/E41A, E36D/E41A) > single mutations (H31R, E36D, E41A).
- **The results suggest that His31 and Glu41 residues in flexible loops and Glu36 residue in b-strand III are involved in binding to the sweet receptor, and these residues play an important role in the multi-point interaction between brazzein and the sweet taste receptor**. The saturation transfer difference NMR spectroscopy study also suggested a multi-point interaction between brazzein and the sweet receptor by study on mutagenesis and chimeras of the receptor. Taken together, we conclude that brazzein binds to multisites of the sweet taste receptor.

Source

Back to Contents

His31, Glu36 and Glu41 amino acids modification in brazzein (4/4)

Application

- The method is applicable for producing novel brazzein with enhanced sweetness content and low calorie content.

Conventional Solutions	Advantages
The conventional brazzein had low sweetness.	The method is capable of producing brazzein with higher sweetness.
The conventional brazzein had high calorie content and pH sensitivity.	The brazzein produced by this method had low calorie amount and effective at 5.4 pH.

Comment

- The method produces brazzein with higher sweetness index then conventional one and with low calorie amount.

Source

Back to Contents

1.11 Mass production of Brazzein by FRI srl **

Back to Ranking

Mass production of Brazzein by FRI srl (1/3)

The Solution To The Problem Is Invented By Food Res and Innovation srl

Patent Published in 2013

Solution

Application in food & beverages.

- **The reference discloses about a method for preparing brazzein protein comprising at least the steps of:**
- **Transforming one or more cells of Pichia pastoris with an expression vector comprising a promoter sequence, a coding nucleotide sequence for a brazzein, a terminator sequence and optionally a secretion signal sequence.**
- **Growing the transformed cell or cells in a fermentation medium under aerobic conditions,**
- Further removing the cells from the fermentation medium is done by means of centrifugation or microfiltration;
- Diluting the supernatant and adjusting the pH.
- Separating the expressed brazzein protein by ion-exchange chromatography.
- **Concentrating and purifying the protein by diafiltration.**
- Optionally, subjecting the isolated protein to lyophilization.

- **The coding nucleotide sequence for brazzein is selected from the nucleotidic sequences SEQ ID NO:1 , SEQ ID NO:2 and SEQ ID NO:3.**
- **The promoter sequence is selected from pFLD1 , pILC1 , pTEF1 , pPGK1 , pPEX8, pAOX1 and pGAP.**
- **The terminator sequence is selected from AOX1 and GAP.**
- **The secretion signal sequence is selected from a-mating factor of Saccharomyces cerevisiae, acid phosphatase of Pichia pastoris (PH01) and invertase of Saccharomyces cerevisiae (SUC2).**

Source

Back to Contents

Mass production of Brazzein by FRI srl (2/3)

Tests

Characterisation of the product:

- **At the end of the fermentation, the protein in the surnatant is separated by gel electrophoresis and the brazzein concentration is estimated after staining with Comassie and densitometric analysis.**
- **The purity of the protein following the ion- exchange chromatography step is established by means of RP-HPLC analysis.**
- The absorbance profile of a reversed-phase chromatography **HPLC was monitored at 280 nm** and the purity of the brazzein was reported as the relative percentage of peak elution area of the brazzein with respect to the total area subtended by the chromatogram.
- **An aliquot of Fraction III was loaded into a Phenomenex Jupiter 5u C4 column; the eluent A used consists of 0.1 % TFA in H 2 0 milliQ, while the eluent B is formed by 0.085% TFA in Acetonitrile**.
- The chromatographic run exploits a non-linear increasing gradient of Eluent B with a stream at 0.6 ml/min.

Results

Characterisation of the product:

- **Mass spectrometry of the purified protein has given a molecular mass of 6500.89 ±0.67 Da as result, a result comparable to the expected one of 6501 Da.**
- It can be inferred that the brazzein is not degraded by proteases present in the supernatant and that the only post-transcriptional changes present are four disulphide bridges typical of the native structure of the protein; other modifications, such as, for example, glycosylations, which can alter the structure of the protein or mask any proteolytic cutting sites thereof, are not present.
- **The purity of the protein at 280 nm was estimated to be 99.9% using a diode-array Agilent 8453 UV-visible spectrophotometer interfaced with Agilent Chemostation software.**

Source

Back to Contents

Mass production of Brazzein by FRI srl (3/3)

Application

- The method is applicable for producing a protein sweetener brazzein with high purity and the yield production is also more than conventional methods.

Conventional Solutions

The conventional methods of brazzein production produce less pure brazzein and the production capacity is also less.

Advantages

The method produces brazzein with high purity and greater yield.

Source

Back to Contents

1.12 E9K and V7R brazzein variants

Back to Ranking

E9K and V7R brazzein variants (1/4)

Solution

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

Non-patent Published in 2020

- ❑ The solution relates to a **Method for improving the sweetness of the recombinant expressed sweet-tasting protein brazzein.**
- ❑ **In the first step constructs** are being prepared by following steps:
- The full-length of des-pGlu brazzein gene was synthesized and its condons were optimized to facilitate the expression in E. coli.
- The gene was then cloned into the vector pET-SUMO with the restriction enzyme sites BamH I and Xho I at the N- and C-terminus, respectively.
- These placed a large His + SUMO tag at the N-terminus.
- All variants were constructed according to the standard PCR based mutagenesis strategy.
- The PCR product was digested by the restriction enzyme Dpn I, and then, transformed into E. coli DH5α cells.
- The correct vector harboring the mutated gene was verified by DNA sequencing.

Application in food & beverages.

- ❑ ***In the second step Expression and purification of the recombinant des-pGlu Brazzein is done:***
- The pET-SUMO expression vector harboring the des-pGlu brazzein gene was transformed into **E. coli** BL21-Codon Plus (DE3)-RIL.
- The cells were cultured in LB medium at 37°C until the OD600 reached 0.6, and then, induced with 0.5 mM IPTG at 25°C for 24 hr.
- The cells were harvested by centrifuge at 10,000 rpm/min for 10 min, resuspended in PBS buffer, and then, disrupted by sonication.
- The soluble proteins obtained were loaded on the nickel column (Ni Sepharose™ High Performance).
- The column was washed with distilled water, binding buffer, washing buffer, and then, was plugged with a plug, followed by addition of 2 ml cleavage buffer (1 unit SUMO protease and 20 µl 10 × SUMO protease buffer diluted in distilled water, pH 6.8).
- After incubating at 37°C for 24 hr, the plug was removed and the His + SUMO tag-removed proteins were obtained.
- The purified proteins were dialyzed in distilled water (pH 6.8), quantified with the BCA protein concentration detection kit and analyzed by 15% SDS-PAGE.
- ❑ ***After the above described steps the structure analysis and sweet test evaluation is carried out.***

Source

Back to Contents

E9K and V7R brazzein variants (2/4)

Tests

Secondary structure analysis of the wild-type and mutated proteins:

- Circular dichroism (CD) spectra were measured from 190–260 nm at 25°C with a spectropolarimeter.
- The samples were centrifuged before the assay to avoid the probable precipitation or aggregation, and then, diluted in distilled water (pH 6.8).
- Each protein sample (200 µl, 0.1 mg/ml) was recorded as a three-scan average value with a 1 mm optical path length, a time constant of 1 s, a 1 nm band width, and a scan rate of 60 nm/min.
- Molar ellipticity per mean residue was calculated from the equation: [θ](deg–1 · cm–2 · dmol–1) = θobs × Mar/10 × C × l, where θobs is the measured ellipticity (in degrees) at wavelength obs, Mar is the mean molecular mass of amino acid of the protein, C is the protein concentration (in g/ml) and l is the optical path length (in cm).
- The secondary structure composition of the proteins was analyzed with the CDNN program based on the CD spectra.

Sweet taste evaluations:

- A **double-blind assay was applied to evaluate the sweetness thresholds of recombinant brazzein and its mutants**.
- The tasters were five males and five females, 20–40 years old with reportedly good health conditions and normal sense of taste.
- The **stored protein samples were centrifuged before the test, and then, diluted to a series of concentrations from 0.1 to 10 µg/ml (0.1 µg/ml concentration interval) with distilled water (pH 6.8) at room temperature**. A 4 mg/ml sucrose solution was served as reference.
- The assessors first tasted the reference and then, the sample in plastic cups.
- The assessors held 2 ml of each sample in the apex of the tongue for no less than 15 s, and then, spit it out.
- After each test, the tasters rinsed their mouth with distilled water until no residual taste was detected.
- The sweet potency of each sample was scored as the following criterion: 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.
- The data were averaged in three independent tests, and the sweetness threshold was designated as the lowest concentration at which the protein sample scored 2.

Source

Back to Contents

E9K and V7R brazzein variants (3/4)

Brazzein variants (E9K and V7R) are positively charged and thus can bind to negatively charged sweet taste receptor to produce sweetness.

Results

- **No obvious change in secondary structure** was found between the CD spectra of the wild type and its three mutants, indicating that the global configuration and folding of the mutants were retained.
- Furthermore, the secondary structure composition of the wild type was similar to those of the mutated proteins, as shown in the CDNN analysis.
- However, differences of **small negative peaks at 198 nm and 217–218 nm were presented between the wild type and E9D, suggesting that this mutation could lead to subtle structural changes in the β-strands and loops region, which may be correlated to the drastic decrease of the sweetness of this mutant**.
- It should be noted that the CD spectra profile of the proteins is different to those of the previously reported recombinant brazzein expressed in E. coli. For example, an **obvious negative peak in previous spectra was replaced by a positive one at 205 nm as well as emergence of a new negative peak around 195 nm in the present CD**.
- The **sweetness threshold of wild type was obviously lower than the previously reported values 4, 11, or 25 μg/ml of recombinant brazzein in E. coli, indicating the significant enhancement of sweetness**.
- The **improved sweetness was further validated by expression of three mutants E9K, E9D, and V7R** with modified sweetness, respectively.
- The **V7 and E9 sites are located at the short β1 strand and its adjacent flexible loop 9–19 on the protein surface, respectively**.
- It has been described that there is electrostatic interaction at the interface between the sweet protein (positive) and sweet taste receptor (negative), which determines the sweetness of the sweet proteins.
- The **E9K and V7R mutants increased the surface positive charge of sweet protein brazzein**, **while E9D not**, which led to the enhanced sweetness of E9K and V7R but the reduced sweetness of E9D.

Samples	Sweetness threshold[a] (μg/ml)	Sweetness in comparison to sucrose
Sucrose	4×10^3	1
Wild type	1.5 ± 0.1	2,666
V7R	1.2 ± 0.3	3,333
E9K	1.0 ± 0.2	4,000
E9D	4.0 ± 0.5	1,000

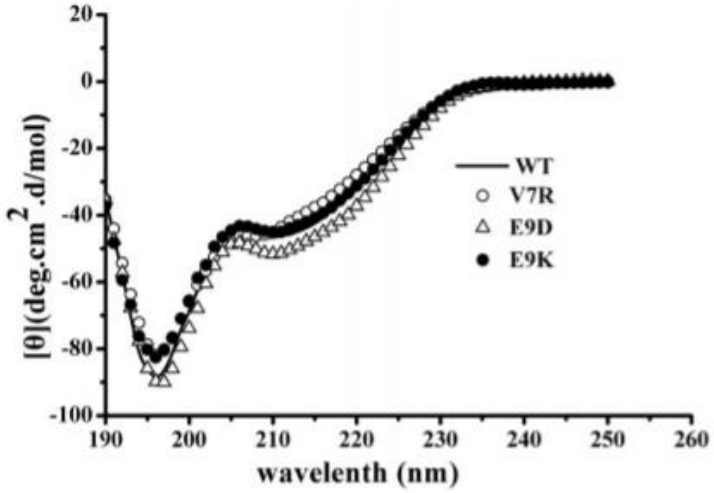


FIGURE 3 Circular dichroism spectra of recombinant wild-type (wt) brazzein and its mutants

Source

Back to Contents

E9K and V7R brazzein variants (4/4)

Application

- The method is applicable for producing novel brazzein with a sweetness threshold of 1.5 µg/ml which is which is the sweetest brazzein protein reported up to now.
- This method produced low-, or non-caloric and nutritive brazzein.

Conventional Solutions

The conventional brazzein had high caloric content.

The conventional brazzein had less sweetness.

Advantages

The method is capable of producing brazzein with low or no caloric content.

The brazzein produced by this method had the highest sweetness threshold of 1.5 µg/ml which is highest up to now

Comment

- The method also produces brazzein with highest sweetness threshold of 1.5 µg/ml which is highest up to now.

Source

Back to Contents