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Efficient Production and Characterization of the Sweet-Tasting Brazzein Secreted by the Yeast *Pichia pastoris*

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Supporting Information

ABSTRACT: Brazzein is a small, heat-, and pH-stable sweet protein present in the fruits of the West African plant *Pentadiplandra brazzeana* Baillon. It exists in two forms differing in sweetness intensity. The major form, called pyrE-bra, contains a pyroglutamic acid at its N-terminus, while the minor form, called des-pyrE-bra, lacks this residue. Here we describe the heterologous expression in the methylotrophic yeast *Pichia pastoris* of two natural forms of brazzein, pyrE-bra and des-pyrE-bra, and an additional form, called Q1-bra, which is not naturally occurring in the fruit. Q1-bra differs from pyrE-bra in having a glutamine residue instead of pyrE at its N-terminus. Over an expression period of 6 days, we obtained approximately 90, 30, and 90 mg/L of purified recombinant pyrE-bra, Q1-bra, and des-pyrE-bra brazzein forms, respectively. Recombinant proteins were purified and submitted to mass spectrometry and ^{1}H NMR spectroscopy. The data indicate that the recombinant brazzein forms were properly folded. Moreover, they activated the human sweet receptor in vitro and evoked sweetness in vivo with properties similar to those of the two natural brazzein forms.

KEYWORDS: *brazzein, calcium assay, heterodimeric sweet receptor, sweet protein, psychophysical assay, Pichia pastoris*

INTRODUCTION

Brazzein is a small (6.5 kDa) sweet-tasting protein naturally occurring in berries of *Pentadiplandra brazzeana* Baillon, a plant found in West Africa. Isolated from its natural source, brazzein exists in two forms differing in sweetness intensity.[1] The major form (54 amino acids, ∼80%), called pyrE-bra, contains a pyroglutamic acid (pyrE) at its N-terminus, while the minor form (53 amino acids, ∼20%), called des-pyrE-bra, lacks the N-terminal pyrE. It has been reported that des-pyrE-bra is twice as sweet as pyrE-bra.[2] The three-dimensional structure of brazzein has been solved by NMR spectroscopy.[3] Its structure is composed of a short α helix and three antiparallel β-strands held together by four disulfide bridges. A number of point mutations has been made to identify brazzein residues that are important for its interaction with the sweet taste receptor.[2,5,6] It has been proposed that the site of interaction includes two surface loops and the disulfide bond that connect the N- and C-termini.[2,7−9]

The mass production of sweet-tasting proteins, such as thaumatin, monellin, mabinlin, brazzein, and curculin, for commercial use has been investigated for many years.[10−13] Brazzein is one of the most characterized and promising sweet-tasting proteins for food application.[5] For instance, brazzein could be suitable to increase the sweetness of soft drinks, syrups, chewing gum, or pharmaceuticals. Brazzein combines a long history of human consumption, high sweet potency (from 500- to 2000-fold sweeter than a sucrose solution on a weight basis and from 9500- to 38 000-fold on a per-molecule basis), high water-solubility, and exceptional thermostability.[2,5,14,15] In addition, brazzein tastes purely sweet with no sourness, saltiness, or bitterness, making it a good alternative to artificial sweeteners.[5]

Because of the difficulties in obtaining brazzein from its natural source, brazzein has been expressed using various recombinant expression systems. Heterologous production of brazzein is complicated by the fact that the protein contains four disulfide bridges and requires a specific N-terminal sequence to be fully active. Using *Escherichia coli* as expression host, brazzein expression was limited by the initial insolubility of the protein product, the requirement of protein refolding, and the difficulty of removing the fusion tag, leading to proteins with a modified N-terminus.[4,16] *Lactococcus lactis* represent attractive host cells for recombinant protein expression due to their "generally recognized as safe" (GRAS) status. Unfortunately, using the *L. lactis* system, recombinant brazzein was poorly expressed with a low-intensity sweetness.[17−19] Lamphear et al.[20] obtained transgenic corn that produced high yields of recombinant brazzein with a sweet phenotype. However, the major drawback to the use of plant-derived protein for research purpose is the low purification yields of recombinant proteins, which limit the use of this production system.

The methylotrophic yeast *Pichia pastoris* has become an important tool for recombinant protein expression. *P. pastoris* is

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A

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pyrE-bra      pyrEDKCKKVYENYPVSKCQLANQCNYDCKLDKHARSGECFYDEKRNLQCICDYCEY 54
des-pyrE-bra    -DKCKKVYENYPVSKCQLANQCNYDCKLDKHARSGECFYDEKRNLQCICDYCEY 53
Q1-bra          QDKCKKVYENYPVSKCQLANQCNYDCKLDKHARSGECFYDEKRNLQCICDYCEY 54
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B

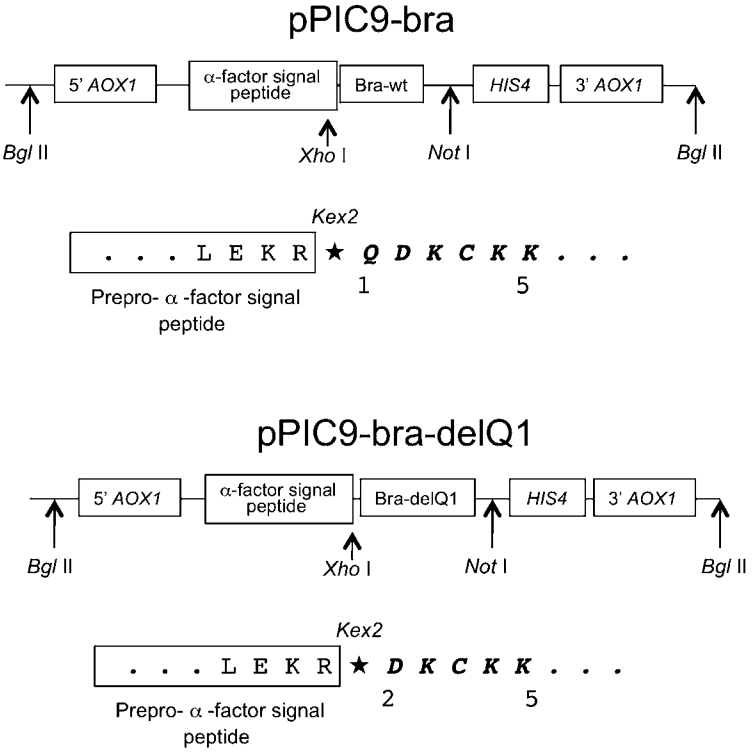

Figure 1. Amino acid sequences and constructions of expression vectors for brazzein forms. (A) Alignment of the two sweet-tasting constituents isolated from the fruit of *P. brazzeana* Baillon with the recombinant product, called Q1-bra. The major form (pyrE-bra) contains a pyroglutamic acid residue (pyrE), while the minor form (des-pyrE-bra) lacks the N-terminal pyrE residue, yielding a 53-amino acid type brazzein. Recombinant Q1-bra differs from the pyrE-bra molecule by a N-terminal glutamine residue (Q1) instead of a pyrE residue. (B) Plasmid constructs used for the expression of recombinant brazzein. The linearized expression pPIC9-bra and pPIC9-bra-delQ1 vectors are composed of the promoter of the gene encoding AOXI alcohol oxidase (5′ *AOX1*), HIS4 gene for selection of transformants, and the 3′ *AOX1* sequence from the AOX1 gene. The processed N-termini are indicated in bold italics; the last four amino acids of the removed signal sequences are boxed. The asterisk shows the cleavage site processed by the KEX2 protease.

known to be safe with no known associated toxigenic or pathogenic properties and has a long history of safe use to produce recombinant proteins.[21,22] High-level expression, high density of cells, easy scaling up, and strong and tightly regulated promoters have all been implied as the main advantages of *P. pastoris*. In addition, this system exhibits much of the structure and function of the eukaryotic secretory system and has the capacity to fold, to process proteolytically, to glycosylate, and to secrete large quantities of disulfide-bonded proteins.[23] The *P. pastoris* expression system has been already successfully used to express the sweet-tasting protein thaumatin[24,25] and the sweet-taste-suppressing protein gurmarin.[26] Finally, since overexpressed proteins are directly secreted in the minimum medium, protein purification is greatly facilitated. These features make *Pichia* a very useful protein expression system suitable for industrial production. Thus, the aim of the present study is to produce brazzein in *P. pastoris* to avoid the shortcomings associated with purifying natural brazzein and to provide an initial functional characterization of the produced recombinant protein.

MATERIALS AND METHODS

Strains and Materials. The pPIC9 shuttle vector and *P. pastoris* strain GS115 (his4) were purchased from Invitrogen. Media components were purchased from BD Difco (BD Biosciences). *E. coli* strain DH5*α* was used for DNA subcloning and propagation of the expression plasmids. Oligonucleotides were synthesized by Eurogentec. Synthetic brazzein cDNA cloned in pGEM::Bra-wt plasmid[17] was used as the template for polymerase chain reaction (PCR). Restriction endonucleases and T4 DNA ligase were purchased from Promega. The origin of other chemicals is indicated in the text.

Construction of the Expression Vectors. The following different expression constructs were made as outlined in Figure 1. The first vector, called pPIC9-bra, was constructed to express pyrE-bra brazzein form, while the second one, named pPIC9-bra-delQ1, was designed to express des-pyrE-bra brazzein form. Since it is known that the side chain of an N-terminal glutamine residue (Gln) can be converted into pyrE residue by a natural chemical reaction, we constructed pyrE-bra brazzein with a Gln residue at its N-terminus. The synthetic cDNA encoding pyrE-bra brazzein was amplified by PCR using the following primers: 5′ primer, 5′-CTCGAGAAAAGA-CAGGACAAATGTAAAAAAGTATACGAAAACTACCCG-3′; 3′-primer, 5′-GGAAGCGGCTGACTGCGGCCGCTCAGTATTCG-CAGTAGTCGCAGAT-3′. After subcloning into pSTC1.2 vector (Eurogentec), the PCR-amplified fragment was inserted into the *Xho*I

and *Not*I sites of pPIC9, generating the construct pPIC9-bra. In this construct, two Glu-Ala repeats have been deleted between the α-factor mating prepropeptide and the mature brazzein sequence. pPIC9-bra-delQ1 vector was generated through the subsequent introduction of point mutations using PCR-based direct mutagenesis (QuikChange Multi Site-Directed Mutagenesis Kit, Stratagene) using the following primers: 5′-GGGGTATCTCTCGAGAAAAGAGACAAATG-TAAAAAAGTATACG-3′ and 5′-CGTATACTTTTTTA-CATTTGTCTCTTTTCTCGAGAGATACCCC-3′. The integrity of both constructs was checked by automated DNA sequencing.

Transformation of *P. pastoris* and Screening for Brazzein Expression. The expression plasmids were linearized with *Bgl*II and transferred into the *P. pastoris* yeast host by the electroporation method as described in the manual (version 3.0) of the *Pichia* Expression Kit (Invitrogen). His^+ and methanol utilization slow (Mut^S) transformants were inoculated in 10 mL of buffered minimal glycerol (BMGY) medium (1% w/v yeast extract, 2% w/v peptone, 1.34% w/v yeast nitrogen base with ammonium sulfate without amino acids (YNB), 4 mg/mL D-biotin, 100 mM potassium phosphate, pH 6.0, 1% v/v glycerol) in sterile 50 mL Erlenmeyer flasks, which were then incubated at 29 °C, 300 rpm for 2 days. Induction of protein expression was achieved by harvesting the cells by centrifugation at 3000*g* for 5 min at room temperature and resuspending them in 2 mL of buffered minimal methanol (BMM) medium (1.34% w/v YNB, 4 mg/mL D-biotin, 100 mM potassium phosphate, pH 6.0, 1% v/v methanol) before continuing the incubation as earlier. After 2 days, samples were centrifuged at 12 000*g* for 10 min at room temperature to pellet the cells, and 100 μL of the supernatant was removed for sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) analysis. Having identified the best brazzein-producing transformants, large-scale protein production was achieved as previously described,[27] except that the protein was secreted for only 6 days. During the induction period, methanol was fed twice a day in order to maintain a concentration of 1% v/v.

Purification of Recombinant Brazzein. The supernatant containing recombinant brazzein was chilled and clarified by centrifugation at 6000*g* for 30 min at 4 °C and by filtration (0.22 μm). The protein solution was then dialyzed against water adjusted to pH 4.0 with acetic acid for 2 days at 4 °C, using a dialysis tube with a 3500 Da cutoff (Spectra/Por, Dutscher, France). After dialysis, the yeast culture filtrate containing recombinant brazzein was loaded on cation-exchange chromatography column using a HiTrap SP-sepharose column (5 mL, GE Healthcare) equilibrated with 50 mM sodium acetate buffer, pH 4.0. The elution was achieved using a linear gradient with the same buffer from 0 to 1 M NaCl in 50 min at a flow rate of 1 mL/min. Since brazzein contains no tryptophan residues, its absorbance was recorded at 275 nm. The fractions containing purified proteins were identified using SDS−PAGE analysis. Brazzein-containing fractions were pooled, dialyzed extensively against Milli-Q water, and stored at −20 °C. Brazzein concentrations were determined using UV spectroscopy employing a molar extinction coefficient of 9440 M^{-1} cm^{-1} at 280 nm calculated from the web-based ProtParam software.[28]

SDS−PAGE, Mass Spectrometry and NMR Analysis. SDS−PAGE (16% acrylamide) was performed using a Mini-Protean II system (Bio-Rad) according to the method of Schagger and von Jagow[29] with the Polypeptide SDS−PAGE standards (Bio-Rad). The proteins were stained with Bio-Safe colloidal Coomassie Brillant Blue G-250 (Bio-Rad). Mass spectra were acquired using a PE Biosystems Voyager-DE STR^+ time-of-flight spectrometer (Applied Biosystems). Two microliters of purified brazzein were mixed with 2 μL of matrix solution (saturated solution of sinapinic acid in 30% v/v acetonitrile, 0.2% v/v trifluoroacetic acid). One microliter of the mixture was applied to a stainless steel sample plate and allowed to air-dry. The spectra were externally calibrated using horse heart cytochrome C [$(M + H)^+$ = 12 361.1 Da].

One-dimensional (1D) ^1H NMR spectroscopy was used to check that each recombinant brazzein form was properly folded. NMR spectra were recorded with a Bruker DRX-500 NMR spectrometer in a 5-mm ^1H probe at 300 K. Each brazzein form (0.7 mM) was dissolved in 10% ^2H_2O prepared in 50 mM sodium acetate, pH 4.0.

Functional Characterization of the Three Brazzein Forms Using Functional Sweet Taste Receptor Expression System. In order to validate the genuine taste properties of the three produced brazzein forms, we employed a cell-based heterologous expression system for the human sweet taste receptor heteromer hT1R2/hT1R3. HEK293T cells stably expressing the chimeric G protein subunit Gα16gust44[30] were cultured under regular conditions at 37 °C, 95% humidity, and 5% CO_2 in Dulbecco's modified Eagle medium (D-MEM) high glucose (Invitrogen) supplied with FCS (10%), penicillin G (10 000 Units/mL), streptomycin (10 mg/mL), and 1% L-glutamine (2 mM) and selected for stable expression of Gα16gust44 with G418 (50 μg/mL, Invitrogen).

Prior to the experiment, cells were seeded into 96-well plates coated with poly(D)-lysine for optimal adherence. Cells were cotransfected with plasmids coding for the human sweet taste receptor subunits hT1R2 (in pcDNA5/FRT, Invitrogen) and hT1R3 (in pcDNA3, Invitrogen) using Lipofectamine 2000 (Invitrogen) in D-MEM low glucose GlutaMAX (Invitrogen). Mock cells transfected with empty vector served as control for unspecific reactions of the cellular background. After allowing expression of the transfected constructs for 30 h, cells were loaded with Fluo-4 AM (2 μM, Molecular Probes, in D-MEM low glucose GlutaMAX with 2.5 mM probenecide) for 1 h at 37 °C and washed with bath solution (130 mmol/L NaCl, 5 mmol/L KCl, 10 mmol/L HEPES, 2 mmol/L $CaCl_2$, 10 mmol/L pyruvate, pH 7.4) subsequently. Brazzein stock solutions were diluted in bath solution. After ∼40 min incubation at room temperature, cells were washed again. Intracellular calcium levels upon automated application of test compounds were monitored using the FLIPR Tetra (Molecular Devices).

To analyze the efficacy of brazzein forms to stimulate hT1R2/hT1R3-mediated cellular responses in detail, we calculated concentration−response functions. Fluorescence signals of receptor-expressing cells were reduced by and normalized to baseline fluorescence ($\Delta F/F$) using Screenworks 2.0 (Molecular Devices) and SigmaPlot (Systat Software, Inc.). Ratios were plotted semilogarithmically against substance concentration. Half-maximal effective agonist concentrations (EC_{50}) were calculated by nonlinear regression to the sigmoidal function $f(x)$ = min + (max − min/1 + $[x/EC_{50}]$ Hill slope) using Sigma Plot. Experiments were conducted in duplicate and repeated three times. Statistics were performed with GraphPad Prism 5.00 (GraphPad Software, Inc.). One-way analyses of variance (ANOVA) was performed to compare EC_{50} values and maximum receptor response amplitudes upon brazzein isoforms, followed by a Tukey posthoc means comparison test with 5% α-risk level.

Sensory Characterization of the Recombinant Brazzein Forms. In order to assess the sweetness properties of the brazzein forms produced using the yeast *Pichia pastoris* (pyrE-bra, des-pyrE-bra, and Q1-bra), psychophysical assays approved by the Ethics Committee of the University of Potsdam (Potsdam, Germany) were performed. Twenty-four healthy volunteers, without overt taste pathologies or other obvious health problems (20 women, 4 men; age range 23−60 years, mean age 34.8 years, SD = 2.4) were recruited. Pregnant and breast-feeding women were excluded. All subjects provided written informed consent before the experiment. The sweet taste stimuli were the three brazzein forms and sucrose used as control substance (Sigma-Aldrich Inc.), diluted in 1 mM sodium phosphate buffer pH 7.0 (disodium hydrogen phosphate, sodium dihydrogen phosphate; Sigma-Aldrich Inc.) with 0.2 μm sterile-filtered deionized water (Siemens Water Technologies Corp.). All subjects participated in six sessions: one training session, three sessions devoted to threshold measurements and two sessions devoted to suprathreshold intensity ratings. Tests were carried out with nose clip. Numerous samples of 0.5 mL were presented in amber-colored 2 mL tubes (Eppendorf AG). Samples had to be expectorated. Subjects had to rinse their mouth with mineral water (Evian; Danone, France) after each sample or each test and to wait a mandatory 30 s between trials. For data acquisition, a PC-network and Fizz sensory software (Biosystemes) were used.

Threshold Determination. Subjects received an ascending series of four-alternative forced choice (4-AFC) tests composed of 12 concentration steps ranging from 0.025×10^{-3} to 50×10^{-3} g/L for the brazzein forms and from 0.013 to 25 g/L for sucrose. Four coded samples were simultaneously presented for each test: one with a test substance, either sucrose or one of the three brazzein forms, diluted in sodium phosphate buffer, and the three other ones with sodium phosphate buffer alone. Subjects had first to identify the different sample and second had to specify if the quoted sample had a sweet taste; these collected data allowed us to assess the detection and the recognition threshold, respectively. Two measurements per brazzein form were performed over three sessions and the control substance sucrose was tested at the end of every three sessions. Detection and recognition probabilities were obtained for every concentration step per repetition and averaged per subject. The relationship between detection probabilities, respective recognition probabilities, and concentrations were fitted by a logistic regression model using the maximum-likelihood method in order to estimate the threshold value and the slope of the logistic curve. (Matlab; The MathWorks, Inc.). Statistics were performed with SPSS (International Business Machines Corp.). One-way ANOVA (substance) was performed with subjects as the random effect with or without sucrose included, followed by a Bonferroni posthoc means comparison test with 5% α-risk level.

Intensity Rating. Sweet intensity was directly rated on the general labeled magnitude scale (gLMS).[31,32] Subjects monadically evaluated six samples of each brazzein form (3.1×10^{-3}, 6.3×10^{-3}, 12.5×10^{-3}, 25×10^{-3}, 50×10^{-3}, and 100×10^{-3} g/L) and six control samples, one with the sodium phosphate buffer alone and the other ones with the sweet control substance sucrose (5, 10, 25, 50, and 100 g/L). The whole session was replicated once and results were averaged per subject. The relationship between rated intensities (i.e., percent of the whole-length scale) and concentrations was fitted by nonlinear regression (SigmaPlot; Systat Software, Inc.) using the equation model corresponding to the Stevens' power law.[33] Two-way ANOVA (substance, concentration) was then performed with subjects as random effect (SPSS; International Business Machines Corp.), followed by a Bonferroni posthoc test. Finally, sweetness potencies equivalent to sucrose at 20, 50, and 100 g/L were calculated for each subject. One-way ANOVA (substance) was then performed at 20, 50, and 100 g/L with subjects as the random effect (SPSS; International Business Machines Corp.), followed by a Bonferroni posthoc test.

RESULTS AND DISCUSSION

Construction of Expression Vectors and Transformation into *P. pastoris*. The methylotrophic yeast *P. pastoris* was reported to be an alternative host for high-level expression.[22] The *P. pastoris* expression system was chosen because it allows the production of large quantities of soluble disulfide-bonded proteins.[21−26] To compare the production level and the sensory properties of the two naturally occurring forms of brazzein called pyrE-bra and des-pyrE-bra (Figure 1A), we constructed two different *Pichia* expression plasmids. Both vectors were designed to direct secretion of brazzein from the yeast cell using the yeast prepropeptide signal from the *S. cerevisiae* α-mating factor. Since it is known that the side chain of an N-terminal Gln can undergo the cyclization reaction with the amino terminus of the peptide backbone to form pyrE, pPIC9-bra plasmid was constructed by inserting the full mature (54 amino acid residues including a N-terminal Gln) synthetic coding sequence of brazzein downstream from the methanol inducible alcohol oxidase (AOXI) promoter between the *Xho*I and *Not*I restriction sites (Figure 1B). pPIC9-bra-delQ1 vector aimed at expressing des-pyrE-bra form (53-amino acid residues) was created from pPIC9-bra site-directed mutagenesis. For transformation of yeast, the plasmids pPIC9-bra and pPIC9-bra-delQ1 were digested with *Bgl*II to give an integrative fragment containing the brazzein cDNA and the selectable marker of the gene encoding HIS4 histidinol dehydrogenase (HIS4). Approximately 300 His^+ transformants of the GS115 strain were obtained after electroporation of both plasmids. Fifty transformants corresponding to Mut^S phenotypes were isolated. They were grown in the BMGY medium and induced at 29 °C for 2 days in the BMM medium buffered to pH 6.0. For each construct, the higher producing clone was screened by determining the amount of brazzein secreted in the extracellular medium by SDS−16% polyacrylamide gel. Having identified the best brazzein-producing transformants, large-scale protein production was achieved using 2-L shake flasks as described under Materials and Methods. Samples of the expression medium supernatants, taken at various time intervals, were also analyzed by SDS−PAGE to determine the optimal induction time. For both constructs, only a recombinant protein, migrating at about 6.5 kDa, was detectable by Coomassie Blue staining (Figure 2), while no protein expression was observed in the culture supernatant of nontransformed GS115 cells (data not shown). Protein bands corresponding to recombinant brazzein were excised and digested with trypsin, and the resulting peptides were analyzed by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-ToF) for protein identification. This analysis confirmed that brazzein was the secreted protein (data not shown).

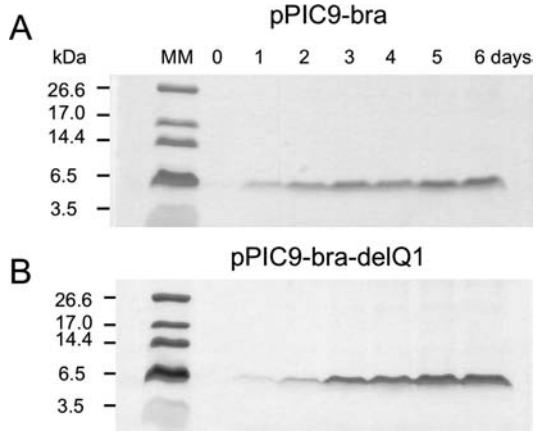

Figure 2. Kinetics of recombinant expression of brazzein secreted by *P. pastoris*. SDS−PAGE analysis of recombinant brazzein secreted by *P. pastoris* using pPIC9-bra (A) and pPIC9-bradelQ1 (B) constructs. Lanes 1−7 are 50-μL aliquots of culture supernatants from days 0−6. Molecular mass standards (MM) are indicated on the left. Proteins were visualized by Coomassie Brillant Blue G-250 staining.

Purification and Characterization of Recombinant Brazzein Forms. The high-level secretion of brazzein from *P. pastoris* facilitated the development of a simple purification method in the absence of any sequence alteration such as the addition of a His-tag. To determine the chromatographic conditions, after centrifugation at 10 000*g* for 30 min, the supernatant was clarified by filtration, submitted to dialysis, and then applied to a SP-Sepharose column equilibrated with 50 mM sodium acetate at pH 4.0. Elution was achieved using a linear gradient (dashed line) from 0.0 to 1.0 M NaCl in 50 min. For the pPIC9-bra construct, the chromatographic profile (see Supporting Information, Figure S1A) reveals that two proteins could be separated. The first protein (corresponding to approximately 75% of the total protein amount, based on UV

absorbance) eluted at 0.5 M NaCl, while the second protein (representing approximately 25%) eluted at 0.6 M NaCl. Maldi-ToF mass spectrometry analysis was used to measure the mass of each purified protein (see the Supporting Information). The first eluting protein was assigned to the pyrE-bra form. This protein exhibited a mass of 6490.7 Da (m/z 4491.7), in perfect agreement with that deduced from the cDNA sequence with a pyrE amino acid residue at the N-terminus resulting from the chemical cyclization of N-terminal Q1 and four disulfide bridges formed (theoretical average mass of 6490.3 Da). The second eluting protein showed a mass of 6509.3 Da (m/z 6510.3) corresponding to a mass excess of 18 Da. This protein was assigned to the same molecule with a Gln residue instead of pyrE at its N-terminus. We named this recombinant brazzein form, which is not naturally occurring in the fruit, Q1-bra. For the pPIC9-bra-delQ1 construct (see Supporting Information, Figure S1B), the resulting protein eluted as a single peak at higher ionic strength (0.7 M NaCl), in agreement with its calculated p*I*. As demonstrated by Maldi-ToF analysis, this protein corresponded to the des-pyrE-bra form (see Supporting Information, Figure S2C). Indeed, this protein exhibited a mass of 6369.4 Da (m/z 6370.4), in perfect agreement with that deduced from the cDNA sequence without pyrE at its N-terminus, four disulfide bridges formed, and the first N-terminal Gln lacking (theoretical mass 6370.2 Da). These results indicated that, using both constructs, the yeast α-factor prepropeptide signal from the *S. cerevisiae* had been properly removed from the N-terminus of the three recombinant brazzein forms. In addition, we produced a third brazzein form (Q1-bra), which is not naturally occurring in the fruit, corresponding to the pyrE-bra molecule (54 amino acid residues) with a Gln residue instead of pyrE amino acid residue at its N-terminus. Over an expression period of 6 days, we obtained approximately 90, 30, and 90 mg/L of purified recombinant pyrE-bra, Q1-bra, and des-pyrE-bra brazzein forms, respectively. To investigate the folding of these three brazzein forms, we performed ^{1}H NMR spectroscopy. The respective spectra are presented in the Supporting Information. The ^{1}H- NMR spectra display the key signals for folded proteins, with sharp methylene/methyl peaks upfield of 0.5 ppm and a good dispersion of amide proton resonances. Altogether, our data indicated that the three expressed recombinant brazzeins were greater than 95% pure and in perfect agreement with the expected features. These results illustrate that *P. pastoris* is perfectly compatible with the correct folding of brazzein, including the formation of the four disulfide bonds.

Activation of the Sweet Taste Receptor by Recombinant Brazzein. To verify the functional integrity of the three brazzein forms, we tested their ability to activate the human sweet taste receptor hT1R2/hT1R3, heterologously expressed in HEK293T Gα16gust44 cells.[34−36] Cells were loaded with the Ca^{2+}-indicator dye Fluo-4 AM, and intracellular calcium responses upon automated application of increasing concentrations of recombinant brazzein were monitored using the FLIPR Tetra system. Similar functional assays have been successfully employed to demonstrate the importance of the cysteine-rich region of T1R3 for the sensitivity of the human sweet taste receptor to brazzein[37] or to identify amino acid residues involved in the interaction between brazzein and sweet receptor.[6] As shown in Figure 3, application of each brazzein form on hT1R2/hT1R3-expressing cells evoked calcium responses in a dose-dependent manner, while no fluorescence

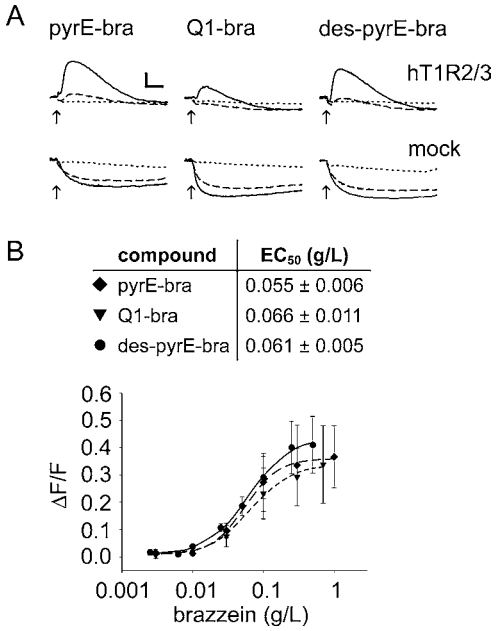

Figure 3. Response of hT1R2/hT1R3-expressing HEK293T Gα16-gust44 cells to different recombinant brazzein forms. (A) Calcium traces of hT1R2/hT1R3-expressing cells (upper panels) and mock-transfected cells (lower panels) upon bath application (arrows) of pyrE-bra, Q1-bra, and des-pyrE-bra, respectively. Displayed substance concentrations were 0 g/L (dotted lines), 0.03 g/L (dashed lines), and 0.1 g/L (solid lines), respectively. Scale: *y*, 60 counts; *x*, 50 s. (B) Concentration−responses of hT1R2/hT1R3-expressing cells on different brazzein forms.

signals were observed from mock cells. We determined concentration−response curves for each brazzein form to compare their actions on the functionally expressed hT1R2/hT1R3. The half-maximal effective concentrations (EC_{50}) of pyrE-bra (0.055 ± 0.006 g/L), Q1-bra (0.066 ± 0.011 g/L), and des-pyrE-bra (0.061 ± 0.005 g/L) did not statistically differ, although the concentration−response curve for des-pyrE-bra is left-shifted relative to that of the other forms. Moreover, also the efficacy of des-pyrE-bra was slightly greater as seen from the trend for greater signal amplitudes.

The pharmacological properties of the brazzein forms determined in the present report are consistent with those obtained by Assadi-Porter et al.[6] and Dittly et al.,[9] who obtained recombinant brazzein from bacterial expression. In contrast to previous studies carried out on the natural brazzein isoform mixture[37] or recombinant des-pyrE-brazzein from bacteria,[6,9] recombinant expression in yeast enabled us to evaluate the impact of the N-terminal residue on the sweetness properties of brazzein. The concentration−response curves showed that the three brazzein forms did not differ significantly in their potency and efficacy to evoke responses from the functionally expressed human sweet taste receptor. However, pyrE-bra shows a tendency to be more potent than the other two brazzein forms, as reflected by their similar EC_{50} values. Thus, our data support the previous hypothesis of Dittly et al.[9] that the N-terminus of the brazzein molecule is not crucial for interaction with the sweet receptor.

Sweetness Properties of the Recombinant Brazzein Forms. To examine the sweetness properties of the three recombinant brazzein forms and to compare them with that of the control substance sucrose, we performed psychophysical tests in which we determined the detection and recognition

A

	Threshold ± SEM (g/L)		Sweetness potencies equivalent to sucrose		
	detection	recognition	20 (g/L)	50 (g/L)	100 (g/L)
Sucrose	2.21 ± 0.25 a	2.43 ± 0.38 a	1	1	1
pyrE-bra	$5.31 \times 10^{-3} \pm 1.11 \times 10^{-3}$ b	$8.90 \times 10^{-3} \pm 2.17 \times 10^{-3}$ b	422.59 ± 51.66 a	473.39 ± 37.69 a	545.37 ± 45.03 a
Q1-bra	$3.63 \times 10^{-3} \pm 0.96 \times 10^{-3}$ b	$3.09 \times 10^{-3} \pm 0.80 \times 10^{-3}$ c	922.59 ± 144.87 b	790.33 ± 71.76 b	759.71 ± 48.24 b
des-pyrE-bra	$2.73 \times 10^{-3} \pm 0.43 \times 10^{-3}$ b	$3.07 \times 10^{-3} \pm 0.64 \times 10^{-3}$ c	1568.08 ± 266.19 c	1075.66 ± 95.26 c	875.49 ± 51.32 b

B

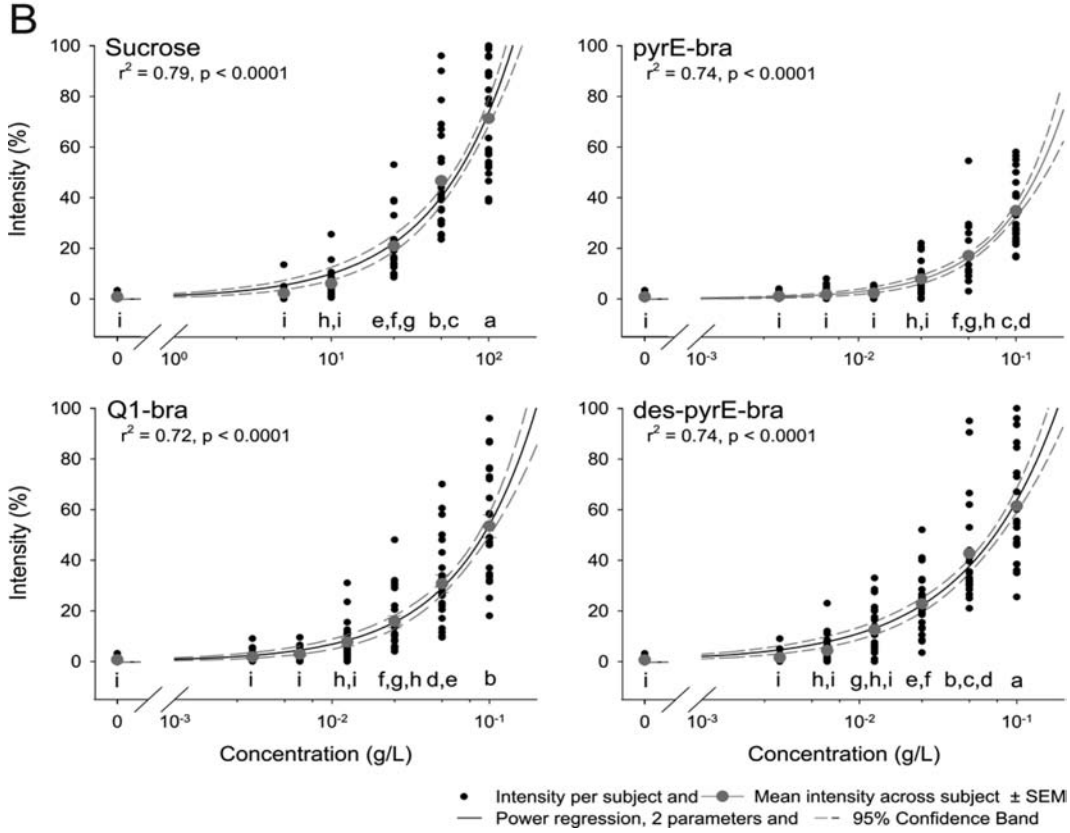

Figure 4. Sweetness properties of the recombinant brazzein forms. (A) Detection and recognition thresholds obtained for each brazzein form and sucrose following a 4-AFC procedure and 12 concentration levels. Detection and recognition probabilities were fitted by a logistic regression model. Sweetness potencies equivalent to sucrose were calculated on the basis of the concentration−sweetness psychophysical curves (B) of each subject. Different letters indicate significant differences at 5% α-risk level after ANOVA and Bonferroni posthoc tests. (B) Concentration−sweetness psychophysical curves for each brazzein form and sucrose. Relationships between sweet taste intensity and stimulus concentration were fitted to the Stevens' power law by nonlinear regression. Different letters indicate significant differences at 5% α-risk level after ANOVA and Bonferroni posthoc tests.

thresholds, as well as psychometric curves. For both detection and recognition thresholds (Figure 4A), we observed significant differences between the four substances, i.e., the three brazzein forms and the control substance sucrose, with $[F(3,69) = 310; p < 0.001]$ and $[F(3,69) = 513; p < 0.001]$, respectively. The control substance sucrose was detected and recognized as sweet at concentrations of 2.21 and 2.43 g/L, respectively. These concentrations were much higher than those of the three brazzein forms, which are in the milligram range. Between the three brazzein forms, no differences were observed for the detection threshold $[F(2,46) = 1.97; p = 0.15]$, with threshold values of 5.31, 3.63, and 2.73 mg/L for the forms pyrE-bra, Q1-bra, and des-pyrE-bra, respectively. However, significant differences were observed for the recognition threshold $[F(2,46) = 10.04; p < 0.001]$. The form pyrE-bra has a significantly higher recognition threshold of 8.90 mg/L than Q1-bra and des-pyrE-bra forms, with 3.09 and 3.07 mg/L, respectively.

At suprathreshold intensities (Figure 4B), psychometric curves fitted to the Stevens' power law revealed that the brazzein forms were rated significantly sweeter than the control sample for concentrations exceeding 50 mg/L for pyrE-bra, 25 mg/L for Q1-bra, and 25 mg/L for des-pyrE-bra. In marked contrast, the lowest concentration of sucrose that scored significantly sweeter than the control sample was 25 g/L. Remarkably, this concentration is 500−1000-fold higher than those of the brazzein forms. A comparison of the recombinant brazzein forms shows that Q1-bra and des-pyrE-bra have

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similar perceived sweetness, except for the highest concentration of 100 mg/L. However, the pyrE-bra form was significantly perceived as less intense sweet than Q1-bra and des-pyrE-bra. This difference is evident for the two highest concentrations (50 and 100 mg/L) when pyrE-bra and Q1-bra are compared and for the three highest concentrations (25, 50, and 100 mg/L) when pyrE-bra and des-pyrE-bra are compared.

In comparison to sucrose, the equivalent sweetness potencies of the three brazzein forms (Figure 4A) were between 400 and 1500, which means that these proteins were perceived as 400−1500-fold sweeter than sucrose on a weight basis. We found that pyrE-bra had significantly the lowest sweetness potency with 422, 473, and 545 at 20, 50, and 100 g/L of sucrose, respectively. Q1-bra brazzein had significantly an intermediate sweetness potency with 923, 790, and 760, whereas des-pyrE-bra brazzein had significantly the highest sweetness potency with 1568, 1076, and 875, at 20, 50, and 100 g/L of sucrose, respectively. Only the sweetness potencies of Q1-bra and des-pyrE-bra forms at 100 g/L of sucrose were not significantly different.

The detection and recognition thresholds we obtained were 4−5 times lower than the previously reported thresholds for all substances (brazzein forms and sucrose).[2] This is most likely due to the use of a different methodology. The recent report computed these values from psychometric curves, whereas we determined the thresholds directly. Indeed, our measured detection and recognition thresholds for the control substance sucrose were consistent with several other studies.[38−42] Our ratios of the recognition threshold values for the three brazzein forms and the control substance sucrose were consistent with those of Assadi-Porter et al.[2] These authors observed, in line with our results, that the threshold for des-pyrE-bra brazzein produced using *E. coli* was 2 times lower than that of the natural brazzein, which is ∼80% pyrE-bra and ∼20% des-pyrE-bra.[5] Moreover, the sweetness potencies determined here correspond well with those previously indicated by Izawa et al.,[14] ranging from 500 to 2000, and with those determined by Assadi-Porter et al.,[2] being approximately 500 for natural brazzein and 1000 for des-pyrE-bra brazzein produced by *E. coli* relative to a sucrose solution of 20 g/L.

Taken together, the sensory characterization of the brazzein forms indicated that the recombinant pyrE-bra and des-pyrE-bra produced by *P. pastoris* are approximately 400- and 1500-fold sweeter than sucrose on a weight basis, respectively. A similar difference is also seen in the case of the data from the receptor assays. The EC_{50} values for the three brazzein isoforms are ∼10 μM, whereas the EC_{50} value for sucrose is ∼25 mM (data not shown). Moreover, the sensory experiments revealed that our recombinant des-pyrE-bra was a little sweeter than the other brazzein forms, being 3.7-, 2.2-, and 1.6-fold sweeter than the recombinant pyrE-bra for low, intermediate, and high concentrations, respectively. In the receptor assay, the concentration−response curve for des-pyrE-bra is slightly left-shifted relative to those of the other brazzein forms and reaches higher signal amplitudes. This suggests that des-pyrE-bra is also somewhat more potent than the other brazzein forms in the receptor assay, even though the recorded EC_{50} values do not differ significantly. Thus, the in vitro and in vivo data agree reasonably well, although the oral milieu appears to account for the observed slight differences. Finally, our results indicate that the N-terminal variations of the molecule do not significantly impact the sweetness of brazzein at the detection threshold. In contrast, as soon as brazzein is perceived as sweet at recognition thresholds or at suprathreshold concentrations, the N-terminal variations of the molecule influence the sweetness of the protein.

To our knowledge, this is the first expression of both natural forms of brazzein being highly expressed in soluble form with the correct N-termini. This expression system opens the possibility for site-directed mutagenesis of specific residues to investigate and further define the relationships between the structure and the function of this sweet-tasting protein. In addition, this study demonstrates that *P. pastoris* is an economical expression system for both research studies and future large-scale production of brazzein.

■ ASSOCIATED CONTENT

Supporting Information

Purification (Figure S1), MALDI-ToF mass spectra (Figure S2), and ^{1}H NMR spectra (Figure S3) of the three purified recombinant brazzeins. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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