

Brazzein, a new high-potency thermostable sweet protein from *Pentadiplandra brazzeana* B.

Ding Ming, Göran Hellekant*

Department of Animal Health and Biomedical Sciences, University of Wisconsin at Madison, 1655 Linden Dr., Madison, WI 53706, USA

Received 13 October 1994

Abstract We have discovered a new high-potency thermostable sweet protein, which we name brazzein, in a wild African plant *Pentadiplandra brazzeana* Baillon. Brazzein is 2,000 times sweeter than sucrose in comparison to 2% sucrose aqueous solution and 500 times in comparison to 10% of the sugar. Its taste is more similar to sucrose than that of thaumatin. Its sweetness is not destroyed by 80°C for 4 h. Brazzein is comprised of 54 amino acid residues, corresponding to a molecular mass of 6,473 Da.

Key words: Sweet protein; Brazzein; *Pentadiplandra brazzeana*; Thermostable protein

1. Introduction

It was once thought that compounds with molecular masses over 2,500 would generally be tasteless [1]. Researchers did not think that macromolecules such as proteins could elicit taste activities similar to small ones, e.g. sugars, until the discovery of miraculin, a taste modifying glycoprotein which acts by adding sweetness to sour taste [2] as if sugar has been added. Later, two sweet tasting proteins, monellin and thaumatin, which in themselves taste sweet, were isolated and studied extensively [3–5]. Recently, curculin, a taste modifying and sweet protein [11], and other sweet proteins, including mabinlin [6–8], pentadin [9], and one from chick eggs [10], have been reported. This article reports a new sweet protein, brazzein, isolated from *Pentadiplandra brazzeana* B. [12]. Brazzein combines small size with high sweet potency and exceptional thermostability.

2. Materials and methods

2.1. Materials

Fruits of *Pentadiplandra brazzeana* from West Africa were used. Each fruit has a reddish nutshell-like epicarp, under which three to five reniform seeds are located, surrounded by a thick soft layer of red pulp which is sweet and containing brazzein.

2.2. Protein isolation

We found that the best method to extract brazzein from the pulp was to use 0.1 M phosphate buffer at pH 7.0 containing 5% glycerol, 0.1 mM DTT, 20 µg/ml PMSF, 0.1 mM EDTA and 0.5% (w/v) PVP at 4°C. The proteins, which precipitated between 30 and 85% ammonium sulfate saturation, were separated on a Sephacryl S-100 column (XK 26/100; Pharmacia, Piscataway, NJ) in 50 mM phosphate buffer at pH 7.0. Finally, brazzein was purified on a CM-Sephacryl CL-6B column (XK 16/70; Pharmacia, Piscataway, NJ) by a NaCl gradient of 0.1 to 0.4 M in 20 mM sodium citrate at pH 3.6.

2.3. Protein characterization

A tricine system [13] was used in SDS-PAGE. ESI-MS was carried out at the Analytical Chemistry Center of the Medical School of the University of Texas in Houston. An expert taste panel (NutraSweet R&D, Mt. Prospect, IL) compared brazzein with a series of sucrose concentrations. Thermostability assay was carried out by incubating aqueous solutions of 1 mg/ml brazzein in water bathes at 80°C. Every 15 min an aliquot was analyzed by a lab bench taste panel.

2.4. Sequence determination

S-Pyridylethylation [14] and S-carboxymethylation [15] were performed. S-Pyridylethylated brazzein was dot-blotted on PVDF membrane [16], and treated with pyroglutamate aminopeptidase [17] for N-terminal deblocking. S-Carboxymethylated brazzein was digested by trypsin (TPCK treated) and *Staphylococcus aureus* V8 protease. The internal peptides were separated by RP-HPLC [15] and sequenced on an ABI 470 protein sequencer. Time-course hydrolysis of S-carboxymethylated brazzein with carboxypeptidase Y was performed [14] and amino acids released were analysed by Pico-Tag amino acid analysis kit (Waters, Milford, MA) [18].

3. Results

3.1. Protein purification and characterization

Table 1 summarizes the results of brazzein purification. The results of SDS-PAGE (Fig. 1) show the molecular weight of brazzein to be about 6,500 Da. Brazzein is a single chain polypeptide as shown by the result of SDS-PAGE and gel filtration. The molecular mass of brazzein determined by ESI-MS is 6473 Da. Brazzein is 2,000 times sweeter than sucrose in comparison to 2% sucrose solution (w/v), and 500 times to 10% of the sugar (w/v). It has a more sucrose-like temporal profile than other sweet proteins, and it cross-adapts with other sweeteners. The sweetness of brazzein remained after incubation at 80°C for 4 h.

3.2. Amino acid sequence

The results of tryptic and *Staphylococcus aureus* V8 protease digestion of S-carboxymethylated brazzein are shown in Fig. 2. Time-course hydrolysis of S-carboxymethylated brazzein with carboxypeptidase Y shows that the C-terminal amino acid is a tyrosine (Fig. 3). Fig. 4 shows the complete amino acid sequence of brazzein. This amino acid sequence has been confirmed by mass spectrometry analyses (to be published). Sequence computational analysis shows that curculin [11] is the

*Corresponding author. Fax: (1) (608) 262-7420.

Abbreviations: EDTA, ethylenediamine tetra acetic acid; ESI-MS, electrospray ionization mass spectrometry; M_r , molecular weight; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; PVP, polyvinylpyrrolidone; RP-HPLC, reverse-phase high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Tris, Tris(hydroxymethyl)amino-methane.

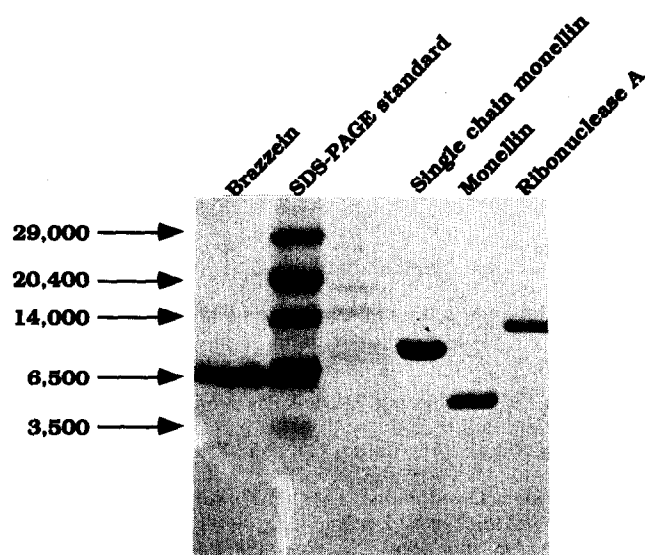


Fig. 1. SDS-PAGE of brazzein. Protein samples were treated for about 30 min at 50°C in 4% SDS, 8 M urea, 12% glycerol (w/v), 50 mM Tris and 0.01% Bromophenol blue solution adjusted with HCl to pH 6.8, to which 2% (v/v) β -mercaptoethanol was added. The compositions of separation, spacer, and stacking gels were 15% total acrylamide concentration (T; w/v), 3% ratio of cross-linking reagent (C; w/v); 10% T, 3% C; and 4% T, 3% C. The numbers listed in the figure represent the molecular weight values of the protein standards used in the electrophoresis.

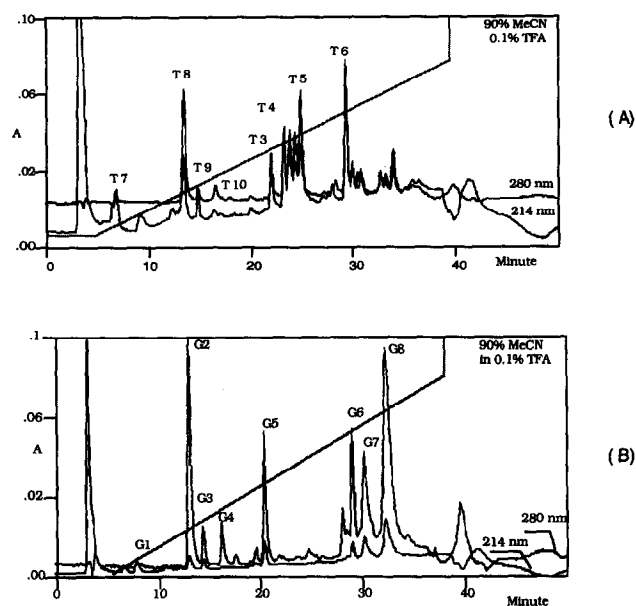


Fig. 2. Reverse-phase HPLC chromatograms showing the peptide fragments resulted from (A) tryptic digestion and (B) *Staphylococcus aureus* V8 protease digestion of the carboxymethylated brazzein. The brazzein was denatured, reduced and *S*-carboxymethylated with 50 mM iodoacetamide in 8 M urea. The digestions were undertaken 24 h at 37°C at an enzyme/substrate ratio of 1:100 (w/w). The separations were carried out on a Vydac C18 column (8 × 100 mm) with a linear gradient of 0.1% TFA to 90% acetonitrile in 0.1% TFA in 60 min. The eluants were monitored with a Waters 490E programmable multiwavelength detector (Waters, Milford, MA) at both 214 nm and 280 nm. T_N refers to the peptides from tryptic digest and G_N refers to the peptides from *Staphylococcus aureus* V8 protease digest.

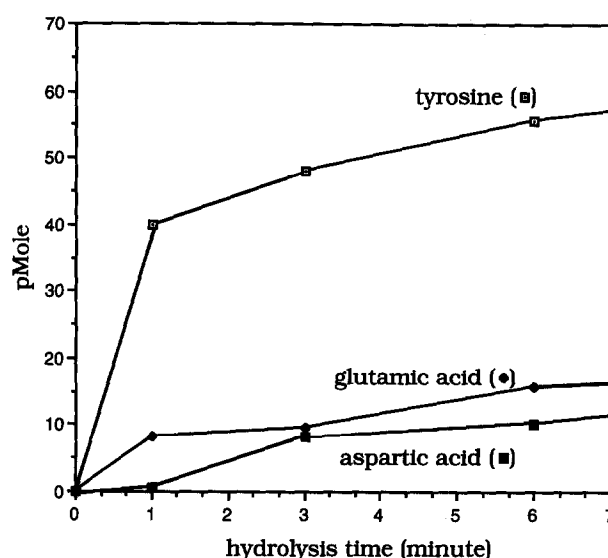


Fig. 3. C-Terminal amino acid determination by time-course hydrolysis with carboxypeptidase Y. The brazzein was *S*-carboxymethylated before the treatment by carboxypeptidase Y. x-axis stands for the digestion time, and y-axis stands for the quantities of free amino acids released from the C-terminal of the polypeptide.

most homologous one to brazzein among the sweet proteins: they share 20% identity in a 50 amino acid overlap.

4. Discussion

In comparison with other sweet proteins, brazzein at higher concentration is much sweeter than thaumatin [19]. Sensory analysis shows that its taste differs from that of thaumatin [20]; it has a more phasic response and a faster adapting tonic phase (to be published). The molecular weight of brazzein is 6473 Da, which is less than thaumatin (M_r 22,206), monellin (M_r 11,086), curculin (M_r 12,491) and mabinlin (M_r 12,441). Thus, brazzein is the smallest protein sweetener discovered so far. Water solubility is one of the crucial criteria for high potency sweeteners in commercial applications [21]. The water solubility of brazzein is at least 50 mg/ml, i.e. more than 7.7 mM. Thus, it seems to be the most water soluble member in sweet protein family. In addition, brazzein is extremely thermostable. All sweet proteins except mabinlin lose their sweetness in a few minutes at 80°C [3,6,22], but brazzein's sweetness remains for

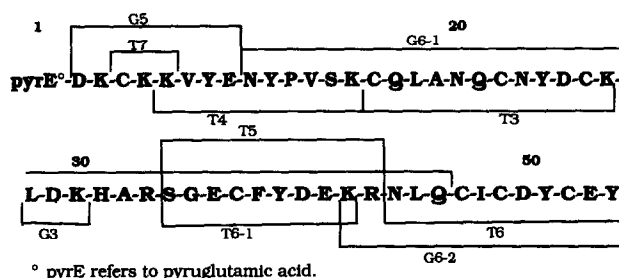


Fig. 4. Amino acid sequence of brazzein. T_N 's are peptide fragments from tryptic digestion, and G_N 's are peptide fragments from *S. aureus* V8 protease digestion. Automated Edman degradation was performed on 100 pmol aliquots of each peptide and on about 400 pmol of the entire protein.

Table 1
Summary of brazzein purification from 200 g fruit

	Brazzein preparation (mg)	Yield (percentage of fruit, w/w)	Purity ^a	Sweetness ^b
Buffer extraction	2100	1.05	≥ 15	100
Salting out	1700	0.85	≥ 8	1,000
Gel-filtration	1460	0.73	≥ 3	1,500
Ion-exchange	720	0.36	1	2,000
Overall	720	0.36	1	2,000

^a Purity was defined the number of bands on SDS-PAGE.

^b Sweetness, as indicated by the numbers, was defined by comparing each protein preparation to a series of solutions on a weight basis.

hours at this temperature. Brazzein has 8 cysteines out of 54 residues. Considering its high thermostability, brazzein may tightly cross-linked by an disulfide-bonded network.

In summary, we have discovered a new natural sweet protein which we named brazzein. From the point of view of its sweetness, heat stability, high water solubility and minimum molecular weight, brazzein is the most superior protein sweetener presently known.

Acknowledgements: We thank Dr. C.M. Hladik and his associates at the Laboratoire D'Ecologie Generale in France for the *Pentadiplandra brazzeana* fruit; we thank the expert taste panel at the NutraSweet R&D (Mt. Prospect, IL) for the sensory analyses; we thank Dr. J. Caprioli of the University of Texas in Houston for MS analyses. Finally, we thank Dr. J. Markley and his associates at the Department of Biochemistry, University of Wisconsin at Madison, for helpful suggestions and discussions.

References

- [1] Ney, K.H., (1972) *Z. Lebensm. Unters. Forsch.* 149, 321.
- [2] Kurihara, K. and Beidler, L.M. (1968) *Science* 161, 1241–1243.
- [3] van der Wel, H., (1991) in: *Development in Food Proteins*, vol. 4 (Hudson, B.J.F., ed.) pp. 219–245, Elsevier, Amsterdam.
- [4] Kohmura, M., Nio, N. and Ariyoshi, Y. (1992) *Biosci. Biotech. Biochem.* 56, 1937–1942.
- [5] Somoza, J., Jiang, F., Tong, L., Kang, C., Cho, J. and Kim, S.-H. (1993) *J. Mol. Biol.* 234, 390–404.
- [6] Ming, D. and Hu, Z. (1986) *Acta Botanica Yunnanica* 8, 2, 181–192.
- [7] Kurihara, Y. (1992) *Critical Rev. Food Sci. Nutrition* 32, 231–251.
- [8] Nirasawa, S., Liu, X., Nishino, T. and Kurihara, Y. (1993) *Biochim. Biophys. Acta* 1202, 277–280.
- [9] van der Wel, H., Larson, G., Hladik, A., Hladik, C.M., Hellekant, G. and Glaser, D., (1989) *Chem. Senses* 14, 75–78.
- [10] Eguchi, I. (1988) Japanese Patent Application 63–48298 (to Ajinomoto Kaisha), Feb. 29.
- [11] Harada, S., Otani, H., Maeda, S., Kai, Y., Kasai, N. and Kurihara, Y. (1994) *J. Mol. Biol.* 238, 286–287.
- [12] Hutchinson, J. and Palziel, J.M., (1927) *Flora of West Tropical Africa*, Crown Agents, London, UK, Vol. 1, 71.
- [13] Schagger, H. and Jagow, G. von, (1987) *Anal. Biochem.* 166, 368–379.
- [14] Shively, J.E., (1986) in: *Methods of Protein Microcharacterization*, pp. 41–86, Humana Press, Clifton, NJ.
- [15] Stone, K.L. and Williams, K.R. (1993) in: *A Practical Guide to Protein and Peptide Purification for Microsequencing* (Matsudaira, P. ed.) pp.45–66, Academic Press, San Diego, CA.
- [16] Aebersold, R., in Matsudaira, P. (1993) in: *A Practical Guide to Protein and Peptide Purification for Microsequencing* (Matsudaira, P. ed.) pp.45–66, Academic Press, San Diego, CA.
- [17] Podell, D.N. and Abraham, G.N. (1978) *Biochem. Biophys. Res. Commun.* 81, 176–185.
- [18] Cohen, S.A., Meys, M. and Tarvin, T.L. (1989) *The Pico-Tag Method: A Manual of Advanced Techniques for Amino Acid Analysis*, pp. 2–26, Millipore Co., Bedford, MA.
- [19] Schiffman, S.S. and Gatlin, C.A. (1993) *Neurosci. Biobehav. Rev.* 17, 313–345.
- [20] Hellekant, G. (1994) in: *Thaumatococcus* (Witty, M. and Higginbotham, J.D., ed.) pp. 99–114, CRC Press, Boca Raton, FL.
- [21] Muller, G.W., Madigan, D.L., Culbertson, J.C., Walters, D.E., Carter, J.S., Klade, C.A., DuBois, G.E. and Kellogg, M.S. (1991) in: *Sweeteners: Discovery, Molecular Design, and Chemoreception* (Walters, D.E., Orthoefer, F.T. and Dubois, G.E., eds.) pp. 113–125, ACS Symposium Series 450, American Chemical Society, Washington DC.
- [22] Kim, S., Kang, C. and Cho, J. (1991) in: *Sweeteners: Discovery, Molecular Design, and Chemoreception* (Walters, D.E., Orthoefer, F.T. and Dubois, G.E., eds.) pp. 28–40, ACS Symposium Series 450, American Chemical Society, Washington DC.