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耐热甜味蛋白 Brazzein 的特性和化学修饰

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摘要 Ming and Hellekant (1994) 从西非洲热带野生植物 *Pentadiplandra brazzeana* Baill.的果实中，分离出一种甜味蛋白质，命名为 brazzein。它是由 54 个氨基酸残基组成的单链多肽；甜度是蔗糖的 2000 倍。本实验测得 Brazzein 分子量为 6.5 kD，等电点为 5。Brazzein 含有 8 个半胱氨酸，构成分子内的双硫键；其水溶液经 80℃、4 h 处理，甜味和电泳行为不变。虽然 Brazzein 的氨基酸序列与芥菜蛋白酶抑制剂 MTI-2 有高的同源性，但未测出有蛋白酶抑制剂活性。根据氨基酸序列的计算机分析表明：Brazzein 是亲水性多肽；二级结构主要是 β-折迭和 β-转角。蛋白质印迹分析显示，Brazzein 抗血清与甜味蛋白 curculin, mabinlin 和 miraculin 有强的交叉反应。Brazzein 的 S-烷基化修饰引起甜味的丧失，同时也丧失与抗血清的免疫反应。Brazzein 中赖氨酸 ε-氨基的酰化、酪氨酸酚羟基的碘化、组氨酸咪唑基和精氨酸胍基的修饰均导致甜味的丧失或降低；但赖氨酸 ε-氨基的甲基化只改变其电泳行为，却不降低甜味。这表明 Brazzein 分子的表面电荷对于其甜味性质是重要的。

关键词 *Pentadiplandra brazzeana*, 甜味蛋白质, 化学修饰

CHARACTERIZATION AND CHEMICAL MODIFICATION OF BRAZZEIN, A HIGH POTENCY THERMOSTABLE SWEET PROTEIN FROM PENTADIPLANDRA BRAZZEANA

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Abstract A sweet protein, brazzein, has been discovered from the fruits of *Pentadiplandra brazzeana* B., a tropical wild plant in west Africa. Brazzein has 54 amino acid residues; it is 2000 times sweeter than sucrose (Ming and Hellekant, 1994). In this article, further characterization shows that its molecular weight is 6.5 kD and isoelectric point is 5. It has 8 cysteines and they form four disulfide bonds. After treating its aqueous solution at 80 ℃ for 4 hrs, no changes in sweetness and behavior of electrophoresis are found. Secondary structures predictions suggests that beta turns and beta sheets are the dominant structures in the protein. Although brazzein shares 13 out of 31 amino acid residues with mustard trypsin inhibitor 2, no trypsin inhibitory activity of it is

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detected. The tests of all sweet related natural sweet proteins by dot blot show that curculin, mabinlin and miraculin cross- react strongly with a native brazzein antiserum. Acetylation, succinylation and pyridoxalization of the ε-amino group of Lys residues, iodination of the hydroxyl of Tyr residues and the modification of Arg and His residues all result in the abolition or diminution of the sweetness. However, the methylation of the Lys -amino group does not change the sweetness although the electrophoresis mobility is changed. Thus, chemical modifications of brazzein indicate that the charge in the surface of molecule is important for its sweetness.

Key words *Pentadiplandra brazzeana*, Sweet protein brazzein. Chemical modification

INTRODUCTION

We discoved of a new high potency sweet protein, which we named brazzein, from the fruit of *Pentadiplandra brazzeana* Baillon, a wild tropical plant in west Africa (Ming *et al*, 1994). Compared to other sweet proteins including curculin, mabinlin, monellin, pentadin and thaumatin, brazzein is the smallest in size, the sweetest in high concentration , and one of the most thermostable in aqueous solution. Brazzein comprises 54 amino acid residues, based on the peptide mapping and Edman degradation analysis of derived peptides. In this article, new biochemical data are presented. We also probe its structure-function relationship by selective chemical modification and immunochemical analyses with a native brazzein antiserum from rabbit.

MATERIALS AND METHODS

Materials

The fruits of *Pentadiplandra brazzeana* B. were collected by Dr. C. M. Hladik and his associates in west Africa. The brazzein- rich red pulp was separated from the other part of the fruit and the total water soluble proteins were extracted quickly with 0.1 mol / L phosphate buffer at pH 7.0, containing 5% glycerol, 0.1 mol / L DTT, 20 μg / ml PMSF (phenylmethylsulfonyl floride), 0.1 mol / L EDTA and 0.5% PVP (polyvinylpolypyrrolidone) at 4℃ . Then proteins were precipitated by adding ammonium sulfate to 90% saturation.

Buffer salts used in the experiments were mostly from Sigma (St.Louis). Organic solvent were from Aldrich (Milwaukee,WI). Chromatography and electro phoresis reagents were from Pharmacia (Piscataway, NJ). Reagents for immuno chemical analysis were from Pierce (Rockford,IL), and protein blot membrances from Millipore (Milford, MA). Monellin was kindly offered by Dr. J. Brand at the Monell Chemical Senses Center (Philadelphia, PA), and a single chain monellin was from Dr. S. Kim. Thaumatin was from Dr. J. L. Welckmann of the Xoma, Inc.(Santa monica,CA). Gurmarin, an antisweet protein (Kamei et al.,1990) was supplied from Dr. Y. Ninomiya at Asahi university (Gifu, Japan). Curculin and miraculin were kindly supplied by Dr. Y. Kurihara at the Yokohama National University of Japan. Pentadin was prepared from smoked fruits of *Pentadiplandra brazzeana*. A miraculin antiserum from rabit was from Dr H. van del wel at the Unilever Research (Vlaardingen, Netherlands).

Protein purification

The purification of brazzein was carried out as described earlier (Ming *et al*. 1994). Briefly, the total

proteins were separated by gel filtration on a Sephacryl S-100 column, and then purified by ion exchage chromatography on a CM-sepharose CL-4B column at pH 3.5 to give a pure protein which was showed to be a single band in SDS-PAGE. The yield was 0.36 g from 100 g fruit.

Protein characterization

SDS-PAGE and nondenaturing PAGE We carried out SDS-PAGE in a tricine system as described earlier. The nondenaturing PAGE was carried out in an alkaline buffering system (Bollag *et al*, 1991).

Gel filtration analysis We performed analytical gel filtration on a G-75 Sephadex column (1.5 × 100 cm). The eluent was 0.5 mol / L sodium chloride in 0.05 mol / L phosphate buffer at pH 7.0. Bovine serum albumin (BSA, Mr 66000), carbonic anhydrase (CA, Mr 29000), cytochrome C (CytC, Mr 12400) and aprotinin (APR, Mr 6500) were used as molecular weight standards.

pI determination The isoelectric point (pI) was determined by pH dependent binding analyses according to Yang and Langer (1985). The protein concentrations were determined by measurement of absorbance values at 280 nm and by BCA protein Assay Kit (Pierce).

Sulfhydryl group determination Free sulfhydryl groups and sulfhydryl groups produced from disufide bond by sodium borohydride reduction (Cavalliini *et al*, 1966) were detected by titration with 5,5-dithiobis (2-nitrobenzoic acid) (DTNB). In the determination of sulfhydryl groups produced from disulfide bond, the concentration of sodium borohydride was increased to 2.5% and the incubation time was increased to three hours. The exact number of total sulfhydryl groups in brazzein has been determined by a new method combining selective chemical modification and electrospray ionization mass spectrometry (ESI-MS) analysis (Ming *et al*. 1995).

Thermostability assay We analyzed the thermostability of brazzein as following: 1 mg / ml brazzein aqueous solution was incubated at various temperature, an aliquot was taken out and cool to room temperature about every 15 min, then the sample was analysed for its sweetness and by nondenaturing PAGE.

Amino acid sequence computational analysis The hydrophobility and the secondary structure prediction was done by computational analyses of the amino acid sequence (Ming *et al*, 1994). The analyses were carried out by using the sequence analysis software GCG package from the Genetic Compute Group,Inc. and performed on an Utrix DEC-500 computer with version 7.1.1-Unix and a VAx 4400 with VMS version 5.5-2 at University of Wisconsin-Madison.

Tryptic inhibition activity assay We used measurement of tryptic hydrolysis of BAPNA (benzoylarginine p-nitroanilide) which resulted in a change in absorbance at 410 nm for the tryptic inhibition assay (Mikola et al, 1969). In our experiment, sweet protein mabinlin was used as a negative control, and ovomucoid was used as a tryptic inhibitor control.

Selective chemical modification

S-alkylation of cysteines S-pyridylethylation of brazzein was performed with the method of Shively (1986). The unreacted reagents were removed by dialyzing the products in water at 4 ℃ S-carboxyamidomethylation was carried out based on the method of Stone *et al* (1993).

ε-amino group modification Acetic anhydride was used for acetylation of brazzein (Riordan *et al*. 1972a). The products were separated from the reagents by passing through a sephadex G-25 column. Aqueous formaldehyde solution was used for reductive methylation of brazzein (Means, 1977). Pyridoxal-5-phosphate was used for the pyridoxal modification of lysine residues (Nishigori *et al*. 1979). The amount of pyridoxl bound irreversibly to each brazzein molecule was determined

spectrophotometrically from the absorbance value at 325 nm, assuming a molar extinction coefficient of 9710 for the reduced pyridoxal phosphate derivative. Succinylation of brazzein with succinic anhydride was also carried out based on the procedure of Meighem *et al* (1971).

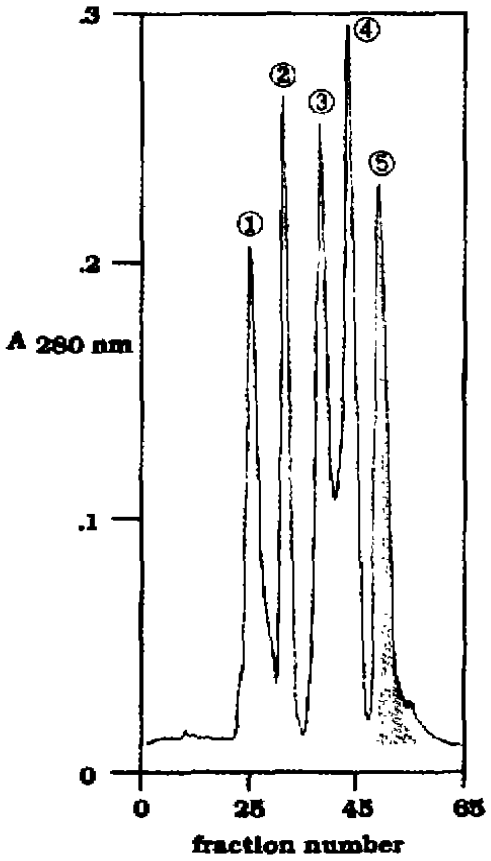

Fig 1. Estimation of the molecular weight of brazzein by gel filtration. The eluent was 0.5 mol / L NaCl in 0.05 mol / L phosphate buffer, pH 7.0 The flow rate is about 15 mL / hour. Peak 1 is blue dextran (Mr 2 000 000), peak 2 is BSA (Mr 66 000), peak 3 is carbonic anhydrase (Mr 29 000), peak 4 is cytochrome C (Mr 12 400) and peak 5 is the mixture of aprotinin (Mr 6 500). The shaded area in the figure indicates the fraction which tasted sweet after dialyzing out salt.

Tyrosine modification We used Pierce solid phase iodination method (Markwell, 1982) to process the iodination of brazzein. The reaction was stoped by separating the solid beads out of the reaction mixture. The excess NaI was removed by gel filtration. N-acetylimidazole was also used to modify the tyrosine residues in brazzein (Riordan et al, 1972 b).

Arginine modification p-Hydroxypheny-lglyoxal was used to modify the quanidyl group of arginine residues in brazzein. We used a bicarbonate- carbonate buffer system to prevent the side reaction with the alpha amino group (Cheung *et al*, 1979). In addition, 1,2-cyclohexanedione was also used to modify the arginine residues in borate buffer (Pattthy et al, 1975).

Histidine modification Diethylpyrocarbonate was diluted seven folds with dry alcohol. Then 10 μL of the reagent solution was added to 1 mL of 50 mmol / L Mes / NaOH buffer (pH 6.0) solution containing 6.4 mg brazzein and the mixture was incubated at room temperature for 30 min under stirring (Miles, 1977).

Brazzein antiserum preparation and analyses

Purified brazzein was dissolved to make a solution of about 100 μg protein per mL of phosphate buffered saline, and emulsified with complete Freunds adjuvant. Two 4 month old New Zealand white rabbits weighing about 3 kilograms were used for immunization. About 1 mg brazzein was used per rabbit for primary immunization. Additional four consecutive boosters of 0.3 mg antigen with incomplete Freunds adjuvant injected every three weeks. Antisera were initialy checked with double diffusion tests and then with dot blot assays (Harlow *et al*, 1988).

We used the brazzein antiserum to analyze chemically modified brazzeins as well as all natural sweet related proteins in Western and dot blots. In addition, we used a miraculin antiserum in the dot blot assay of all natural sweet related proteins. In blot assays with this antiserum, the nonspecific binding sited on the

blot membrance were blocked with 3% BLOTTO (Pierce). An alkaline phosphatase coupled anti-rabbit IgG was used as the secondary antibody and bromochloroindolyl phosphate / nitroblue tetrazolium (BCIP / NBT, Sigma) were used as substrates in the enzymatic detection.

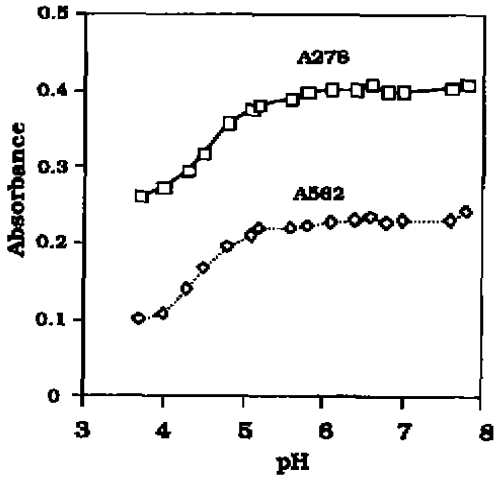

Fig 2 pI Determination of pH dependent binding assay. Brazzein was tested to bind with Sephadex C-50 under different pH values. A278 was the absorbance of the supernatants at 278 nm, A562 was the absorbance values of the supernatants in the measurement of BCA at 562 nm. Both values were used to indicate the quantities of the protein left in the solution

RESULTS

Brazzein characterization

The analytical gel filtration result shows the molecular weight of brazzein is about 6500 (Fig.1). This result is in accordance with the results of ESI-MS analysis and SDS-PAGE (Ming *et al*, 1994). The result of pH dependent binding analyses show that brazzein has an isoelectric point of 5 (Fig. 2).

DTNB titration showed that native brazzein had no free sulfhydryl group. This was true even in the presence of 8 mol / L urea. After brazzein was reduced by sodium borohydride, 7.2 sulfhydryl groups were found based on DTNB titration and spectrophotometric measurements. The positive control, lysozyme with 8 sulfhydryl groups in its sequence, showed 7.8 sulfhydryl groups

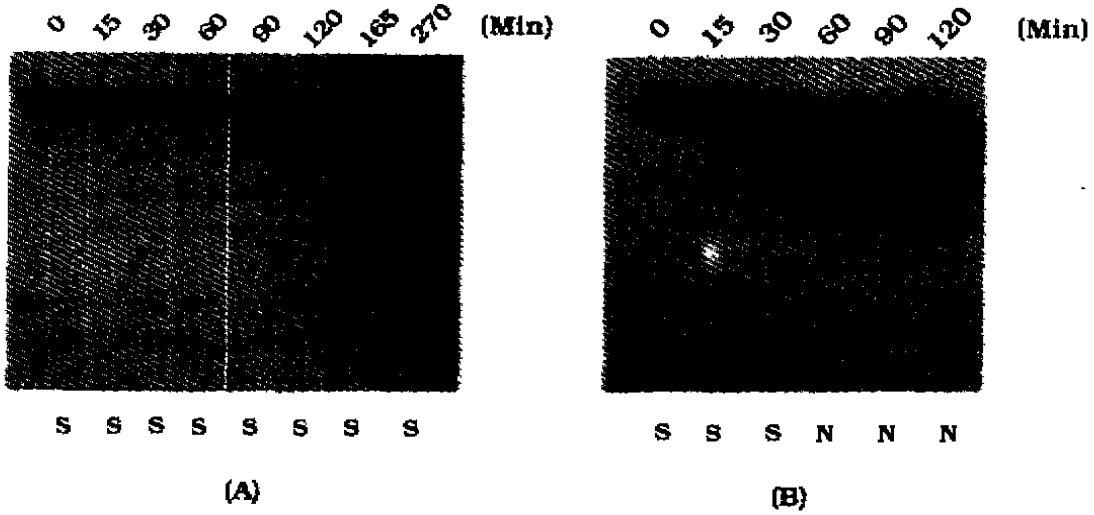

Fig. 3 Heat denaturation time course analysis of brazzein. All brazzein sample in (A) were incubated at 80℃, and all brazzein sample in (B) were incubated in boiling water. A nondenaturing Tris / glycine buffer system was used in the PAGE. S refers to sweet; N means non-sweet.

under the same condition. The ESI-MS analysis (Ming *et al*, 1995) indicated that the molecular mass of S-pyridylethylated brassein was 7323 mass units, the mass difference (6473 mass units) of pyridylethylated brazzein with native brazzein (6473 mass unit) accounts for exactly 8 reaction sites with 4-vinylpyridine; in addition, the mass difference (8 mass units) of reduced brazzein (6481 daltons) and native brazzein demonstrates that all sulfhydryl groups are connected with each other as intramolecular disulfide bonds in the native brazzein.

The thermostability analyses indicated that the sweetness of brazzein remained after incubation at 80℃ for four hours. The changes of the migration behaviors in nondenaturing PAGE fit well with the changes of the sweetness(Fig.3).

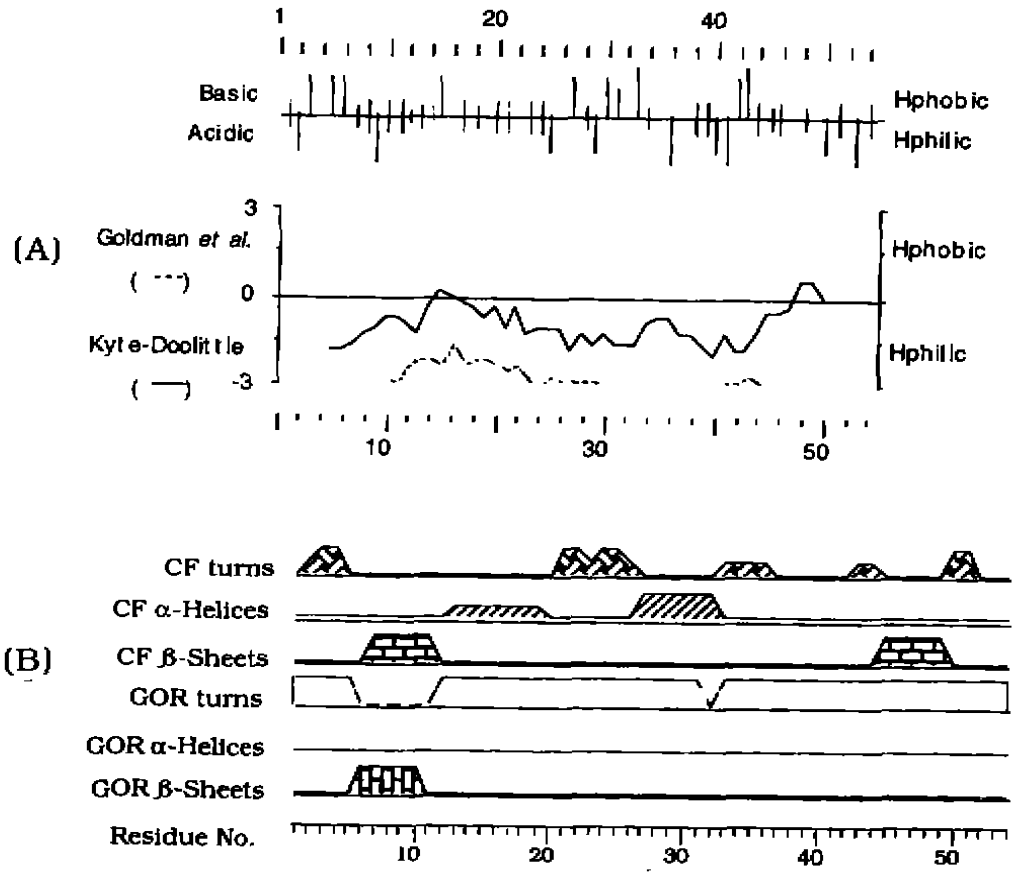

Fig. 4 Computational analyses of the amino acid sequence of brazzein. (A)Hydrophobicity analyses. The analysis was based on the Kyte and Doolittle hydropathy measure(Kyte *et al*, 1982), and Goldman,Engelman and Steiz (GES) curve(Engelman *et al*, 1986). The thickness of the amino acid residue indicators in the upper part of the figure refers to the polarity of the residue and the orientation of these indicators indicates the alkalinity of each residues;(B) Brazzein secondary structures prediction The computation were based on Garnier-Osguthorpe-Robson algorithm (GOR) (Garnier *et al*, 1978) and Chou-Fasmanalgorithm (CF)(Chou *et al*, 1978).

Hydrophobicity analysis of the protein suggests that it is a hydrophilic polypeptide (Fig.4A). Fig.4B shows the secondary structure of brazzein predicted by GOR and CF prediction algorthms, and the domi-

nant structures are beta sheets and turns. Computational analyses show that brazzein is strikingly homologous to a trypsin inhibitor MTI-2, a serine protease inhibitor from the seeds of white mustard (Menegatti, 1992). They share 13 amino acid residues in a region with 31 residues (42%). In addition, 20 out of the 31 residues (64%) can be matched in two sequences regarding the similar natures of the corresponding residues (Fig.5). But neither trypsin inhibitory nor tryptic activities were detected in the brazzein

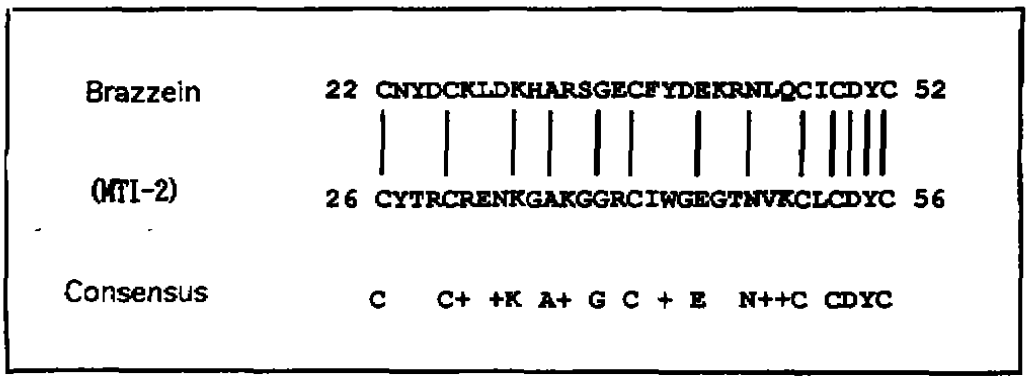

Fig. 5 Homological sequence of brazzein versus mustard trypsin inhibitor 2 (MTI-2)

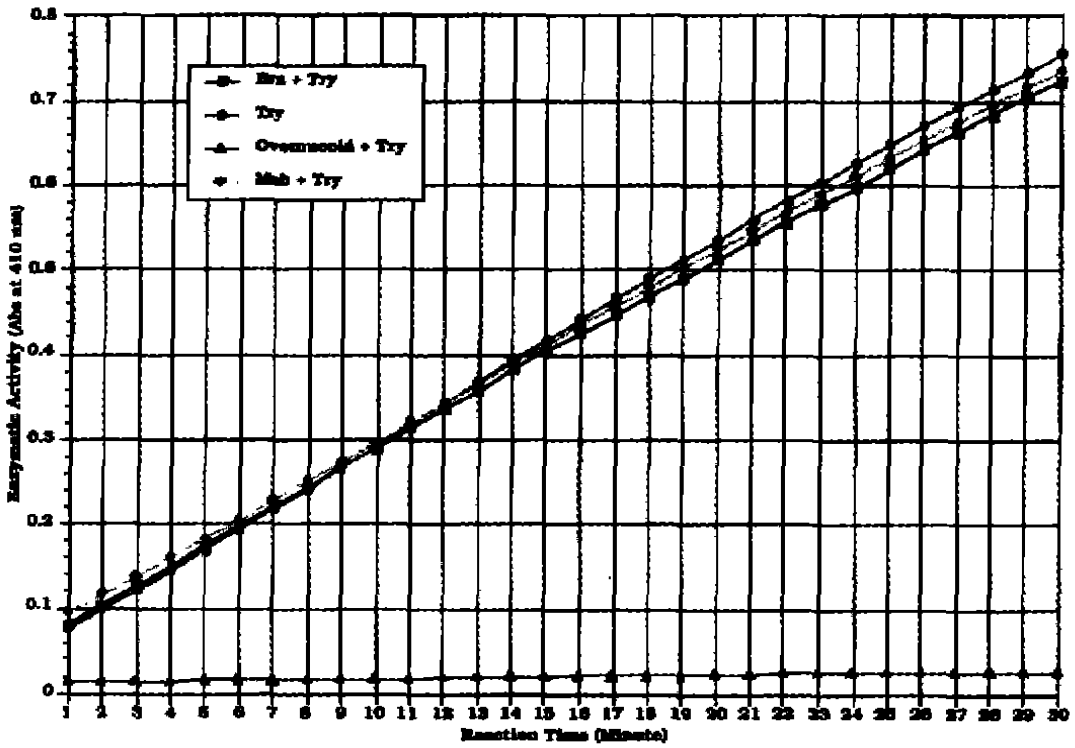

Fig. 6 Trypsin inhibitory analyses. Brazzein (Bra), mabinlin (Mab) and ovomucoid were used in 5:1 molar ratio to trypsin (Trp). After trypsin wasincubated with the protein in 50 mmol / L sodium acetate, pH 5.4 containing 20mmol / L $CaCl_2$ for 1 hour at room temperature, 0.5 mL of mixture was added to 5 ml of mmol / L Tris, pH 8.2 containing 20 mmol / L $CaCl_2$ 0.435 mg / mL BAPNA. The absorbance at 410 nm were measured continuously for 30 minutes.

preparation as shown in Fig.6.

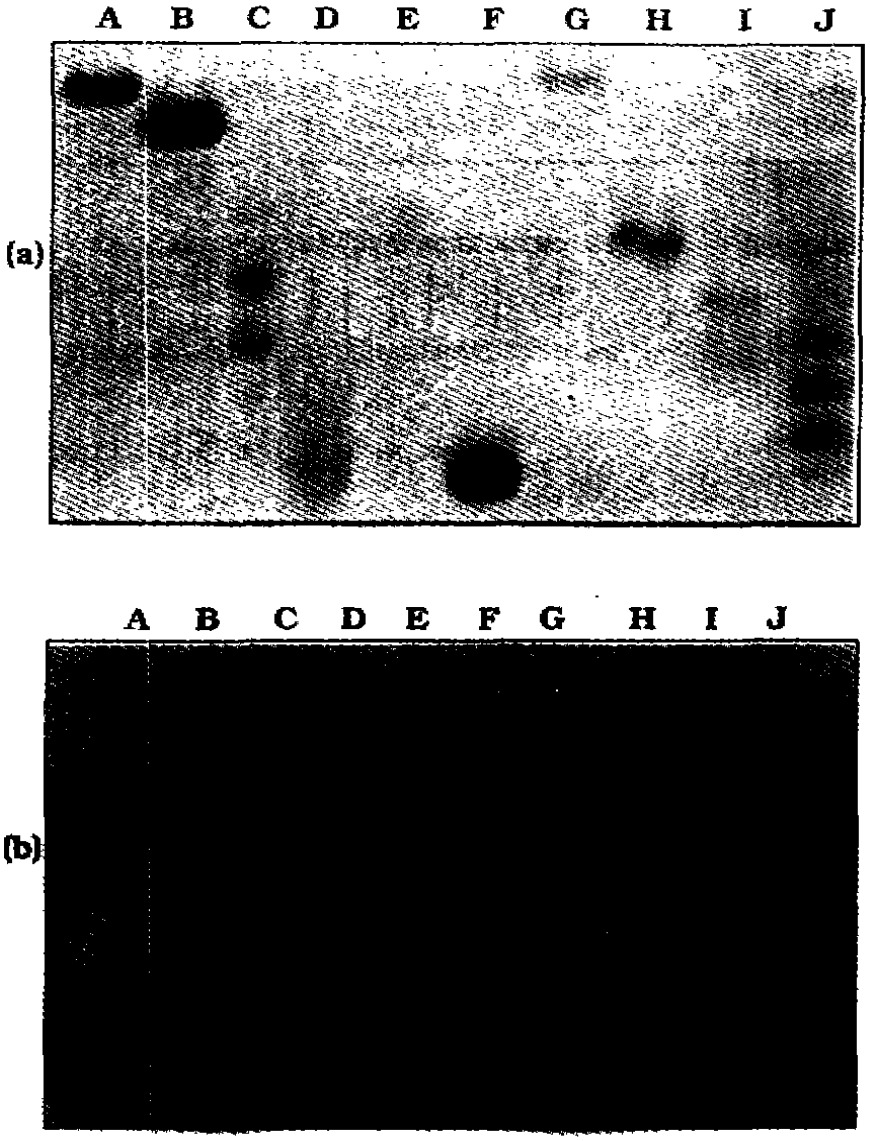

Fig. 7 Nondenaturing PAGE (a) and a Western blot assay (b) of chemically modified brazzeins. A shows native brazzein; B shows the product of reductive methylation; C shows the products from acetylation; D shows the products from pyridoxal modification, E is the product from succinylation; F is the succinylated S-pyridylethylated brazzein; G shows the S-pyridylethylated brazzein; H shows the products from brazzein iodination; I and J show the O-acylated and diethylpyrocarbonated brazzeins respectively. A dilution of 1 500 of the antiserum raised against native brazzein was used in the blot.

Selective chemical modification of brazzein

No sweetness was detected from the products of S-alkylation. After the guanidine -HCl or urea was removed from S-pyridylethylation reaction mixture, the S-pyridylethylated brazzein precipitated out of the

solution and this precipitate was only soluble in 0.5% acetic acid solution although the carboxylmethylated brazzein was soluble in water.

Reductive methylation of brazzein resulted in a product which was as sweet as native brazzein although the electrophoresis mobility of the product was different from native brazzein (Fig. 7). The amino acid composition analysis showed that five out of seven -amino groups were modified.

Acetylation, succinylation and pyridoxalization resulted in a loss of the sweetness of brazzein. Amino acid composition analysis and spectrophotometric assay showed that at least one lysine residue was modified in pyridoxalization Although several products resulted from acetylation (Fig.7A), the mixture is no taste sweet The uniform product of succinylation, in which five out of ε-amino groups were modified, based on the titration of free amino groups by trinitrobenzenesulfonic acid (Habeeb, 1966), was also tasteless.

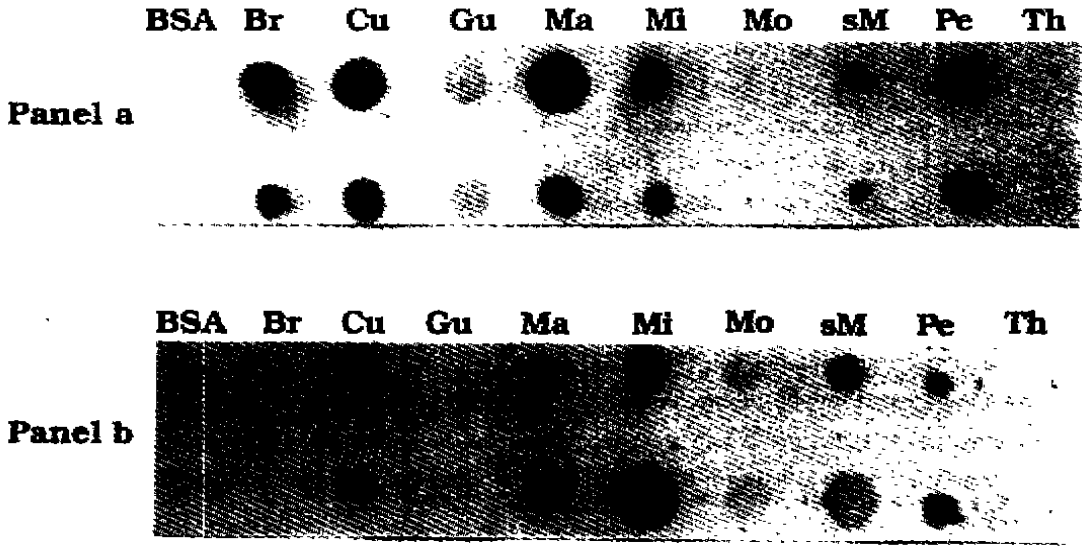

Fig. 8 Dot blot analyses of all known sweet taste related natural proteins by a brazzein antiserum (panel a) and a miraculin antiserum (panel b). BSA is bovine serum albumin; Br is brazzein; Cu is curculin; Gu is gurmarin; Ma is mabinlin; Mi is miraculin; Mo is monellin; sM is a single chain monellin; Pe is pentadin; Th is thaumatin.

The iodination of brazzein abolished its sweetness. The same results were observed with N-acetylimidazole modification of tyrosine and modification of histidine. Based on the analyses of ESI-MS, at least six iodine atoms were attached to a brazzein molecule. The sweetness of brazzein diminished after only one of the two arginine residues was modified by p-hydroxylphenolglyoxal. Based on the result of amino acid composition analysis, 1,2-cyclohexanedione modified all of the arginine residues in the protein, as well as abolished its sweetness.

Immunochemical analyses with a brazzein antiserum

The chemically modified brazzeins were separated in a nondenaturing PAGE. Our brazzein antiserum cross reacted with all modified brazzeins except the ones from S-alkylation in a Western blot (Fig. 7B).

As shown in Fig. 8, curculin, mabinlin, miraculin and pentadin showed strong cross reactions with the brazzein antiserum, while thaumatin and gurmarin did not cross react with the antiserum. Only curculin has

a strong cross reaction with the miraculin antiserum used in the experiment.

DISCUSSION

It is interesting to know that brazzein has an acidic isoelectric point value. This is different from all of other sweet prteins which have an electric point of above 7. There are no free sulfhydryl groups in the molecule, and its eight cysteines form four disulfide bonds. This is similar to sweet protein mabinlin, curculin and thaumatin, in all of which eight cysteines form four disulfide bonds (Nirasawa *et al*, 1994; Yamachita *et al*, 1990; Van del wel *et al*, 1984).

The protein sequence computational analyses show that brazzein is a hydrophilic polypeptide. The water solubility is at least 50 mg / mL. The secondary structure predictions suggest that brazzein is a polypeptide dominated by beta turn and beta sheet and with only a few alpha helical segments. Brazzein may be wrapped up tightly by its disulfide bonds and hydrophobic interaction to form a compact structure at the tertiary level. This may explain the extreme thermostability of brazzein.

Among brazzein derivatives produced by chemical modification, only reductive methylated brazzein was as sweet as native one. In addition, S-alkylated brazzeins were not recognized by a native brazzein antiserum. It is obvious that only the S-alkylation altered overall molecular conformation while the other modifications affected only the surface of the protein. Among these surface modifications, only reductive methylation, which did not change the charge of the molecule, maintained its sweetness. Thus, it seems that the surface charge of the molecule is important to its sweetness. This result agrees with earlier data on thaumatin and monellin (Van del wel, 1983).

The strong cross reactivity of curculin, mabinlin and miraculin to the brazzein antiserum showed that these proteins shared structures which were recognized by the antiserum. Futher studies including site directed mutagenesis may shed light on the relationship between these shared structures and the sweetness of these proteins.

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