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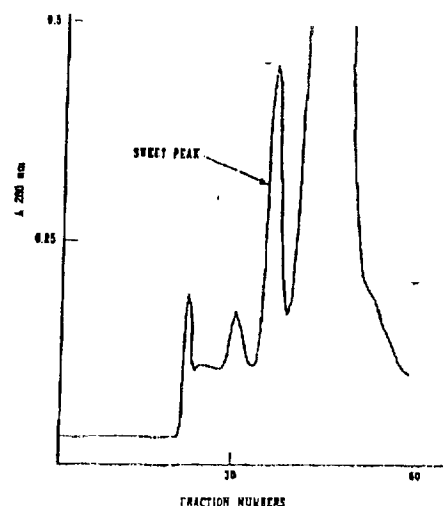
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54 Titre: Brazzein sweetener.

57 Abrégé:

Disclosed herein is a protein sweetener that has been isolated from *Pentad plandra brazzeana baillon*. The sweetener is thermostable, lysine rich, and has a relative long lasting taste. Also disclosed is a recombinant host capable of producing the sweetener in large quantities. Compositions of this sweetener with other sweeteners are also disclosed.



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BRAZZEIN SWEETENERTechnical Field

The present invention relates to the identification and isolation of a sweet protein from the plant Pentadiplandra brazzeana Baillon. It also relates to the sequencing of that protein and the creation of cDNA
5 capable of producing the protein artificially.

Background Art

The most widely used natural sweetener, sugar, has problems associated with its use (especially causing weight gain by users). Many other sweeteners either have
10 undesirable side effects or are deficient in certain respects. For example, aspartame loses its sweetness when exposed to elevated temperatures for long periods. This renders aspartame unsuitable for use in most baking applications.

15 Another problem is that existing artificial sweeteners have temporal sweetness profiles which do not exactly match that of sugar. For example, the sweetness may die out sooner. It may therefore be desirable to mix an existing artificial sweetener with another sweetener
20 having a different temporal profile (so as to create a mixed sweetener that more closely matches the overall temporal sweetness profile of sugar).

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Thus far, only relatively few sweet proteins (as distinguished from sweet carbohydrates) have been found in nature. See e.g. J.A. Morris et al., Biochem. Biophys. Acta. 261:114-122 (1972) (Monellin); H. Van Der Wel, FEBS Letters 21(1):88-90 (1972) (Thaumatin); D. Ming et al., Acta Botanica Yunnanica 8(2):181-192 (1986) (Mabinlin); and H. Yamashita et al., J. Bio. Chem. 265 (26):15770-15775 (1990) (Curculin). The disclosure of these articles and of all other articles referred to herein are incorporated by reference as if fully set forth herein. These sweet proteins are many times sweeter (for a given weight) than sugar, and are lower in calories.

The search for non-carbohydrate sweeteners has led to an examination of various plants which are known to be sweet. In H. van der Wel, et al., 14 Chemical Senses 75-79 (1989) the authors reported the existence of a sweet protein in Pentadiplandra brazzeana Baillon of about 12,000 Daltons per subunit that they named "Pentadin". This plant is a climbing shrub found in tropical Africa, especially in Gabon. It bears red globular berries of approximately five centimeters in diameter. Under their thick epicarp, these berries contain one to five reniform seeds surrounded by a thick soft layer of red pulp. The fruit used by these authors in their isolation attempt was a smoked and dried form of the plant. Unfortunately, attempts to isolate purer forms of Pentadin were unsuccessful.

As such, it can be seen that the need exists for an improved low calorie sweetener, especially one that is heat stable and has a different temporal sweetness profile from known artificial sweeteners.

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Disclosure Of The Invention

One aspect of the present invention provides the protein "Brazzein" in a form that is essentially free of other Pentadiplandra brazzeana Baillon plant material
5 besides Brazzein. Brazzein is a second, previously unknown, sweet protein isolated from this plant.

In another aspect, the invention provides a composition of Brazzein mixed with another sweetener. Preferably, the other sweetener has a sweetness temporal
10 profile which provides a faster sweetness response than Brazzein.

In another aspect, the invention provides a recombinant host that contains a nucleotide sequence coding for the protein Brazzein.

15 The invention involves the isolation of a new sweet protein from Pentadiplandra brazzeana Baillon, herein named "Brazzein". The protein is about 5,890 Daltons in size and is about 2,000 times sweeter in 2% solution than a 2% sucrose solution. Thermostability tests indicate
20 that this protein has greatly desired heat stability characteristics (thus making it much more suitable for use in connection with baking). In this regard, the protein still tasted very sweet after two hours of incubation at 98°C.

25 Moreover, Brazzein elicited a relatively long lasting sweetness response. Thus, Brazzein is especially suitable to being mixed with other known artificial sweeteners (e.g. acesulfameK) that elicit a quick, but short sweetness response.

30 Brazzein has been sequenced and that amino acid sequence is provided as SEQ ID NO:1. DNA sequences coding for Brazzein are listed at SEQ ID NO:2. A DNA sequence coding for Brazzein which is specially designed for use in an E. coli expression system host is provided

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at SEQ ID NO:3. It will be appreciated that various other nucleotide sequences coding for Brazzein can be developed using conventional techniques based on the SEQ ID NO:2 sequence provided herein. In this regard, for many expression systems there are optimal codons for each amino acid. To optimize Brazzein production in another specific host, one would create a nucleotide sequence encoding Brazzein that incorporated these preferred, optimal codons.

10 The protein of the present invention should also be useful as a flavor enhancer (apart from its sweetness). Further, when mixed with enzymes it is expected to increase their overall thermostability.

Also, the cDNA of the present invention should be suitable to be inserted into hosts other than bacteria. For example, if the cDNA were inserted in a tomato or carrot gene (using what are now well known techniques) a sweet plant would result. See generally M. Chilton, 248 Scientific American 50-59 (1983) (carrot). Similarly, animals could have their genes modified so that foods derived from them would be sweeter. See B. Wang et al., 51 Cancer Res. 2642-2648 (1981) (genes in bovine mammary epithelial cells - modified milk).

25 It is an object of the invention to provide a protein sweetener that is stable when exposed for long periods of time to elevated temperatures.

It is another object to provide a sweetener which has a temporal profile which involves a slow but sustained sweetness response.

30 It is yet another object of the invention to provide Brazzein in large quantities, at low cost, by artificial means.

It is yet another object of the invention to provide a gene coding for Brazzein.

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Still other objects and advantages of the present invention will become apparent from the following description. It should be appreciated that what follows is merely the preferred embodiments of the invention.

- 5 Various other embodiments of the invention are also intended to be within the scope of the claims. Thus, the preferred embodiments should be understood merely as examples of the present invention.

Brief Description Of The Drawings

- 10 Fig. 1 shows a gel filtration chromatography graph; and Fig. 2 shows a cation exchange chromatography graph.

Best Modes For Carrying Out The Invention

Materials

- Our initial source for Brazzein is the fruit of
15 Pentadiplandra brazzeana, a species described as a Tiliaceae by Baillon in 1868, and which is presently classified in the Pentadiplandraceae, a family created by Hutchinson. See J. Hutchinson, Pentadiplandraceae, In Hutchinson, J. and Dalziel, J.M. (eds)., Flora of West
20 Tropical Africa I.461 (1928).

- Under its nutshell-like epicarp, the berries of this plant contain three to five reniform seeds surrounded by a thick soft layer of pulp, which turns from green to red during ripening. Brazzein is distributed in the pulp
25 from epicarp to seed and the content of Brazzein in the ripe fruit appears to be roughly 0.2% to 0.05% by weight.

- The chemicals we used in our experiment were mostly from Sigma Chemical Co., St. Louis, MO. The organic solvents such as acetonitrile, trifluoroacetic acid, 4-
30 vinylpyridine, methanol, ethanol, etc. were from Aldrich;

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Liquid chromatography equipment was from Pharmacia, Pharmacia LKB Biotechnology, Piscataway, NJ.; HPLC system was from Waters, Millipore Corp., Bedford, MA.; Ultrafiltration equipment was from Amico Corp., Danvers, Mass.; and the electrophoresis equipment was from Pharmacia LKB Biotechnology, and Novex Experimental Technology, San Diego, CA.

Brazzein Purification

Protein Extraction

1.0 The soft pulp was separated from the epicarp and seeds and the pulp was extracted with extracting buffer (1:40 w/v) containing 0.05 M sodium acetate, pH 5.0; 0.1 mM dithiothreitol; 5% glycerol; and 0.5% PVP for 30 min. We then filtered the extracting solution using Whatman 1.5 filter paper #54. The obtained filtrate was centrifuged at 10,000 x g for 20 min and the supernatant (containing the protein of interest) was saved.

Salt Precipitation

2.0 Solid ammonium sulfate was added to the supernatant to 30% saturation. We also added a drop of 1 M sodium hydroxide per 10 grams of ammonium sulfate added. After this we store the solution on ice for 30 min. At this point we centrifuged the solution and discarded the precipitate.

2.5 We then added solid ammonium sulfate to the supernatant to 90% saturation, and stored the container at 4°C overnight. An additional precipitate was then collected by centrifugation at 10,000 x g for 40 min. This time the precipitate was kept.

Gel Filtration

3.0 A Sephadex G-75 (Pharmacia) column (2.0 cm X 80 cm) was pre-equilibrated with 0.5 M NaCl; 0.02 M NaAc, pH 5.0. This solution was also used as the elution buffer.

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The second precipitate from the above procedure was dissolved in 0.5 M NaCl in 0.02 M NaAc, pH 5.0. Seven ml of the sample solution was loaded on for each run and the flow rate was controlled at -0.5 ml per min. Fractions were collected and screened for sweetness by tasting. Fractions 35-43 were found to be sweet and were therefore pooled for further purification. Fig. 1 is a chart of the Sephadex column fractions versus absorbance at 280 nm.

When used as a way to estimate the molecular weight of the protein, a G-75 Sephadex column (1.5 X 100 cm) was set up as above, but the flow rate was slowed down to half of the value above. We used Albumin (BSA, 66,000), Carbonic Anhydrase (CA, 29,000), Cytochrome C (CytC, 12,400) and Aprotinin (APR, 6,500) as the molecular weight standards.

Cation Exchange Chromatography

A CM-Cellulose CM 52 (Whatman) column (2.5 cm X 40 cm) was pre-balanced with 0.02 M NaAc, pH 5.0 overnight. The pooled sample (fractions 35-43) from the Sephadex gel filtration was diluted with double distilled water five fold and then loaded on the column directly. We then eluted the protein with a linear gradient of NaCl generated by Gradient Mixer GM-1 (Pharmacia) from 0.05 M to 0.5 M. Fractions comprising the major peak, as detected by absorbance at 280 nm, were pooled and tasted to confirm that the major peak was sweet. Fig. 2 is a diagram of the column fractions versus absorbance at 280 nm.

Anion Exchange Chromatography

A DEAE-Cellulose column was equilibrated with 0.05 M Tris-HCl (pH 8.5). The major peak fraction from the cation exchange column was concentrated and desalted by ultrafiltration with an Amico 8400 ultrafiltration unit with a YM3 membrane. The concentrated and desalted

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sample was loaded on the anion exchange column. The peak ("Brazzein") was eluted with 0.1 M NaCl in 0.05 M Tris-HCl.

Preparative Reverse Phase HPLC

5 As an alternative to the anion exchange chromatography step, a C₄-DYNAMAX RP column (4.6 X 250 mm) can be used in further purification. Elutant A is 0.1% Trifluoroacetic acid (TFA) and elutant B is 80% acetonitrile in 0.086% TFA. Linear gradient elution was
10 used here and flow rate was controlled at 1 ml/min.

Cysteine Alkylation

To study Brazzein conformation, the protein's cysteines can be alkylated. Alkylation with 4-vinylpyridine (see C.S. Fullmer, Anal. Biochem. 142, 336-
15 339 (1984)) can be done in 0.5 M N-ethylmorpholine, pH 8.3 with 6 M Guanidinium HCl. The protein is reduced with 25 mM DTT first for four hours, then 8 ul 4-VP can be added per 100 nmole protein and incubated under room temperature overnight.

20 In the alternative, one can alkylate with iodoacetamide. The reaction will be similar except that 8 M urea was used instead of 6 M Guanidinium HCl.

When alkylated Brazzein was electrophoresed, no subunit aggregation was observed. This phenomenon
25 strongly suggests that aggregation happens through disulfide bonds.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

To further characterize the protein's molecular
30 weight and its relative purity or abundance, a modified Tricine-SDS-PAGE system (see H. Schagger et al., Anal. Biochem. 166:368-379 (1987)) was used in a Novex precasted gel cassette. The composition of the separation gel, the spacer gel, and the stacking gel was
35 16.5%T, 3%C, 10%T, 3%C, and 4%T, 3%C. "T" is the weight

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percentage of total monomer (acrylamide plus cross-linker, in grams per 100 ml) and "C" is the proportion of cross-linker as a percentage of total monomer in the gel. The molecular weight was calculated according to 3 groups of molecular weight standards. Generally, the alkylated or unalkylated protein samples were treated for about 30 min at 50°C in 4% SDS, 12% glycerol (w/v), 50 mM Tris, 0.01% Bromophenol Blue adjusted with HCl to pH 6.8 with or without 2% mercaptoethanol (v/v) depending on the circumstances. The electrophoresis was carried out under the condition of 5V/cm. When electrophoresis is completed, the gel was stained in Comassie Brilliant Blue R-250 staining Solution A (30 mg dye in 1 L of fixative solution of 65% water, 25% isopropanol and 10% acetic acid) for 15 min, and then changed to Solution B (30 mg dye in 1 L of staining solution of 80% water, 10% isopropanol and 10% acetic acid). The gel was left in Solution B overnight. The molecular weight was estimated at a value very close to the theoretical molecular weight of SEQ ID NO:1.

UV Absorption Spectrum

The UV/Vis Absorption spectrum of Brazzein was recorded on a Perkin-Elmer 559A UV-VIS spectrophotometer with 1 mg/ml Brazzein in water. The wavelength range is from 190 nm to 700 nm, and the scanning speed is 20 nm/min. The absorption exhibited confirmed that what we had isolated was a protein.

Thermostability Assays

A thermostability assay was done (2 hours at 98°C) at a concentration of 0.5 mg/ml Brazzein at the following different pH levels: 2 (Glycine-HCL buffer); 4 (Acetate buffer); 6 (Phosphate buffer); and 8 (Tris-HCL buffer). All of the buffers were in concentration of 50 mM. After the incubations, the samples were cooled down to room temperature and the sweetness of the samples was tasted

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with a comparison to the original protein solution. The protein exhibited excellent thermostability.

Amino Acid Composition Analysis

5 A preliminary amino acid analysis was done by using the Pico-Tag system of Waters. The purified protein sample was hydrolyzed in 6N HCl under 110°C for 24 hours first and the derivatization was done by using PITC according to the standard procedure. See S.A. Cohen et al., The Pico-Tag® Method, A Manual Of Advanced
10 Techniques For Amino Acid Analysis, Millipore Co. (1989). The amino acid composition indicated a high incidence of lysine.

Protein Sequence Determination

15 The purified Brazzein was then sequenced on an ABI 470A Protein Sequencer. The sequence was obtained from 400 picomoles observed sample.

In our preferred experiment we dissolved the Brazzein in 8 M urea, 0.4 M NH_4HCO_3 with 5 mM dithiothreitol; incubated at 50°C for 15 min; then added
20 iodoacetamide to 10 mM after cooling to room temperature; and then incubated for another 15 min. Finally, we diluted the urea in whole reaction system to 2 M and added trypsin (TPCK treated) in ratio of 1 to 50 of Brazzein. We then incubated the reaction at 37°C for 24 hours and
25 stopped the reaction by injecting directly onto a reverse phase HPLC system. Trypsin-digested peptides were isolated by manual collection (see J.E. Shively, Reverse Phase HPLC Isolation and Microsequencing Analysis in
30 "Methods of Protein Microcharacterization, (Humana Press 1986).

Brazzein Sequence Analysis

In order to confirm the degree of uniqueness of Brazzein, BLAST and FASTA in the Gen Bank were used to carry out a homology search. See S.F. Altschul et al.,
35 J. Mol. Biol. 215:403-410 (1990); W.R. Pearson et al.,

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PNAS U.S.A. 85:2444-2448 (1988). NBRF/PIR V.31 and Swiss-Prot V.21 were used as searching databases. No significant sequence homology was found.

As indicated by SEQ ID NO:1, nearly one seventh of the amino acid composition of Brazzein is lysine, an essential amino acid. Thus, not only does this sweetener have desirable heat stability and temporal characteristics, it is a possible source of lysine.

Note that Brazzein is so sweet that only a very small amount of it will be needed to sweeten coffee, tea, or the like. For such uses, it can be blended with a bulky filler (e.g. lactose) to give the user a feeling of perceived value.

Expression Of cDNA

If one desires to produce the sweetener artificially, one could synthesize a SEQ ID NO:2 sequence such as Sequence ID NO:3 by combining standard cloning and an automated synthesizer techniques (e.g. 380 B ABI DNA synthesizer). The gene could then be cloned in one of the well known E. Coli expression vectors such as pGEMEX®-1 (Promega), at the T7 gene 10 site (using conventional techniques). The vector could then be inserted in host JM109 (DE3) (Promega), with expression in the usual manner.

Brazzein could then be harvested as part of a fusion protein. If desired, modifications could be made in the usual way to reduce or eliminate undesired portions of the fusion proteins.

Industrial Applicability

The present invention provides a sweet protein that could be added to consumable items to impart a sweet flavor.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Hellekant, Bengt Göran
Ming, Ding (n.m.i.)
 - (ii) TITLE OF INVENTION: BRAZZEIN SWEETENER
 - (iii) NUMBER OF SEQUENCES: 3
 - (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Carl R. Schwartz, Quarles & Brady
 - (B) STREET: 411 East Wisconsin Avenue
 - (C) CITY: Milwaukee
 - (D) STATE: Wisconsin
 - (E) COUNTRY: U.S.A.
 - (F) ZIP: 53202
 - (v) COMPUTER READABLE FROM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version 1.25
 - (vi) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Schwartz, Carl R.
 - (B) REGISTRATION NUMBER: 29,437
 - (C) REFERENCE/DOCKET NUMBER: 96-296-9186-1
 - (vii) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (414) 277-5715
 - (B) TELEFAX: (414) 277-5591
- (2) INFORMATION FOR SEQ ID NO:1:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 52 amino acids
 - (B) TYPE: amino acid
 - (C) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: protein
 - (iii) HYPOTHETICAL: NO
 - (iv) ANTI-SENSE: NO
 - (vi) ORIGINAL SOURCE: Pentadiplandra brazzeana Baillon

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Asp Lys Cys Lys Lys Val Tyr Glu Asn Tyr Pro Val Ser Lys Cys Cln
1 5 10 15

Ser Gly Glu Cys Phe Tyr Asp Glu Lys Arg Asn Leu Gln Cys Ile Gly
35 40 45

Asp Tyr Cys Gly
50

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 156 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GAYAARTGYA	ARAARGTNTA	YGARAAYTAY	CCNGTNWSNA	ARTGYCARYT	NGCNAAYCAR	60
TGYAAYTAYG	AYTGYAARYT	NGAYAARCAY	GCMNMGWSNG	GNGARTGYTT	YTAYGAYGAR	120
AARMGNAAYY	TNCARTGYAT	HGGNGAYTAY	TGYGGN			156

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 156 base pairs
- (B) TYPE; nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GACAAATGCA	AAAAAGTTTA	CGAAACTAC	CCGGTTCCA	AATGCCAGCT	GGCTAACCAG	60
TGCAACTACG	ACTGCAAACT	GGACAAACAC	GCTCGTTCCG	GTGAATGCTT	CTACGACGAA	120
AAACGTAACC	TGCAGTGCAT	CGGTGACTAC	TGCGGT			156

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Claims

We claim:

1. The protein Brazzein which is essentially free of Pentadiplandra brazzeana Baillon plant material other than Brazzein.
2. A composition comprising the protein Brazzein mixed with another sweetener obtained from other than Pentadiplandra brazzeana Baillon.
3. The composition of claim 2, wherein the other sweetener has a sweetness temporal profile which provides a faster sweetness response than Brazzein.
4. A recombinant host capable of expressing the protein Brazzein.
5. Brazzein that has been produced by the host of claim 4.
6. A DNA sequence coding for the protein Brazzein that is not in a Pentadiplandra brazzeana Baillon cell.
7. A recombinant vector having the DNA sequence encoding the protein Brazzein.
8. A sweet protein containing an amino acid sequence essentially the same as that shown in SEQ ID NO:1, the protein being essentially free of Pentadiplandra brazzeana plant material other than Brazzein.
9. The protein of claim 8, wherein the protein is Brazzein.

10. A composition comprising the protein of claim 8 mixed with another sweetener obtained from other than *Pentadiplandra brazzeana* Baillon.

11. A sweet protein containing an amino acid sequence essentially the same as that shown in SEQ ID NO:1, wherein the protein has been produced recombinantly and is essentially free of *Pentadiplandra brazzeana* plant material other than Brazzein.

12. The protein of claim 11 wherein the protein comprises residues 1-48 of SEQ ID NO:1.

13. The sweet protein of claim 11, wherein the protein comprises a sequence of at least 52 amino acid residues, the sequence having a fragment consisting essentially of residues 1-47 of SEQ ID NO:1.

14. The sweet protein of claim 11, wherein the sequence is at least 52 amino acid residues of which at least 48 amino acid residues consist essentially of those residues in SEQ ID NO:1.

Project n°

Project n°

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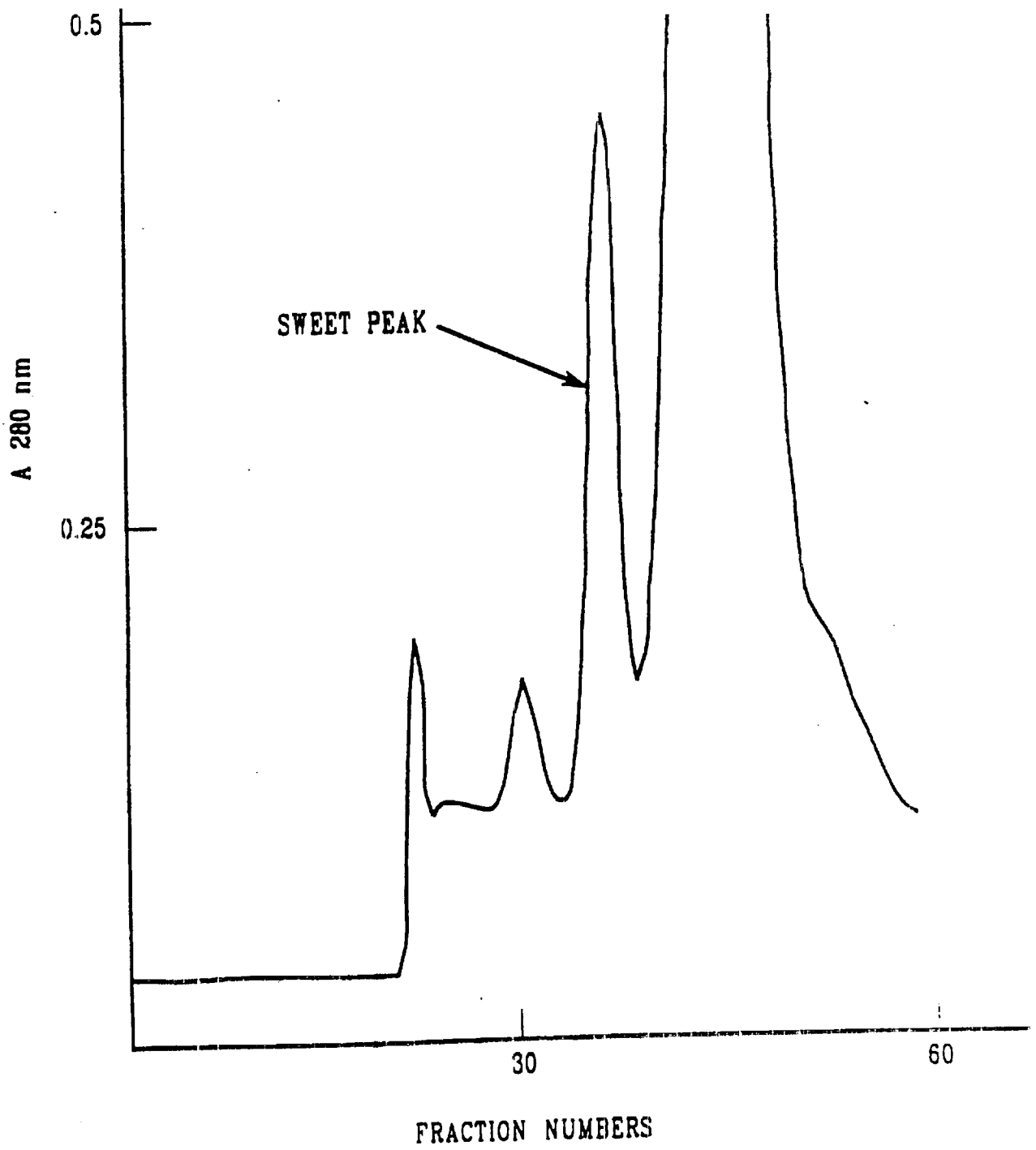


FIG. 1

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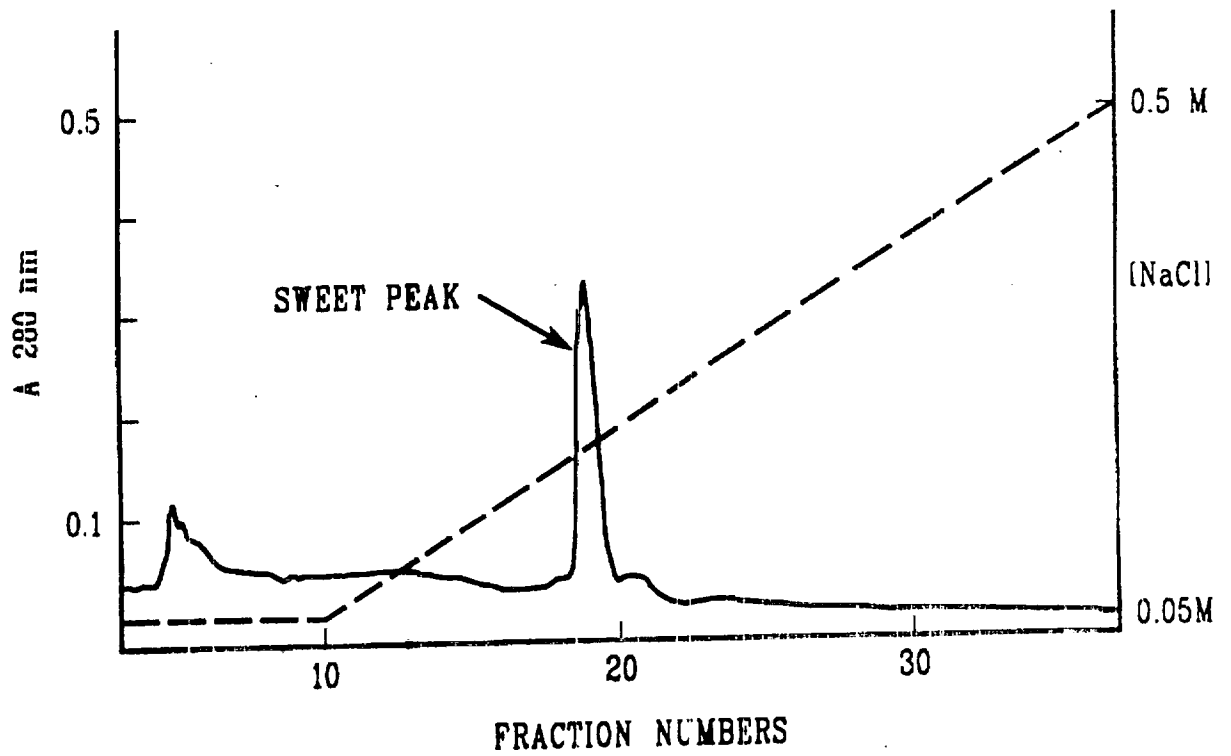


FIG. 2