Solid-Phase Synthesis and Crystallization of [Asn²², Gln²⁵, Asn²⁶]-A-Chain-[Asn⁴⁹, Glu⁵⁰]-B-Chain-Monellin, an Analogue of the Sweet Protein Monellin

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The sweet protein monellin consists of two noncovalently associated polypeptide chains, the A chain of 44 amino acid residues and the B chain of 50 residues. The [Asn²², Gln²⁵, Asn²⁶]-A chain, an analogue of the A chain, was synthesized by the stepwise Fmoc-solid-phase method in an overall yield of 13.4%. The synthetic A chain analogue was combined with the [Asn⁴⁹, Glu⁵⁰]-B chain, which was left over from a previous work, to give [Asn²², Gln²⁵, Asn²⁶]-A-chain-[Asn⁴⁹, Glu⁵⁰]-B-chain-monellin in a yield of 26.2%. This synthetic monellin analogue was approximately 550 times sweeter than sucrose. Changing the carboxyl groups of Asp²², Glu²⁵ and Asp²⁶ of the A chain to amide groups significantly decreased the sweetness potency. Crystallization was performed by a vapor diffusion method.

In an attempt to deduce the mode of interaction between sweet peptides and the receptor, we have synthesized a number of sweet peptide analogues.¹⁾ However, it is difficult to deduce this mode of the interaction, since peptides can assume a variety of conformations in water. We, therefore, began efforts to employ sweet proteins as tools for studying the mode of interaction, since proteins are generally of much-reduced conformational mobility. The binding site of a sweet protein may be determined by studying structure–taste relationships of the sweet protein. The three-dimensional structure of the binding site may be then determined by an X-ray analysis of the sweet protein. The mode of interaction between the binding site and the receptor can be deduced from these results. As the tool, we chose the sweet protein monellin.^{2,3)}

The sweet protein monellin has been isolated from the fruit of the West African plant, *Dioscoreophyllum cumminsii* (Stapf) Diels, and is 3000 times sweeter than sucrose.^{2,3)} Monellin consists of two noncovalently associated polypeptide chains, the A chain of 44 amino acid residues and the B chain of 50 residues.

Two different primary structures had been reported for each of them.^{4–6)} Very recently, we determined the amino acid sequences of the A and B chains.⁷⁾

As a first step to study the structure–taste relationship of monellin, in our previous paper,⁸⁾ we synthesized monellin to the structure proposed by Frank and Zuber.⁵⁾ The synthetic monellin was termed [Asn⁴⁹, Glu⁵⁰]-B-chain-monellin, since the amino acid residues at positions 49 and 50 were different from those of natural monellin, in which they were Glu and Asn, respectively.⁷⁾

In this paper, we synthesized the A chain to the structure proposed by Hudson and Biemann.⁶⁾ This peptide was termed [Asn²², Gln²⁵, Asn²⁶]-A chain, since the amino acid residues at positions 22, 25 and 26 were different from those of natural monellin, in which they were Asp, Glu and Asp, respectively. It was demonstrated that Asp²² (A chain) bound to Leu³⁵ (A chain) and Arg³⁶ (A chain) through hydrogen bonding, and the Glu²⁵ (A chain) bound to Arg³² (A chain) through ionic bonding^{9,10)} (for the sequence, see ref. 7). In the synthesis, Asn was used for Asx³⁸ in the

proposed sequence for the A chain,⁶⁾ because position 38 was assigned to Asn.⁷⁾

Peptide synthesis was carried out manually by the stepwise solid-phase method with a semi-automated peptide synthesizer, using the base-labile fluoren-9-ylmethoxycarbonyl (Fmoc) group¹¹⁾ for protecting the α -amino group, and such acid-labile groups as the *tert*-butyl (Bu^t) for the hydroxyl and carboxyl groups, the 4-methoxy-2,3,6-trimethylbenzenesulfonyl (Mtr)¹²⁾ for the guanidino group of Arg, the *tert*-butoxycarbonyl (Boc) for the ϵ -amino group of Lys, and the 4,4'-dimethoxybenzhydryl (Mbh)¹³⁾ for the amide groups of Asn and Gln. The peptide was assembled on a *p*-alkoxybenzyl alcohol resin.¹⁴⁾

The protection scheme for the stepwise solid-phase synthesis of the [Asn²², Gln²⁵,

Fig. 1. Protection Scheme for the Stepwise Solid-Phase Synthesis of the [Asn²², Gln²⁵, Asn²⁶]-A Chain.

Asn²⁶]-A chain is shown in Fig. 1. Fmoc-Pro-Pro-OCH₂-C₆H₄-OCH₂-resin was synthesized by coupling Fmoc-Pro-Pro-OH with H-Pro-OCH₂-C₆H₄-OCH₂-resin as described in our previous paper.⁸⁾ The Fmoc group was removed with 50% piperidine in *N,N*-dimethylformamide (DMF). Elongation of the peptide chain was carried out by the dicyclohexylcarbodiimide (DCC)/1-hydroxybenzotriazole (HOBt) method¹⁵⁾ in CH₂Cl₂-DMF or in *N*-methyl-2-pyrrolidone (NMP), or by the symmetrical anhydride method. The coupling reaction and deprotection of the Fmoc group were monitored by the Kaiser test.¹⁶⁾

After the last coupling step, the Fmoc group was removed. The peptide-resin was treated with CH₂Cl₂-anisole-thiophenol-trifluoroacetic acid (TFA), and the resulting peptide was further treated with thiophenol-thioanisole-TFA (Fig. 2A), and then purified by preparative reversed-phase high-performance liquid chromatography (HPLC) on a C₁₈ column to give the pure A chain (Fig. 2B) in an overall yield of 13.4%, based on the amino acid content of the starting Fmoc-Pro-resin.

The purity of the peptide was further confirmed by analytical HPLC, fast-atom bombardment (FAB) mass spectrometry, a quantitative amino acid analysis after hydrolyzing in constant-boiling HCl containing 1% phe-

Fig. 2. HPLC on an Analytical Reversed-Phase Inertsil ODS-2 5 μ m C₁₈ Column (4.6 $\times$ 250 mm) of the Synthetic [Asn²², Gln²⁵, Asn²⁶]-A Chain Directly after Deprotection and Cleavage (A), and after Purification (B).

Solvent A, 0.05% aqueous TFA; solvent B, acetonitrile containing 0.05% TFA. The sample was eluted with a linear gradient of 20% B to 50% B obtained in 30 min at a flow rate of 1.0 ml/min. The elution profile was monitored at 210 nm.

nol for 24 hr at 110°C, and by a sequence analysis with an automatic Edman degradation method. HPLC analysis of the peptide gave satisfactory results (Fig. 2B). An FAB-mass spectrometric analysis of the A chain gave a protonated monoisotopic molecular ion at m/z 5245.6 for a calculated value of m/z 5245.8 (Fig. 3). For this spectrum, the observed isotopic distributions were in good agreement with the patterns calculated from the elemental compositions. An amino acid analysis of the peptide gave the expected values. Sequencing data for the intact synthetic A chain and a tryptic peptide from its tryptic digest fully supported

the expected sequence (data not shown).

Combination of the synthetic A chain analogue and the [Asn⁴⁹, Glu⁵⁰]-B chain⁸⁾ (Fig. 4A), and subsequent HPLC purification gave [Asn²², Gln²⁵, Asn²⁶]-A-chain-[Asn⁴⁹, Glu⁵⁰]-B-chain-monellin (Fig. 4B) in a yield of 26.2%. In the HPLC procedure using a TSKgel Phenyl-5PW column, the synthetic monellin, and the A chain and B chain analogues were significantly adsorbed on the solid support. A quantitative amino acid analysis of the monellin analogue gave satisfactory results. This monellin analogue was approximately 550 times sweeter than sucrose, and its taste property was very similar to that of [Asn⁴⁹, Glu⁵⁰]-B-chain-monellin,⁸⁾ which was 4000 times as sweet as sucrose.

The monellin analogue was found to be susceptible to the HPLC conditions. This fact may suggest that Asp²² and Glu²⁵ of the A chain participated in intramolecular binding, as was reported by Kim *et al.*¹⁰⁾ The peak eluting at 5.6 min (Fig. 4B) was collected and lyophilized to give a powder, which was not sweet. An FAB-mass spectrometric analysis of the powder gave average molecular ions at m/z 5249.4, 5851.5 and 5835.7, vs. the calculated values of m/z 5249.1, 5851.7 and 5835.7 for the respective [Asn²², Gln²⁵, Asn²⁶]-A, [MetO⁴², Asn⁴⁹, Glu⁵⁰]-B and [Asn⁴⁹, Glu⁵⁰]-B chains. The sulfur atom of Met⁴² of the B chain was susceptible to air oxidation to partly form the

Fig. 3. FAB-Mass Spectrum of the Synthetic [Asn²², Gln²⁵, Asn²⁶]-A Chain.

Fig. 4. HPLC on a TSKgel Phenyl-5PW Column (7.5×75 mm) of the Synthetic [Asn²², Gln²⁵, Asn²⁶]-A-Chain-[Asn⁴⁹, Glu⁵⁰]-B-Chain-Monellin (after 20 hr, A) Prepared by Mixing the [Asn²², Gln²⁵, Asn²⁶]-A Chain and [Asn⁴⁹, Glu⁵⁰]-B Chain (1:1, w/w), and after Purification (B).

Solvent A, 0.1% aqueous TFA; solvent B, acetonitrile containing 0.1% TFA. The sample was eluted with a linear gradient of 10% B to 60% B obtained in 10 min at a flow rate of 1.0 ml/min. The elution profile was monitored at 277 nm.

Fig. 5. Crystals of the Synthetic [Asn²², Gln²⁵, Asn²⁶]-A-Chain-[Asn⁴⁹, Glu⁵⁰]-B-Chain-Monellin Grown by the "Hanging Drop" Vapor Diffusion Method and Photographed under a Polarized Microscope within a Crystallizing Well.

corresponding sulfoxide. When the mixture was analyzed by HPLC under the conditions described in Fig. 2, the [Asn²², Gln²⁵, Asn²⁶]-A, [MetO⁴², Asn⁴⁹, Glu⁵⁰]-B and [Asn⁴⁹, Glu⁵⁰]-B chains were eluted at 21.5, 28.3 and 29.6 min in a ratio of approximately 70:6:24, respectively. The peak eluting at 5.6 min contained mostly the A chain analogue.

To detect the rest of the B chain analogue, a large quantity of the synthetic monellin analogue was applied to a TSKgel Phenyl-5PW column (data not shown). A very broad peak eluting at 14.6 min was collected and lyophilized to give a powder. When the powder was analyzed by HPLC under the conditions described in Fig. 2, it was eluted at 29.5 min. An FAB-mass spectrometric analysis of this gave an average molecular ion at m/z 5835.1 vs. the calculated value of m/z 5835.7 for the [Asn⁴⁹, Glu⁵⁰]-B chain. Thus, the peptide eluted at 14.6 min was determined to be the [Asn⁴⁹, Glu⁵⁰]-B chain, which had been formed by dissociation during the HPLC procedure. When the A and B chain analogues were separately analyzed by HPLC under the conditions described in Fig. 4, the former was eluted at 5.2 min, and the latter was eluted at 8.2, 10.1 and 14.9 min as broad peaks. Such chromatographic behavior of proteins is well known.¹⁷⁾ Thus, the B chain analogue eluting

at 5.6 min (Fig. 4B) might have been loosely associated with the A chain analogue, or its conformation was different from that of the B chain analogue eluting at 14.6 min.

Changing the carboxyl groups of Asp²², Glu²⁵ and Asp²⁶ to the amide groups significantly decreased the sweetness potency and stability under the HPLC conditions. However, it could not be confirmed whether some of these residues participated in binding to the receptor. A further study on replacing the amino acid residues is in progress.

The synthetic monellin analogue was crystallized by the "hanging drop" vapor diffusion method (Fig. 5).

Experimental

The protected amino acids were obtained from Kokusan Chemical Works Ltd., Bachem Feinchemikalien AG or Novabiochem AG. Fmoc-Pro-OCH₂-C₆H₄-OCH₂-resin was purchased from Bachem Feinchemikalien AG. Fmoc-Pro-Pro-OH was left over from a previous work.⁸⁾ All the solvents were of reagent or HPLC grade. The peptide synthesis was carried out manually by the stepwise solid-phase method with a semi-automated Labortec SP 640 peptide synthesizer. Coupling and deprotection were performed at room temperature unless otherwise stated. HPLC was carried out on a Waters M600 instrument under linear gradient conditions [C₁₈ reversed-phase columns, Inertsil ODS-2, 5 μ m, 4.6 $\times$ 250 mm, Gasukuro Kogyo (for analytical HPLC) and Inertsil ODS, 5 μ m, 16.2 $\times$ 250 mm,

Gasukuro Kogyo (for preparative HPLC), UV detection at 210 nm; TSKgel Phenyl-5PW column, 7.5 × 75 mm, Tosoh (for the monellin analogue), UV detection at 277 nm]. Peptide hydrolyses were performed in constant-boiling HCl containing 1% phenol for 24 hr (for the A chain analogue), and for 24 and 96 hr (for the monellin analogue) at 110°C in sealed ampules under high vacuum. Quantitative amino acid analyses were carried out on either a Hitachi 835 or a Beckman 6300 E amino acid analyzer. FAB-mass spectra were taken on a JEOL JMS HX110/HX110 tandem mass spectrometer. Sequencing was performed on a 470A automated protein sequencer from Applied Biosystems.

Synthesis of the [Asn²², Gln²⁵, Asn²⁶]-A chain. In the following synthesis cycle, Fmoc-Pro-Pro-Pro-OCH₂-C₆H₄-OCH₂-resin was prepared by coupling Fmoc-Pro-Pro-OH with H-Pro-OCH₂-C₆H₄-OCH₂-resin, because treatment of Fmoc-Pro-Pro-OCH₂-C₆H₄-OCH₂-resin with piperidine resulted in elimination of the dipeptide from the resin support. Fmoc-Pro-OCH₂-C₆H₄-OCH₂-resin (0.65 mmol/g, 192 mg, 0.125 mmol) was suspended in DMF (15 ml) in a 50-ml reaction vessel and shaken for 15 min to swell the resin.

The following synthesis cycle was used:

(a) Fmoc cleavage: (i) shaking in DMF (15 ml) for 1 min to swell the resin; (ii) repeated treatment with 50% piperidine in DMF (15 ml) while shaking; the reaction period was 3 + 10 min for residues 44 and 42–29, 3 + 15 min for residues 28–17, and 3 + 20 min for residues 16–1.

(b) Washing: (i) DMF (15 ml, four times, 30 sec each); (ii) isopropanol (15 ml, twice, 1 min each); cleavage was monitored by the Kaiser test.

(c) Coupling: washing with DMF (10 ml, twice, 1 min each); (i) residues 42–43 (Fmoc-Pro-Pro-OH), 41–28, 24, 21, 19–13, 11 and 10 were coupled by the DCC/HOBt method, in which 1 M DCC (0.413 ml) in CH₂Cl₂ was added to a solution of an appropriate Fmoc-amino acid derivative (0.375 mmol) and HOBt (0.45 mmol) in a mixture of DMF (1 ml) and CH₂Cl₂ (9 ml), the mixture being shaken for 90 min at room temperature. For residues 30, 28 and 10, the monitoring data from the Kaiser test indicated that a second coupling was necessary. Thus, for these three residues, a second coupling was carried out by the DCC/HOBt (in NMP) method, in which an appropriate Fmoc-amino acid derivative (0.375 mmol) and HOBt (0.45 mmol) were dissolved in NMP (10 ml) and then treated with 1 M DCC (0.413 ml) in CH₂Cl₂ while shaking for 3 hr; (ii) residues 27–25 and 7–4 were coupled by the DCC/HOBt (in NMP) method under the conditions just described; (iii) residues 23, 22, 20, 12, 9, 8 and 3–1 were coupled by the symmetrical anhydride method, in which the anhydrides of Fmoc-amino acid derivatives were prepared by the reaction of an appropriate Fmoc-amino acid derivative (0.75 mmol) with 1 M DCC (0.375 ml, in CH₂Cl₂) in a mixture of DMF (5 ml) and CH₂Cl₂ (5 ml) at room temperature for 15 min. Coupling was performed

for 90 min. For residues 9 and 8, the monitoring data from the Kaiser test indicated that a second coupling was necessary. Thus, these two residues were further coupled by the DCC/HOBt (in NMP) method under the conditions already described. Coupling was performed for 2 hr while shaking, and then the mixture was kept standing overnight.

(d) Washing: (i) DMF (15 ml, once, 1 min); (ii) isopropanol (15 ml, twice, 1 min each); (iii) coupling was monitored by the Kaiser test.

After the last coupling step, the Fmoc group was cleaved according to procedures (a) and (b). The peptide–resin was washed with CH₂Cl₂ (15 ml, three times, 1 min each), and then treated with CH₂Cl₂–anisole–thiophenol–TFA (12.3:2.0:0.7:20.0, v/v, 35 ml) for 2 hr while shaking at room temperature. After filtering, the filtrate was concentrated to a small volume under reduced pressure at a bath temperature of 50°C. The peptide was precipitated with ether, filtered off, dried and weighed (730 mg). The peptide thus obtained was further treated with thiophenol–thioanisole–TFA (0.5:1.5:13.5, v/v, 15.5 ml) for 5 hr while stirring at room temperature. A sufficient amount of ether was added to the reaction mixture. The resulting precipitate was collected by filtration, dried and weighed (820 mg). The crude peptide (820 mg) was dissolved in 8.5 ml of 58.8% TFA and filtered. The solution (400 μl) was loaded onto a preparative HPLC column, and eluted with 0.05% aqueous TFA and acetonitrile containing 0.05% TFA under a linear gradient of 20% to 33% acetonitrile obtained in 30 min at a flow rate of 9 ml/min. The analytical HPLC elution profile is shown in Fig. 2A. This procedure was carried out 22 times to purify the whole amount of the peptide (820 mg), the fractions being checked by analytical HPLC. The fractions of the main peak were collected, pooled, concentrated to a small volume under reduced pressure, and lyophilized to give 102.6 mg of the A chain analogue. The peptide (102.6 mg) was dissolved in 2 ml of 20% aqueous acetonitrile containing 0.05% TFA. The solution (200 μl) was loaded onto the same column and further purified under the same conditions as those just described. This procedure was carried out 10 times to purify the whole amount of the peptide (102.6 mg). The pure fractions were collected, pooled, concentrated under reduced pressure, lyophilized, and dried over NaOH pellets *in vacuo*. Overall yield, 88.2 mg (13.4%). The analytical HPLC elution profile is shown in Fig. 2B.

Amino acid analysis, numbers in parentheses being theoretical values: Asp, 4.03 (4); Thr, 0.97 (1); Ser, 1.81 (2); Glu, 3.82 (4); Pro, 3.82 (4); Gly, 2.96 (3); Ala, 2.08 (2); Val, 1.93 (2); Ile, 1.92 (2); Leu, 4.09 (4); Tyr, 4.76 (5); Phe, 2.04 (2); Lys, 3.90 (4); Arg, 4.81 (5).

Combination of the synthetic [Asn²², Gln²⁵, Asn²⁶]-A chain and [Asn⁴⁹, Glu⁵⁰]-B chain. The synthetic A chain analogue (2.33 mg) was dissolved in 0.1% acetic acid (291 μl). The synthetic B chain analogue (2.17 mg)⁷⁾ was then dissolved in this solution (271 μl). The mixture was

stirred for 1 min with a mixer under an atmosphere of nitrogen, and then kept standing for 20 hr at room temperature. The HPLC elution profile of the mixture is shown in Fig. 4A. The mixture (90 μ l) was loaded onto a TSKgel Phenyl-5PW column and purified by linear gradient conditions (see the legend to Fig. 4A). The procedure was carried out three times to purify the whole amount of the protein, and the fractions of the main peak were collected, pooled and lyophilized. The protein thus obtained was dissolved in water (100 μ l), shaken for 2 hr at room temperature, and then lyophilized to give the desired protein, which was dried over NaOH pellets *in vacuo*. Yield, 1.35 mg (26.2%). The HPLC elution profile of the protein is shown in Fig. 4B.

Amino acid analysis (hydrolyzed for 24 hr; 96 hr for the Ile-Ile bond*), numbers in parentheses being theoretical values: Asp, 10.04 (10); Thr 3.88 (4); Ser, 1.80 (2); Glu, 11.67 (12); Pro, 6.00 (6); Gly, 8.04 (8); Ala, 3.02 (3); Val, 3.80 (4); Met, 0.98 (1); Ile*, 7.64 (8); Leu, 6.29 (6); Tyr, 7.02 (7); Phe, 5.14 (5); Lys, 9.31 (9); Arg, 7.31 (7). Trp and Cys were not determined.

Crystallization of the synthetic [Asn²², Gln²⁵, Asn²⁶]-A-chain-[Asn⁴⁹, Glu⁵⁰]-B-chain-monellin. Crystallization was carried out by a combination of the methods described by Tomlinson and Kim,¹⁸⁾ and by Wlodawer and Hodgson,¹⁹⁾ using the "hanging drop" vapor diffusion technique. The dishes used were Linbro tissue culture trays with 24 separate wells (Flow Laboratories Inc., U.S.A.). Each well was filled with 1 ml of a precipitant solution (aqueous polyethylene glycol), and a protein solution (10 μ l) was placed on a clean, siliconized cover glass with a pipette. The rim of each well was coated with silicone grease, and the cover glass was put on the well in such a way that a droplet faced the solution. In a typical run, a solution containing the protein (4 mg/ml) in a 14% (w/w) solution of polyethylene glycol and 10 mM phosphate buffer at pH 7.2 was equilibrated with a 28% (w/w) solution of polyethylene glycol and 10 mM phosphate buffer at 4°C. Crystals appeared after 13 days (Fig. 5). No further attempt was made to obtain better-shaped crystals.

Sweetness evaluation. The sweetness value of the synthetic [Asn²², Gln²⁵, Asn²⁶]-A-chain-[Asn⁴⁹, Glu⁵⁰]-B-chain-monellin was organoleptically determined by a panel of five untrained people. The evaluation was carried out at concentrations near the threshold value to avoid confusion arising from the persistently lingering sweet taste characteristic of protein sweeteners. Thus, the sweetness evaluation was carried out by matching a threshold concentration of the synthetic protein with that (0.6%, w/v) of sucrose. The synthetic [Asn²², Gln²⁵, Asn²⁶]-A-chain-[Asn⁴⁹, Glu⁵⁰]-B-chain-monellin (0.60 mg) was dissolved in water (10 ml), this concentration corresponding to a 0.006% (w/v) solution of the synthetic monellin

analogue. This solution was diluted with water to several concentrations (4, 5, 5.5 and 6 times), and the sweetness intensity of each was compared with that of the 0.6% sucrose solution. The solutions from the 4- and 5-fold diluted solutions were sweeter than the 0.6% sucrose solution. The sweetness intensity of the 5.5-fold dilution was almost equal to that of 0.6% sucrose, and the 6-fold dilution was judged faintly sweet or not sweet. These results indicate that the synthetic monellin analogue was approximately 550 times sweeter than sucrose, although no attempt was made to quantitate the sweetness potency.

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