Solid-Phase Synthesis of Crystalline Monellin, a Sweet Protein

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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 B chain was synthesized by the stepwise Fmoc solid-phase method in an overall yield of 6.2%. The synthetic B chain was combined with the synthetic A chain, which was left over from a previous work, to give monellin in a yield of 25.7%. The synthetic monellin was approximately 4000 times as sweet as sucrose, while the previously synthesized [Asn49, Glu50] B-chain monellin was also approximately 4000 times as sweet as sucrose. Exchanging Glu49 and Asn50 in the B chain did not affect the sweetness potency. Crystallization was performed by a vapor diffusion method.

The sweet protein, monellin, has been isolated from the fruit of the West African plant, Dioscoreophyllum cumminsii (Stapf),1,2) and 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 have been reported for each of them.3-5) Recently, the primary structure of monellin was unambiguously determined by us.6)

In an attempt to delineate the binding site(s) of monellin to the receptor by means of a structure–activity relationship, we previously synthesized two monellin analogues, [Asn49, Glu50] B-chain monellin7) and [Asn22, Gln25, Asn26] A-chain [Asn49, Glu50] B-chain monellin,8) which were 4000 and 550 times as sweet as sucrose, respectively. In this paper, we report the synthesis of monellin with the natural amino acid sequence as the standard compound in our series of syntheses.

The peptide synthesis was performed manually by the stepwise solid-phase method with a semi-automated peptide synthesizer, using the fluoren-9-ylmethoxycarbonyl (Fmoc) strategy9) as described previously.7,8)

The protection scheme for synthesizing the B chain is shown in Fig. 1. The Fmoc group of Glu49, which was removed with 20% piperidine in DMF. Elongation of the peptide chain was performed by the dicyclohexylcarbodiimide (DCC)/1-hydroxybenzotriazole (HOBT) method12) 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.13)

After the last coupling step, the peptide resin was treated with CH2Cl2-anisole-1,2-ethanedithiol-trifluoroacetic acid (TFA), and then a sufficient amount of ether was added to the reaction mixture. The resulting peptide was further treated with m-cresol-1,2-ethanedithiol-thioanisole-TFA, and then trimethyl-

![Fig. 1. Protection Scheme for the Stepwise Solid-Phase Synthesis of the B Chain of Monellin.](image-url)
silylbromide (TMSBr)\textsuperscript{14} was added to the reaction mixture. The crude B chain was precipitated with ether, and treated with 2-mercaptoethanol and 1 m NH$_4$F\textsuperscript{14} in water (Fig. 2A). As can be seen in Fig. 2A, several major peaks appeared. These peaks were separately collected by preparative reversed-phase high-performance liquid chromatography (HPLC) on a C$_{18}$ column, concentrated and lyophilized to give peptides. Their fast atom bombardment (FAB) mass spectrometric analyses revealed that the main peak (indicated by an arrow) contained the B chain; the molecular weights of the other peptides were less than that of the B chain (data not shown). Thus, the peptide obtained from the main peak was further purified by preparative reversed-phase HPLC to give the B chain (Fig. 2B) in an overall yield of 6.2%.

The purity of the peptide was further confirmed by analytical HPLC, FAB mass spectrometry and a quantitative amino acid analysis after hydrolyzing in constant-boiling HCl containing 1% phenol for 24 and 96 hr at 110°C. HPLC analysis of the peptide gave a satisfactory result (Fig. 2B). An FAB mass spectrometric analysis of the B chain gave a protonated monoisotopic molecular ion at m/z 5832.2 for a calculated value of m/z 5832.0. An electrospray ionization (ESI) mass spectrometric analysis of the B chain gave a mean molecular weight of 5834.2 vs. the calculated value of 5834.7 for the B chain. An amino acid analysis of the B chain gave the expected values (see the Experimental section).

The synthetic B chain was combined with the A chain, which was left over from a previous work.\textsuperscript{7} When the A and B chains were mixed in a ratio of 1:1 (w/w), a considerable amount of the A and B chains remained unreacted (Fig. 3), which is surprising in view of its close structural similarity to [Asn$^{49}$, Glu$^{50}$] B-chain monellin, which was almost completely formed simply by mixing the A and [Asn$^{49}$, Glu$^{50}$] B chains (1:1, w/w).\textsuperscript{7} Bohak and Li\textsuperscript{5} have reported that the sweetness potency of a 1:1 (w/w) mixture of the separated A and B chains was significantly weaker than that of natural monellin. This result suggests that the sep-
arated A and B chains recombined in a low yield.

It is well known that peptides and proteins are sometimes eluted as broad asymmetrical peaks or as multiple peaks with different retention times by altering their conformation on the hydrophobic surfaces of a chromatographic column. In our previous paper, the [Asn49, Glu50] B chain was eluted as multiple peaks on HPLC.

When the synthetic A and B chains were separately analyzed by HPLC under the conditions specified in Fig. 3, the A and B chains were each eluted as two broad peaks at 6.2 and 15.9 min in a ratio of 89:11, and at 7.1 and 14.9 min in a ratio of 53:47, respectively. Liquid secondary ion (LSI) mass spectrometric analyses of the peptides obtained from the peaks eluting at 6.2, 15.9, 7.1 and 14.9 min gave average molecular ions at m/z 5250.7, 5250.7, 5834.6 and 5834.6, respectively, which corresponds to the calculated values for the A, A, B and B chains, respectively. Circular dichroism (CD) spectra of these peptides revealed that they existed in the unfolded state.

When the peptides obtained from the peaks eluting at 15.9 and 14.9 min were separately analyzed by HPLC under the conditions specified in Fig. 3, they were each eluted as two peaks with the same retention times as those already described, although the ratio was different from that of the original one. Thus, the peaks eluting at about 15.3 and 16.5 (shoulder) min in Fig. 3 could be assigned to the B and A chains, respectively; the peak eluting at about 6.5 min will be described later. On the other hand, when the peptides obtained from the peaks eluting at 6.2 and 7.1 min were separately analyzed by HPLC under the conditions specified in Fig. 3, they were each eluted as a single peak with the original retention times.

When a mixture of the peptides obtained from the peaks eluting at 6.2 and 15.9 min was analyzed by HPLC under the conditions specified in Fig. 2, it was eluted as a single peak with the same retention time as that of the A chain. A similar HPLC analysis of a mixture of the peptides obtained from the peaks eluting at 7.1 and 14.9 min gave a single peak with the same retention time as that of the B chain.

The synthetic A and B chains were confirmed to be pure as already described. Therefore, it can be concluded that some of the A and B chains was altered in conformation to another unfolded form on the hydrophobic surfaces of the TSKgel Phenyl-5PW column.

For the reason already described, the B chain was predominantly used over the A chain in the combination procedure. Mixing the A and B chains in a ratio of 1:1.9 (w/w), and subsequent HPLC purification gave monellin in a yield of 25.7%, based on the A chain (Figs. 4A and 4B). As can be seen in Fig. 4A, the peak eluting at about 6.5 min in Fig. 3 hardly appeared when the B chain was excessively used over the A chain. Thus, the peak eluting at about 6.5 min in Fig. 3 could be assigned to the A chain. The peaks eluting at 14.8 and 16.7 min in Fig. 4A were found by LSI mass spectrometry.
Fig. 5. HPLC on an Analytical Reversed-phase Inertsil ODS-2 5 \mu m C_{18} \text{ Column (4.6} \times \text{250 mm)} \text{ of the B Chain Separated from the Synthetic Monellin.}

The sample was eluted with a linear gradient of 20\% B to 50\% B obtained in 30 min at a flow rate of 1 ml/min, employing the same solvent systems as those used in Fig. 2.

Fig. 6. FAB Mass Spectrum of the B Chain Separated from the Synthetic Monellin.

spectrometry to contain the B (m/z, 5834.9) and A (m/z, 5251.9) chains, respectively. A quantitative amino acid analysis of the synthetic monellin gave a satisfactory result (see the experimental section). The B chain of the synthetic monellin was separated by HPLC on a C_{18} column, and its purity was confirmed by HPLC, an amino acid analysis and FAB mass spectrometry. HPLC analysis of the B chain gave a satisfactory result (Fig. 5), and its amino acid analysis gave the expected values (see the experimental section). An FAB mass spectrometric analysis of the B chain gave a protonated monoisotopic molecular ion at m/z 5831.7 against a calculated value of m/z 5832.0 (Fig. 6). For this spectrum, the observed isotopic distribution was in good agreement with patterns calculated from the elemental composition. These results indicate that the separated B chain was free from any impurities, confirming that the synthetic monellin was highly pure.

No comparison of the synthetic monellin with the natural material could be made, since natural monellin consists of heterogeneous A and B chains, and separation of the concomitant peptides from these chains was successful for the A chain, but not for the B chain, as described previously.6)

The synthetic monellin was approximately 4000 times as sweet as sucrose, while [Asn^{49}, Glu^{50}] B-chain monellin was also 4000 times as sweet as sucrose.7) Thus, exchanging the amino acid residues at positions 49 and 50 of the B chain did not affect the sweetness potency.

The synthetic monellin was crystallized by the “hanging drop” vapor diffusion method7,8) (Fig. 7).

Fig. 7. Crystals of the Synthetic Monellin Grown by the “Hanging Drop” Vapor Diffusion Method and Photographed under a Polarized Microscope within a Crystallizing Well.

Experimental

The protected amino acids were obtained from Kokusan Chemical Works Ltd., Bachem Feinchemikalien AG or Novabiochem AG. Fmoc-Asn(Mbh)-OCH_{2}-C_{8}H_{4}-OCH_{2}-resin was purchased from Bachem Feinchemikalien AG. All solvents were of reagent or HPLC grade. The peptide synthesis was performed by the stepwise
solid-phase method with semi-automated equipment (Labortece 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 \([C_{18} \text{ reversed-phase column, Inertil ODS-2}, 5 \mu m, 4.6 \times 250 \text{mm}, \text{Gasukuro Kogyo (for analytical HPLC) and Inertil ODS, } 5 \mu m, 16.2 \times 250 \text{mm, Gasukuro Kogyo (for preparative HPLC)}]\), UV detection at 210 nm; TSKgel Phenyl-SPW column, 7.5 \times 75 mm, Tosoh (for monellin), UV detection at 277 nm. Peptide hydrolysates were performed in constant-boiling HCl containing 1% phenol for 24 hr and 96 hr (for the Ile–Ile peptide bond) at 110°C in sealed ampules under high vacuum. Quantitative acid analyses were carried out on either a Hitachi 835 or a Beckman 6300E amino acid analyzer. FAB and LSI mass spectra were taken on a JEOL JMS HX110/110 tandem mass spectrometer, and ESI mass spectra were taken on a VG Masslab BIO-Q mass spectrometer. CD spectra were recorded on a Jasco J-500A spectropolarimeter equipped with a DP-500N data processor. Monellin crystals were photographed under a polarized microscope (Olympus BH-2) within a crystallizing well. 

**Synthesis of the B chain.** Fmoc-Asn(Mbh)-OCH2-C6H4-OCH2-resin (0.48 mmol/g, 635 mg, 0.30 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 50 and 48-41, 3 + 15 min for residues 39-15, and 3 + 20 min for residues 40 and 14-2; the Fmoc group of Glu(OBul) at position 49 was cleaved by repeated treatment (3 + 10 min) with 20% piperidine in DMF (15 ml).

(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: (i) shaking in DMF (10 ml, twice, 1 min each) to swell the resin; (ii) residues 49-40, 36-34, 32, 30, 24, 16 and 9 were coupled by the DCC/HOBt method, in which 1 m DCC (0.99 ml in CH2Cl2) was added to a solution of an appropriate Fmoc-amino acid derivative (0.9 mmol) and HOBt (1.08 mmol) in NMP (10 ml); the reaction period was 1.5 hr for residues 49-40, 36-34, 32 and 30-26, and 3 hr for residues 25, 24, 16 and 9. For residues 24 and 25, procedure (c) was repeated to complete the coupling reaction. Residues 39–37, 33, 31, 23–17, 15–10 and 8–2 were coupled by the symmetrical anhydride method, in which anhydrides of Fmoc-amino acid derivatives were prepared by the reaction of an appropriate Fmoc-amino acid derivative (1.2 mmol) with 1 m DCC (0.6 ml in CH2Cl2) in a mixture of DMF (5 ml) and CH2Cl2 (5 ml) in an ice-bath for 15 min; the reaction period was 1.5 hr for residues 38, 37, 23-17, 15-10 and 8-2, and 4 hr for residues 39, 33, 31 and 5. The Kaiser test data for residues 39-37, 33, 31, 21, 19-17, 14-12 and 8-2 indicated that a second coupling was necessary for quantitative incorporation. The second coupling was carried out by the DCC/HOBt method, in which the coupling reaction was performed for 2 hr, and the reaction mixture was then 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 removing the Fmoc group of residue 2 according to procedures (a) and (b), the peptide-resin was washed with isopropanol four times, and then dried in vacuo, weighing 2.79 g. The peptide-resin (1.86 g) was suspended in NMP (15 ml) and coupled with Boc-Gly-OH (158 mg, 0.90 mmol) by the DCC/HOBt method already described. The reaction was performed for 3 hr. After washing the peptide-resin according to procedure (d), the peptide-resin was further washed with DMF (15 ml, twice, 1 min each), and then with isopropanol (15 ml, four times, 1 min each), and finally dried in vacuo, weighing 2.13 g. The peptide-resin (2.13 g) was treated with CH2Cl2-anisole-m-cresol-1,2-ethanedithiol-TFA (12.3 : 2.0 : 0.5 : 0.2 : 20.0, v/v, 35.0 ml) while shaking for 2 hr. After filtration, the filtrate was concentrated to a small volume at a bath temperature of 50°C under reduced pressure. The peptide was precipitated by adding ether, collected by filtration, and dried, to yield 1.30 g. The crude peptide was dissolved in a mixture of m-cresol-1,2-ethanedithiol–thioanisole–TFA (0.75 : 0.3 : 3.52 : 21.47, v/v, 26.04 ml), stirred for 10 min, and then cooled in an ice-bath. To this ice-cooled, stirred mixture was added dropwise TMSBr (3.96 ml), stirring being continued for 6 hr while cooling. A sufficient amount of ether was added to the reaction mixture, and then the resulting precipitate was collected by filtration and dried. The crude peptide (1.68 g) thus obtained was suspended in water (20 ml) and cooled in an ice-bath. 2-Mercaptoethanol (400 μl) was added to the suspension, which was then adjusted to pH 8 with triethylamine while stirring and cooling. To this suspension was added 1 m NH4F (800 μl). The mixture was stirred for 30 min while cooling and then adjusted to pH 5 with acetic acid. The resulting precipitate was collected by filtration and dried, weighing 1.16 g. The precipitate (580 mg) was dissolved in TFA (5 ml) and filtered. The filtrate (400 μl) was applied to a preparative HPLC column (Inertil ODS) and eluted with a linear gradient of 20% to 50% acetonitrile containing 0.05% TFA obtained in 50 min at a flow rate of 9 ml/min, the elution profile being monitored at 210 nm. The procedure was repeated 13 times to purify the whole amount of the peptide. The analytical HPLC elution profile of the filtrate is shown in Fig. 2A. The fractions of the main peak (indicated by an arrow) and several peaks were separately collected, concentrated under reduced pressure to a small volume, and lyophilized to give peptides. Since the FAB-mass spectra of these peptides revealed that the main peak contained the desired B chain, the peptide from the FAB-mass spectra of these peptides revealed that the main peak contained the desired B chain, the peptide from...
the main peak was dissolved in 2 ml of 20% acetonitrile containing 0.05% TFA and further purified by preparative HPLC, in which the solution (500 μl) was applied to a preparative HPLC column (Inertsil ODS) and eluted with a linear gradient of 20% to 43% acetonitrile containing 0.05% TFA obtained in 50 min at a flow rate of 9 ml/min. The elution profile was monitored at 210 nm. The procedure was repeated four times to purify the whole amount of the peptide. The fractions thus obtained were collected, concentrated under reduced pressure to a small volume and lyophilized, and finally dried over NaOH pellets in vacuo to give 36.25 mg (6.2% overall yield) of the B chain (Fig. 2B).

Amino acid analysis (hydrolyzed for 24 hr, with 96 hr for the Ile-Ile peptide bond*), numbers in parentheses being theoretical values: Asp, 6.05 (6); Thr, 2.72 (3); Glu, 7.63 (8); Pro, 2.08 (2); Gly, 5.52 (5); Ala, 1.16 (1); Val, 1.91 (2); Met, 0.81 (1); Ile*, 5.81 (6); Leu, 2.18 (2); Tyr, 1.81 (2); Phe, 3.22 (3); Lys, 4.53 (5); Arg, 2.27 (2). Trp and Cys were not determined.

FAB-MS: m/z 5832.2 (M + H)+; calculated value, m/z 5832.0 (M + H)+.

ESI-MS: Measured mean MW, 5834.2; calculated value, 5834.7.

Combination of the synthetic monellin A and B chains. The synthetic A chain (2.40 mg), which was left over from a previous work,7) was dissolved in 0.1% acetic acid (300 μl). The synthetic B chain (4.56 mg) was then dissolved in this solution. The resulting mixture was stirred for 1 min with a mixer under an atmosphere of nitrogen, and then kept standing for 20 hr. The mixture (100 μl) was applied to a TSKgel Phenyl-5PW column and eluted with a linear gradient of 10% to 60% acetonitrile containing 0.1% TFA obtained in 10 min at a flow rate of 1 ml/min. The elution profile was monitored at 277 nm and is shown in Fig. 4A. The procedure was repeated three times to purify the whole amount of the protein, and the fractions of the main peak were collected, concentrated and lyophilized. The protein thus obtained was dissolved in water (200 μl), shaken for 2 hr, lyophilized, and finally dried over NaOH pellets in vacuo.

Yield, 1.3 mg (25.7%).

Amino acid analysis (hydrolyzed for 24 hr, with 96 hr for the Ile-Ile peptide bond*), numbers in parentheses being theoretical values: Asp, 6.05 (6); Thr, 2.72 (3); Glu, 7.63 (8); Pro, 2.08 (2); Gly, 5.52 (5); Ala, 1.16 (1); Val, 1.91 (2); Met, 0.81 (1); Ile*, 5.81 (6); Leu, 2.18 (2); Tyr, 1.81 (2); Phe, 3.22 (3); Lys, 4.53 (5); Arg, 2.27 (2). Trp and Cys were not determined.

FAB-MS: m/z 5831.7 (M + H)+; calculated value, m/z 5832.0 (M + H)+.

ESI-MS: Measured mean MW, 5834.2; calculated value, 5834.7.

Sweetness evaluation. The sweetness value of the synthetic monellin was organoleptically determined by a panel of five untrained people. The evaluation was carried out at concentrations near the threshold to avoid confusion arising from the persistent lingering sweet taste of monellin. Thus, the sweetness evaluation was carried out by matching a threshold concentration of the synthetic monellin with that (0.6%, w/v) of sucrose. The synthetic monellin (0.6 mg) was dissolved in water (10 ml), this concentration corresponding to a 0.006% (w/v) monellin solution. This solution was diluted to several concentrations (30, 40, 45 and 50 times), and the sweetness intensity of each was compared with that of the 0.6% sucrose solution. It was found that the sweetness intensity of the 40-fold dilution was almost equal to that of 0.6% sucrose. This result indicates that the synthetic monellin was approximately 4000 times as sweet as sucrose.

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