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Characterization of Monellin, a Protein That Tastes Sweet*

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SUMMARY

Monellin, an intensely sweet substance from the fruit of the tropical plant *Dioscoreophyllum cumminsii*, has been clearly established to be a protein.

The molecular weight of monellin was estimated by two independent physical methods. The values were 10,000 and 10,500 and agreed with minimal molecular weights based on chemical analyses. The molecular weight calculated from amino acid analysis is 10,700, and the protein consists of a single polypeptide chain of approximately 91 amino acids. The protein contains no histidine, and only 1 residue each of tryptophan, cysteine, and methionine are present. It contains no mono- or dimethyl derivatives of lysine or arginine. Fifteen basic residues (Lys + Arg) and 22 acidic residues (Asp + Glu) are present, but up to 6 of the latter may be in the amide form. Isoelectric focusing established the isoelectric point of monellin to be 9.3.

Monellin is a protein that tastes sweet. Using standard methods of protein purification including salt precipitation, dialysis, and ion exchange chromatography on substituted celluloses, we obtained a protein from the fruit of *Dioscoreophyllum cumminsii* that is homogeneous by disc gel electrophoresis and which has an intense and persistent sweet taste (1). Chemical evidence indicated that monellin is a carbohydrate-free protein, in contrast to a previous report by Inglett and May (2) that the sweet principle in *D. cumminsii* is a carbohydrate. In a recent preliminary report, van der Wel (3) independently confirmed our finding that monellin is a protein.

In this paper we present data on the molecular weight, amino acid composition, isoelectric point, and spectral characteristics of monellin.

EXPERIMENTAL PROCEDURE

Materials

SDS[1], hemoglobin (bovine, Type I, twice crystallized), and α-chymotrypsin (bovine pancreas, Type II, three times crystallized) were purchased from Sigma; ovalbumin (egg white, twice crystallized), chymotrypsinogen A (beef pancreas, six times crystallized), myoglobin (sperm whale, crystallized), and cytochrome *c* (horse heart, amorphous powder) were from Schwarz-Mann; and trypsin (bovine pancreas, twice crystallized) was from Worthington. Sephadex G-100 and blue dextran 2000 were purchased from Pharmacia Fine Chemicals. *p*-Dimethylaminobenzaldehyde was obtained from Fischer Scientific, and DL-tryptophan from Eastman. All other chemicals were reagent grade.

Fresh berries (*D. cumminsii*) were procured and the sweet protein monellin was extracted and purified as described previously (1). The protein was dialyzed against deionized water, lyophilized, and stored at −15°.

Methods

SDS-Gel Electrophoresis—The apparent molecular weight of monellin was estimated by electrophoresis in 10% polyacrylamide gels containing SDS according to the method of Dunker and Rueckert (4). Gels approximately 6.5 cm long were pre-

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[1] The abbreviation used is: SDS, sodium dodecyl sulfate.

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pared in glass columns (7.5 cm × 0.5 cm, internal diameter). Electrophoresis was carried out in 0.05 M sodium phosphate buffer, pH 7.1, in the presence of 0.1% (w/v) SDS, at a constant current of 4 ma per gel. Protein samples were prepared at a concentration of 2.0 mg per ml; 10 to 40 μg of individual proteins were applied to a single gel. Gels were stained with Coomassie brilliant blue R250 (Colab Laboratories, Chicago Heights, Illinois) and destained as described by Weber and Osborn (5). Electrophoresis was carried out on various mixtures of the standard proteins with and without monellin present. The identity of each protein was first established by running it alone (with chymotrypsinogen A). The relative mobility of each protein was calculated as described (4).

Gel Filtration—Gel filtration was carried out according to the method of Andrews (6), on a column (2.5 × 88 cm) of Sephadex G-100 at 4° in 0.01 M sodium phosphate buffer, pH 7.26, containing 0.2 M KCl. Flow rate was maintained at 15 ml per hour, and fractions of 30 drops (1.95 ml) were collected. The absorbance of the effluent at 280 nm was monitored continuously and the A_{280} of each fraction was also measured at the completion of the experiment. To minimize possible protein-protein interactions, the proteins (10 mg in 1.0 ml) were applied to the column in the order of decreasing molecular weight. Each was followed by a 10-ml aliquot of the equilibrating buffer. Monellin was added last. Appropriate corrections in elution volumes were made for the additions of buffer. The location of the monellin peak was confirmed by its sweet taste.

Standard Amino Acid Analysis—Samples of protein (1.0 mg) were hydrolyzed in 1.0 ml of constant boiling HCl containing 4% (v/v) thioglycolic acid (7) for 24 hours at 110° in an evacuated desiccator (8). Hydrolysates were evaporated to dryness over NaOH pellets *in vacuo* and dissolved in 2.0 ml of 0.2 N sodium citrate, pH 2.2, frozen, and stored at −10°. Analyses were carried out with a Beckman 120C amino acid analyzer utilizing Beckman AA-27 and AA-15 resins. The standard 4-hour system operated at high sensitivity was used. Peak integration was performed with an Infotronics CRS 110A integrator with digital printout. Samples of 250 μl (equivalent to 125 μg of protein) were applied to the columns with Beckman manual sample injectors.

Tryptophan content was estimated in the intact protein by the method of Spies and Chambers (9). Assays were performed according to Procedure K, and the tryptophan content was calculated from the standard curve prepared with free DL-tryptophan. The spectral procedure of Edelhoch (10) was also used to determine the contents of tyrosine and tryptophan in the intact protein.

Analysis for Methylated Basic Amino Acids—A sample of protein (2.0 mg) was subjected to acid hydrolysis as described above, but without thioglycolic acid. The hydrolysate was then evaporated as described above and dissolved in 1.0 ml of the citrate buffer. A 250-μl sample (equivalent to 0.5 mg of protein) was chromatographed on a column (0.9 × 30 cm) of Durrum DC-2A resin.[2] Traces of N^{ϵ}-monomethyllysine, N^{ϵ}-dimethyllysine, 3-methylhistidine, N^{G},N^{G}-dimethylarginine, N^{G},N'^{G}-dimethylarginine, and N^{G}-monomethylarginine are readily identified and quantitated in acid hydrolysates of proteins by this procedure.

Performic Acid Oxidation—Performic acid oxidation of a sample of monellin (1.0 mg) was carried out as described by Hirs (11).

[2] G. E. Deibler and R. E. Martenson, manuscript in preparation.

Isoelectric Focusing—The isoelectric point of monellin was determined by isoelectric focusing in an LKB ampholine column, model 8100 (LKB-Produkter AB, Sweden); the experiments were performed at 3.9° ± 0.1° according to the manufacturer's instruction manual. Voltage was held constant at 600 volts. Focusing was terminated when the current reached a constant level of 0.40 ma (216 hours). Following the run, fractions (30 drops) were collected, and the pH (at 4°) and A_{277} were measured. Appropriate fractions were dialyzed overnight against deionized water and assayed for sweetness (1). Electrode solutions were 1% (w/v) NaOH (cathode) and 1% (v/v) H_2SO_4 (anode). Ampholyte concentration was 1% (w/v).

Spectral Measurements—The ultraviolet absorption spectrum of monellin was measured with a Cary 14 recording spectrophotometer at room temperature (22°). For determining the extinction coefficients, lyophilized monellin was dried to constant weight (4 days) under vacuum with P_2O_5 as the drying agent. The protein (weighed to ±0.1%) was then dissolved in 0.01 M sodium phosphate buffer, pH 7.20. This stock solution was diluted with the same buffer to give a solution with an absorbance (at 277 nm) in the range of 0.7 to 1.0. Absorption measurements of triplicate samples were made in a Zeiss PMQ II spectrophotometer. Samples for measurements at pH 12.8 were prepared by diluting the stock solution with 0.1 N NaOH instead of buffer. Fluorescence spectra were measured with a Perkin-Elmer model MPF-2A fluorescence spectrophotometer at 20.0° ± 0.2°, and are uncorrected.

RESULTS

Molecular Weight—After treatment of monellin with SDS and mercaptoethanol, a procedure known to dissociate proteins into their constituent polypeptide chains (16), only a single electrophoretic component was observed. This observation is consistent with monellin being a single polypeptide chain. It is conceivable from this evidence alone, however, that monellin could consist of two or more polypeptide chains of identical length. The molecular weight of the polypeptide chain(s) estimated by SDS-gel electrophoresis (Fig. 1) is 10,500.

The molecular weight of native monellin was examined by gel filtration through Sephadex G-100 (Fig. 2). A single experiment showed that the sweet-tasting activity was associated with a protein having a molecular weight value of 10,000. Since the native protein and the "reduced and dissociated" protein have virtually the same molecular weight, the molecule of monellin must consist of a single polypeptide chain. The minimal molecular weight determined from the content of tryptophan in the intact protein was found to be in good agreement with the above values. Tryptophan analyses carried out according to the method of Spies and Chambers (9) on five samples of monellin yielded a value of 1.00 mole of tryptophan per 11,240 ± 240 g (S.D.) of protein; a single analysis carried out according to the method of Edelhoch (10) yielded a value of 1.00 mole of tryptophan per 8,939 g of protein.

Amino Acid Analysis—Acid hydrolysis of monellin was generally carried out in the presence of thioglycolic acid (4% v/v) to preserve tryptophan (7) and prevent oxidation of methionine and tyrosine (7, 17). Calculation of the moles of each amino acid recovered relative to 1.00 mole of methionine (Table I, Column 1) yielded a minimal molecular weight for the protein of 10,700. This value, in conjunction with those obtained from SDS-gel electrophoresis (10,500), gel filtration (10,000), and tryptophan content (11,200 and 8,900), demonstrates that the polypeptide chain contains a single residue of methionine. The

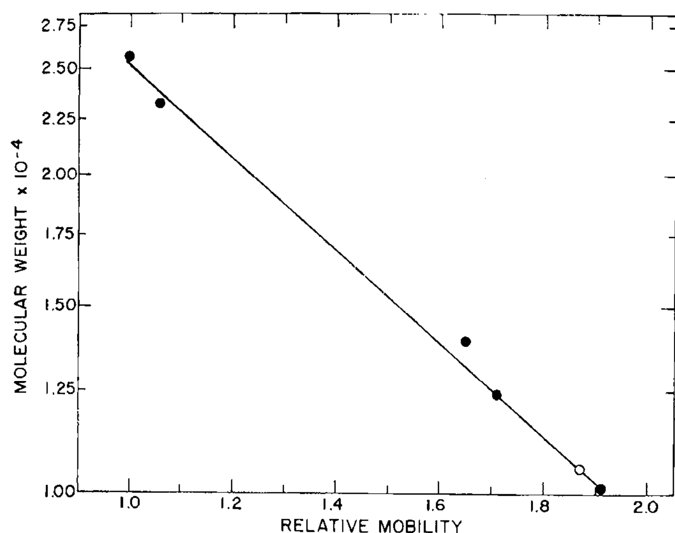

FIG. 1. Estimation of molecular weight of monellin by electrophoresis in SDS-polyacrylamide gels. Sample preparation and electrophoresis conditions are described under "Methods." The data are the results from three separate experiments, and each point on the standard curve represents the mean of at least 17 individual gels. The relative mobility of monellin is indicated by the *open circle*. The marker proteins used, and their molecular weights, from left to right, are: chymotrypsinogen A, 25,800 (Reference 12); trypsin, 23,300 (calculated from data in Reference 13); chymotrypsin B chain, 13,900 (Reference 12); cytochrome *c*, 12,400 (Reference 14); chymotrypsin C chain, 10,200 (Reference 12).

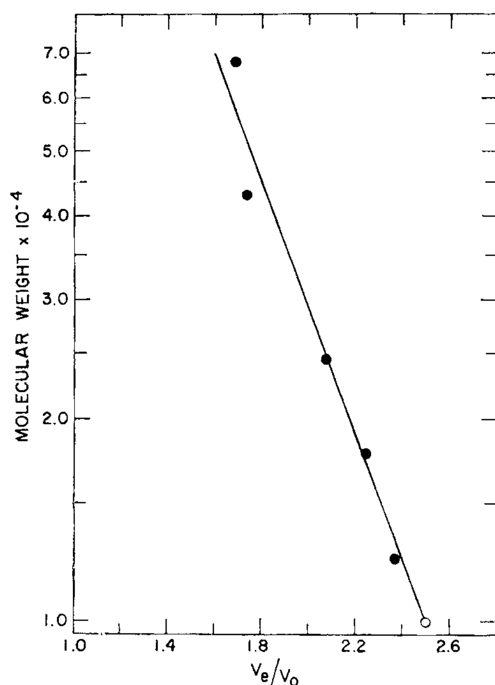

FIG. 2. Estimation of molecular weight of monellin by gel filtration through Sephadex G-100. The procedures are described under "Methods." The elution volume (V_e) is the volume eluted prior to the emergence of the peak fraction for each marker protein. The void volume (V_0) is the volume eluted prior to the peak fraction of blue dextran 2000. The ratio ($V_e:V_0$) for monellin is indicated by the *open circle*. The marker proteins and their molecular weights used are, from left to right: hemoglobin, 66,300 (Reference 15); ovalbumin, 43,000 (Reference 15); α-chymotrypsin, 25,300 (Reference 12); myoglobin, 17,800 (calculated from data in Reference 13); and cytochrome *c*, 12,400 (Reference 14).

data in Table I also show that the molecule contains 3 residues of alanine, an amino acid that is known to be recovered quantitatively under these conditions. No histidine was detected by standard amino acid analyses.

Chemical (9) and spectrophotometric (10) determinations of tryptophan in the intact protein showed the presence of 0.95 ± 0.02 (S.D.) and 1.20 moles of tryptophan, respectively, per 10,700 g of protein. The somewhat high value obtained in the standard analysis for tryptophan shown in Table I (Column 1) appears to have been due to incomplete separation of this amino acid from lysine during chromatography on the short AA-27 resin column.

Another sample of monellin was oxidized with performic acid. The oxidized protein and an untreated control were hydrolyzed in the absence of thioglycolic acid. These amino acid analyses (Table I, Columns 2 and 3) showed the presence of very nearly 1 mole each of cysteic acid and methionine sulfone per 3 moles of alanine (*i.e.* per mole of oxidized protein). The recovery of cysteic acid was 89% and compares favorably with values of 90 to 95% reported by Hirs (11). Tyrosine was completely destroyed upon treatment of the protein with performic acid. Loss of tyrosine was accompanied by the appearance of a relatively large peak of 570 nm-absorbing material that emerged from the AA-27 resin column just prior to lysine in the region of tryptophan and which caused an erroneously high integration of lysine due to overlap. The recoveries of all of the other amino acids were essentially identical in both performic acid-oxidized and control proteins (Table I, Columns 2 and 3).

In order to determine if any of the lysyl or arginyl residues in monellin are methylated, analysis for basic amino acids was carried out on a column of Durrum DC-2A resin. The only peaks observed were those corresponding to lysine, arginine, ammonia, and a trace of histidine; no mono- or dimethyl derivatives of lysine or arginine were present. Trimethyllysine, if present, would probably have been detected in this system. The results of the standard analyses (Table I, Columns 1 to 3) indicated the presence of 7 arginyl residues per molecule of protein. Using the long DC-2A column, the quantities of lysine and histidine relative to 7.00 moles of arginine were 7.64 and 0.32 moles, respectively. The histidine must therefore have arisen from a contaminating protein. Use of the short AA-27 resin column, loaded with an acid hydrolysate of monellin equivalent to 0.5 mg of protein should be as effective as the long DC-2A column for detecting small amounts of histidine, if present, and should serve an an excellent method of assaying the purity of subsequent preparations of this protein.

The amino acid composition of monellin arrived at by averaging the results of several standard analyses is presented in Table I (Column 4). In many cases close to integral values were obtained. The lysine values obtained in the standard analyses ranged from 8.3 to 8.6 moles per mole of protein; the corresponding value obtained from the analysis of a larger amount of hydrolysate on the Durrum DC-2A resin column was 7.6 moles per mole of protein. Accordingly, a value of 8 was chosen for the probable number of lysyl residues in the protein. The maximum recoveries of threonine and serine were 3.8 and 1.8 moles per mole of protein, respectively. Assuming minimal losses of threonine and serine (5% and 10%, respectively (18)) upon acid hydrolysis, the data indicate the presence of 4 threonyl and 2 seryl residues in the protein. Whether there are 6 or 7 prolyl residues in the protein is uncertain at the present time; the value of 6 was chosen arbitrarily. Leucine recoveries ranged from 5.3 to 5.8 moles per mole of protein. Since this

amino acid may be liberated slowly during acid hydrolysis, a value of 6 was chosen. The presence of 7 residues of tyrosine determined from amino acid analysis data was confirmed by the results of spectrophotometric studies (10) on the intact protein, which revealed a tyrosine to tryptophan ratio of 7.0. These data indicate that monellin consists of approximately 91 amino acid residues and has a molecular weight of approximately 10,700. Additional analyses of samples that have been hydrolyzed for

TABLE I

Amino acid composition of monellin

Amino Acid	Residues/mole			
	Native[a]	Oxidized[c]	Native[c]	Native Averages[f]
Tryptophan	1.42	-	0.31	1.0 (1)
Lysine	8.35	10.15	8.29	8.4 (8)
Histidine	0	0	0	0 (0)
Ammonia	7.52	-	5.47	-
Arginine	7.03	7.24	7.17	7.1 (7)
Aspartic acid	10.05	10.03	9.78	10.0 (10)
Threonine	3.34	3.77	3.68	3.5 (4)
Serine	0.91	1.78	1.78	1.4 (2)
Glutamic acid	12.67	11.86	11.88	12.1 (12)
Proline	6.32	6.36	6.62	6.5 (6)
Glycine	7.75	7.88	7.78	7.8 (8)
Alanine	2.96	3.00	3.00	3.0 (3)
Half-cystine	0[b]	0.89[d]	0	0.9 (1)
Valine	3.71	3.72	3.79	3.7 (4)
Methionine	1.00	0.94[e]	0.89	0.9 (1)
Isoleucine	6.24	5.94	5.94	6.1 (6)
Leucine	5.62	5.26	5.45	5.5 (6)
Tyrosine	6.69	0	6.63	6.7 (7)
Phenylalanine	5.48	4.57	4.86	5.0 (5)
Total number of residues				(91)
Calculated molecular weight				10,700

[a] The result represents the average of analyses carried out in duplicate on a single sample of hydrolyzate. Hydrolysis was performed in the presence of thioglycolic acid as described in "Methods." The residues per mole of protein were calculated by normalizing to methionine.

[b] Analyses of proteins hydrolyzed in the presence of thioglycolic acid revealed a peak that emerged 5-7 min after authentic cystine and whose absorbance was primarily at 440 nm. The presence of cysteine could not be detected.

[c] Samples of native and oxidized proteins from the same preparation of monellin were hydrolyzed simultaneously in the same desiccator in the absence of thioglycolic acid as described in "Methods." Each result represents the average of analyses carried out in duplicate on a single hydrolyzate. The residues per mole were calculated on the basis of 3 residues of alanine per mole of protein.

[d] Determined as cysteic acid.

[e] Determined as methionine sulfone.

[f] The data represent the average of six standard analyses, including the data in columns 1-3 of this Table. (Not included in the average are the values of tryptophan obtained in the absence of thioglycolic acid and of lysine and tyrosine obtained with the performic acid oxidized protein.) The residues per mole are calculated on the basis of 3 residues of alanine per mole of protein. Numbers in parentheses indicate the probable integral number of residues.

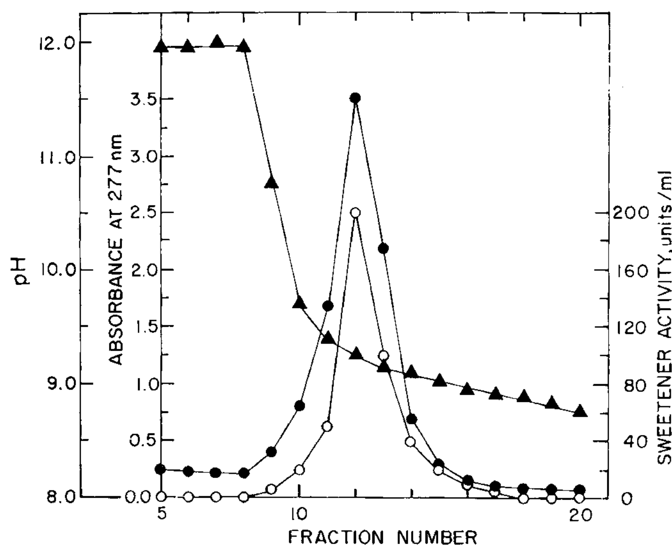

FIG. 3. Determination of the isoelectric point of monellin by isoelectric focusing. The experiment was carried out as described under "Methods." Fifteen milligrams of monellin were incorporated into the pH gradient (pH 7 to 10). ●——●, absorbance at 277 nm; ○——○, sweetener activity in arbitrary units per ml; ▲——▲, pH.

differing lengths of time will be necessary, of course, to determine the amino acid composition more precisely.

Isoelectric Point—The isoelectric point was determined by isoelectric focusing in a pH gradient from pH 7.0 to pH 10.0. Data from one experiment are shown in Fig. 3; the isoelectric point here is 9.26. In a second experiment, the value obtained was 9.31. The relatively high isoelectric point is surprising in view of the amino acid composition of the protein. The maximal possible number of amide groups estimated from the minimal quantity of ammonia found in acid hydrolysates is 6 per mole of protein.[3] Since 22 moles of acidic amino acids per mole of protein were recovered in the acid hydrolysates (Table I, Column 4), it follows that the minimum number of side chain carboxyl groups in the protein is 16. If the NH_2 terminus is free, then the protein would bear a net charge of -1 at neutral pH, since there are 15 basic residues in the protein. While these calculations are admittedly only approximate, the conclusion seems inescapable that the relatively high isoelectric point of monellin cannot be solely, or perhaps even primarily, due to the amidation of glutamyl and aspartyl residues. Further studies will be required to clarify this point.

Spectral Measurements—The ultraviolet absorption spectrum of monellin (Fig. 4) is similar to those of many other proteins. The λ_{max} occurs at 277 nm. Significant absorption occurs at 288 nm, which is characteristic of the tryptophan indole chromophore (10). The values for $E_{1\,cm}^{1\%}$ and the molar extinction coefficients (mol wt 10,700) at pH 7.20 and 12.8 are presented in Table II. The shift in λ_{max} from 277 nm to 290 nm at pH 12.8 due to ionization of tyrosine is also accompanied by a complete loss of the sweet taste, but any connection between the ionization of tyrosine and loss of activity remains to be determined.

The fluorescence emission maximum (excitation at 277 nm) of monellin is at 337 nm due to tryptophan; a shoulder at 300 nm due to tyrosine could be partially resolved (Fig. 5). In 0.1 N NaOH the emission maximum is shifted to 348 nm, with a marked loss in tryptophan fluorescence intensity due to quenching by ionized tyrosine and by hydroxyl ions.

[3] This value is based on the lowest amount of ammonia found in the several standard amino acid analyses and also includes ammonia formed during the destruction of serine and threonine plus traces of ammonia that could have been picked up by the sample.

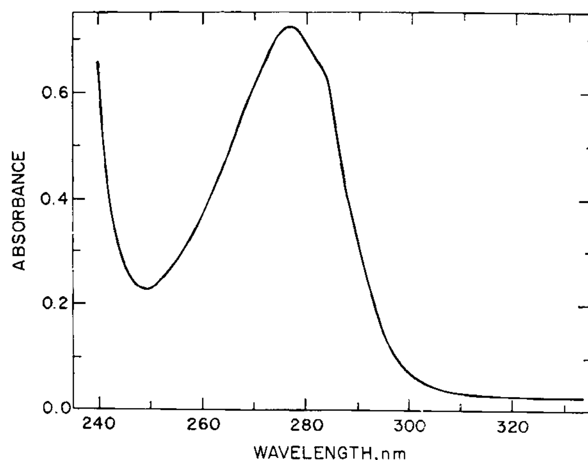

FIG. 4. Ultraviolet absorption spectrum of monellin. Protein concentration was 0.519 mg per ml. The spectrum was measured in 0.01 M sodium phosphate buffer, pH 7.20, in quartz cuvettes of 1-cm path length against a phosphate buffer blank.

TABLE II

Extinction coefficients of monellin

pH	Absorption maximum	$E_{1\,cm}^{1\%}$	$\epsilon(M^{-1}\,cm^{-1})^a$
	nm		
7.20	277	13.7	1.47×10^4
12.8	290	17.1	1.83×10^4

[a] A molecular weight value of monellin of 10,700 was used in the calculations.

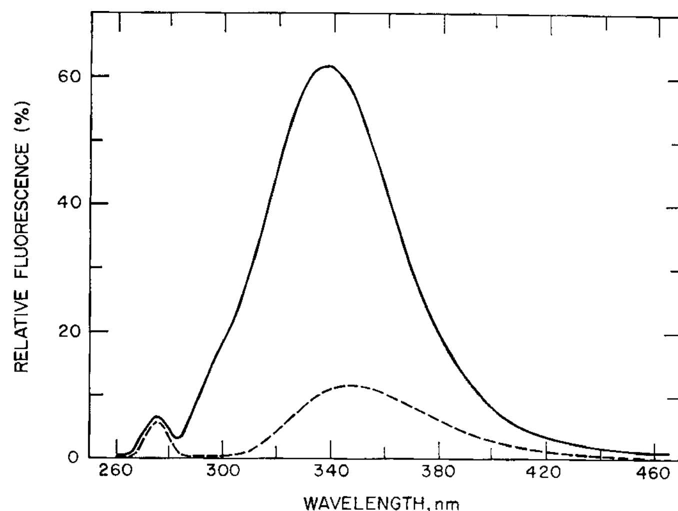

FIG. 5. Fluorescence emission spectra of monellin. Protein concentration was 0.1 mg per ml. Excitation was at 277 nm. ——, emission spectrum of monellin in water; - - -, emission spectrum of monellin in 0.1 N NaOH.

DISCUSSION

Little is known of the molecular basis of either taste quality or intensity of various taste stimuli. Sweet compounds encompass a wide variety of chemical structures, and until recently (1) it was not known that a protein could have a sweet taste. Shortly following the report of the intense sweet taste of the fruit of the

tropical plant *D. cumminsii* (19), the chemical nature of the sweet principle in this fruit was reported to be a "carbohydrate type substance" and "not a protein or polypeptide" (2). The evidence presented here proves that the sweet principle monellin is, in fact, a protein. Additional confirmation of the idea that a protein can function as a chemical stimulus in man was recently reported. The sweet principle (thaumatin) from *Thaumatococcus daniellii* was obtained in a highly purified form, and evidence was presented[4] that it is a protein. The glycoprotein miraculin, although not sweet itself, is capable of altering the taste of acids (sour) so that they taste sweet (20, 21) after the tongue is treated with the glycoprotein.

The agreement is excellent between the values for the molecular weight of monellin determined by two independent physical methods. Using SDS-gel electrophoresis a value of 10,500 was obtained, and using gel exclusion chromatography the value was 10,000. The molecular weight calculated from the amino acid composition is 10,700. Monellin is therefore an order of magnitude larger than previously known sweet molecules. The molecular weight of thaumatin, the other known sweet protein, was estimated[4] by gel exclusion chromatography to be 14,000.

The sweet taste of a small molecule is usually extremely sensitive to chemical modification. Often very minor changes in the structure result in complete loss of sweetness. The relatively large size of monellin and its variety of side chains should permit a number of chemical modifications in attempts to elucidate the chemical features essential for its biological activity as well as to attach particular labeling groups for binding studies.

Native (sweet) monellin displays a fluorescence emission maximum at 337 nm (Fig. 5), and at high pH the emission maximum shifts to 348 nm. The sweet taste is also lost at high pH, whereas the activity of the protein appears to be reasonably stable at a pH as low as 2.[5] The relatively large red shift observed in the tryptophan emission upon treatment of monellin with alkali may be indicative of a transfer of the indole chromophore from a region of relatively low polarity to one of high polarity (22) and suggests that a considerable disorganization of the protein's tertiary structure may have resulted. Whether there is any connection between the ionization of tyrosine *per se* and loss of activity at high pH is under investigation. While the importance of the tertiary structure for the sweet taste is suggested, it is, of course, possible that a smaller portion of the polypeptide chain is sufficient to elicit a sweet sensation. Several D-amino acids are known to be sweet (23), although their L enantiomorphs are usually tasteless or bitter. Several α-amide derivatives of L-aspartic acid are also sweet (24), and the dipeptide ester L-Asp-L-Phe-Me is intensely sweet (25), but only certain restricted substitutions are allowable for retention of the sweet taste.

[4] Presented by H. van der Wel at the Fourth International Symposium on Olfaction and Taste, Starnberg, Germany, August, 1971.

[5] J. A. Morris and R. H. Cagan, unpublished observations.

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