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Methylation of the Lysine Residues of Monellin[1] (40019)

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Monellin, a sweet-tasting protein, has been isolated in homogeneous form and characterized (1-10). Prior to the present study, a number of unsuccessful attempts were made in our laboratory to derivatize monellin without destroying its sweet taste. In this paper we describe methylation of the lysyl residues, a portion of which can be so modified with retention of the sweetness of the protein. Through this reaction a radioactively labeled derivative of monellin has been prepared, which will now enable direct binding studies of it to taste receptors.

Materials and methods. Monellin was isolated from the fruit of *Dioscoreophyllum cumminsii* (1, 4). Reagents and their sources were as follows: fluorescamine, Roche Laboratories; 37% formaldehyde, Fisher Scientific; [^{3}H]formaldehyde (0.1 Ci/mmole, lot No. 728-029), New England Nuclear Corp.; N^{ϵ}-trimethyl-L-lysine-bis(*p*-hydroxyazobenzene-*p'*-sulfonate) $2H_2O$ (A Grade), Calbiochem; N^{ϵ}-methyl-L-lysine HCl and N^{ϵ}-dimethyl-L-lysine HCl, Cyclo Chemical. Other chemicals were reagent grade.

Methylation of monellin (11). The protein was dissolved in 0.2 *M* sodium borate, pH 9.0, at 10 mg/ml (1 m*M* monellin, or 11 m*M* in primary amino groups). Concentrated $NaBH_4$ in water (10 mg/ml) was added to give a final concentration of 0.5 mg/ml, which is an approximate equimolarity of amino groups and borohydride. The mixture was kept at 0°. Small aliquots of formaldehyde (1-5 μl of 6-8 *M* formaldehyde/ml of monellin solution) were added at 5-min intervals with stirring. Just prior to each addition, an aliquot of the mixture was removed for chemical determination of the extent of reaction and for taste testing. The extent of methylation was measured by assaying the free amino groups remaining (12).

Tritium-labeled methylated monellin was prepared with [^{3}H]formaldehyde (6 μCi/μmole). The unreacted formaldehyde was removed with an Amicon ultrafiltration apparatus (Model 202, UM2 filter) at room temperature. The monellin solution was filtered and then alternately diluted (with 0.01 *M* sodium phosphate buffer, pH 7.2) and concentrated until the radioactivity in the effluent was reduced to background levels. The ^{3}H-labeled methylated monellin was chromatographed on a column of CM-cellulose. The fractions were assayed for protein concentration, for free amino groups, and for radioactivity by liquid scintillation counting (13) with a Packard TriCarb Model 3375 at a counting efficiency of 37%.

Amino acid analyses. Samples of monellin were hydrolyzed *in vacuo* in constant boiling HCl for 22 hr at 110°. A Beckman 121 automatic analyzer equipped with a Beckman System AA computing integrator was used. The standard basic column (8-cm Beckman PA-35 resin) was eluted with 0.35 *N* sodium citrate buffer, pH 5.28, at 53.5° at a flow rate of 70 ml/hr. On this column monomethyllysine and dimethyllysine were not separated. For their separation, a 20-cm column of Durrum DC-6A resin was used. It was eluted initially with 0.35 *N* sodium citrate buffer, pH 5.70, at 28°. At 70 min the temperature was raised to 56° and the buffer changed to 0.35 *N* sodium

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citrate, pH 4.70. The flow rate was 70 ml/hr throughout. Elution times of the standards to the nearest minute were as follows: lysine, 67; monomethyllysine, 76; dimethyllysine, 81; trimethyllysine, 84; histidine, 92; ammonia, 100; and arginine, 195.

Gel electrophoresis. Electrophoresis was carried out in polyacrylamide gels under several conditions: pH 6.9 (0.05 *M* ammonium acetate), pH 7.5 (0.05 *M* ammonium acetate + 8 *M* urea + 0.1% 2-mercaptoethanol), pH 8.4 (0.05 *M* Tris/acetate), and pH 10.0 (0.05 *M* sodium glycinate). In gels of both 7.5 and 10% (w/w) acrylamide the ratio of acrylamide to *N,N'*-methylenebisacrylamide was 30:1. Electrolyte buffers were identical with those in the gels except that none of them contained urea. Proteins were stained for 1 to 2 hr in a solution of amido black (1%) and Coomassie brilliant blue G250 (0.25%) in 7% acetic acid, or alternatively with Coomassie brilliant blue G250 (0.25%) alone (14). Gels were scanned at 560 or 600 nm.

The two chains of monellin were dissociated by incubation for 2 hr at 25° in 8 *M* urea, 0.01 *M* Tris/acetate buffer, pH 8.4, and 1% mercaptoethanol (final pH, 8.5). Separation was achieved by polyacrylamide gel electrophoresis in the presence of 8 *M* urea + 0.1% mercaptoethanol.

Column chromatography and other procedures. Chromatography on CM-cellulose was carried out in 0.01 *M* sodium phosphate buffer, pH 7.2, using elution with a linear gradient of 0 to 0.05 *M* NaCl in the buffer (1). Gel filtration was carried out on Sephadex G-75 in 0.05 *M* Tris/acetate buffer, pH 8.4, which contained 0.2 *M* KCl to prevent adsorption of the protein. Protein fluorescence (2, 9) and sweetness (1) were determined as described earlier. Protein concentrations were calculated by comparison with the fluorescence emission of a standard solution of native monellin.

Results and discussion. Methylation and sweet taste. Monellin that was successively methylated showed successive decreases in primary amino groups (Fig. 1), 20 to 40% of which could be methylated without appreciable loss of sweetness. Additional methylation resulted in decreased sweetness. Even after extensive methylation under denaturing conditions (2% sodium dodecyl sulfate or pH 11.5), about 20% of the amino groups remained fluorescamine reactive.

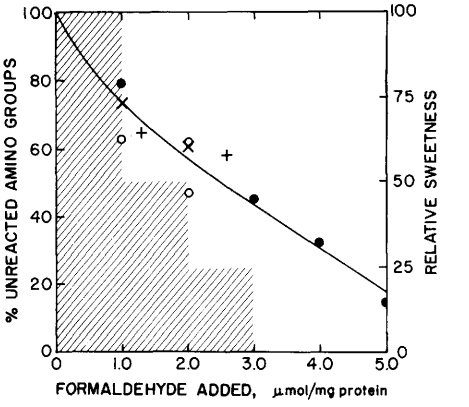

FIG. 1. Methylation of monellin. Aliquots of formaldehyde were added as shown on the abscissa. The ordinate shows sweetness level (shaded area) and fluorescamine reactivity remaining in four experiments (○, x, ●, +). The sweetness of the samples was evaluated at three concentrations (5, 10, and 20 μg/ml); the lowest concentration that tasted sweet is indicated on the scale as 100 (i.e., 100 means sweet at 5 μg/ml, while 0 means not perceptibly sweet at 20 μg/ml).

Chromatography of methylated monellin. In order to determine the possible extent of heterodispersity, the CM-cellulose chromatogram was analyzed for protein fluorescence, primary amino groups, and sweet taste. The elution profile (Fig. 2) showed a single peak for which these parameters were coincident. The ratio of the fluorescence parameters in the more slowly eluting material indicated somewhat less extensive methylation. A mixture of methylated and unmethylated monellin gave essentially the same elution profile, although it appeared to be slightly broader.

The half-width of the fluorescence emission for the fractions in the midportion of the elution peak (Fig. 2) was relatively constant at 62 ± 2 nm (range) (native monellin is 60 nm) (9). The fractions beyond number 215 showed an emission peak at 304 nm due to tyrosine. The data therefore indicate some conformational alteration of the protein due to methylation.

Amino acid composition of methylated monellin. The amino acid compositions of the six pooled fractions (see Fig. 2) were very similar (Table I), and those of pools

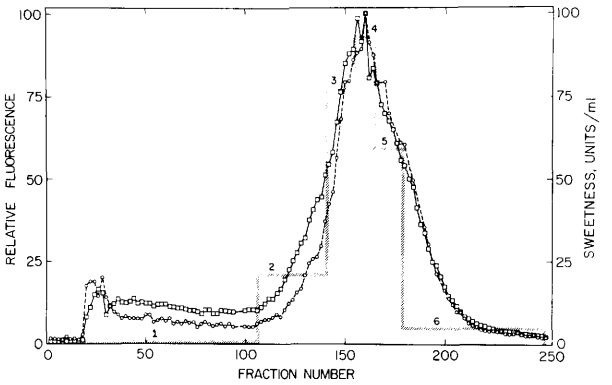

FIG. 2. Chromatography of methylated monellin on CM-cellulose. Methylated monellin (40 mg) (approximately 50% methylation) was chromatographed on a 2 × 20-cm column of CM-cellulose. The sample was applied in 0.01 *M* sodium phosphate buffer, pH 7.2, the column washed with 40 ml (1 column volume) of the same buffer, and the protein eluted with a 300-ml linear gradient of 0 to 0.05 *M* NaCl in the phosphate buffer. Fractions of 1 ml were collected at a flow rate of 15 ml/hr. Protein fluorescence (protein concentration) (□——□) and fluorescamine fluorescence (primary amino group concentration) (○- - -○) are expressed as relative fluorescence; curves normalized to the peak fraction as 100. The hatched lines are the sweetness values, with the numbers there indicating the six pools. The data for methylated lysines in these six pools are shown in Table II.

TABLE I. AMINO ACID COMPOSITIONS OF CHROMATOGRAPHIC FRACTIONS OF METHYLATED MONELLIN.

Residue	Pool number[a] (mole%)						Combined pools No. 3 to 6 (mole/mole of protein)[b]	Lit. ref. 2	Lit. ref. 8
	1	2	3	4	5	6			
Lys (total)	9.3	9.6	9.6	9.6	9.6	9.7	8.22 ± 0.14	8	9
His[c]	Trace	0.3	0.2	Trace	Trace	Trace	0.03 ± 0.07	0	–
Arg	6.1	6.2	6.9	7.0	6.9	6.6	5.85 ± 0.13	7	6
Asx	11.6	10.4	11.1	11.2	11.2	11.3	9.58 ± 0.14	10	10
Thr	4.4	4.7	4.5	4.5	4.3	4.3	3.77 ± 0.04	4	4
Ser	3.3	5.2	3.2	2.8	2.6	3.7	2.79 ± 0.57	2	2
Glx	15.0	14.4	14.2	14.2	13.8	14.0	12.00	12	12
Pro	6.1	5.5	6.0	6.1	6.5	6.3	5.32 ± 0.27	6	5
Cys/2	0.4	0.0	0.5	0.7	0.4	0.4	0.43 ± 0.12	1	1
Gly	10.9	11.2	10.1	10.0	9.8	10.4	8.59 ± 0.19	8	8
Ala	3.0	4.5	3.5	3.3	3.4	3.6	2.95 ± 0.12	3	3
Val	3.6	3.3	3.7	3.6	4.1	3.9	3.17 ± 0.11[d]	4	4
Met	0.8	0.8	0.8	0.9	1.0	0.8	0.75 ± 0.07	1	1
Ile	8.2	6.7	7.1	7.1	7.3	7.1	6.12 ± 0.13[d]	6	8
Leu	5.9	6.0	6.4	6.5	6.4	6.2	5.46 ± 0.08[d]	6	6
Tyr	6.2	6.3	7.0	7.2	7.2	6.4	5.95 ± 0.34[d]	7	7
Phe	5.2	4.9	5.2	5.3	5.5	5.3	4.55 ± 0.15	5	5
Trp	–	–	–	–	–	–	– –	1	1

[a] Pools from the chromatography column shown in Fig. 2. Average values from duplicate analyses.
[b] Mean ± SD.; values normalized to Glx at 12.00.
[c] The presence of His reveals a trace contamination.
[d] Low recoveries of Val, Ile, Leu, and Tyr are probably due to the presence of Ile-Ile, Val-Ile, Ile-Tyr, Val-Tyr, Leu-Tyr, and Tyr-Val bonds in the protein (7, 8).

No. 3 to 6 were identical within experimental error (cf. 2, 8). This demonstrated that the two chains of monellin had not separated. The two chains would not have eluted together on this column and the sweet taste activity would have been lost.

Total methyllysine among the protein pools was similar (Table II). The lower methyllysine content in the trailing edge (pool No. 6) agrees with the fluorescence data (see above) and is accounted for by a decrease only in the dimethyl derivative.

TABLE II. EXTENT OF METHYLATION OF LYSINES IN METHYLATED MONELLIN.

Pool No.[b]	Percentage of total lysine[a]			
	Methyl-lysine[c]	Methylly-sine[d]	Monome-thyllysine[d]	Dime-thylly-sine[d]
1	48.5	49.6	10.3	39.3
2	54.0	61.3	6.4	54.8
3	51.7	53.6	8.2	45.4
4	47.5	48.0	7.0	41.0
5	45.4	46.2	8.2	38.0
6	36.0	38.0	8.8	29.3

[a] Averages of duplicate analyses. Trimethyllysine was not present.

[b] Pools from the chromatography column shown in Fig. 2.

[c] PA-35 resin, 8-cm column.

[d] DC-6A resin, 20-cm column.

The constant percentage of monomethyllysine in pools No. 3 to 6 and the progressive decrease in dimethyllysine in these pools argue against the possibility of admixtures of methylated and unmethylated monellin.

The formation of mainly dimethyllysine in monellin is similar to the results with other proteins; reductive methylation of chymotrypsin, chymotrypsinogen, transferrin, lysozyme, and ovomucoid with formaldehyde leads to formation of dimethyllysine as the principal product (11).

^{3}H-Labeled methylated monellin. A sample of ^{3}H-labeled methylated monellin (27 mg) was chromatographed on a column of CM-cellulose (1.3 × 30 cm). It eluted in a single peak similar to that in Fig. 2, with the radioactivity, protein fluorescence, and fluorescamine fluorescence coincident across the elution peak. The specific radioactivity was 7.9 μCi/μmole (±10%) in the major portion of the peak, and for a second preparation under similar, but not identical, conditions the specific radioactivity was 14 μCi/μmole.

A mixture of ^{3}H-labeled methylated monellin (50 μg) and unmethylated monellin (2.3 mg) was chromatographed on a Sephadex G-75 column (1.5 × 41 cm). The small quantity of the ^{3}H-labeled derivative did not interfere with the protein fluorescence from the native monellin, enabling detection of the two molecular species by independent measures. The two elution peaks were therefore separately observable. Each was homogeneous with respect to molecular size. The methylated monellin eluted somewhat earlier than the native monellin, indicating a slightly larger Stokes radius.

Polyacrylamide gel electrophoresis of native and methylated monellins. The six pools of methylated monellin (Fig. 2) and an additional preparation (66% methylated, which tasted sweet) were compared with the native protein by electrophoresis toward the cathode at pH 8.4 on 7.5% gels. The mobility of the methylated protein was in every case less than that of the native, thereby demonstrating the absence of unmodified protein. The mobility decreased from pools No. 6 to 1, consistent with decreasing net positive surface charge. Thus the orders of electrophoretic mobilities and affinities for the cation exchanger were in complete agreement.

Both the native and 66% methylated proteins migrated toward the anode at pH 10.0 (Fig. 3, left), with the methylated protein showing higher mobility. At pH 6.9 (Fig. 3, right) the converse order of mobilities was observed. Because all lysyl residues, whether methylated or unmodified, are fully charged at pH 6.9, this result can be explained either by deamidation or by a methylation-induced conformational change, resulting possibly in increased anion binding. Aggregation could not explain the findings.

Conformational changes that affect electrophoretic mobility should be abolished under denaturing conditions whereas changes in the net charge of the protein should not. Accordingly, native and methylated proteins were denatured and examined by electrophoresis. Monellin was dissociated into its two chains (Fig. 4), one bearing a net positive charge at pH 7.5 (the smaller subunit) and the other a net negative charge (the larger subunit). Methylation had no significant effect on the electrophoretic mobility under denaturing conditions. Thus the change in electrophoretic mobility of the intact protein after methylation must be related to a change in its conformation rather than to deamidation.

The evidence to date indicates that the tertiary structure of monellin is important for its sweet taste activity (1–10). The present work shows that a portion of the lysyl residues can be methylated without appreciable loss of sweet taste activity; further

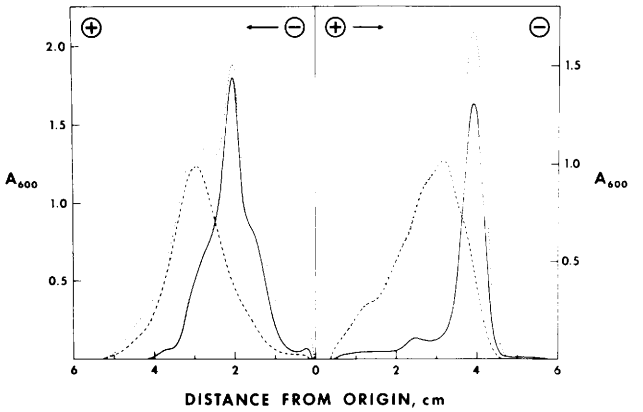

FIG. 3. Left: Electrophoresis in parallel of 50 μg of native monellin (——), 50 μg of 66% methylated monellin (-----), and a mixture (50 + 50 μg) of the two samples (· · · ·) toward the anode in 10% (w/w) polyacrylamide gels at pH 10.0 (6 hr, 3 mA/gel). Stained with Coomassie brilliant blue G250. Right: Electrophoresis in parallel of 25 μg of native monellin (——), 50 μg of 66% methylated monellin (-----), and a mixture (25 + 50 μg) of the two (· · · ·) toward the cathode in 7.5% (w/w) polyacrylamide gels at pH 6.9 (10 hr, 5 mA/gel). Stained with an amido black–Coomassie brilliant blue G250 mixture.

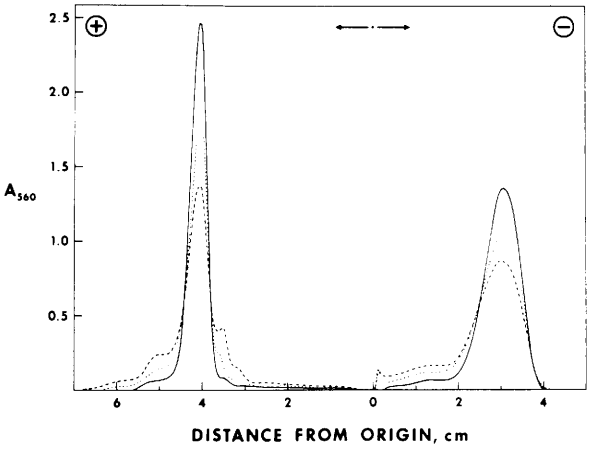

FIG. 4. Electrophoresis in parallel of 50 μg of native monellin (——), 46 μg of 66% methylated monellin (-----), and a mixture (25 + 23 μg) of the two (· · · ·) in 7.5% (w/w) polyacrylamide gels containing 8 *M* urea + 0.1% mercaptoethanol, pH 7.5 (24 hr, 2.5 mA/gel). The two monellin chains were dissociated by incubation for 2 hr at 25° in 8 *M* urea + 1% mercaptoethanol, pH 8.5, prior to electrophoresis. Stained as in Fig. 3, right.

methylation results in a decline in sweetness. Methylated monellin shows evidence of a slight increase in Stokes radius, a change in electrophoretic mobility, and a small increase in fluorescence emission half-width, all of which are consistent with a conformation change that includes some expansion of the molecule.

Summary. The ε-amino groups of the lysyl residues of monellin were reductively methylated with formaldehyde and sodium borohydride; 20–40% of the lysines could be methylated with essentially complete retention of the sweetness of the protein. The methylated protein yielded dimethyllysine and monomethyllysine upon acid hydrolysis. ^{3}H-Labeled methylated monellin was also prepared with [^{3}H]formaldehyde as the methyl donor; this derivative could be useful in binding studies to taste receptors. The methylated monellin was studied by ion-exchange chromatography, gel filtration, fluorescence spectroscopy, polyacrylamide gel electrophoresis, and amino acid analysis. Although sweetness was maintained after limited methylation, some

change in conformation of the protein did occur.

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