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The Sulfhydryl Group of Monellin: Its Chemical Reactivity and Importance to the Sweet Taste (39457)

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The chemostimulatory protein monellin, a basic protein of molecular weight 10,700, tastes intensely sweet (1-4). A combination of evidence from amino acid analyses and gel exclusion chromatography shows that the species of minimal molecular weight tastes sweet and contains a single cysteine residue (2). Van der Wel and Loeve (3), however, reported finding no free —SH in the protein using Ellman's reagent. Rather, they reported that reduction of monellin with 0.5 *M* cysteine or ethanethiol eliminated the sweet taste, and they suggested that a —SS— bond is involved in its sweetness. The tertiary structure does appear to be important for the sweet taste of monellin (4, 5).

The earlier findings that the monellin molecule contains a single cysteine residue per active (sweet) molecule (2) are, therefore, at variance with the reported presence of a —SS— bond. The possibility that monellin might be a dimer of molecular weight 20,000 in its sweet-tasting form was ruled out (2). In this paper we demonstrate that a single free -SH is present in native monellin, and that chemical modifications of the -SH lead to a concomitant decrease in sweetness of the protein.

Materials and methods. Iodoacetic acid, 2-mercaptoethanol (Eastman), high purity guanidine-hydrochloride (Gdn-HCl) (Heico, Inc., Delaware Water Gap, Pa.), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), *p*-hydroxymercuribenzoate (sodium salt) (PHMB), cysteine (free base), dithioerythritol, sodium dodecyl sulfate (Sigma), and ultrapure urea (Schwarz-Mann) were used without further purification. Monellin was purified as described previously (1, 5), and solutions were prepared from lyophilized protein held over P_2O_5 *in vacuo* at 4°. Other chemicals were reagent grade.

The —SH content of native, denatured, or carboxymethylated monellin was determined by titration with PHMB using the procedure of Boyer (6) as described by Riordan and Vallee (7). Titrations were performed at room temperature (24-25°) in an initial reaction volume of 4 ml, buffered at either pH 7.0 (0.01 *M* sodium phosphate) or pH 4.5 (sodium acetate). The monellin for the sample in acetate was prepared in 1.5 ml of 0.1 *N* acetic acid and added to the cuvette containing 2.5 ml of 0.33 *M* Na acetate, final pH 4.5. The appropriate denaturant was in the cuvette, and the reference cuvette had only the protein omitted. After addition of each 10-μl aliquot of PHMB (containing approximately 10 nmoles), the absorbance was measured until it reached a constant value. The absorbance at either 250 nm (pH 7.0) or 255 nm (pH 4.5) was measured in 1-cm path length quartz cuvettes with a Zeiss PMQ II spectrophotometer. Protein concentration was adjusted to between 60 and 80 nmoles of monellin per 4 ml, and was determined for each titration from the absorbance at 277 nm (2). The reactivity of the PHMB was confirmed by titration of 2-mercaptoethanol and of dithioerythritol.

Monellin was titrated with DTNB (24°) according to the method of Ellman (8) in either the absence or presence of denaturants. The sample cuvette contained, in a total volume of 3 ml, 150 μmoles of sodium phosphate buffer (pH 8.0), 0.2 μmole of DTNB, and usually about 75 nmoles (0.8 mg) of monellin. The reference cuvette contained the same mixture without protein. The increase in absorbance at 412 nm was

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followed in 1-cm path length quartz cuvettes in the Zeiss PMQ II. As controls, 2-mercaptoethanol or dithioerythritol were titrated similarly in the absence and presence of the denaturants.

Monellin was carboxymethylated with iodoacetate (9) in 0.05 *M* Tris-HCl buffer (pH 8.4), in the absence and presence of 6 *M* Gdn-HCl. Monellin was dissolved in the Tris buffer or the Tris-guanidine solution immediately before use. Carboxymethylation of protein samples of approximately 10 mg was carried out at ambient temperature (26–28°) in a total volume of 2 ml with magnetic stirring for 2 hr. The reaction was initiated by adding the iodoacetate (10 μmoles in 0.1 ml of the buffer). A control sample stirred in buffered Gdn-HCl was included. The pH was maintained between 8.4 and 8.5 by adding small aliquots of 1 *N* NaOH. After 2 hr, the reaction mixture was cooled in ice and dialyzed overnight at 4° against 4 liters of 0.01 *M* sodium phosphate buffer (pH 7.1). During dialysis of the guanidine-containing samples (carboxymethylated and control), small amounts of protein precipitated. This precipitated protein, which amounted to less than 5% of the original samples, was removed by centrifugation and the supernatant solutions were used for the taste assays and —SH titrations. The sedimented material was dissolved in 1.5 ml of 0.1 *N* acetic acid (precipitates were not soluble in neutral solutions) and also analyzed for —SH content, but adequate taste evaluation was difficult because of the high acid concentration relative to the concentration of monellin.

Samples of monellin (1 mg) were treated with 2-mercaptoethanol (7 m*M*, 0.05 *M*, or 0.71 *M*), cysteine (0.5 *M*), or dithioerythritol (0.05 *M*) (10), in either 0.01 *M* sodium phosphate buffer (pH 7.0), or 0.01 *N* acetic acid (pH 2.9). Thiols were present in 15- to 1500-fold molar excess over the protein. Reductions were carried out at room temperature (24°) for 1 hr (cysteine) or 18 and 24 hr (mercaptoethanol or dithioerythritol).

For air-exposure of monellin, samples were dissolved (5 mg/ml) in either 0.01 *M* sodium phosphate buffer (pH 7.0) (control), 6 *M* Gdn-HCl, or 6 *M* Gdn-HCl containing 0.14 *M* mercaptoethanol. The latter two solutions were prepared in the phosphate buffer and titrated to pH 7.0 with NaOH. The thiol-containing sample was flushed with nitrogen and kept covered with parafilm, while the other two samples were left exposed to the air. The samples were magnetically stirred for 2 hr at ambient temperature (24–26°). The two samples left exposed to the air were then dialyzed overnight at 4° against 2 liters of 0.1 *N* acetic acid; the thiol-containing sample was dialyzed against 1 liter of 0.1 *N* acetic acid that contained 14 m*M* mercaptoethanol. The dialyzed samples were assayed for sweetness and protein concentration.

Protein concentration was estimated from the absorbance of monellin at 277 nm ($\epsilon_{277\,nm} = 1.47 \times 10^4\ M^{-1}\ cm^{-1}$ [2]). Sweetener activity was estimated as described previously (1).

Results. Content of free —SH. Reaction of native monellin with PHMB was very slow, demonstrating that the —SH is relatively inaccessible. Complete reaction of each aliquot of added PHMB (approximately 10 nmoles) required 30 min or longer (Table I), in contrast to the essentially instantaneous reaction of identical aliquots of PHMB with small molecule thiols (e.g., mercaptoethanol). At either pH 7.0 or pH 4.6 the presence of 1 mole of titratable —SH per mole of protein was found, confirming the presence of one cysteine residue that was established by amino acid analyses (2). Monellin titrated with PHMB in the presence of each of three common protein denaturants showed close to 1 titratable —SH per molecule of monellin (Table I), and in contrast to the slow reaction with native monellin, denatured monellin reacted very rapidly with each aliquot of added PHMB. This clearly demonstrated the increased accessibility of the —SH.

The presence of a single titratable —SH in monellin was also confirmed by titration with DTNB (Ellman's reagent) (Table II). As with PHMB, DTNB reacted extremely slowly with native monellin; complete reaction would require more than 25 hr, compared with 2 to 5 min for complete reaction of DTNB with mercaptoethanol or dithioerythritol. The long time period required for the —SH of native monellin to react ex-

TABLE I. SULFHYDRYL CONTENT OF MONELLIN BY PHMB TITRATION.

Titration conditions[a]	Nanomoles of monellin	Nanomoles of —SH	Moles —SH/ mole of monellin	Reaction time[b]
Sodium phosphate, pH 7.0	76.0	76.0	1.00	30 min
Sodium acetate, pH 4.5	49.6	46.5	0.94	45 min
1% Sodium dodecyl sulfate	70.3	58.5	0.83	30 sec
6 *M* Gdn-HCl	67.8	44.0	0.65	30 sec
8 *M* Urea	69.0	51.5	0.75	30 sec

[a] Titrations in denaturants were carried out in the phosphate buffer (pH 7.0) as described under Materials and Methods. (The first sample is also the first sample in Table III.)

[b] Reaction time for the first two samples, without denaturant, is the minimum time required for each aliquot of added PHMB to react (except for the initial addition, which was somewhat more rapid). The reaction time indicated for each of the three samples in denaturants is the maximum time required for complete reaction of each added aliquot. For these samples, reaction of each aliquot of PHMB was essentially complete in the time required to mix the sample and place it into the spectrophotometer.

TABLE II. SULFHYDRYL CONTENT OF MONELLIN BY DTNB TITRATION.

Titration conditions[a]	Nanomoles of monellin	Nanomoles of —SH	Moles —SH/ mole of monellin	Reaction time[b]
0.05 *M* Na phosphate, pH 8.0	70.4	52.5	0.74	>25 hr
1% Sodium dodecyl sulfate	70.4	62.4	0.89	10 min
5.7 *M* Gdn-HCl	70.4	48.2[c]	0.69[c]	10 min
7.6 *M* Urea	70.4	58.6	0.83	50 min

[a] Titrations were carried out in the phosphate buffer (pH 8.0) as described under Materials and Methods. The original denaturant concentrations for the last two samples were 6 *M* (Gdn-HCl) and 8 *M* (urea), but addition of aliquots of protein (in deionized water) and DTNB (in 0.05 *M* sodium phosphate buffer, pH 7.0) resulted in the final concentrations indicated. (The titration curves for the samples in denaturants are shown in Fig. 1.)

[b] The reaction time is the approximate time to reach the maximal absorbance at 412 nm (see Fig. 1). Even after 25 hr the absorbance for the first sample (native) had not reached a maximum.

[c] DTNB titrations proved to be less reliable in the presence of Gdn-HCl because the colored reaction product was unstable (see Fig. 1 and inset).

plains the absence of —SH in monellin reported by van der Wel and Loeve (3). In the presence of a denaturant, the —SH group of monellin reacted much more rapidly (Table II, Fig. 1), and titration with DTNB showed the presence of approximately 1 —SH per molecule (Table II). DTNB titrations in Gdn-HCl are less reliable than in the other denaturants because the colored reaction product is unstable in Gdn-HCl (Fig. 1). The maximal absorbance reached before the color began to fade was usually 70 to 80% of theoretical. This chromophore instability, which was encountered both with monellin and small molecule thiols (inset to Fig. 1), has been reported previously (11) and is doubtless due to the Gdn-HCl or a trace of impurity in it. For example, monellin that was first denatured in Gdn-HCl and then dialyzed, subsequently showed a normal titration in the presence of dodecyl sulfate.

Monellin no longer tastes sweet after complete titration with either PHMB or DTNB. When native monellin had been titrated, the loss of sweetness was not reversed by dialysis against phosphate buffer (pH 7.0) containing a large excess (10^4 to 10^6 molar excess) of either mercaptoethanol or dithioerythritol. However, full reversal was achieved under the following conditions. Monellin in the presence of 8 *M* urea was inactivated by a 20-fold molar excess of PHMB; the protein was then dialyzed first against 0.1 *N* acetic acid containing 7 m*M* mercaptoethanol, then against large volumes of 0.1 *N* acetic acid alone, and finally against 0.01 *M* sodium phosphate buffer (pH 7.0). The recovered protein tasted sweet. (The final two dialysis steps are probably not essential for recovery of sweetness, but were included in this particular experiment because of other studies that were carried out on the protein sample.)

Carboxymethylation of monellin. Treat-

ment of native monellin with iodoacetate for 2 hr resulted in only a slight decrease in PHMB-titratable —SH content (Table III). The retention of 88% of the titratable —SH corresponded quite well with the 82% retention of sweetness. Monellin retains its sweet taste following treatment with 6 *M* Gdn-HCl at acid pH, although some loss in sweetness occurs after denaturation at neutral or slightly alkaline pH (5). Advantage was taken of the latter observation. Monellin that was carboxymethylated in the presence of Gdn-HCl at alkaline pH was completely devoid both of titratable —SH and of sweetness (Table III), while the control sample incubated in Gdn-HCl had retained 52% of the sweetness, closely paralleling the 60% retention of titratable —SH.

Reduction and air-oxidation. It was recently reported that reduction of monellin with thiols resulted in loss of sweetness (3). This has not been confirmed. Monellin maintained at neutral pH in the presence of 0.5 *M* cysteine (1 hr), 7 m*M* – 0.71 *M* mercaptoethanol (18 hr), or 0.05 *M* dithioerythritol (24 hr) retained full sweetness activity. Exposure to the latter two reducing agents (0.05 *M*) at low pH for 24 hr was also ineffective in decreasing sweetness.

The somewhat low —SH values determined in the presence of denaturants are possibly due to oxidation of the —SH (5).

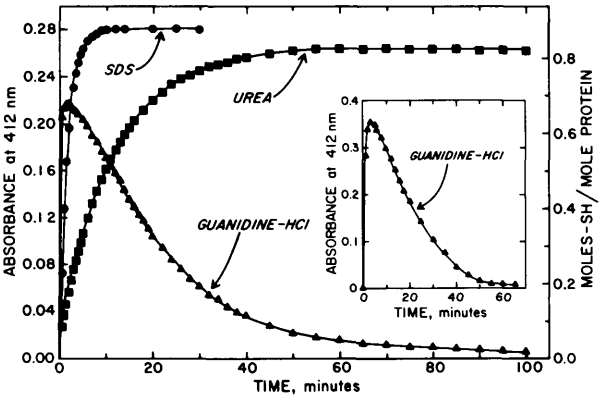

FIG. 1. DTNB titration of denatured monellin. A stock solution of monellin was prepared in deionized water and aliquots (0.16 ml containing 70.4 nmoles) were titrated in the presence of the indicated denaturants in 0.05 *M* sodium phosphate buffer (pH 8.0). The concentrations of denaturants were 1% (w/v) sodium dodecyl sulfate, 5.7 *M* Gdn-HCl, and 7.6 *M* urea. These are the samples shown in Table II. The *inset* shows the titration curve for 2-mercaptoethanol (100 nmoles) in 6 *M* Gdn-HCl.

TABLE III. CARBOXYMETHYLATION OF MONELLIN.

Treatment	Sweetener activity (units/mg)	Percent of native	Theoretical –SH[a] (nmoles)	Observed –SH[b] (nmoles)	Percent of native
Native	174	100	76.0	76.0	100
Carboxymethylated in buffer, pH 8.4	142	82	65.6	57.5	87.5
Buffered 6 *M* Gdn-HCl, pH 8.4	90[c]	52	74.0	44.5[c]	60.2
Carboxymethylated in buffered 6 *M* Gdn-HCl, pH 8.4	0[c]	0	80.8	0[c]	0

[a] Calculated from the absorbance at 277 nm (2) and the presence of 1 cysteine residue per 10,700 g of protein.

[b] Determination of –SH was by PHMB titration. Each 10 μl aliquot of added PHMB (approximately 10 nmoles) was allowed to react for at least 30 min.

[c] Values are for the soluble protein remaining after the small amounts of precipitates (5%) were removed by centrifugation (see Materials and Methods). The dissolved (in 0.1 *N* acetic acid) precipitates were not sweet; slight sweetness could have been masked by the sour taste of the acetic acid. PHMB titration of these dissolved precipitates revealed no titratable –SH.

TABLE IV. TIME-DEPENDENT LOSS OF TITRATABLE SULFHYDRYL OF DENATURED MONELLIN[a].

Conditions	Incubation time	Nanomoles of monellin	Nanomoles of −SH[b]	Moles -SH/mole of monellin
6 *M* Gdn-HCl	15 min	42.2	27.1	0.64
8 *M* Urea	15 min	42.3	33.3	0.79
6 *M* Gdn-HCl	2 hr	43.7	26.2	0.60
8 *M* Urea	2 hr	42.9	16.0	0.37
6 *M* Gdn-HCl	6 hr	39.3	22.2	0.56
8 *M* Urea	6 hr	42.9	5.6	0.13
6 *M* Gdn-HCl	24.5 hr	43.5	13.3	0.31
8 *M* Urea	24.5 hr	39.2	0	0

[a] Samples (nominally 5 mg/ml) were prepared in 0.05 *M* sodium phosphate buffer (pH 7.0) that contained either 6 *M* Gd-HCl or 8 *M* urea, and kept, without stirring, throughout the experiment at room temperature (24°).

[b] For determining −SH content, aliquots (0.1 ml) were withdrawn at the times indicated and titrated with DTNB in phosphate (pH 8.0) which also contained the indicated denaturant. Not more than 30 min was required for maximal color development and in most cases the time required was much less (approximately 2.5 min). Values for samples titrated in Gdn-HCl are undoubtedly low because of interference of the Gdn-HCl with color development (see Fig. 1 and inset). Calculations are based on the maximal absorbance reached.

The results in Table IV support this interpretation, where titratable —SH decreased with time in the presence of either denaturant. To further examine the possibility of a close relationship between free —SH and the sweetness of monellin, the protein was exposed to oxidation by air under conditions closely paralleling those of the carboxymethylation experiments in Table III. Monellin stirred in 6 *M* Gdn-HCl to expose it to air lost 51% of its sweetness in 2 hr (thus, comparing closely with the loss of 48% in the experiment in Table III); in the presence of 0.14 *M* mercaptoethanol the loss was only 24%.

Discusson. The molecular basis for the sweet taste of the chemostimulatory protein monellin is only beginning to be understood. The evidence available previously is consistent with the sweet taste requiring the intact native protein (4), and recent evidence using denaturants (5) strengthens the idea that the tertiary structure is important for sweetness.

Amino acid analyses (2) showed that a single cysteine residue is present in the active (sweet) molecule of monellin of molecular weight 10,700. In contrast, van der Wel and Loeve (3) reported that maintenance of tertiary structure was aided by the presence of a —SS— bond. This was based on the reported absence of free —SH and the loss of sweetness upon reduction of the protein by thiols. The results of the present study contradict those of van der Wel and Loeve (3) and point to an important role of the —SH group, not an —SS—, in allowing maintenance of the sweet taste of monellin.

Determination of free —SH by titration with either PHMB or DTNB (Ellman's reagent) gave values approaching 1 mole of —SH per mole (10,700 g) of monellin (Tables I, II). The absence of —SH reported by van der Wel and Loeve (3), who used Ellman's reagent, is readily explained by our finding that the —SH in the native protein is relatively inaccessible to titration. The accessibility of the —SH of monellin to titration increases dramatically in the presence of protein denaturants. The inaccessibility of the —SH in native monellin was also evident when carboxymethylation was attempted, while complete reaction was achieved in the presence of 6 *M* Gdn-HCl. Inaccessibility of —SH to various chemical modifications has been reported for certain other proteins (e.g., 11-13).

The —SH appears to be essential for the sweet taste of monellin, a conclusion supported by several lines of evidence. Monellin is completely inactive (not sweet) after titration with PHMB or DTNB, or when carboxymethylated. Loss of sweetness parallels loss of titratable —SH, and mercaptoethanol helps to protect against loss of sweetness. Inactivation by PHMB is reversed by removing the mercurial with excess mercaptoethanol under appropriate conditions, an observation that also offers the strongest evidence to date that the pro-

tein itself tastes sweet. Despite the critical role of the —SH for the sweet taste of monellin, there is no evidence for a direct role of the —SH in eliciting sweetness by interacting with a binding site on taste receptor cells (14), and this type of interaction seems unlikely because the —SH in the native monellin is buried. We postulate that the —SH of monellin must remain unaltered in order for the protein to maintain its tertiary structure and hence its sweet taste.

Summary. The presence of a single cysteine in the sweet-tasting protein monellin was confirmed by titrations with *p*-hydroxymercuribenzoate (PHMB) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). The sulfhydryl group in native monellin reacts very slowly with each of these reagents, indicating that the sulfhydryl is relatively inaccessible. In the presence of either 6 *M* guanidine-HCl, 8 *M* urea, or 1% sodium dodecyl sulfate, the rate of reaction of the sulfhydryl group with titrant is dramatically increased. Under a variety of conditions, the presence of 1 mole of sulfhydryl per mole of protein (of molecular weight 10,700) was found.

Reaction of the sulfhydryl by titration with PHMB or DTNB leads to loss of sweetness. The free sulfhydryl is also lost by carboxymethylation of monellin in the presence of guanidine-HCl, yielding a protein that is not sweet. Exposure to air in the presence of denaturant leads to a decrease in the sweetness of monellin. Sweetness of the PHMB-reacted monellin can be recovered upon treatment of the protein with mercaptoethanol, and the partial loss of sweetness that occurs with air exposure is lessened in the presence of mercaptoethanol. It is postulated that alteration of the single sulfhydryl group of monellin leads to a change in the tertiary structure of the protein and hence its sweet taste.

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