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Effects of Denaturants on the Sweet-Tasting Protein Monellin (39017)

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(Introduced by M. R. Kare)

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The specific structural features of the chemostimulatory protein monellin (1–3) that are required for its intense sweet taste are not known. Although the precise role of the tertiary structure in eliciting sweetness is unknown, it appears to be important from the somewhat fragmentary evidence to date (4). In this paper we report studies, carried out with common protein denaturants, on the denaturation of monellin and concomitant changes in sweetness that support the hypothesis that the tertiary structure of monellin is important to its sweetness.

Materials and methods. Ultrapure urea (Schwarz–Mann), high-purity guanidine hydrochloride (Gdn–HCl) (Heico, Inc., Delaware Water Gap, Pennsylvania), and sodium dodecyl sulfate (Sigma) were used without further purification. Solutions of urea were freshly prepared. Monellin was purified according to Morris and Cagan (1) and solutions were prepared from lyophilized protein held over P_2O_5 *in vacuo* at 4°. For some experiments monellin that had been additionally purified by chromatography on a column of Sephadex G-50 was used. This additional purification step was necessary for some preparations of monellin because of the presence of a trace amount of contaminating protein that was not removed by the previously described procedure. Sephadexes G-25 (50–150 μm particle size), G-50 (50–150 μm particle size), and G-75 (40–120 μm particle size) were purchased from Pharmacia. *p*-Hydroxymercuribenzoate, sodium salt (PHMB) was obtained from Sigma. Other chemicals were reagent grade. The dialysis tubing (A. H. Thomas Co., Philadelphia, Pennsylvania or Spectrapor TM_2 from National Scientific Co., Cleveland, Ohio; stated molecular weight cutoff: 12,000–14,000) was washed (5) prior to use.

For neutral and slightly alkaline solutions the denaturants urea (8 *M*) and Gdn–HCl (6 *M*) were prepared in 0.01 *M* sodium phosphate buffer (pH 7.0) and titrated with NaOH or H_3PO_4 to between pH 7 and 8 as required. To obtain pH values in the acid range, the denaturants were dissolved in 0.1 *N* acetic acid and used without further adjustment of pH. Denaturation was carried out at room temperature (24°) with the protein at about 5 mg/ml for 2 hr (cf. 6).

Denaturants were removed by dialysis overnight (18–24 hr) at 4° against at least 4 liters of 0.01 *M* sodium phosphate buffer (pH 7.15) or 0.1 *N* acetic acid (pH 2.88). Precipitates which formed in samples dialyzed against the phosphate buffer were removed easily by centrifugation and the supernatants were drawn off for assays of protein and sweetness. The pellets were dissolved in 1 ml of 0.1 *N* acetic acid. In one case (Table II) the redissolved pellets were centrifuged again and the second pellets (very small) were discarded.

Protein concentration was determined either from the weight of the sample or from the absorbance at 277 nm ($\epsilon_{277\ nm} = 1.47 \times 10^4\ M^{-1}\ cm^{-1}$) (2). This extinction coefficient was used for all samples. (It decreased by not more than 5% in 0.1 *N* acetic acid, and 5–10% in the presence of 8 *M* urea or 6 *M* Gdn–HCl). Measurements were made in 1-cm path length quartz cuvettes in a Zeiss PMQ II spectrophotometer. Fluorescence spectra were measured as described previously (2).

Preliminary screening for sweetness was performed on 1:10 dilutions in deionized water. Samples that were not sweet at that dilution were recorded as tasteless. Those samples with a sweet taste at the 1:10 dilu-

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tion were subsequently taste-tested as described previously (1).

The aggregation of monellin was investigated by chromatography on Sephadex G-75 (7). Two columns were used: one column (1.5 × 38 cm) was equilibrated with 0.01 *M* sodium phosphate buffer (pH 7.15) containing 0.2 *M* KCl, and the second column (1.5 × 40 cm) was equilibrated with 0.1 *N* acetic acid containing 0.2 *M* KCl (pH 2.92). Both columns were equilibrated and eluted at 4° at a flow rate of 7–8 ml/hr. Fraction size was 20 drops (approx. 1.3 ml). Optical density at 277 nm of each fraction was measured on a Zeiss PMQ II spectrophotometer.

Content of free –SH groups was determined with PHMB using the method of Boyer (8) as described by Riordan and Vallee (9). Titrations were carried out at room temperature (24°) in 0.33 *M* sodium acetate buffer (pH 4.6) containing 1% (w/v) sodium dodecyl sulfate. The increase in absorbance at 255 nm was measured in 1-cm path length quartz cuvettes in the Zeiss PMQ II. The presence of the detergent permitted more rapid titration of the protein than is possible in the buffer alone (unpublished results).

Results and discussion. Preliminary survey of effects of denaturants on monellin. Sweetness was lost irreversibly in monellin treated for 24 hr at room temperature (24°) with 8 *M* urea or 1% (w/v) sodium dodecyl sulfate, in 0.01 *M* sodium phosphate buffer (pH 7.1–7.2). Sweetness was not regained after dialysis or gel filtration on Sephadex G-25 in the phosphate buffer. Surprising in light of those results was the full recovery of sweet taste activity after treatment in this manner with 6 *M* Gdn–HCl (pH 5.4).

Because the sweet taste of monellin cannot be assayed in the presence of the denaturants, they were removed in these preliminary studies by either conventional dialysis or gel filtration. Using the latter method the protein could be recovered free of denaturant within 1 hr, and taste assays could be performed within 2 hr. Though this might be expected to decrease the probability of refolding to the native conformation (e.g., 10) compared with the slower removal of denaturants by dialysis, the guanidine-treated sample was sweet after this treatment as well. It is difficult to remove sodium dodecyl sulfate bound to protein (10, 11), hence no further studies with this reagent were conducted.

Effects of pH during denaturation and renaturation (dialysis). Because the recovery of sweetness of guanidine-treated monellin in the preliminary studies above could have resulted from the lower pH (5.4) of these samples, we investigated more closely the effect of pH on both the denaturation and the renaturation (by dialysis) of monellin. In an initial series monellin was treated with urea or Gdn–HCl under acidic, neutral, or slightly alkaline conditions. Retention of sweetness was markedly pH-dependent. Monellin that was denatured in urea under neutral or slightly alkaline conditions lost approximately 80–90% of the sweetness, but when it was denatured and dialyzed under acid conditions it lost only 20% of the sweetness. The samples treated with Gdn–HCl retained higher levels of sweetness; as with urea, the one treated at the lowest pH retained the highest sweetener activity.

The effects of pH during denaturation itself and during subsequent dialysis were therefore studied further. When monellin was exposed to urea or Gdn–HCl at low pH, the retention of sweetness was essentially complete; the pH of the dialysis solution had no effect (Table I). Monellin was also exposed to urea or Gdn–HCl at neutral pH (Table II). These data, when taken in conjunction with those in Table I, show that what loss of sweetness occurs in these denaturants is dependent upon the pH of the denaturing solution. Recovery of sweetness following denaturation is clearly favored when the denaturation occurs at low pH, while the pH of the renaturing (dialysis) solution has no measurable effect on recovery of sweetness.

The difference in the ability of monellin to remain sweet after treatment with the two denaturants (urea and Gdn–HCl) is unexplained. For example, a similar result has also been reported for the enzymatic activity of carbonic anhydrase (12) although the conformations of most proteins are probably disrupted more in Gdn–HCl than in urea

TABLE I. FULL RECOVERY OF SWEETNESS OF MONELLIN THAT WAS DENATURED UNDER ACID CONDITIONS[a].

Sample	Denaturant[b]	Denaturation pH	Dialysis pH[c]	Retentate protein: Total recovered (%)	Retentate protein: Soluble (%)	Sweetener activity[d] (units/mg)	Percentage of control[e]
1	None: 0.1 *N* acetic acid	2.88	2.88	81.7	98.7	229	100
2	None: 0.1 *N* acetic acid	2.88	7.14	72.2	97.4	239	100
3	8 *M* urea	4.17	2.88	59.4	98.7	244	107
4	8 *M* urea	4.17	7.14	71.5	96.9	231	97
5	6 *M* Gdn-HCl	2.77	2.88	79.5	98.3	212	93
6	6 *M* Gdn-HCl	2.77	7.14	90.3	94.1	235	98

[a] Samples (5.13–5.69 mg) were treated with the denaturants at room temperature (24°) for 2 hr and then dialyzed overnight at 4° against sodium phosphate buffer or acetic acid.

[b] Denaturants were dissolved in 0.1 *N* acetic acid.

[c] Samples were dialyzed against either the phosphate buffer or acetic acid.

[d] Sweetener activity values are those of soluble protein. Taste assays of dissolved precipitates were not possible because of the high acetic acid content relative to the protein concentration.

[e] Percentage sweetness values are based on the activities of the control samples (1 and 2), with each control as 100%. Samples 3 and 5 are compared with Sample 1, and Samples 4 and 6 with Sample 2.

TABLE II. EFFECT ON SWEETNESS OF MONELLIN BY DENATURATION AT NEUTRAL pH[a].

Sample	Denaturant[b]	Denaturation pH	Dialysis pH[c]	Retentate protein: Total recovered (%)	Retentate protein: Soluble (%)	Sweetener activity[d] (units/mg)	Percentage of control[e]
1	None: 0.01 *M* Na phosphate	7.00	2.88	64.7	99.0	214	100
2	None: 0.01 *M* Na phosphate	7.00	7.14	78.7	91.8	198	100
3	8 *M* urea	7.03	2.88	73.9	99.5	120	56
4	8 *M* urea	7.03	7.14	87.6	62.7	89	45
5	8 *M* urea-stirred	7.03	7.14	70.1	46.8	127	64
6	6 *M* Gdn-HCl	7.04	2.88	76.2	99.2	187	87
7	6 *M* Gdn-HCl	7.04	7.14	74.6	89.8	192	97

[a] Samples (4.85–6.12 mg) were treated as described in Table I.

[b] Denaturants were dissolved in 0.01 *M* sodium phosphate buffer (pH 7.0), and pH adjustments were made with either NaOH or H_3PO_4. Sample 5 was stirred on a magnetic stirrer in an uncovered tube to maximize exposure to the air.

[c] Samples were dialyzed against either the phosphate buffer or acetic acid.

[d] See footnote *d* to Table I.

[e] Percentage sweetness values are based on the activities of the control samples (1 and 2), with each control as 100%. Samples 3 and 6 are compared with Sample 1; Samples 4, 5, and 7 are compared with Sample 2.

(6). Under no conditions of Gdn–HCl treatment was there an appreciable loss of sweetness of the recovered protein.

Precipitates appeared when the denatured samples were dialyzed against phosphate buffer (pH 7.15), but those dialyzed against acetic acid remained completely soluble (Tables I and II). The loss of protein observed in various instances, however, is not attributable to precipitation because a sample with no precipitation could also show poor protein recovery (e.g., Sample 1, Table II). Significant loss of protein sometimes occurred when native monellin was dialyzed (Tables I and II). Although we have not noticed unusual losses of monellin during

its routine purification, which includes dialysis, it appears that slight changes in dialysis conditions, such as pore size of the dialysis membrane, could cause significant losses. The molecular weight of monellin (10,700) is slightly below the stated cutoff point of the dialysis membrane (molecular weight cutoff: 12,000–14,000), and it is perhaps surprising that difficulties with greater losses of protein have not been encountered previously.

Aggregation of monellin. As noted above, formation of large aggregates of monellin was evidenced by precipitation under some conditions (Table II). In the presence of denaturant, it is favored at or above neutral pH, and is especially marked with urea. Very little precipitation occurred in samples denatured or dialyzed at low pH. Exposure to low pH at any point clearly reduces the likelihood of aggregation of monellin. In all cases where taste assays could be carried out, the aggregated protein was found to be tasteless.

One cysteine residue is present per molecule of monellin (2). Disulfide bond formation is expected to be favored above neutral pH (6), where aggregation of monellin was in fact pronounced. This possibility was therefore explored by direct –SH titrations of urea-treated and guanidine-treated monellin (samples in Table II). Measurements made on the soluble portion of the dialyzed samples showed the –SH content of some samples to be decreased by 20–30% (Samples 3, 4, 5, and 7) when compared with the native protein. Particularly revealing were the titrations made on the protein that precipitated (from Samples 4 and 5). Only a small amount of –SH was found in the precipitate (about 13% of the theoretical), and this could be accounted for by the contaminating monomer present (see Fig. 2). The large aggregates that form therefore contain no free –SH. It is hypothesized that oxidation of –SH to –SS– can occur under denaturing conditions that lead also to loss of sweetness.

The aggregated monellin was studied further by gel filtration chromatography. The samples shown in Table II were chromatographed on Sephadex G-75 in the presence of 0.2 *M* KCl (Figs. 1 and 2). Aliquots (0.5 ml) of samples that had been dialyzed against phosphate buffer were chromatographed in the same buffer; samples in acetic acid were chromatographed in that solvent. Two control samples of monellin were run on each column, one freshly prepared and the other the control sample shown in Table II. Since their elution volumes were the same for each column, only the latter controls are shown.

In addition to the precipitation that oc-

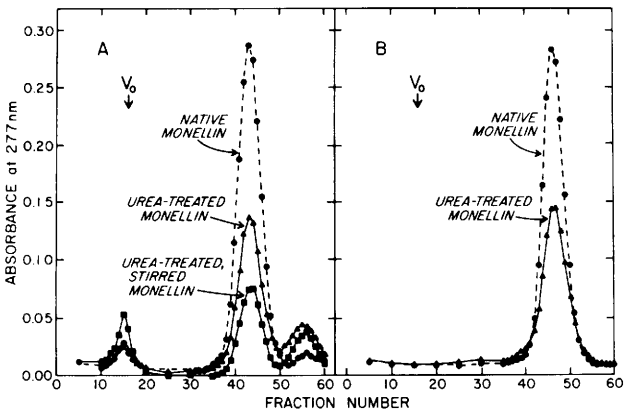

FIG. 1. Chromatography of urea-treated monellin on Sephadex G-75. Samples are those shown in Table II. (A) Native monellin (1.76 mg), monellin treated with urea (1.12 mg) (treated and dialyzed at neutral pH), and monellin treated with urea with stirring (0.79 mg). The column was equilibrated and eluted with 0.01 *M* sodium phosphate buffer (pH 7.15) containing 0.2 *M* KCl. (B) Native monellin (1.63 mg) and monellin treated with urea (1.25 mg) (treated at neutral pH, dialyzed at acid pH). The column was equilibrated and eluted with 0.1 *N* acetic acid (pH 2.92) containing 0.2 *M* KCl.

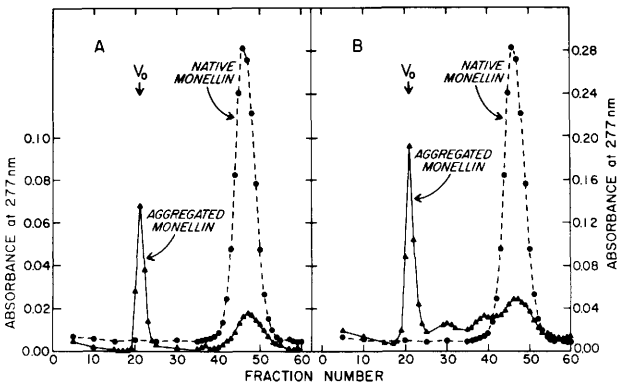

FIG. 2. Chromatography of urea-treated monellin on Sephadex G-75. Samples are protein that had precipitated (see Table II). (A) Native monellin was Sample 1. Aggregated monellin was insoluble protein from the sample (No. 4) treated and dialyzed at neutral pH. It was dissolved in 0.1 *N* acetic acid containing 0.2 *M* KCl. (B) Native monellin was the same as in A. Aggregated monellin was insoluble protein from Sample 5 treated with stirring and dialyzed at neutral pH. It was dissolved in 0.1 *N* acetic acid containing 0.2 *M* KCl. The column used for A and B was equilibrated and eluted with 0.1 *N* acetic acid (pH 2.92) containing 0.2 *M* KCl. The scale on the right-hand ordinate applies to native monellin (dotted lines) and that on the left-hand ordinate to the urea-treated (aggregated) protein.

curred during dialysis of monellin at neutral pH, the presence of aggregated protein (mol wt $\geq$ 70,000) that remained in solution was demonstrated by the elution of a small protein peak (10–20%) in the void volume (Fig. 1A). The control also showed a trace amount of aggregate. The majority of the protein (55–80%) eluted at the position of native monellin. No evidence of dimers was observed, but surprisingly a third peak eluted following native monellin (Fig. 1A), indicating the presence of a smaller ultraviolet-absorbing species. The peak was present in both guanidine-treated and urea-treated monellin, but was more marked with the latter. The fluorescence emission spectrum of a fraction from this peak was markedly different from that of native monellin and similar to, but not identical with, that of denatured monellin. The fraction from the column showed emission maxima at 303 and 347 nm. Samples dialyzed at acid pH showed a single peak whose elution volume was similar to that of native monellin (Fig. 1B).

The presence of a large amount of aggregated monellin was clearly demonstrated (Fig. 2) in the protein that precipitated (urea-treated samples) and which was then dissolved in acid. The acid-soluble aggregated protein was not sweet although low sweetness levels could have escaped detection because of the sour taste of the acetic acid. Approximately half of the protein recovered eluted as a sharp peak in the void volume. In one sample the remainder was in a small broad peak eluting at the position of native monellin (Fig. 2A). The sample that had been stirred during urea denaturation was similar (Fig. 2B), but in addition showed a shoulder on the leading edge of the broad monomer peak, suggesting an apparent molecular weight of approximately 20,000. A small peak immediately following the void volume suggested the presence of an oligomer of molecular weight approximately 40,000. These may represent the dimer and tetramer, respectively, but were the only direct evidence for their existence found in this series of experiments.

Formation of dimers and larger aggregates of other proteins in the presence of denaturants has been reported. For example, irreversible denaturation of β-lactoglobulins can occur in which the degree of irreversibility increases as the exposure time in urea increases (13). The likely involvement of –SH/–SS– interchange in that case was pointed out, and the formation of aggregates was demonstrated by gel filtration chromatography (14).

Whether a dimer and tetramer could be intermediates in the formation of large aggre-

gates or dissociation products of previously aggregated protein is not known. Possibly an equilibrium exists among three or more species. Ikai and Tanford (15) proposed that a denatured protein may form an incorrectly folded state which is not an intermediate between the native and denatured conformations. Kinetic evidence supported this hypothesis for cytochrome c (16) but not for lysozyme (17). Based also on kinetic analyses, it was suggested to occur in renaturation of carbonic anhydrase after its denaturation in Gdn–HCl (12).

As expected from the full recovery of taste activity and absence of precipitate in the samples treated under acid conditions (Table I), these eluted at the same position as did the control samples during chromatography under conditions similar to those described above. In only one case (Sample 4, Table I) was there present a small amount of aggregated protein.

The results of gel filtration therefore indicate that most of the soluble protein following denaturation and dialysis is similar in size to native monellin. Some high molecular weight aggregated monellin is present in solution, although most of the aggregated monellin precipitates and is not sweet.

Summary. Effects of the denaturants urea and guanidine–HCl on the sweet-tasting protein monellin have been studied. The pH at which monellin is initially treated with denaturant is an important factor in retention of sweetness, but the pH maintained during subsequent removal of denaturant by dialysis has no effect on activity. Recovery of sweetness of denaturant-treated monellin is favored when denaturation occurs at acid pH. Monellin treated with either 6 *M* guanidine–HCl or 8 *M* urea at acid pH retains all of its sweetness following removal of denaturant, but urea treatment at neutral pH leads to some irreversible loss of sweetness.

Monellin precipitates from solution under some conditions during removal of denaturant by dialysis, and the precipitated protein is no longer sweet. Precipitation is least under acid conditions. Aggregated protein was demonstrated by gel filtration chromatography. The single sulfhydryl group of monellin was not demonstrable in the precipitated protein, having apparently become oxidized during denaturation and formation of the aggregated protein.

The data support the hypothesis that the tertiary structure is important in the ability of monellin to elicit a sweet sensation.

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