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Formation of Oligomeric Monellin in Protein Denaturants (40876)[1]

JAMES A. MORRIS[2] AND ROBERT H. CAGAN[3]

Veterans Administration Medical Center and Monell Chemical Senses Center, Philadelphia, Pennsylvania 19104

Abstract. Monellin is a protein with an intense sweet taste. It is known to consist of two dissimilar polypeptide chains that are tightly but noncovalently bound. Monellin is also known to contain a single sulfhydryl group; in native monellin it appears to be buried within the interior of the protein. An oligomer of monellin is demonstrated to form in the presence of protein denaturants. It appears to involve dimerization through formation of a disulfide linkage. Formation of larger aggregates, which occurs during removal of denaturant, is postulated to involve the molecular species $[monellin]_2$ as an intermediate.

The single cysteine residue (1) of the sweet-tasting protein monellin (2, 3) is buried within its interior and is relatively inaccessible to reaction (4, 5). Under some conditions of denaturation and renaturation, a portion of the monellin in solution forms large aggregates which precipitate from solution; the aggregated protein is not sweet and has lost its titratable -SH (6). We postulated that large aggregates might begin to form through disulfide-linked $[monellin]_2$ (mol. wt. 22,000), based on tentative evidence (6) suggesting the presence of this size protein. In the previous experiments (6), however, denaturants were removed by dialysis prior to estimating molecular size by gel exclusion chromatography. In the present study, we examined the chromatographic behavior of monellin in the presence of protein denaturants, in order to eliminate possible protein renaturation or precipitation upon removal of denaturant. We thereby demonstrate the presence of an oligomer of monellin of $\simeq$ 22,000 molecular weight.

Materials and methods. Chemicals and suppliers were as follows: urea, Schwarz/Mann (ultrapure) and Fisher Scientific (reagent grade); high purity guanidine hydrochloride (Gdn–HCl), Heico, Inc., Delaware Water Gap, Penn; sodium dodecyl sulfate (SDS), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), dithioerythritol (DTE) (sodium salt), *p*-hydroxymercuribenzoate (sodium salt) (PHMB), and Tris, Sigma; 2-mercaptoethanol, Eastman; Sephadex G-75 (particle size 40–120 μm), Pharmacia; and trypsin (bovine pancreas, twice crystallized), Worthington. Monellin was purified as described previously (2, 6) and solutions were prepared from lyophilized monellin stored over P_2O_5 *in vacuo* at 4°. Other chemicals were reagent grade.

The denaturants urea (8 and 6 *M*) and Gdn–HCl (6 and 4 *M*) were prepared in 0.2 *M* sodium phosphate buffer (final pH 7.4; Gdn–HCl solutions adjusted with NaOH). Monellin was dissolved in the denaturant solution at 5 mg/ml and stirred (24°) in an uncovered vessel to maximize exposure to the air, unless noted otherwise. An aliquot containing 2.5 mg of protein was withdrawn after 2 hr and applied to the gel filtration column. The remainder of the sample was retained for a total of 24–26 hr and then applied to the same column.

The Sephadex G-75 column (1.5 × 41.5 cm) was equilibrated with either 6 *M* urea or 4 *M* Gdn–HCl, each of which were in 0.2 *M* sodium phosphate buffer (pH 7.4). Samples of approximately 0.5 ml (2.5 mg protein) were applied to the column and eluted with the appropriate solvent at a flow rate of 4 to 6 ml/hr (24°). Fractions of 20 drops were collected and protein was measured on

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[2] Present address: International Flavors and Fragrances, Union Beach, N.J. 07735.

[3] Address correspondence to Dr. Robert H. Cagan at the Monell Chemical Senses Center, 3500 Market Street, Philadelphia, Penn. 19104.

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each fraction by absorbance at 277 nm on a Hitachi Model 191 spectrophotometer. In examining formation of the postulated oligomer, the estimate of molecular size was made by employing trypsin (mol. wt. 23,300) (7) as a marker protein, which is close in size to the expected oligomer. The trypsin was incubated for 2 hr in either 8 *M* urea or 6 *M* Gdn–HCl and chromatographed under conditions identical with those used for the comparable monellin samples. Where samples contained DTE, the chromatography column was equilibrated with 6 *M* urea containing 1 m*M* DTE.

Dialysis was carried out at 4° against 7 liters of each of the following: step 1, 2 hr against 0.01 *N* acetic acid + 7 m*M* mercaptoethanol; step 2, 16 hr against a fresh portion of the same solution; step 3, 7 hr against 0.01 *N* acetic acid; step 4, 2 days against 0.01 *M* sodium phosphate buffer (pH 7.0). The -SH content was determined using DTNB by the method of Ellman (8) as described earlier (4). Reactions were carried out in urea or Gdn–HCl as appropriate or, when denaturants had been removed by dialysis, in the presence of 1% (w/v) SDS buffered at pH 7.4 with 0.2 *M* sodium phosphate. Protein concentration was routinely estimated from the absorbance at 277 nm with $E^{1\%}_{1cm}$ = 13.7 (1) or with a corrected value of the extinction coefficient in denaturing solutions ($E^{1\%}_{1cm}$ = 13.2). Sweetness was estimated as described previously (2).

Results and discussion. Formation of oligomeric monellin. The molecular weight of the active (sweet-tasting) monellin molecule is 11,000, which contains a single cysteine residue by amino acid analysis (1), accounting for the single titratable -SH group (4, 5). Some irreversible loss of sweetness can occur upon treating monellin with urea, depending upon the specific conditions (6). Aggregation of monellin, along with loss of its titratable -SH, can also occur. It was postulated that disulfide-linked $[monellin]_2$ occurs as an intermediate in formation of large aggregates, but gel filtration chromatography showed only suggestive evidence after treatment with urea but not with Gdn–HCl (6). The denaturants had been removed by dialysis prior to chromatography. Therefore, to test the hypothesis chromatography was carried out in the presence of denaturants in order to eliminate the possibility of reformation of native monellin or precipitation of large protein aggregates.

The elution profile of samples treated with urea and chromatographed in the presence of urea showed protein peaks with apparent molecular weights near 22,000 (Peak I) and 11,000 (Peak II) (Fig. 1). Peaks I and II represented 53 and 47%, respectively, of the protein recovered estimated by A_{277}. Identical results were obtained for samples exposed to urea for 2 and 26 hr (Fig. 1). In addition, a sample applied immediately to the chromatography column, within 5 min following exposure to 8 *M* urea, showed a similar elution profile. The elution profiles of guanidine-treated monellin chromatographed in the presence of Gdn–HCl (Fig. 2) were qualitatively similar to those with urea, but in guanidine Peak I was considerably smaller than Peak II. Irreversible loss of sweetness occurs to a lesser extent in 6 *M* Gdn–HCl than in 8 *M* urea (4), although the rate and extent of denaturation (studied in acidic medium) is greater in the guanidine solution (9). The relative amount of Peak I increased with time (Fig. 2). The proportions of protein in Peaks I and II were estimated at 36 and 64% (2-hr sam-

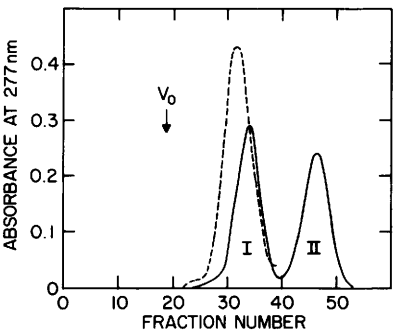

FIG. 1. Chromatography of urea-treated monellin on Sephadex G-75. Monellin was incubated for 2 or 26 hr in 8 *M* urea and chromatographed in 6 *M* urea. (Dotted line) trypsin (mol. wt. 23,300) used as a molecular weight marker; (solid line) monellin.

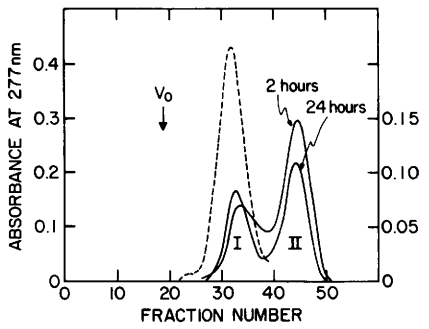

FIG. 2. Chromatography of guanidine-treated monellin on Sephadex G-75. Monellin was incubated for 2 or 24 hr in 6 *M* Gdn–HCl and chromatographed in 4 *M* Gdn–HCl. The right-hand ordinate refers to the latter sample, which was one-half of the usual sample size. (Dotted line) trypsin; (solid line) monellin.

ples) and 43 and 57% (24-hr samples), respectively. These results agree with our previous observation (4) that at neutral pH loss of titratable -SH is slower in the presence of Gdn–HCl than it is in urea. Although the 2-hr guanidine-treated sample revealed titratable -SH, the quantitation was questionable because of anomalous titration behavior (see below). Sweetness was tested (following dialysis) in the 2-hr guanidine-treated sample, and showed approximately 60% of the original sweetener activity in Peak II and none in Peak I.

Effect of sulfhydryl protection reagents. The results with added -SH protection reagents support the hypothesis that $[monellin]_2$ is formed by intermolecular -SS- bonding. Monellin was dissolved in 8 *M* urea that contained either 10 m*M* DTE or 10 m*M* PHMB (approximately twofold molar excess). Loss of sweetness after treatment with some -SH reagents had been shown to be reversible (4, 5). The DTE-containing sample was incubated (under nitrogen) for 2 hr and chromatographed in 6 *M* urea containing 1 m*M* DTE. That containing PHMB was incubated in air for 18 hr and chromatographed in 6 *M* urea. Upon chromatography the elution profiles of the DTE- and PHMB-treated samples showed no evidence of a 22,000 molecular weight species (no peak I); a single molecular species emerged near the position of monellin itself (peak II), showing only a slight shift in its elution position compared with unreacted monellin. The PHMB remained bound to the protein as judged by the increased absorbance at 250 nm. No sweetness was detected (following dialysis) in unprotected samples treated with urea (cf. 4, 6), but essentially full sweetener activity was recovered in both of the protected samples.

DTNB titration of monellin in 1% SDS is generally complete within 10 min (4). In the present titrations the increase in absorbance at 412 nm was followed for at least 10 min. It was uniformly low and stable for the samples in urea ($\leq$0.010 absorbance units; 0–0.2 mole -SH/mole protein). The -SH content of the protected samples was higher, 0.5–0.6 mole/mole protein. It was necessary, however, to monitor the latter titrations for an extended period (3 hr) because the A_{412} steadily increased. Even after 3 hr. the maximal absorbance was not reached, and the estimate is therefore low. This slow titration of the -SH in denaturant (1% SDS) is unlike our earlier findings (4), and might suggest that the conformation of the protein changed upon renaturation.

The data support the hypothesis that denatured monellin begins to aggregate through formation of a $[monellin]_2$ species. Fluorescence studies show that the conformation of monellin is disrupted in denaturants (9) such that some separation of the two polypeptide chains (5, 10, 11) appears to occur. Circular dichroism measurements (12) also show the concomitant loss of tertiary structure and loss of sweetness under denaturing conditions. Protein aggregation is a complex process, and the studies reported here on monellin represent only an initial examination of one aspect of the process. The conformational changes in denaturants apparently involve exposure of the buried -SH group on subunit II, which is postulated to react with a second molecule of monellin to form the oligomer. The -SH/-SS- reaction would not be involved directly in formation of the larger aggregates, but it appears that the 22,000 molecular weight species tends itself to aggregate when the denaturant is removed. Formation of larger aggregates is in fact facilitated upon removal of denaturant (6),

but the complex mechanisms involved are not addressed in the present study. Other examples of dimerization and formation of larger aggregates are known. For example, β-lactoglobulin forms aggregates in urea, which likely involves -SH/-SS- interchange (13, 14). The present study suggests that $[monellin]_2$ is an intermediate during aggregation.

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