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# Conformational Changes of the Sweet Protein Monellin as Measured by Fluorescence Emission (42066)

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*Abstract.* Monellin is a protein that tastes sweet. In the native state it is a dimer composed of two dissimilar noncovalently associated polypeptides. The conformation of the protein is a determinant of its sweetness, and the present investigation takes advantage of the fluorescence spectrum being a sensitive index of its conformation. The emission spectrum is used to evaluate the ability of temperature and pH to alter the conformation and the sweetness of the protein. When monellin dissolved in water is heated in discrete steps from 25 to 100°C, its sweetness decreases. The halfwidth of the fluorescence emission band increases in parallel with the loss of sweetness. The increase in halfwidth is due primarily to an increase in the intensity of tyrosine emission that may be the result of the two dissimilar polypeptides of monellin beginning to separate. Tyrosine residues are present in both chains, while the single tryptophan occurs in only one. Monellin is less susceptible to denaturation by increasing temperature when dissolved in sodium acetate buffer at pH 4 than it is at pH 3 or 7. When the pH of a solution containing monellin in 0.1 *M* KCl is varied from 2 to 13, there is a broad pH range (pH 4 to 9) where monellin's conformation is not markedly altered. Below pH 3.5 and above pH 10.5, however, the emission spectra indicate that substantial denaturation occurs. However, monellin can be partially renatured following pH 12 treatment with only minimal loss of sweetness. The sweetness of monellin under these two types of denaturing conditions, temperature and pH, can be predicted by the fluorescence emission spectrum of the protein. In addition, this study confirms that the biological activity of monellin, its sweetness, is a function of quaternary structure of the protein. 

The protein monellin (1–3) consists of two dissimilar, noncovalently associated polypeptides. One of these polypeptides contains the single tryptophan, while the seven tyrosine residues are distributed between the two polypeptides (4–6). This distribution of the aromatic amino acids allows the fluorescence emission spectrum of monellin to reflect changes in conformation (6). Studies of monellin under various conditions have led to the conclusion that its biological activity, sweetness, is dependent upon the tertiary and quaternary structures of the protein (1–3, 6–13).

Monellin is labile to traditional protein denaturants such as urea, guanidine hydrochloride, high temperature, and extremes of pH (1, 3, 6–13). The extent to which the sweet taste of the protein is altered by these treatments helps in defining the manner in which the tertiary and quaternary structures of the protein dictate its biological activity, sweetness. Denaturation with urea or guanidine hydrochloride leads to an altered conformation of the protein (6) and presumably to loss of sweetness (7). Removal of denaturants restores sweetness (except after certain treatments with urea (7)), although nonsweet dimers form under these conditions (12). During denaturation with urea or guanidine hydrochloride the fluorescence emission spectrum of monellin changes dramatically (6). Two convenient parameters with which to monitor the course of denaturation are the fluorescence emission maximum, $\lambda_{max}$, and the halfwidth of the emission band, $\Delta\lambda_{1/2}$. The changes in these parameters suggest that when monellin is chemically denatured, polypeptide chain separation as well as alterations in the conformations of the individual chains occur. We use these fluorescence indices in the present work to monitor the conformation of monellin during changes in

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temperature and pH, thus providing physical correlates to the changes in sweetness of the protein.

**Materials and Methods.** *Materials.* Monellin was isolated as previously described (1, 7). DL-Tryptophan (lot No. 712G) was purchased from Eastman and Sephadex G-75 (40–120 μm) from Pharmacia. Other chemicals were reagent grade.

*Fluorescence measurements.* Fluorescence spectra were recorded (6) on a Perkin-Elmer Model MPF-2A spectrophotofluorometer equipped with a thermostatted cuvette holder. Excitation and emission slit widths were between 4 and 7 nm. Spectral measurements were uncorrected for instrumental parameters. Water was charcoal filtered and deionized to a resistance of at least 18 MΩ.

*Temperature studies.* Monellin was dissolved in water at 0.1 mg/ml. A series of 4-ml aliquots of the protein solution was incubated at several temperatures (25, 40, 50, 60, 70, 75, 80, or 100°C) in a thermostatted water bath. The temperature of the sample was monitored directly with a thermister probe attached to a tele-thermometer (Model 42SC, Yellow Springs Instrument Co.). The limiting temperature was reached within 1 min of immersing the sample into the bath, and the incubation was then carried out for 15 min. Only in the case of the 100°C bath was the limiting temperature lower, i.e., 98°C. Following the heating regimen, the sample was placed into an ice bath in order to bring the protein solution to 25°C as rapidly as possible. Fluorescence spectra were recorded within 1 hr of heating; excitation was at 260 nm at a temperature of 25 ± 0.2°C. The absorbance of each protein solution at the excitation wavelength was determined using either a Zeiss PMQII or a Hitachi 191 spectrophotometer. To determine the susceptibility of the protein, when dissolved in water, toward loss of sweetness with temperature, the identical heating regimen with monellin at 1.0 mg/ml was employed, and the taste tests were performed as previously described to measure the sweet recognition threshold (1).

Considerable opalescence was observed upon heating water-solubilized monellin at 80°C and above. To monitor the appearance of opalescence, a Rayleigh scattering peak was recorded at 500 nm. Samples of monellin dissolved in water and subjected to temperatures of 80 and 100°C were clarified by centrifugation in a clinical centrifuge at 800*g*. The pellets resulting from this centrifugation were dissolved in 0.1 *M* sodium acetate, pH 3.5, combined, and diluted with the acetate buffer to an absorbance of 0.075 at 260 nm. The fluorescence emission spectra of both this dissolved pellet (in acetate) and of the clarified supernatant fraction (in water) were recorded. The amount of protein in the clarified solution was determined (14) and the remainder of the sample was used for taste tests.

In previous studies (6, 7) we demonstrated that in acetate, precipitation of denatured monellin is retarded but this buffer does not itself alter either the fluorescence characteristics or sweet taste of the protein. Temperature denaturation studies were conducted in sodium acetate at pH 3.0, 4.0, and 7.0, with monellin at 0.1 mg/ml. Preliminary work had indicated that heating monellin in 0.1 *M* sodium acetate, pH 4 or 7, for 15 min in a boiling water bath was insufficient to increase the halfwidth of the emission band of monellin to its limiting value. A time period of 45 min was therefore chosen, since by that time large differences were observed between the samples at pH 4 and at pH 7. The procedures used were identical with the studies of monellin in water (above) and used water bath temperatures of 25, 35, 45, 55, 65, 75, 85, and 100°C.

*pH studies.* Values of pH in the alkaline range were monitored with a high-pH-sensitive electrode (Model 4094-L60, A. H. Thomas Company), and those in the acid range were monitored using a combination electrode (Model 445, Markson Science). The pH was recorded using an Accumet Model 320 Expanded Scale Research Meter (Fisher Scientific).

Monellin was dissolved in 0.1 *M* KCl at 0.1 mg/ml. Temperature was held at 25°C. The pH was varied from neutrality to alkalinity, using a single sample of monellin (2.5 ml) in the fluorescence cuvette, by discrete additions of microliter volumes of 0.1 *N*, 1.0 *N*, and 10 *N* NaOH. When a desired pH was attained, the emission spectrum was recorded using excitation wavelengths of 260, 268, and

295 nm, after which the titration was continued. Once the extreme pH was attained (13.05), the fluorescence spectra were recorded and the solution was back-titrated to neutrality with 12 *N* and 1.0 *N* HCl. Similarly, a second sample of monellin was titrated in the fluorescence cuvette from neutrality to acidity using 0.1 *N*, 1.0 *N*, and 12 *N* HCl. Fluorescence spectra (260-, 268-, and 295-nm excitation) were recorded at discrete pH steps. Once the extreme pH (1.94) was reached the fluorescence spectra were recorded and the sample was back-titrated to neutrality with 10 *N* and 1.0 *N* NaOH.

*Chromatography.* A separate sample of monellin, 3 mg/ml in water, was subjected to a pH of 12.0 (by titrating with microliter volumes of 10 *N* NaOH) for 10 min and then back-titrated to pH 6.9 with 1.0 *N* and 0.1 *N* HCl. The precipitate which formed was removed by centrifugation and dissolved in 0.1 *M* sodium acetate, pH 3.5. The supernatant fraction was chromatographed on a column of Sephadex G-75 (40 × 1.5 cm) in 0.01 *M* Tris–HCl, pH 7.0, containing 0.1 *M* NaCl. Fractions (2.5 ml) were collected at 5-min intervals using a flow rate of 0.5 ml/min. Following elution, a control sample of monellin (3 mg/ml) that had not been subjected to pH 12 treatment was chromatographed in the same manner. The collection of fractions was in each case monitored by absorbance at 278 nm.

Fluorescence emission spectra were recorded of the alkaline-treated monellin that was eluted from the column, of the control monellin chromatographed in the same manner, and of the percipitate dissolved in sodium acetate. The eluted fractions from each peak were pooled, a portion was analyzed for protein (14), and the remainder was taste-tested (1).

**Results and Discussion.** We previously reported (6) that the fluorescence emission spectrum of monellin is a sensitive index of the biological activity, sweetness, of the protein. These observations confirmed earlier results showing the importance of conformation on sweetness (3, 5, 7–9, 13) and paralleled observations made by Jirgensons (9) demonstrating circular dichroic spectral changes of monellin under conditions leading to various retentions of sweetness. In Table I are listed the fluorescence emission parameters ($\Delta\lambda_{1/2}$ and $\lambda_{max}$) and percentage sweetness of monellin subjected to various conditions. The table is intended as a reference summary for the results of each of the following sections.

*Effects of temperature on the conformation of monellin.* As the temperature of monellin in water is increased from 25 to 100°C, the value of $\Delta\lambda_{1/2}$ measured after monellin's return to 25°C increases concomitant with a decreased sweetness (Fig. 1). Not shown in Fig. 1 is relative light scatter, which also parallels the increase in $\Delta\lambda_{1/2}$. The increase in $\Delta\lambda_{1/2}$ and percentage loss of sweetness are both sigmoidal, with an inflection point between 70 and 80°C. The limiting value of $\Delta\lambda_{1/2}$ for monellin after being held in water at 100°C is approximately 83 nm. The complete emission spectra that result after the protein was held at four of the temperatures studied are shown in the inset to Fig. 1. The broadening of the emission spectrum of monellin during this heating regimen is due primarily to the appearance of tyrosine fluorescence at 304 nm, and to a lesser extent to an increase in tryptophan bandwidth (6). Complete loss of sweetness is not observed even after holding the protein at the highest temperature. It is possible that monellin may partially renature during its return to 25°C, but if it does, higher temperatures obviously make this renaturation less effective (but, see below). This regimen of heating the protein and returning it to 25°C was chosen because it is impossible to taste-test the solutions at the higher temperatures.

The fluorescence spectra of the supernatant fraction (in water) and of the precipitate fraction (in acetate) of monellin after heating at 80 and 100°C are shown in Fig. 2. The precipitate samples heated at these two temperatures were combined for spectral analyses. The acetate-solubilized precipitate displays a $\lambda_{max}$ at 340 nm, a prominent shoulder near 304 nm, and a $\Delta\lambda_{1/2}$ of 87 nm (Table I), values that indicate that this precipitate is highly denatured (6).

The clarified supernatant fraction of the 100°C sample (Fig. 2) displays a $\lambda_{max}$ of 337 nm, a slight shoulder near 304 nm, and a $\Delta\lambda_{1/2}$ of 79 nm, values that suggest that following heating at 100°C the protein in

TABLE I. FLUORESCENCE EMISSION PROPERTIES OF MONELLIN UNDER VARIOUS CONDITIONS[a]

| Condition | Excitation (nm) | $\Delta\lambda_{1/2}$ (nm) | $\lambda_{max}$ (nm) | % Sweetness |
|---|---|---|---|---|
| Water | 260 | 60.5 | 338 | 100 |
| | 295 | 56.0 | 339 | |
| 0.1 *M* NaCl[b] | 260 | 60.5 | 340 | 100 |
| | 295 | 56.0 | 339 | |
| 0.1 *M* KCl | 260 | 60.5 | 337 | 100 |
| | 268 | 61.0 | 337 | |
| | 295 | 56.5 | 338 | |
| 0.1 *M* acetate, pH 3.5[b] | 260 | 59.0 | 338 | 100 |
| | 295 | 56.0 | 340 | |
| Water, 100°C | 260 | 83.0 | 336, 304 (sh) | 15 |
| Water, 100°C, clarified | 260 | 79.0 | 337, 304 (sh) | 25 |
| Water, 80° and 100°C, precipitate dissolved in 0.1 *M* acetate, pH 3.5 | 260 | 87.0 | 340, 304 (sh) | — |
| 0.1 *M* KCl, pH 1.94 | 260 | 73.5 | 336, 301 (sh) | — |
| | 268 | 74.5 | 335, 301 (sh) | |
| | 295 | 58.0 | 339 | |
| Back titration from pH 1.94 to pH 7[c] | 260 | 66.0 | 338 | 75 |
| | 268 | 66.5 | 337 | |
| | 295 | 59.0 | 339 | |
| 0.1 *M* KCl, pH 13 | 268 | 71.0 | 351 | — |
| | 295 | 75.0 | 355 | |
| Back titration from pH 13 to pH 7[c] | 260 | 89.0 | 305, 337 (sh) | — |
| | 268 | 79.0 | 304, 337 (sh) | |
| | 295 | 64.0 | 340 | |
| Back titration from pH 12 to pH 7, supernatant[d] | 260 | 61.0 | 338 | 90 |

[a] The fluorescence properties, which were measured as described under Materials and Methods, are uncorrected for instrumental parameters.

[b] Values were obtained from Ref. (6). All other values are from present report.

[c] Sample was in 0.1 *M* KCl. Values are for protein previously held at the extreme pH values for 15 min (while recordings were being made). The sample back-titrated from pH 13 was slightly turbid.

[d] Values recorded following chromatography using Sephadex G-75. See Materials and Methods.

solution, perhaps surprisingly, is not completely denatured. The limiting value of $\Delta\lambda_{1/2}$ for the unclarified 100°C sample was artificially high (by 4 nm), probably due to light scattering from the opalescent solution. The present findings are in accord with our observation (10) that heating monellin at 100°C for 15 min does not result in complete loss of sweetness.

In 8 *M* urea, the $\Delta\lambda_{1/2}$ for monellin is 91 nm (6), which is very close to a theoretical maximum calculated by assuming the emission spectrum is derived only from tyrosine and tryptophan in an aqueous environment. If we assume that a $\Delta\lambda_{1/2}$ of 83 nm is limiting (monellin after being held at 100°C in water, Table I), the halfwidth of 79 nm predicts a sweetness retention of 18% compared with the native value. If we assume that the limiting value is 91 nm (monellin in 8 *M* urea in acetate) (6), a sweetness retention of 39% is predicted. A taste recognition threshold test (1) performed on the 100°C-heated supernatant sample indicated approximately 25% retention of sweetness on a protein basis when compared with native monellin. This value is in agreement with the expected range.

The decreased sweetness indicates a critical heating point near 60–70°C where monellin (in water) begins to denature and lose its

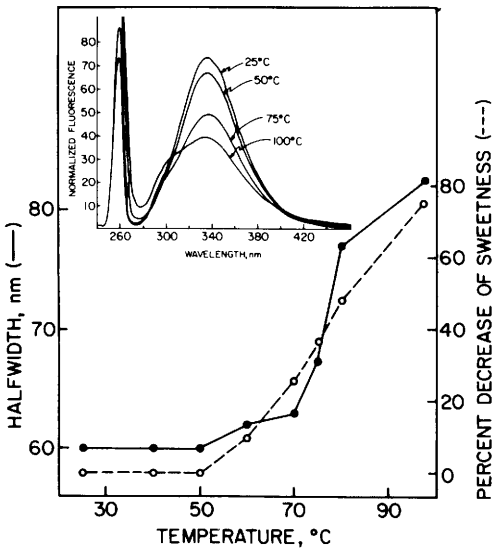


FIG. 1. Effect of temperature on monellin in water. Fluorescence spectrum halfwidth (solid line) and loss of sweetness (dashed line) are shown. Samples of monellin in water reached the temperatures indicated within 1 min of the sample being immersed in the water bath and were held at that temperature for 15 min prior to measuring fluorescence or sweetness. For the values of $\Delta\lambda_{1/2}$, monellin was dissolved at 0.1 mg/ml; for sweetness determination, monellin was dissolved at 1.0 mg/ml. The inset displays emission spectra of monellin at several water bath temperatures used in the temperature versus $\Delta\lambda_{1/2}$ experiment. Excitation for the spectra was at 260 nm.

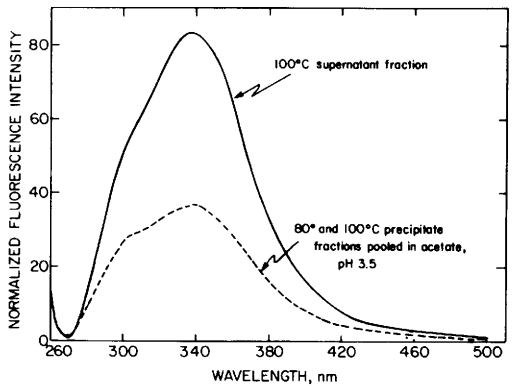


FIG. 2. Fluorescence emission spectrum of monellin in water after heating. The sample was heated to 100°C for 15 min and then clarified by centrifugation (solid line). The precipitates from monellin that was heated in water to 80°C and to 100°C were collected by centrifugation and pooled, and then solubilized in 0.1 *M* sodium acetate, pH 3.5 (dashed line). Excitation was at 260 nm. Fluorescence intensity was normalized to sample absorbance at 260 nm. Both samples displayed an absorbance of less than 0.1.

taste stimulus activity (Fig. 1). This fairly high temperature for heat denaturation agrees with earlier studies showing that monellin is a relatively stable protein toward denaturation (6, 7, 9, 10). It was earlier suggested that the large amount of $\beta$-structure in monellin is partially responsible for this stability (9, 10). The close parallel between sweetness and conformational measures of monellin (both circular dichroism and fluorescence) indicates that the tertiary and quaternary structures of monellin must exist within a narrow range for sweetness to be retained.

The temperature effects on fluorescence of monellin in 0.1 *M* sodium acetate are shown in Fig. 3. As described earlier (6, 7), the acetate solvent inhibits precipitation and opalescence during denaturation of monellin, yet its presence does not affect the sweetness (7) or the overall fluorescence characteristics (6). Mildly acidic pH values (e.g., pH 4) tend to stabilize the protein toward chemical denaturation (7). The data of Fig. 3 show that of the three pH values studied, the protein is most resistant to temperature-induced denaturation at pH 4.0 as indicated by halfwidth measurements. At pH 7, slight opalescence

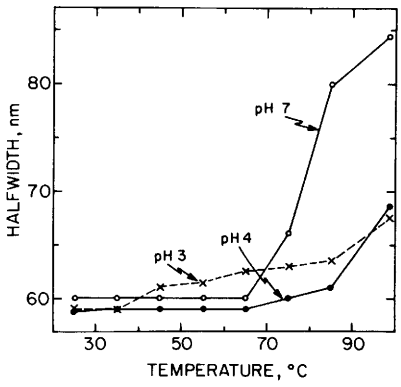


FIG. 3. Fluorescence spectral halfwidth versus temperature for monellin in 0.1 *M* sodium acetate at three pH values. Individual monellin samples were immersed in a water bath for 45 min to reach the temperatures indicated. The samples were then cooled to room temperature and the fluorescence measurements (excitation 260 nm) were made.

was observed in samples heated for 45 min at 85 and 100°C. Apparently, the acetate solvent itself further stabilizes monellin to the effects of acid denaturation, because even at pH 3 in acetate, monellin exhibits a fluorescence spectrum indicative of the native conformation and retains its sweetness.

*Effects of pH on the conformation of monellin.* The effects of pH on fluorescence of monellin (in 0.1 *M* KCl) from two excitation wavelengths are shown in Fig. 4. The excitation wavelength of 268 nm was chosen because when monellin is titrated from the acidic to the alkaline range, the absorption spectra exhibit an isosbestic point at 268 nm. Excitation at 268 nm induces fluorescence from both tyrosine and tryptophan, and excitation at 295 nm induces fluorescence primarily from tryptophan. Variations of the parameter $\Delta\lambda_{1/2}$ were parallel using 260 nm (not shown) and 268 nm excitation.

Increasing the pH from near neutrality to pH 8.8 results in little change of $\Delta\lambda_{1/2}$. Above pH 8.5 tyrosine begins to ionize to a nonfluorescent species (15); $\Delta\lambda_{1/2}$ begins to rise with 295-nm excitation but falls (transiently) with 268-nm excitation between pH 10 and 11. Further changes in $\Delta\lambda_{1/2}$ are parallel for both 268- and 295-nm excitation (pH 10.5 to 13.0). Upon titration toward the acid range, $\Delta\lambda_{1/2}$ is stable down to pH 3.8 and changes markedly only below pH 3 (Fig. 4). Because the $\Delta\lambda_{1/2}$ value does not approach its maximum until apparently well below pH 3, sweetness may be maintained below pH 3 but the extreme sourness of the solvent in this pH range makes this hypothesis difficult to taste-test. Back-titration from pH 2 does result in partial recovery of the native conformation, and we have observed partial recovery of sweetness (Table I).

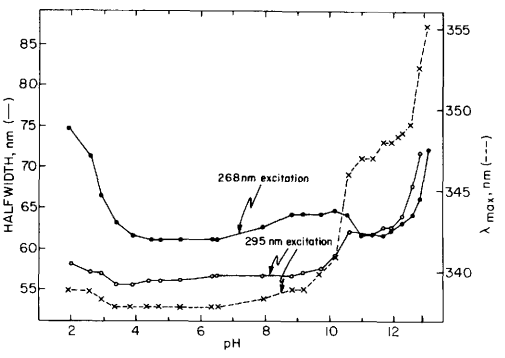


FIG. 4. Fluorescence characteristics of monellin versus pH. Fluorescence spectral halfwidth $\Delta\lambda_{1/2}$ (solid line), and intensity maximum $\lambda_{max}$ (dashed line), versus pH. Two different samples of monellin in 0.1 *M* KCl were used, one for the acidic range (using HCl as titrant), the other for the alkaline range (using NaOH). For the $\Delta\lambda_{1/2}$ determinations, excitation was at both 268 and 295 nm. For the $\lambda_{max}$ determinations, excitation was at 295 nm.

With 295-nm excitation, little change in $\lambda_{max}$ is noted in the acid region but a large shift occurs in the alkaline range, reaching a value of 355 nm at pH 13.05 (Fig. 4; Table I). We measured the fluorescence of free tryptophan in 0.1 *M* KCl, and found that its $\lambda_{max}$ reaches 357 nm at pH 13.07. This suggests that the tryptophan in monellin at pH 13 is completely exposed to the solvent. Back-titration of tryptophan itself from either extreme of pH to near neutrality results in a $\lambda_{max}$ of 349 nm, which is within 2 nm of the initial value. Values of $\lambda_{max}$ of monellin using 260- and 268-nm excitation parallel the results reported in Fig. 4 using 295-nm excitation.

The values of $\Delta\lambda_{1/2}$ and $\lambda_{max}$ after back-titration are shown in Table I. Back-titrating monellin from the acid extreme to neutrality results in a final $\Delta\lambda_{1/2}$ different from the original value (Table I) although considerable renaturation occurs. Back-titrating alkaline-treated monellin results in a high value for $\Delta\lambda_{1/2}$ (Table I) and a spectrum resembling that of monellin that is nearly completely denatured.

The large increases in both $\Delta\lambda_{1/2}$ and $\lambda_{max}$ between pH 12 and pH 13 (Fig. 4) suggest that monellin may not be completely and irreversibly denatured by subjecting it to pH 12, even though its conformation is obviously altered at pH 12. Consequently, monellin was subjected to pH 12 as described under Materials and Methods. Following back-titration, the sample was centrifuged and chromatographed on a column of Sephadex G-75. Based on absorbance at 278 nm, about 15% of the original monellin sample was precipitated following back-titration. There were no differences in the fluorescence emission characteristics among the monellin fractions from the leading edge, the center, or the trailing edge of the single elution peak. All portions of the eluted peak had a $\Delta\lambda_{1/2}$ of 61 nm, indicating substantially native monellin (Table I). Compared with the un-

treated control, the alkaline-treated soluble monellin showed an elution profile slightly retarded relative to the void volume (~3 ml). The eluted alkaline-treated monellin was only slightly less sweet (by about 10%) than the nontreated chromatographed control when compared on a total protein basis. On the other hand, the precipitate, when dissolved in acetate buffer, demonstrated a $\lambda_{max}$ of 337 nm and a $\Delta\lambda_{1/2}$ of 79 nm (260-nm excitation), indicating substantial denaturation of the protein.

In agreement with previous chemical denaturation studies (7), the present results suggest that monellin can be renatured from a previously (partially) denatured state. The transition from pH 12 to pH 13 leads to an abrupt increase in $\lambda_{max}$ and $\Delta\lambda_{1/2}$ of the protein (Fig. 4, Table I). The $\Delta\lambda_{1/2}$ and $\lambda_{max}$ of monellin in 8 *M* urea or 6 *M* guanidine HCl using 295-nm excitation were 63–63.5 nm and 349–350 nm, respectively (6), values similar to those reported here for monellin subjected to pH 12 (Fig. 4). By pH 13, these values increased to 75 and 355 nm, respectively (Table I). Monellin can be renatured to the native (sweet) conformation after removal of urea or guanidine HCl, much as it can be renatured after being subjected to pH 12 (Table I). It is likely that under the conditions of urea, guanidine HCl or pH 12, the protein is not completely denatured. After treatment by pH 13, however, back titration results in a fluorescence spectrum that indicates that the two separate chains of monellin have not reassociated (Table I). Unlike being subjected to pH 12, once subjected to pH 13, both chains of monellin may be denatured to an extent that their renaturation and recombination to the native protein dimer is no longer probable.

We have shown that the conformation and the sweetness of the sweet-tasting protein monellin are sensitive to and vary concomitantly with both temperature and pH. The nature of the solvent also affects its stability; under the conditions we used, monellin is most stable to heat denaturation in acetate buffer at pH 4. Our results support the hypothesis (3) that the conformation of monellin determines the biological activity, sweetness, of this protein.

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