Enzymatic Proteolysis of Monellin. Absence of Sweet Peptides

Enzymatic proteolysis of the sweet-tasting protein monellin with trypsin, α-chymotrypsin, or bromelain failed to produce a sweet-tasting fragment. Monellin is relatively resistant to proteolysis, but prolonged digestion with excess enzyme does result in production of nonsweet fragments.

The tertiary structure of monellin appears to be important in the sweet taste of this protein (Cagan, 1973; Morris and Cagan, 1975; van der Wel and Loeve, 1973). On the other hand, the possibility that a fragment of monellin could retain the sweet taste cannot be discounted, particularly in view of an earlier report. A carbohydrate-containing sweet material of molecular weight around 1500 was obtained after bromelain hydrolysis of partially purified extracts of the berries of *Dioscoreophyllum cumminsii* (Inglett and May, 1969). We did not find carbohydrate released by bromelain hydrolysis of monellin (Morris and Cagan, 1972), although we did not investigate the effect of the enzyme on the extract.

To further investigate the possibility of a sweet-tasting fragment, a survey has been made of the effects on monellin of some common proteolytic enzymes. Native monellin was digested with either trypsin, α-chymotrypsin, or bromelain, and the digests were chromatographed. Evidence for the presence of sweet-tasting peptides was not found, and therefore no attempt was made to optimize the conditions for complete proteolysis or to further characterize the fragments in detail.

EXPERIMENTAL SECTION

Enzymatic Proteolysis. Trypsin (bovine pancreas, twice crystallized, type TRL lot No. 9JB) was purchased from Worthington Biochemical Corp. and bromelain (crude amorphous powder, control No. 9318) from Nutritional Biochemicals Corp. α-Chymotrypsin (bovine pancreas, Type II, three times crystallized, lot No. 51C-8050), *p*-toluenesulfonyl-L-arginine methyl ester hydrochloride (lot No. 81C-0550), and *N*-benzoyl-L-tyrosine methyl ester (lot No. 72C-5040) were purchased from Sigma. The activities of the trypsin and α-chymotrypsin were assayed by the method of Hummel (1959) as outlined in the Worthington Enzyme Manual (Worthington Biochemical Corp., 1972), at an enzyme concentration of 0.7 μg/ml. Activities found were: trypsin, 95.7 units/mg (nominal: 192 units/mg), and α-chymotrypsin, 33.9 units/mg (nominal: 65 units/mg). Monellin was purified as described previously (Morris and Cagan, 1972) and kept as a lyophilized powder in vacuo at 4 °C over P_2O_5.

Solutions of monellin were prepared by dissolving the protein (5 mg/ml) in 0.2 M sodium phosphate buffer (pH 7.0) or (for use with bromelain) in 0.5 M sodium acetate buffer (pH 4.5) containing 20 mM cysteine-HCl. Trypsin was prepared several hours before use (Smyth, 1967) at 10 mg/ml in 1 mM HCl, and α-chymotrypsin was prepared at 10 mg/ml in deionized water immediately before use. Bromelain was dissolved in the acetate buffer at 1 mg/ml or 5 mg/ml immediately before use.

To initiate the reaction, 50 μl of trypsin or chymotrypsin solution (containing 0.5 mg) was added to each of two aliquots (1.11 ml) of monellin, and deionized water to a control sample. The final weight ratio of enzyme to monellin was approximately 1:12. Samples were incubated at room temperature (25 °C, pH 7.0) for 48 h with occasional shaking. At 24 and 48 h 0.1-ml aliquots were withdrawn and assayed for sweetness (Morris and Cagan, 1972). Bromelain hydrolysis was carried out at 25 °C (pH 4.5) for 19 and 72 h using a weight ratio of 1:50 or 1:10.

Chromatography of Enzymatic Digests. Sephadex G-25 (particle size, 50–150 μm) and G-75 (particle size, 40–120 μm) were purchased from Pharmacia Fine Chemicals. Chromatography on Sephadex G-25 was carried out at 4 °C on a column (1.1 cm × 27 cm) which had been equilibrated with 0.01 N acetic acid (pH 3.3). Monellin retains its sweetness at this pH (Morris et al., 1973; Morris and Cagan, 1975). Fractions (15 drops) were monitored by absorbance at 277 nm. Chromatography on Sephadex G-75 was carried out on a column (1.1 × 25 cm) which was equilibrated and eluted at 4 °C with 0.01 N acetic acid (pH 3.3). Fraction size was 15 drops; flow rate was 5 ml/h.

Fluorescence Spectra. Fluorescence spectra were measured on a Hitachi Perkin-Elmer Model MPF-2A fluorescence spectrophotometer at room temperature (25 °C) and were uncorrected. Excitation was at 277 or 295 nm.

RESULTS AND DISCUSSION

The sweetness of native monellin was partially lost after proteolysis. α-Chymotrypsin was more effective than trypsin in reducing sweetness; α-chymotrypsin digestion led to a decrease of 78% of the sweetness and trypsin digestion of 56% after 24 h, with no additional decrement at 48 h. Additional enzyme was not added during the incubations because of the already high concentrations compared with the commonly used enzyme:substrate ratio of 1:100 (Smyth, 1967). With the latter ratio, monellin was essentially completely active (sweet) after 19 h exposure to trypsin or chymotrypsin, though after 72 h with chymotrypsin the sweetness had declined by 85%. After bromelain hydrolysis (weight ratio 1:50) for 19 or 72 h, there was no appreciable loss in sweetness. van der Wel (1972) had reported that digestion with trypsin (weight ratio 1:4) for 24 h led to nearly complete loss of sweetness.

Chromatography of the 48-h digests on Sephadex G-25 showed that some degradation of monellin to smaller peptides had occurred with trypsin or chymotrypsin, and that more fragments were resolvable in the chymotrypsin digest (Figure 1). A substantial portion of the sample eluted in the void volume in each case, so that undegraded monellin could not be distinguished from possible fragments of molecular weight greater than about 5000. Sweetness was detected only in the fractions of the large peak eluting at or near the void volume; no sweetness was detected in the smaller fragments. In each case, the original monellin sample was 4.8 mg. Based on the absorbance at 277 nm, the amount of monellin recovered in the first peak (fractions 12–24) from the trypsin digest was 3.0 mg (62%). The amount recovered in the first peak (fractions 12–25) from the chymotrypsin digest was 2.3 mg (48%). For the total eluate, the recoveries were estimated to be: trypsin digest, 91%; chymotrypsin digest, 99%; and a control sample of monellin (which had been incubated without added enzyme), 96%.

A sample of monellin that was incubated for 24 h with

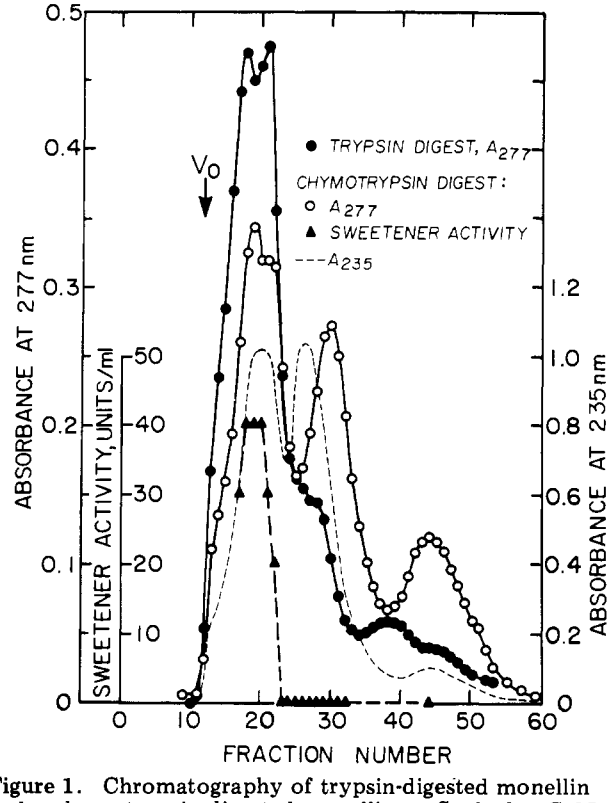

Figure 1. Chromatography of trypsin-digested monellin and α-chymotrypsin-digested monellin on Sephadex G-25.

bromelain (weight ratio of 1:10) was also chromatographed on a Sephadex G-25 column. Most of the protein and all of the recovered sweetness eluted near the void volume. The smaller fragments that eluted following the void volume were not sweet.

Monellin contains a single tryptophan residue per molecule (Morris et al., 1973). Samples from the three major chromatographic peaks obtained from the α-chymotrypsin digest (Figure 1) were examined for the fluorescence emission of tryptophan. The emission maximum (338 nm) of samples at the leading portion of the initial peak appeared to be due to native monellin. The trailing portion of the peak showed an emission λ_{max} of 304 nm due to tyrosine as well as emission due to tryptophan (λ_{max} 340 nm with excitation of 295 nm). The first peak was therefore not homogeneous, although it is possible that a small degree of heterogeneity could be introduced by the protease itself, which comprised around 8% of the original.

The leading edge of the second major peak included a fragment with both tyrosine and tryptophan while the trailing edge had relatively little tryptophan. The leading edge was unusual in having relatively low absorption at 277 nm but high at 235 nm (Figure 1), whereas other fractions showed essentially parallel absorption at both wavelengths. In addition, it had an intense sour taste which was greater than that of the other fractions tested, even though all contained 0.01 M acetic acid. The third major chromatographic peak was smaller and occurred approximately in the region where small molecules should elute (mol wt ≤500). The emission spectrum (emission λ_{max} 353 nm) was virtually identical with that of free tryptophan.

Since most of the material eluted near the void volume of the G-25 column, the fractions from the initial peak of each column were pooled (separately), lyophilized, and dissolved in 0.5 ml of 0.1 N acetic acid. These fractions were then each chromatographed on a Sephadex G-75 column. In each case, a major peak eluted at the position of native monellin, and a small shoulder trailed the large peak (Figure 2). The chymotrypsin digest was recovered to the extent of 55% and the trypsin digest 56%, while the recovery of a control sample was 79%. Pooled fractions from the major peaks tasted sweet (approximately 100% sweetener units/mg of protein), whereas the pooled "shoulder" fractions were tasteless. It therefore appears that a portion of the monellin sample remained undegraded by these proteolytic enzymes and is responsible for the sweetness.

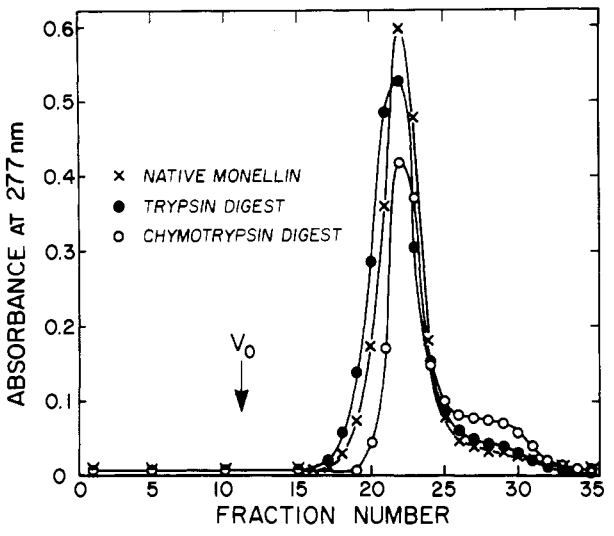

Figure 2. Chromatography of trypsin-digested monellin and α-chymotrypsin-digested monellin on Sephadex G-75. Pooled fractions from the major peak in each case tasted sweet; the pooled "shoulder" fractions were tasteless.

In view of these findings, optimization of the proteolysis conditions was not attempted. Though the apparent resistance of native monellin to proteolysis may simply be due to suboptimal conditions for the enzymatic digestions, it could suggest that the tertiary structure of monellin is compact. Evidence consistent with this interpretation has been obtained (Cagan and Morris, 1976), showing that the single cysteine residue of monellin is inaccessible to chemical reaction unless the protein is denatured.

During these studies, the possibility was also excluded that a small sweet-tasting molecule might occur in crude extracts of the fruit of *Dioscoreophyllum cumminsii*. Aqueous extracts of the pulp of the berries (Morris and Cagan, 1972) were dialyzed extensively against water at 4 °C and the dialysates lyophilized, taken up in a small volume of water, and taste-tested. None of the series of dialysates tasted sweet.

The aim of this investigation was to examine the possibility of producing a sweet-tasting fragment of monellin. That such might be possible is intimately related to the role of the tertiary structure in eliciting sweetness. The data presented indicate that proteolysis of native monellin with either trypsin, α-chymotrypsin, or bromelain each produced fragments, but the fragments did not taste sweet. Our data are consistent with the hypothesis that the tertiary structure of monellin plays an important role in its sweet taste.

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James A. Morris[1]
Robert H. Cagan*

Monell Chemical Senses Center
University of Pennsylvania
and Veterans Administration Hospital
Philadelphia, Pennsylvania 19104
[1]Present address: International Flavors and Fragrances
Union Beach, New Jersey 07735

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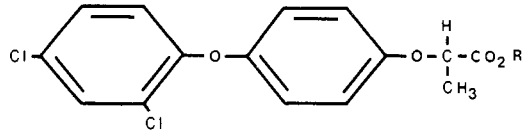
Esterification of the Hydrolysis Product of the Herbicide Dichlorfop-Methyl in Methanol

The acid formed by alkaline hydrolysis of the herbicide dichlorfop-methyl [methyl 2-(4-(2,4-dichlorophenoxy)phenoxy)propionate] was characterized spectroscopically. In methanolic solution, at 25 °C this acid underwent complete esterification within 14 days. At 60 °C methylation was faster and complete within 24 h.

Dichlorfop-methyl [I, R = CH_3; methyl 2-(4-(2,4-dichlorophenoxy)phenoxy)propionate] will be used on the



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Canadian prairies at rates of 1.12 kg/ha as a postemergence treatment for the control of annual grasses in wheat, flax, and rape. Herbicidal esters are known to undergo hydrolysis to their respective acids in moist soils (Burcar et al., 1967; McKone and Hance, 1972; Smith, 1972, 1976; Beynon et al., 1974). Thus, the acid (I, R = H) derived from dichlorfop-methyl was required for soil persistence and degradation studies. This communication reports the preparation of the acid together with the interesting and anomalous reaction of dichlorfop acid whereby it undergoes complete methylation in methanolic solution at room temperature.

MATERIALS AND METHODS

Dichlorfop Acid. A commercial formulation (10 ml) of dichlorfop-methyl (Hoechst Aktiengesellschaft, Frankfurt, Germany) was hydrolyzed at room temperature by treatment with 40 ml of a 50% aqueous methanolic solution containing 4 g of potassium hydroxide. After 24 h the reaction mixture was diluted with 150 ml of water and extracted with 3 × 100 ml portions of ether to remove any unhydrolyzed ester and ether-soluble impurities. The aqueous phase was then acidified with 20 ml of 12 N hydrochloric acid and shaken with 2 × 100 ml volumes of ether. The combined ether extracts were dried over anhydrous sodium sulfate and evaporated, using a rotary evaporator, to yield a red oil. The oil was dissolved in a mixture of ether and *n*-hexane, from which it crystallized as a pink amorphous solid with a melting point of 100–102 °C.

Methanol. Distilled in glass methanol was used in these studies, obtained from Caledon Laboratories Ltd. (Georgetown, Ontario, Canada).

Methylation. A 2 mg/ml solution of the acid was prepared in methanol and 1.0-ml aliquots were measured into 1-ml capacity silylation tubes fitted with screwcaps. Tightly sealed duplicate vials were then incubated at 25 ± 1 °C and 60 ± 2 °C when 20-μl aliquots were removed at regular intervals and added to 100-ml portions of *n*-hexane. After vigorous shaking, the ester content was determined gas chromatographically by comparing the ester peak heights from 5-μl injections with those from an ester standard prepared by treating a 20-μl sample from each vial at every sampling period with diazomethane, using the procedure described by Rivers et al. (1970).

Spectra. Mass spectra were determined using a Finnigan 1015 mass spectrometer utilizing a solid probe for direct insertion.

Gas Chromatographic Analysis. The gas chromatograph used was a Hewlett-Packard 7610A equipped with a nickel electron-capture detector. The 2 m × 3 mm i.d. glass column was packed with a mixture of 2% QF-1 and 3% DC-200 on 60–80 mesh Gas-Chrom Q. Carrier gas was argon containing 5% of methane at a flow rate of 40 ml/min. Injector, column, and detector temperatures were 240, 210, and 300 °C, respectively. Under these conditions the dichlorfop-methyl had a retention time of 2.4 min.

RESULTS AND DISCUSSION

The dichlorfop acid was identified by the fact that it could be remethylated to dichlorfop-methyl and from spectroscopic considerations. The mass spectrum indicated a molecular ion weight of 326 (equivalent to $C_{15}H_{12}O_4Cl_2$) and the presence of 2 chlorine atoms. Ion