**Table II. Per Cent Recovery of $^{14}C$ Extracted from Soils Treated with MBC-2-$^{14}C$[a]**

| Days after treatment | Fargo clay, μg of MBC-2-$^{14}C$/g of soil | | Barnes loam, μg of MBC-$^{14}C$/g of soil | |
|---|---|---|---|---|
| | 10 | 100 | 10 | 100 |
| 0[b] | 79 | 85 | 82 | 91 |
| 20 | 60 | 72 | 61 | 81 |
| 43 | 55 | 64 | 53 | 78 |

[a] Data represent means from duplicate samples. [b] Extracted immediately after application of MBC-$^{14}C$.

may have had an effect on the rate of MBC-$^{14}C$ degradation. However, we have found that concentrations of MBC up to 100 ppm did not significantly change the rate of nitrification and carbon dioxide formation in soil (Lacy *et al.*, 1974).

A portion of the $^{14}C$ applied to soil as MBC-2-$^{14}C$ was not recovered and is assumed to have been retained in the soil in a nonextractable form. The amount not recovered increased with time (Table II) and further extraction with chloroform did not release significant additional amounts of $^{14}C$.

ACKNOWLEDGMENT

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# Purification and Some Properties of Miraculin, a Glycoprotein from *Synsepalum dulcificum* Which Provokes Sweetness and Blocks Sourness

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Miraculin, a taste-affecting glycoprotein from miracle fruit, *Synsepalum dulcificum*, has been purified by ion exchange column chromatography. Purified miraculin contains 6.3% carbohydrate and 14.4% nitrogen and has a mol wt of ~45,000. When denatured and reduced it is cleaved into two fragments, of approximately 28,000 and 17,000 mol wt. The amino acid and carbohydrate compositions of miraculin have been investigated. Twenty micrograms of chromatographically purified miraculin produces a marked increase in sweetness of lemon and concomitantly a marked diminution of sourness.

Miraculin is the name given a taste-affecting glycoprotein found in miracle fruit, *Synsepalum dulcificum*. In the (human) oral environment miraculin causes sour tastes to be appreciated as sweet tastes. Two schemes for preparing the active principle from berries of miracle fruit have been reported (Brouwer *et al.*, 1968; Kurihara and Beidler, 1968). The purification procedure we wish to report results in a higher yield of miraculin than noted previously and the final preparation is free of contaminating polyphenols and proteolytic activity. In addition some chemical and physical properties of the purified miraculin are reported.

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## MATERIALS AND METHODS

Berries of *Synsepalum dulcificum* were obtained from plants cultivated in Florida. The fruits were frozen and shipped by air to our laboratory in Dry Ice. There, berries were stored for periods of as long as 2 years at −40° while the extractability of miraculin apparently remained unaffected.

Insoluble polyvinylpyrrolidone (PVP), "Miracloth," Bio-Gel CM-30, and Bio-Rad ion-exchange resins were obtained from Calbiochem; thin-layer plates of cellulose were obtained from E-M Laboratories; QAE-Sephadex A-50 and other Sephadex materials were obtained from

Pharmacia. Bovine serum albumin was obtained from Armour; ovalbumin was obtained from Pentex; carbonic anhydrase (bovine erythrocytes), soybean trypsin inhibitor, yeast alcohol dehydrogenase, whale skeletal muscle myoglobin, and horse heart cytochrome *c* were obtained from Nutritional Biochemicals; trypsin, chymotrypsin, and chymotrypsinogen were obtained from Worthington; ribonuclease (bovine pancreas) and egg-white lysozyme were obtained from Schwarz/Mann; carboxypeptidase A was obtained from Sigma; and bovine hemoglobin was obtained from Pierce. These proteins were used without further purification. Other reagents were obtained in reagent grade quality.

Protein was determined by absorption at 280 nm and by the Lowry method (Lowry *et al.*, 1951), using bovine serum albumin as a standard. It must be remembered that neither method is specific for protein; polyphenols react with the Folin-Ciocalteu reagent in the Lowry method. In some cases material precipitable in boiling 5% trichloroacetic acid was assayed for protein and nitrogen content.

Nitrogen was determined by the reaction of ammonium ion in Kieldahl digests with ninhydrin. The procedure was similar to that described by Jacobs (1963). Solutions of acid-digested amino acids were used as standards.

Chloride was determined by a colorimetric method (Zall *et al.*, 1956; Technicon AutoAnalyzer Methodology, 1969).

Amino acid analysis was performed on a Beckman Model 121 automatic amino acid analyzer. Half-cystine residues were quantitated by automated amino acid analysis of the *S*-carboxymethylcysteine derivative in hydrolysates of reduced, carboxymethylated miraculin and by a spectrophotometric method employing Ellman's reagent (Ellman, 1959). Disulfide bridges in miraculin were reductively cleaved in a solution of 1% SDS–0.1 *M* dithioerythritol–0.05 *M* sodium phosphate buffer (pH 8.1) by heating for 2 min in a boiling water bath. The protein solution was cooled and passed through a 1 × 16 cm column of Sephadex G-25 fine, equilibrated, and eluted with 0.1% SDS in the phosphate buffer. The protein content of the emergent protein peak was determined by absorption at 280 nm and aliquots of the peak were allowed to react with Ellman's reagent to determine sulfhydryl content. A molar extinction coefficient of 13,600 at 412 nm for the sulfhydryl reaction product was confirmed by the method of Dietz and Rubenstein (1972).

Miraculin was carboxymethylated by reduction of the disulfide bridges with dithiothreitol and reaction with iodoacetic acid (Crestfield *et al.*, 1963). Protein was separated from other reaction products by gel chromatography on a column of Sephadex G-25 fine operated with aqueous 10% acetic acid and was recovered by lyophilization.

The carbohydrate content of miraculin was quantitated by the phenol–sulfuric acid method (Dubois *et al.*, 1956) using an equimolar mixture of xylose and arabinose as a standard. Analysis of monosaccharides was performed by thin-layer chromatography. Lyophilized protein and monosaccharide standards were hydrolyzed in 1 ml of 2.5 *N* trifluoroacetic acid in evacuated sealed tubes for 8 hr at 100° (Yasuda *et al.*, 1971). Each hydrolysate was diluted to 5 ml with water and passed sequentially through 0.5 × 6 cm columns of Bio-Rad AG50W-X8 ($H^+$ form), 200–400 mesh, and Bio-Rad AG1-X2 (formate form), 200–400 mesh (Spiro, 1966). Effluents, including water washes of the columns, were concentrated by rotary evaporation and lyophilized. Each lyophilized residue was redissolved in 20 μl of water and appropriate aliquots were spotted on thin-layer plates of cellulose. Chromatograms were developed three times in a solvent of *n*-butyl alcohol–pyridine–acetic acid–ethyl acetate–$H_2O$ (10:3:3:3:4, by volume) (Scocca and Lee, 1969). For qualitative analysis the thin layers were sprayed with a solution of 5% aniline hydrogen phthalate in glacial acetic acid and heated at 80° (Partridge, 1949). $R_f$ values (and colors) of known sugars were: rhamnose, 0.75 (brown); ribose, 0.65 (red); fucose, 0.63 (brown); xylose, 0.57 (red); arabinose, 0.51 (red); mannose, 0.46 (brown); glucose, 0.38 (brown); and galactose, 0.35 (brown). These sugars were resolvable, except ribose and fucose; these two were differentiated by the color reagent. For quantitative analysis, guide strips at each side of the plate were sprayed and heated while the central portion of the plate was protected by a sheet of plastic film. Unknown, standard, and blank spots were scraped from appropriate areas of the unsprayed section; each was extracted with 0.7 ml of water. After centrifugation a 0.5-ml aliquot was assayed for carbohydrate content by the Park–Johnson method (1949).

Polyacrylamide gel electrophoresis of native miraculin was carried out in a modified cationic system (Reisfeld *et al.*, 1962; Rodbard and Chrambach, 1971). Polyacrylamide gel electrophoresis of denatured, reduced miraculin was carried out in a SDS stacking system (Neville, 1971). Proteins in the gels were stained with Coomassie Blue (Chrambach *et al.*, 1967; Neville, 1971).

Proteolytic activity of crude extract and subsequent fractions was measured by digestion of hemoglobin using the urea-denatured substrate and conditions described by Kunitz (1947) for determination of papain-like proteases.

Molecular weight determinations for native and reduced and denatured miraculin were carried out by gel chromatography on columns of Sephadex G-75 and G-150, respectively. A 2.2 × 108 cm column of Sephadex G-75 was equilibrated and operated with 0.15 *M* NaCl–10 m*M* sodium phosphate buffer (pH 7.4) at a flow rate maintained at 26 ml/hr. Aliquots (approximately 4 ml) of solutions of marker proteins or Blue Dextran (Pharmacia) at concentrations of 0.1–1 mg/ml were chromatographed and the observed elution volumes were employed to plot log mol wt *vs.* $K_{av}$ (Andrews, 1965). A 2.5 × 79 cm column of Sephadex G-150 was equilibrated and operated with 0.1% SDS–0.05 *M* sodium phosphate buffer (pH 8) containing 20 m*M* dithioerythritol, at a flow rate maintained at 6.2 ml/hr. Marker proteins and miraculin were prepared for chromatography by heating each protein solution (0.2–2 mg/ml of column buffer) for 2 min at 100°. Aliquots (1–3 ml) of protein or Blue Dextran solutions were chromatographed and a plot of log mol wt *vs.* $K_{av}$ was prepared. Both columns were operated at room temperature in the reverse flow mode, the outlet at the top of the column leading to a uv monitor and fraction collector.

**Purification of Miraculin.** *Step A.* Miracle fruit berries were transferred from storage at −40° to a beaker containing solidified carbon dioxide. Berries were removed from the beaker and allowed to thaw at room temperature until pulp and skin could be separated from seed; the pulp was returned to another beaker of Dry Ice. Pulp (100 g) was ground briefly in a cooled mortar with 20 g of insoluble (high molecular weight) PVP (Bio-Rad Polyclar AT, etc.); then the mixture was transferrred to a beaker containing 1000 ml of 0.1 *M* sodium carbonate buffer (pH 10.5) and cooled to 0–4°. (Solubilization of miraculin in this buffer was introduced by Kurihara and Beidler (1968).) All succeeding operations also were carried out at 0–4°. The suspension was stirred for 4 or more hours and then suction-filtered on a Büchner funnel using a filter disk of "Miracloth" covered by a layer of Hyflo Super-Cel diatomaceous earth. The filter cake was pressed down to remove small amounts of liquid and then discarded; it contained little miraculin activity.

*Step B.* To the filtrate was added 20 g of insoluble PVP and 13 g of ε-aminocaproic acid (final concentration, 0.1 *M*). The suspension was stirred. Over a half-hour period concentrated acetic acid was added dropwise to bring the pH to 6.0 ± 0.5. The suspension was filtered as in step A.

*Step C.* Bio-Gel CM-30 resin sufficient to form a 300-ml column was equilibrated with 0.1 *M* sodium phosphate

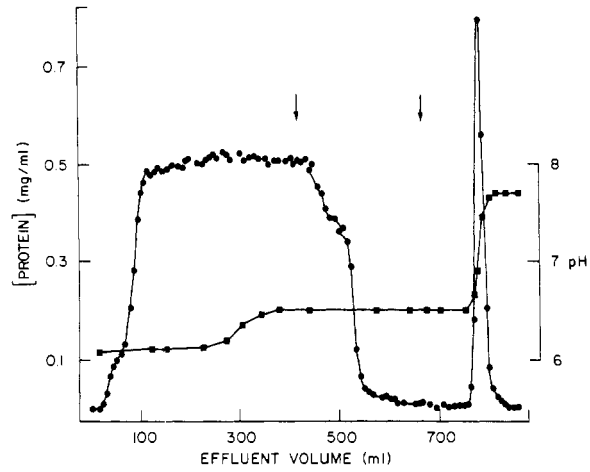


**Figure 1.** Chromatography of miraculin on Bio-Gel CM-30 with stepwise elution. The chromatogram presented is typical of those obtained in step C of the purification scheme (see text). Filtrate (400 ml) (pH 6.4) from 41 g of berry pulp was chromatographed; column dimensions, 4.2 × 8 cm; elution schedule, 250 ml of 0.1 *M* sodium phosphate buffer (pH 6.5), then 200 ml of 0.1 *M* sodium phosphate buffer (pH 7.7) (arrows). Fractions of 7 ml were collected. Protein (and polyphenol) concentration (●) was determined by the Lowry method; effluent pH (■). The sharp peak at the pH discontinuity contained miraculin, while the broad peak contained little or no miraculin activity.

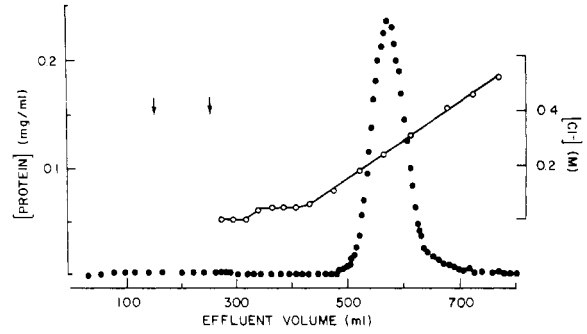


**Figure 2.** Chromatography of miraculin on QAE-Sephadex A-50. This chromatogram was obtained during step D of purification of miraculin from 63 g of berry pulp; volume of step C pool adjusted to pH 10.5 and chromatographed, 100 ml; column dimensions, 2.5 × 22 cm; elution schedule, 110 ml of 0.05 *M* NaCl in 0.1 *M* sodium carbonate buffer (pH 10.5), then 550 ml of linear gradient, 0.05–0.65 *M* NaCl in the carbonate buffer (arrows). Fractions of 4.5 ml were collected. Protein concentration (●) was determined by the Lowry method. Miraculin was eluted over the 0.2–0.3 *M* range of sodium chloride concentration (○).

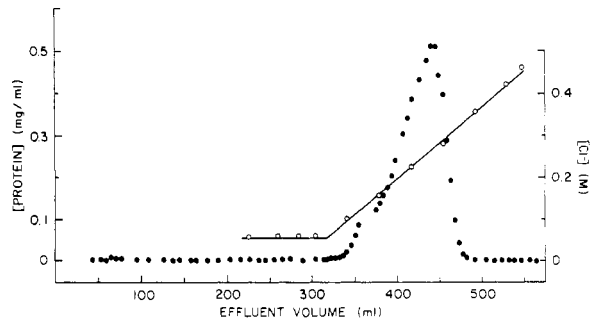


**Figure 3.** Chromatography of miraculin on Bio-Gel CM-30 with gradient elution. A solution of miraculin, 60 ml, deriving from purification of miraculin from 130 g of berry pulp was chromatographed (step E, see text); column dimensions, 2.5 × 21 cm; elution schedule, 150 ml of 0.05 *M* NaCl in phosphate buffer, then 400 ml of linear gradient 0.05–0.55 *M* NaCl in 0.1 *M* sodium phosphate buffer (pH 6.0). Fractions of 4.7 ml were collected. Protein concentration (●) was determined by the Lowry method. Miraculin was pooled from fractions of the protein peak eluted in the 0.2–0.3 *M* NaCl concentration (○) range.

buffer (pH 6.0). Half of the resin was collected by suction filtration and added to the filtrate obtained in step B. This suspension was poured onto a 4-cm diameter column formed from the other portion of resin. Column effluent was collected in fractions under gravity flow conditions. When all the resin and filtrate had been packed into and passed through the column, the column (approximately 4 × 20–25 cm) was eluted stepwise with 0.1 *M* sodium phosphate buffer (pH 6.5) and with 0.1 *M* sodium phosphate buffer (pH 7.7). Elution with pH 6.5 buffer washed from the column nonretained material which was Lowry-positive and absorbed at 280 nm. After approximately one column volume of pH 7.7 buffer had passed through the column, miraculin activity was eluted in a sharp peak, at the pH discontinuity (Figure 1).

*Step D.* Fractions of column effluent containing the sharp peak were pooled and the pH of the solution was raised to 10.5 by addition of 0.1 *N* NaOH in 2% sodium carbonate. QAE-Sephadex A-50 sufficient to form a 150-ml column was equilibrated with 0.1 *M* sodium carbonate buffer (pH 10.5). As in step C, approximately half of the resin was added to the solution of miraculin and the slurry was poured onto a 2.5 cm diameter column formed from the other portion of resin. When all the resin and solution had been packed into and passed through the column, the column (2.5 × 25–30 cm) was washed with one column volume of 0.05 *M* NaCl in 0.1 *M* sodium carbonate buffer (pH 10.5). The wash solution was pumped through the column at a rate of approximately 40 ml/hr by a peristaltic pump. Elution was effected by a linear gradient of 0.05–0.65 *M* NaCl in 0.1 *M* sodium carbonate buffer (pH 10.5). The volume of the gradient was equal to five column volumes; fractions of the column effluent were collected with the flow rate maintained at approximately 40 ml/hr. Miraculin was eluted as is shown in Figure 2. Colored contaminants eluted with the miraculin fraction in step C remained adsorbed to the QAE-Sephadex resin; the active fractions were colorless.

*Step E.* Fractions of column effluent containing the protein peak were pooled. Miraculin was concentrated and solvent was exchanged for 0.1 *M* sodium phosphate buffer (pH 6.0) by pressure dialysis and ultrafiltration (Amicon Corporation cell and PM-10 membrane). The concentrate was pumped onto a 2.5 × 20 cm column of Bio-Gel CM-30 equilibrated with 0.1 *M* sodium phosphate buffer (pH 6.0). At a flow rate maintained at approximately 20 ml/hr, the column was washed with one column volume of 0.05 *M* NaCl in the phosphate buffer. Elution was effected with a linear gradient of 0.05–0.65 *M* NaCl in 0.1 *M* sodium phosphate buffer (pH 6.0). Miraculin was eluted as is shown in Figure 3. Peak fractions were pooled and concentrated by pressure ultrafiltration. This material was studied as purified miraculin.

Miraculin activity was assayed initially by the method of Kurihara and Beidler (1968) in which subjects were asked to select from a series of sucrose solutions one solution that matched in sweetness the taste of a 0.02 *M* citric acid solution. However, untrained subjects, after holding a solution of miraculin in the mouth for 3 min, reported difficulty in comparing the sweet component of a sweet and sour tasting solution of citric acid to solely sweet solutions of sucrose. Most subjects, despite the quantity of miraculin given them for testing, chose as the matching solution sucrose concentrations of intermediate sweetness. The assay we prefer utilized a "forced-scaling" technique (Giroux and Henkin, 1971). Subjects were normal volunteers, aged 20–40, chosen without conscious bias from laboratory personnel and were untrained in taste testing. Each subject was requested to chew a lemon slice cut from a fresh lemon and circulate the resultant lemon juice

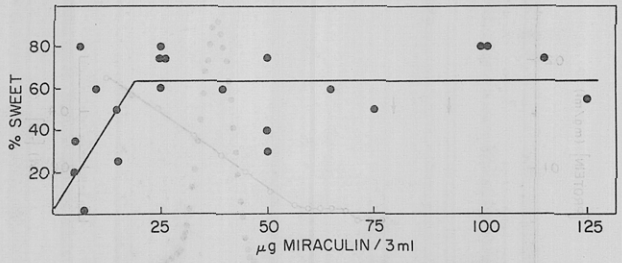


**Figure 4.** Bioassay of miraculin activity. The 21 subjects whose data are presented in this figure generally described lemon juice as sour and evaluated the sourness before exposure to miraculin at 81 and 85%, mean and median values, respectively, and the sweetness at 4% (mean) and 0% (median). One subject reported some bitter taste quality in the lemon slice and none reported a salty taste quality. (Neither salt nor bitter were detected in lemon by any subject after exposure to miraculin.) In this figure are presented sweetness intensity data for lemon slice after exposure of the mouth to a solution of chromatographically purified miraculin. Each datum represents the response of a different subject. At high concentrations of miraculin (greater than 25 μg/3 ml) 14 subjects evaluated sweetness of the lemon slice at 64 and 68% (mean and median, respectively) and sourness at 34 and 22% (mean and median, respectively). A line intersecting the mean value of sweetness response for high miraculin concentrations was drawn from the Y intercept using a line of best fit for response to low miraculin concentrations (less than 25 μg/3 ml). If one unit of activity is defined as the smallest amount necessary to produce maximum sweetness response the activity of purified miraculin may be estimated to be approximately 1 unit per 20 μg of protein.

**Table I. Purification of Miraculin from 100 g of Berry Pulp**[a]

| Step | Vol, ml | Protein, mg[b] | Nitrogen, mg | Act., units[c] |
|---|---|---|---|---|
| A. pH 10.5 extract | 1040 | 750 | 43[d] | 5400 |
| B. Neutralization to pH 6 | 1020 | 510 | 25[d] | |
| C. CM-30 chromatography | 90 | 40 | 5 | |
| D. QAE-Sephadex chromatography | 40 | 30 | 4 | |
| E. CM-30 chromatography | 10 | 22 | 3 | 1100 |

[a] Using data interpolated from several purifications of 40–140 g of pulp from *Synsepalum dulcificum*. [b] Response in Lowry assay. [c] See Figure 4 for definition of assay unit. [d] Determined on trichloroacetic acid precipitate.

throughout his or her mouth. Each was previously instructed to describe the taste as salty, bitter, sweet, or sour or some combination of these tastes. Each was also instructed to grade the taste on a scale from 0 to 100, with 100 representing the most intensely salt, sour, sweet, or bitter taste previously experienced by the volunteer. Three milliliters of a miraculin solution, from neutralized crude extract to purified protein, was circulated by the subject throughout the mouth for 3 min. The solution was spit out, the mouth was rinsed with water, and the taste of a second lemon slice was evaluated in a manner similar to the first. Miraculin fractions obtained during column chromatography were assayed by a few volunteers, essentially to establish the presence or absence of miraculin in any given fraction. Miraculin activity always coincided with the elution of protein peaks as in Figures 1, 2, and 3. These rough data are not included in the figures. Inglett *et al.* (1965) also monitored miraculin activity by tasting lemon slices before and after exposure of the mouth to miraculin preparations. Data from an assay of miraculin activity are illustrated in Figure 4.

## RESULTS

Data characterizing the purification of miraculin are presented in Table I. An apparent overall recovery of one-

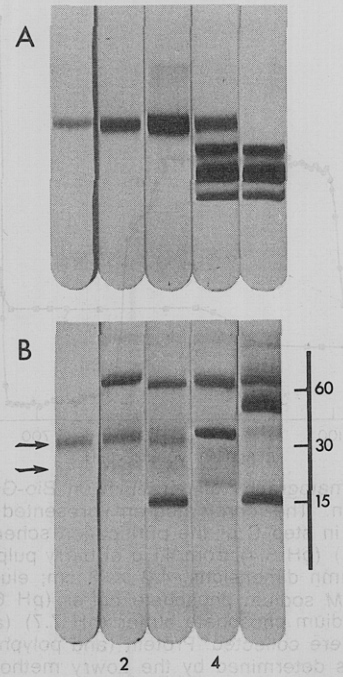


**Figure 5.** Gel electrophoresis of miraculin. (A) Electrophoresis of native miraculin in 7.5% polyacrylamide gels at pH 4.3. Gel 1 (extreme left) was loaded with 3 μg, gel 2 with 7 μg, and gel 3 with 20 μg of purified miraculin. Gel 4 was loaded with 7 μg of purified miraculin, 5 μg of chymotrypsin, 15 μg of RNase, and 2 μg of lysozyme. Gel 5 (extreme right) was not loaded with miraculin, but otherwise was similar to gel 4. Cathode is at the bottom. In gel 4 the bands are, top to bottom, miraculin, chymotrypsin, RNase, and lysozyme. (B) Electrophoresis of SDS-denatured, reduced miraculin in 11% polyacrylamide gels. Gel 1 (extreme left), gel 2, and gel 3 each were loaded with 4 μg of miraculin (arrows indicate strong and faint miraculin bands). Other proteins (and their assumed molecular weights) which were electrophoresed were bovine serum albumin (67,000), 3 μg loaded on gels 2, 3, 4, and 5; ovalbumin (45,000), 12 μg loaded on gel 5; carbonic anhydrase (29,000), 6 μg loaded on gel 4; and hemoglobin (15,500), 6 μg loaded on gels 3 and 5. Anode is at the bottom. The ratios ($R_m$) of distance traveled for each protein relative to the distance traveled by the Bromophenol Blue dye front were calculated and a linear log mol wt *vs.* $R_m$ relationship was established. Data from several other gels in addition to those shown here were used. An approximate molecular weight scale ($\times 10^{-3}$) is indicated. The molecular weight of the strongest staining miraculin band was determined to be $28 \pm 5 \times 10^3$.

fifth of the taste affecting activity present in the crude extract was noted. The overall increase in specific activity was threefold, based on activity per milligram of protein nitrogen.

Purified miraculin was prepared as a concentrated solution from pooled fractions of the second column chromatography with Bio-Gel CM-30. For analytical purposes the protein was desalted on a column of Sephadex G-25 fine eluted with 10 m*M* acetic acid and lyophilized. Miraculin had these properties: 14.4% nitrogen; 6.3% carbohydrate; absorption maximum at 278 nm, with $A_{1cm}^{1\%} = 10.7$; in the Lowry assay it had 81% of the color value of the BSA standard. Polyacrylamide gel electrophoresis at pH 4.3 revealed only a single band (Figure 5). When the reduced, SDS-complexed protein was electrophoresed, the major staining band migrated as if it had a molecular weight of $28 \times 10^3$; a fainter staining band of lower molecular weight also was noted (Figure 5).

The molecular weight of native miraculin was determined by gel chromatography to be $45 \times 10^3$ (Figure 6). When the complex of miraculin and SDS was reduced and chromatographed in another molecular weight determination, two major polypeptides were resolved; they were of $28 \times 10^3$ and $17 \times 10^3$ mol wt, respectively (Figure 6).

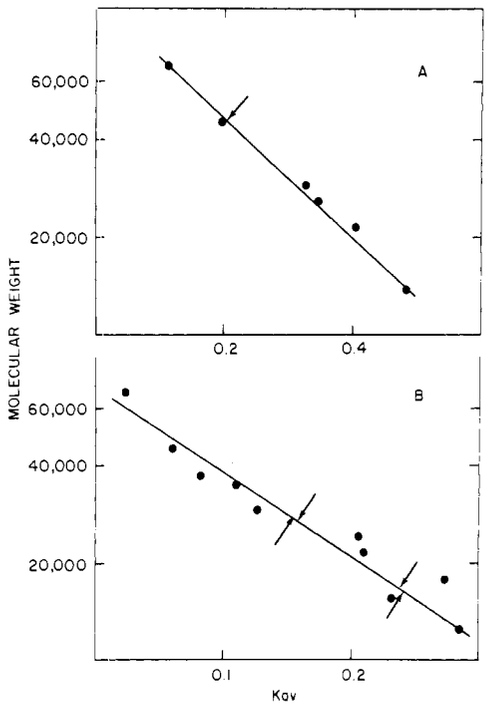


**Figure 6.** Molecular weight determination by gel chromatography. (A) Molecular weight of native miraculin. Proteins used as markers (and their assumed molecular weights) were bovine serum albumin (67,000), ovalbumin (45,000), carbonic anhydrase (29,000), chymotrypsinogen (25,700), soybean trypsin inhibitor (21,500), and ribonuclease (13,700). $K_{av}$ for miraculin (↓) was derived from five determinations of elution volume of native miraculin. The calculated molecular weight was 45 ± 2 × $10^3$. (B) Molecular weight of denatured, reduced miraculin and denatured, reduced, and carboxymethylated miraculin. Marker proteins (and their assumed molecular weights) were bovine serum albumin (67,000), ovalbumin (45,000), yeast alcohol dehydrogenase (37,000, subunit), carboxypeptidase (34,600), carbonic anhydrase (29,000), trypsin (24,000), soybean trypsin inhibitor (21,500), myoglobin (17,800), hemoglobin (15,500, subunit), and cytochrome *c* (12,400). Two polypeptide peaks appearing in the chromatogram of denatured, reduced, and carboxymethylated miraculin (↑) had calculated molecular weights of 27,900 and 16,500; polypeptide peaks in the chromatogram of denatured, reduced miraculin (▾) had calculated molecular weights of 27,200 and 16,800.

**Table II. Amino Acid Composition of Miraculin**[a]

| Amino acid | Mol % | Integral residue value |
|---|---|---|
| Lysine | 6.30 | 22 |
| Histidine | 0.99 | 3 |
| (Ammonia) | | (29) |
| Arginine | 5.72 | 20 |
| Aspartic acid | 12.08 | 42 |
| Threonine[b] | 8.74 | 31 |
| Serine[b] | 6.71 | 24 |
| Glutamic acid | 6.81 | 24 |
| Proline | 7.44 | 26 |
| Glycine | 9.75 | 34 |
| Alanine | 3.72 | 13 |
| Half-cystine[c] | | 12 |
| Valine[d] | 10.44 | 37 |
| Methionine | 0.45 | 2 |
| Isoleucine[d] | 4.20 | 15 |
| Leucine | 5.64 | 20 |
| Tyrosine | 3.65 | 13 |
| Phenylalanine | 7.36 | 26 |
| Tryptophan[e] | | 9 |
| | Σ{100} | |

[a] Eight portions (0.4–0.6 mg) of lyophilized native and reduced, carboxymethylated miraculin, from three miraculin preparations, were each hydrolyzed in 1 ml of constant boiling HCl in evacuated sealed tubes at 110° for 1, 2, and 3 days. Solvent was removed by rotary evaporation *in vacuo* and the residue was dissolved in citrate buffer for analysis. Average recovery values from 1-, 2-, and 3-day hydrolyses are listed, except as noted below. A function of the relative abundance of amino acids was minimized to provide best-fit integral residue values over the region of protein mol wt of 45,000 ± 10,000. The function used was similar to that described by Delaage (1968). The molecular weight of a protein having the residue values listed here is 44.6 × $10^3$, including the contribution of 6.3% carbohydrate. No amino sugars were detected. [b] Extrapolation to zero days of hydrolysis. [c] 12.1 residues/mol as *S*-carboxymethylcysteine; 11.6 residues/mol by the spectrophotometric method (see text). [d] Three day hydrolysis. [e] The ratio Trp/Tyr = 0.72 was determined by the spectrophotometric method of Goodwin and Morton (1946).

These data are consistent with the results obtained by polyacrylamide gel electrophoresis. We have not determined if denatured but not reduced miraculin can be resolved into the lower molecular weight fragments. These fragments derive from the protein as isolated and could conceivably represent products of a single polypeptide chain degraded during purification. The carbohydrate content, the ultraviolet absorption maximum, and the molecular weight of native miraculin are in agreement with previously published data (Brouwer *et al.*, 1968; Kurihara and Beidler, 1968).

A papain-like proteolytic activity was present in crude extracts of miracle fruit. The protease, activated by EDTA and cysteine, hydrolyzed benzoylarginine ethyl ester and acetyltyrosine ethyl ester with optimum activity at pH 6–8 and was inhibited by ε-aminocaproic acid. Assay of this protease throughout the purification procedure showed that approximately half of the activity present in crude extract was lost during the pH reduction step (step B) and little activity remained in the miraculin fraction after the first ion-exchange chromatography (step C). Purified miraculin (step E) had 0.1% or less of the initial proteolytic activity. ε-Aminocaproic acid was included in the purification scheme (step B) with the intent to inhibit degradation of miraculin by this protease, although we have not definitely established that miraculin is susceptible to such degradation.

The amino acid composition of miraculin is listed in Table II. The basic character of the protein is confirmed. Since miraculin chromatographs as an anion at pH 10.5 and as a cation at pH 6.0 its isoelectric point may be near pH 9, as Brouwer *et al.* (1968) reported. No free sulfhydryl groups were found in SDS-denatured miraculin by spectrophotometric assay, hence the 12 half-cystine residues (Table II) must form 6 disulfide bridges. Kurihara and Beidler (1968) reported the amino acid analysis of a 22-hr acid hydrolysate of miraculin. Those data are in only approximate agreement with our data. Their observed values for histidine, glutamic acid, alanine, and methionine content are greater, while their observed values for threonine, half-cystine, valine, and phenylalanine content are lower than what we observed. Our data from 1-, 2-, and 3-day hydrolysates were used to correct for partial destruction or incomplete release of amino acids, which may in part account for some discrepancies.

Qualitative analysis of acid hydrolysates of miraculin revealed the presence of fucose, xylose, mannose, and galactose. No spots on the thin-layer plates corresponding to rhamnose, arabinose, ribose, or glucose were detected. By quantitative analysis total recovery of carbohydrate from the scrapings of the thin-layer plates was 17–25% of that expected, based on the amount of miraculin hydrolyzed in trifluoroacetic acid. The relative molar proportion of individual sugars was observed to be fucose:xylose:mannose:

galactose, 1.0:1.0:2.0:1.1, respectively. These proportions are uncorrected for unique losses of a particular sugar in the analytical procedure. Given its molecular weight and carbohydrate content, one expects miraculin to contain 15–20 monosaccharide residues. When monosaccharide standards were carried through the procedure for quantitative analysis, from hydrolysis onward, the recovery of xylose was only one-fourth that of the three other sugars. This observation would suggest that the ratio of sugars in miraculin is 1:4:2:1 for fucose:xylose:mannose:galactose, respectively. At present we must regard as preliminary our results on the carbohydrate composition of miraculin.

## DISCUSSION

In the initial report on the chemical characteristics of the sweetness-provoking principle from miracle fruit, Inglett *et al.* (1965) were unable to solubilize the active principle from lyophilized berries, but deduced that it was glycoprotein in nature. Later, Brouwer *et al.* (1968) purified (and named) miraculin by extraction in a basic medium, followed by ammonium sulfate fractionation and gel chromatography. One hundred micrograms of protein altered perception of sourness for 1–2 hr. Their preparation released several sugars upon acid hydrolysis: glucose, ribose, arabinose, galactose, and rhamnose. The yield was 50 mg of miraculin per kilo of berries. Kurihara and Beidler (1968) solubilized miraculin and then passed the alkaline extract through a column of DEAE-Sephadex to remove colored impurities followed by ion-exchange chromatography on a column of carboxymethyl-Sephadex. They proposed a model (Kurihara and Beidler, 1969) for the action of miraculin which was based upon arabinose and xylose constituents of the glycoprotein. Maximum sweetening effect was obtained at a protein concentration of $4 \times 10^{-7}$ $M$ (88 $\mu$g/5 ml). The yield was 100 mg per kilo of berry pulp.

We were unable to satisfactorily purify miraculin by either of the published procedures. Removal of polyphenolic substances and of proteolytic activity was inadequate. Miraculin prepared by the present scheme is colorless and free of significant protease activity. The yield of purified miraculin is 200–250 mg per kilo of berry pulp (Table I).

Two classes of taste responses following oral miraculin administration were recognized with the lemon slice assay (Figure 4): one was the *sweetness provoking effect* of the lemon, the other, a *blocking effect*, by which the sour quality of the lemon was markedly attenuated. These same two effects were also observed following miraculin administration with orally placed solutions of citric and hydrochloric acid. This blocking effect also extended to the oral placement of bitter substances; the bitter taste of both urea and phenylthiocarbamide (PTC) was markedly attenuated following miraculin administration.

In the lemon-tasting assay described here, 20 $\mu$g of miraculin was sufficient to produce a maximum sweetness-provoking response (Figure 4). Since in each investigation a different method of assay has been used, it is not possible to compare the specific activity of our preparation with the preparations of Brouwer *et al.* (1968) and Kurihara and Beidler (1968). The increased sweet response and decreased sour response which we observed following exposure to miraculin was in accord with observations of Bartoshuk *et al.* (1969), while Kurihara and Beidler (1969) found that even at maximum levels, miraculin never generated from citric acid a relative sweetness which equalled the sweetest of their reference solutions of sucrose. A solution of purified miraculin has been stored at −20° for 12 months and has apparently retained full activity.

There is general agreement in all reports on the molecular weight of miraculin, on the basicity of the protein, and on its glycoprotein character. The cause of the marked differences in reported carbohydrate composition is not known to us. The complete amino acid composition and properties of the denatured and reduced protein previously have not been published.

We feel that use of insoluble PVP contributes significantly to the improved yield of miraculin which we observed. The presence of leucoanthocyanidins, precursors of condensed tannins, in the pulp of miracle fruit berries was established by a spectrophotometric method (Swain and Hillis, 1959). It is a general observation that many plant proteins form protein–phenol complexes insoluble at pH values less than 7–8 (Jones and Hulme, 1961; Goldstein and Swain, 1965; Loomis and Battaile, 1966). PVP is a commonly used adsorbent of plant phenols and we found that addition of insoluble PVP did increase the solubility of miraculin in crude extracts prepared at various pH values; however, only at high pH was the solubilization complete. We conclude that complete solubilization in the crude extract occurs only above the isoelectric point of miraculin; *i.e.*, above pH 9 (Brouwer *et al.*, 1968). As the pH of the solubilized miraculin is lowered to neutrality miraculin again becomes insoluble, in the absence of PVP. In the presence of PVP miraculin remains soluble at pH 6 (step B in the purification scheme).

We have observed that taste buds on the tongue are relatively unresponsive to the sweetening effect of miraculin; that is, the taste of lemon juice is appreciated there mainly as sour, both before and after exposure of the mouth to miraculin. When lemon juice is directed back over the tongue, taste buds in the posterior portion of the oral cavity (primarily palatal taste buds) seem to be involved with sweet taste provoked by miraculin and acid. This observation implies differentiation among taste buds in their responsiveness both to miraculin and to sweet and sour tastants (Henkin, 1974). Further, the effects of miraculin either can be eliminated completely or markedly decreased by rinsing the mouth with a detergent solution (*e.g.*, 0.1% SDS) following exposure to miraculin. The continued presence of miraculin (in an effective molecular conformation) at the surface of the taste bud may be necessary for the sweetening effect. The duration of the miraculin effect (greater than 2 hr at large doses of miraculin) presumably reflects the slow dissociation of a miraculin–taste bud complex. Further studies on the taste-affecting properties of miraculin are in progress.

Of the proteins recently isolated from tropical African fruits which affect the sense of taste, miraculin is the only one which has no intrinsic taste. Monellin, from *Dioscoreophyllum cumminsii* (Staph) Diels (Morris and Cagan, 1972; van der Wel, 1972; van der Wel and Loeve, 1973), and two proteins from *Thaumatococcus danielli* Benth (van der Wel and Loeve, 1972) are all intensely sweet; miraculin produces a sweet taste only in its association with acidic solutions. Miraculin is the largest of these proteins and it is the only glycoprotein. All these proteins are basic and would be cationic in the oral environment. This observation may have some relevance to the physiological activity of these proteins. Each of these proteins requires native tertiary conformation for activity. It has recently been reported that the application of miraculin to the tongue increases the summated response to acid in the chorda tympani nerve of man and monkey but not of the rat (Diamant *et al.*, 1972). It has also been recently observed that miraculin did not provoke any sweet taste in patients with aglycogeusia (Henkin and Shallenberger, 1971) who exhibit an inability to recognize any sugar as having a sweet taste although its blocking effect, in reducing the recognition of sourness and bitterness, was observed.

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# Breakdown of the Herbicide Dicamba and Its Degradation Product 3,6-Dichlorosalicylic Acid in Prairie Soils

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The degradation of $^{14}$C-ring- and $^{14}$C-carboxyl-labeled dicamba was studied in moist nonsterile heavy clay at 25 ± 1° in Pramer and Bartha (1965) flasks. Gas chromatographic and radiochemical analytical techniques were used to monitor the breakdown. Over 50% of the dicamba was lost within 4 weeks, radioactive carbon dioxide and 3,6-dichlorosalicylic acid being the sole degradation products detected. Decomposition of $^{14}$C-ring-labeled dicamba was compared in moist nonsterile silty clay, heavy clay, and sandy loam soils at 25 ± 1°. In all soils loss was rapid and complete in 3 weeks. A build-up of 3,6-dichlorosalicylic acid was followed by a slow loss which was complete within 9 weeks. Negligible breakdown of dicamba occurred in steam sterilized soils. Soil slurry adsorptive studies with 3,6-dichlorosalicylic acid indicated at least 30% adsorption to all soil types.

Dicamba (2-methoxy-3,6-dichlorobenzoic acid) is used as a postemergence herbicide for the selective control of weeds of the buckwheat family in cereals. The chemical is also applied for the control of broadleaved weeds in pastures and rangeland grasses.

Studies have shown that dicamba is degraded in moist soils (Burnside and Lavy, 1966; Corbin and Upchurch, 1967; Donaldson and Foy, 1965; Parker and Hodgson, 1966; Smith, 1973a,b). This breakdown is considered to be microbial in origin (Parker and Hodgson, 1966; Smith, 1973a), since degradation was inhibited in steam-sterilized soils.

Smith (1973b) using $^{14}$C-carboxyl-labeled dicamba reported that in a warm moist heavy clay the herbicide was transformed into 3,6-dichlorosalicylic acid. Radioactive carbon dioxide was also liberated from the treated soils, indicating that dicamba or the salicylic acid, or both, underwent decarboxylation.

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In the present work the breakdown of $^{14}$C-ring- and $^{14}$C-carboxyl-labeled dicamba was studied in nonsterile heavy clay in Pramer and Bartha (1965) flasks to ascertain whether all the radioactivity applied to the clay could be accounted for after various time intervals. In addition, the isolation and identification of possible decarboxylated metabolites were undertaken. The decomposition of $^{14}$C-ring-labeled dicamba in three different sterile and nonsterile soil types was investigated for comparative purposes in erlenmeyer flasks, to identify the metabolites and to study their persistence in the three soil types. Soil adsorption characteristics of the dicamba soil metabolite, 3,6-dichlorosalicylic acid, were also determined.

## MATERIALS AND METHODS

**Soil.** The composition and physical properties of the soils used in these studies are presented in Table I.

**Chemicals.** $^{14}$C-Carboxyl-labeled dicamba (2-[7-$^{14}$C]methoxy-3,6-dichlorobenzoic acid) and [$^{14}$C]dicamba labeled in the six ring carbon atoms were obtained from the Velsicol Chemical Corporation, Chicago, Ill., as were