THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 263, No. 23, Issue of August 15, pp. 11536-11539, 1988
Printed in U.S.A.

Complete Purification and Characterization of the Taste-modifying Protein, Miraculin, from Miracle Fruit*

(Received for publication, January 29, 1988)

Sarroch Theerasilp‡ and Yoshie Kurihara§

From the Department of Chemistry, Faculty of Education, Yokohama National University, Yokohama 240, Japan

The taste-modifying protein, miraculin, has the unusual property of modifying a sour taste into a sweet taste. Previous attempts to isolate miraculin from deeply colored alkaline extracts of the miracle fruit were unsuccessful. We found that miraculin is extracted with 0.5 M NaCl solution. The extracted solution is colorless and shows the strong sweet-inducing activity. Miraculin was purified from the extracted solution by ammonium sulfate fractionation, CM-Sepharose ion-exchange chromatography, and concanavalin A-Sepharose affinity chromatography. The purified miraculin thus obtained gave a single sharp peak in reverse phase high performance liquid chromatography, indicating that it is highly pure. The sample also gave a single band having molecular weight 28,000 in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This value was much lower than the values reported previously (40,000–48,000). The amino acid composition of the purified miraculin was determined. Sequence analysis of the purified miraculin indicated that it is composed of a pure single polypeptide and identified 20 amino-terminal amino acids. The purified miraculin contained as much as 13.9% of sugars, which consisted of glucosamine, mannose, galactose, xylose, and fucose in a molar ratio of 3.03:3.00:0.69:0.96:2.12.

Richadella dulcifica, a native shrub in tropical West Africa, bears red berries which have the unusual property of modifying a sour taste into a sweet taste. For example, lemons elicit a sweet taste like that of oranges if eaten after the berry has been chewed. Because of this unique property, the berry has been called miracle fruit.

Kurihara and Beidler (1) first isolated the active principle from miracle fruit by ion-exchange chromatographies and showed that it is a basic glycoprotein. Brouwer *et al.* (2) also purified the active principle by gel filtration on a Sephadex column and named it miraculin. Miraculin was also isolated by Giroux and Henkin (3) and Kurihara and Terasaki (4). The molecular weight of miraculin reported by these investigators was 40,000–48,000.

* This work was supported by grants from Ministry of Education of Japan, Mitajiri Chemical Industry Co., and Mishima Science Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Department of Chemistry, Faculty of Science and Technology, Sri-Ayudhya United Colleges, Ayudhya 13000, Thailand.

§ To whom all correspondence should be addressed: Department of Chemistry, Faculty of Education, Yokohama National University, Tokiwadai, Hodogaya-ku, Yokohama 240, Japan.

Miraculin, which exists in the pulp of miracle fruit, is not solubilized by homogenizing the pulp in water. In the above studies, miraculin was extracted with carbonate buffer (pH 10.5) (1, 3, 4) or solutions of highly basic compounds such as salmine or spermine (2). Application of these extraction methods not only led to reduction of the sweet-inducing activity but also solubilized various substances including colored materials which were very difficult to remove. The most serious problem in the previous studies was that the isolated miraculin was not completely pure: *e.g.* SDS-PAGE[1] of the sample obtained gave a broad band together with minor bands.

In the present study, we have found that miraculin can be extracted with 0.5 M NaCl solution at acidic pH. This method has great advantages. It yields miraculin that is very stable in the acidic pH and produces colorless extracts. The extracts are purified by procedures involving two-column chromatographs. Analysis of the purified miraculin by SDS-PAGE, HPLC, and an amino acid sequencer has indicated that it is highly pure. The molecular weight of the miraculin obtained is much lower than the values reported previously. Amino acid and sugar compositions of the purified miraculin are determined.

EXPERIMENTAL PROCEDURES

Purification of Miraculin

Miracle fruits were cultured in a greenhouse at Yokohama National University. Pulps of the fruits free from skin and seed were lyophilized and used for the experiment. The lyophilized pulps were kept at −20 °C before use. All experiments were carried out below 4 °C.

Extraction—20 g of the lyophilized pulps were suspended in 200 ml of water and homogenized for 2 min. The homogenates were centrifuged at 13,000 rpm for 30 min. The pink supernatant which had no sweet-inducing activity was discarded. The sediment was thoroughly washed with water, and then extracted three times with 0.5 M NaCl. In each extraction, the sediment was homogenized for 2 min in 120 ml of 0.5 M NaCl solution and the homogenate was centrifuged at 13,000 rpm for 30 min. The supernatants which showed high sweet-inducing activity were pooled. The pooled solution was colorless and its pH was 4.

Ammonium Sulfate Fractionation—Ammonium sulfate fractionation was carried out by addition of solid ammonium sulfate to the pooled solution to bring about 50% saturation. The precipitate from the solution was collected by centrifugation at 13,000 rpm for 40 min and suspended in water. The solvent was replaced with 0.01 M KH_2PO_4-Na_2HPO_4 buffer (pH 6.8) by means of ultrafiltration using Amicon PM-10, and this solution was used for further purification.

CM-Sepharose Ion-exchange Chromatography—The sample was applied to a column (1.3 × 60 cm, bed volume of 140 ml) of CM-Sepharose CL-4B (Pharmacia LKB Biotechnology Inc.) equilibrated with 0.01 M KH_2PO_4-Na_2HPO_4 buffer (pH 6.8). The column was eluted first with the phosphate buffer, and the adsorbed substances were eluted with a linear gradient of NaCl (0–1.0 M) in the buffer.

[1] The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.

The total volume of the gradient solution was 400 ml and fractions of 5 ml were collected with a flow rate of 20 ml/h. The active fractions were pooled and concentrated. The solvent was replaced with 0.01 M KH_2PO_4-Na_2HPO_4 buffer (pH 6.8) containing 0.5 M NaCl by ultrafiltration. The concentrated solution was subjected to the following affinity chromatography.

ConA-Sepharose 4B Affinity Chromatography—The sample obtained above was applied to a ConA-Sepharose 4B (Pharmacia LKB Biotechnology Inc.) column (1.2 × 9 cm, bed volume of 10 ml) equilibrated with the starting buffer (0.01 M KH_2PO_4-Na_2HPO_4 buffer (pH 6.8) containing 0.5 M NaCl). The column was eluted first with the starting buffer, and the adsorbed substances were eluted with a linear gradient of methyl-α-D-glucoside (Tokyo Kasei Co.) (0–0.15 M) in the starting buffer at a flow rate of 25 ml/h. The active fractions were pooled, exchanged the solvent with water by ultrafiltration, and lyophilized as a pure miraculin sample. 36 mg of the purified miraculin were obtained from 20 g of lyophilized pulps (see Table I).

Reverse Phase HPLC

Reverse phase HPLC was carried out on a TSK gel TMS-250 column (4.6 × 75 mm) for confirming the purity. The protein was eluted from the column by a linear gradient of CH_3CN (20–70%) containing 0.05% trifluoroacetic acid for 40 min at a flow rate of 1 ml/min. Protein was monitored by measuring absorbance at 210 nm.

Gel Electrophoresis

SDS-PAGE was performed in the slab gel electrophoresis apparatus (0.2 × 8.5 × 8 cm) according to the method of Laemmli (5) using the low molecular weight calibration kit (Pharmacia LKB Biotechnology Inc.) as standard marker proteins containing trypsin inhibitor (20,000), carbonic anhydrase (29,000), ovalbumin (43,000), and serum albumin (68,000).

Chemical Analysis

Amino Acid Composition—Purified miraculin was hydrolyzed in 6 N HCl in a sealed tube under vacuum at 110 °C for 22 h. Amino acid analysis was performed on an Atto MLC-703 high performance amino acid analyzer with a 440/570 detector and a Hewlett Packard 3390A integrator. Purified miraculin was also hydrolyzed for 40 and 70 h and the hydrolysates were subjected to amino acid analysis. The content of serine in the hydrolysates was lower than that in the 22-h hydrolysate and hence the value obtained by extrapolating data from three hydrolysates to zero time was listed as content of serine in Table II. The content of valine in the 70-h hydrolysate was highest and hence this content was listed in Table II. The contents of other amino acids including threonine and isoleucine were practically unchanged among three hydrolysates and hence the contents in the 22-h hydrolysate were listed in Table II.

Content of half-cystine was determined by hydrolysis of *S*-carboxyamidomethylated miraculin in 6 N HCl at 110 °C for 22 h on a Pico Tag Work Station (Waters Co.). Amino acids in the hydrolysate were converted into phenylthiocarbamyl-derivatives (6, 7) and their contents were analyzed by HPLC (Toyo Soda PC 8000) on an ODS-80TM column (4.6 × 75 mm). Tryptophan content was estimated in the intact protein by the spectrophotometric method of Edelhoch (8).

Determination of NH_2-terminal Amino Acid Sequence—The NH_2-terminal amino acid sequence of the purified miraculin was determined with an Applied Biosystems 470A Protein Sequencer using 20 μg of the pure miraculin. Phenylthiohydantoin derivatives were identified in a semiquantitative manner by the HPLC system.

Sugar Composition—The sugar composition of the purified protein was determined by HPLC on an ISA-07/S2504 column according to Mikami and Ishida (9). Total sugar content of the purified sample was determined from the ratio of sugar and protein (w/w) in the sample. The amount of protein was determined by amino acid analysis and that of sugar was determined by HPLC as described above.

Measurement of Sweet-inducing Activity

The sweet-inducing activity of miraculin was assayed using four subjects as described previously (10, 11). 5 ml of a miraculin solution were held in the mouth for 3 min and spat out. The mouth was rinsed with water and then 0.02 M citric acid was tasted. The sweetness induced by 0.02 M citric acid was evaluated by comparing its sweetness with that of a series of standard sucrose solutions (0.1–1.0 M).

RESULTS

Purification of Miraculin—The active principle in the lyophilized pulps was extracted with 0.5 M NaCl solution, which led to solubilization of the active principle. The extracted solution was colorless and showed the strong sweet-inducing activity. Practically all activity in the pulps was extracted by 0.5 M NaCl solution (Table I).

The extracted solution was subjected to ammonium sulfate fractionation. Addition of ammonium sulfate to bring about 50% saturation led to precipitation of about 30% of the total protein in the solution, and about 86% of the sweet-inducing activity in the starting solution was recovered in this precipitate (Table I). Hence the 50% fraction was used for further purification of the active principle.

The 50% fraction was subjected to CM-Sepharose ion-exchange chromatography (Fig. 1). *Peak A* in Fig. 1 was eluted with the starting buffer. A linear gradient of NaCl eluted *peaks B*. Only peak B showed the sweet-inducing activity.

TABLE I

Yield of protein and recovery of activity in each step in the purification of miraculin

Amount of protein was determined by the method of Lowry *et al.* (12). The amount of the pulps is expressed by total weight of the lyophilized pulps. The recovery of activity was checked as follows: aliquot of a sample solution was diluted to induce the sweetness equivalent to that of 0.2 M sucrose when 0.02 M citric acid was tasted after the sample. It was estimated that 20 g of the lyophilized pulps give 14 l of solution thus diluted. This value (14 l) is taken as 100 and the recovery of the activity is expressed by relative volume of a solution which induces 0.2 M sucrose-sweetness. -Fold purification was obtained by dividing the recovery of the activity by amount of protein. The value of the pulps is taken as unit.

	Protein	Recovery of activity	Purification
	g		*-fold*
Lyophilized pulps	20	100	1
0.5 M NaCl extracts	0.360	97	54
Precipitates by 50% $(NH_4)_2SO_4$	0.108	83	154
Peak B fraction on CM-Sepharose chromatography	0.040	80	400
Peak I fraction on ConA-Sepharose chromatography	0.036	75	417

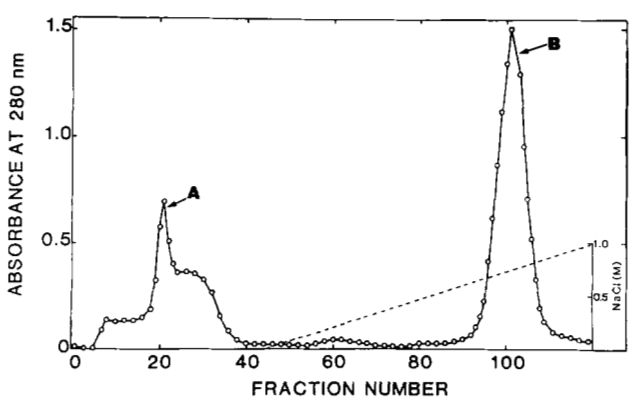

FIG. 1. **Elution profile of chromatography on a CM-Sepharose column.** A sample solution (45 ml) obtained from the 50% ammonium sulfate fraction was applied to a CM-Sepharose column equilibrated with 0.01 M KH_2PO_4-Na_2HPO_4 buffer (pH 6.8). The column was eluted with a linear gradient of NaCl (0–1.0 M) in the phosphate buffer at a flow rate of 20 ml/h. The volume of each fraction was 5 ml. Protein was monitored by measuring absorbance at 280 nm. The active fractions (Nos. 93–110) were pooled.

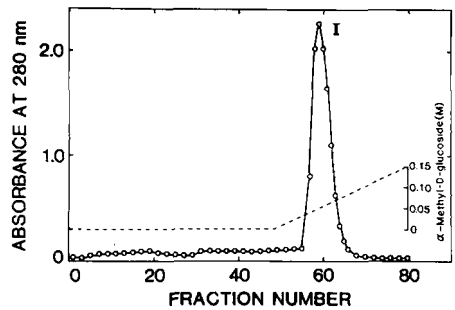

FIG. 2. **Elution profile of affinity chromatography of miraculin on a ConA-Sepharose 4B column.** The active fractions pooled from a CM-Sepharose column were applied to the column. The column was equilibrated with 0.01 M KH_2PO_4-Na_2HPO_4 buffer (pH 6.8) containing 0.5 M NaCl. The column was eluted first with the same buffer and then eluted with a linear gradient of methyl-α-D-glucoside (0–0.15 M) at a flow rate of 25 ml/h. The volume of each fraction was 3 ml. Protein was monitored by measuring absorbance at 280 nm. The active fractions (Nos. 57–64) were pooled.

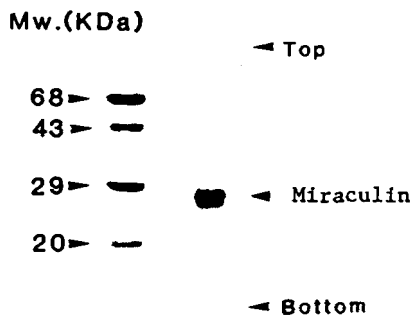

FIG. 3. **SDS-PAGE of the peak I sample from a ConA-Sepharose 4B column.** 8 μg of protein were loaded on 12% polyacrylamide gel in the presence of 0.1% SDS. The molecular weight of miraculin was estimated to be about 28,000 using standard marker proteins.

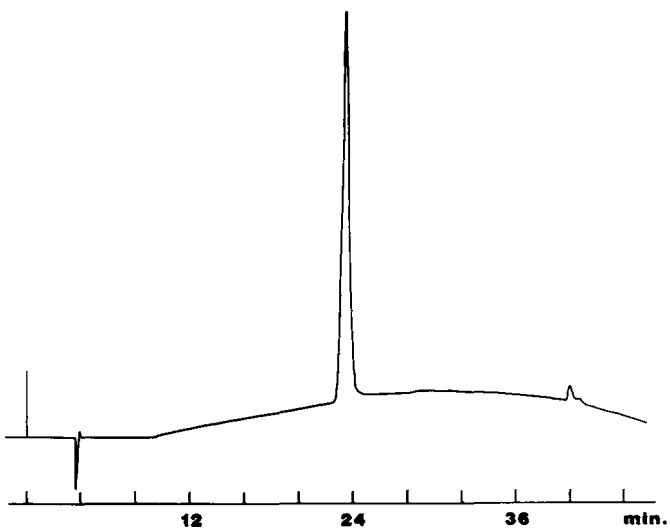

FIG. 4. **Elution profile of the peak I sample in reverse phase HPLC.** HPLC was carried out on a TSK gel TMS-250 column (4.6 × 75 mm). The protein sample was applied to a column equilibrated with 20% acetonitrile containing 0.05% trifluoroacetic acid. The protein was eluted from the column with a linear gradient of CH_3CN (20–70%) containing 0.05% trifluoroacetic acid at a flow rate of 1 ml/min. The protein was eluted at 45% acetonitrile. Protein was monitored by measuring absorbance at 210 nm.

TABLE II
Amino acid compositions of purified miraculin

Amino acids	Mol %[a]	Residues/mol[b]
Aspartic acid	11.0	21
Threonine	7.3	14
Serine	6.3	12
Glutamic acid	6.5	12
Proline	7.9	15
Glycine	8.7	16
Alanine	3.5	6
Half-cystine	2.6	5
Valine	10.8	21
Methionine	0.5	1
Isoleucine	4.3	8
Leucine	5.5	10
Tyrosine	3.7	7
Phenylalanine	7.4	14
Lysine	6.5	12
Histidine	1.0	2
Arginine	5.5	11
Tryptophan	1.0	2

[a] The values represent numbers of amino acid residues per 100 total residues.

[b] The values represent number of residues per 21,200-dalton polypeptide.

The peak B fraction was collected and applied to a ConA-Sepharose 4B column (Fig. 2). The column was eluted with the starting buffer. The absorbance of the effluent at 280 nm is slightly higher than the base line, indicating that certain substances are eliminated by this elution. The column was then eluted with a linear gradient of methyl-α-D-glucoside, which gave a sharp peak (*peak I*). The fractions of the peak I showed the strong sweet-inducing activity; the sweetness induced by 0.02 M citric acid after 10^{-6} M of the sample was kept in the mouth was equivalent to the sweetness of 0.5 M sucrose. Application of the peak I fraction to SDS-PAGE gave a single band with a molecular weight of 28,000 (Fig. 3). The purity of the peak I sample was also checked by reverse phase HPLC. As shown in Fig. 4, the sample gives a sharp single peak, indicating that it is highly pure. 36 mg of pure miraculin were obtained from 20 g of lyophylized pulps by the above method (Table I).

Chemical Analysis—Table II shows amino acid compositions of the purified miraculin (peak I fraction). Amino acids of relatively high content are aspartic acid, valine, glycine, proline, threonine, and phenylalanine.

Application of the purified miraculin to an amino acid sequencer indicated that it is composed of a pure single polypeptide. 20 amino-terminal amino acids were identified, as shown in Table III.

The purified miraculin contained as much as 13.9% of sugar. Analysis of sugar components indicated that it contained glucosamine, mannose, galactose, xylose, and fucose in a molar ratio of 3.03:3.00:0.69:0.96:2.12 (Table IV).

DISCUSSION

Miraculin in the pulps is not extracted with water. In the present study, we have found that miraculin can be extracted with 0.5 M NaCl solution. This extraction method has great advantages when compared with the methods reported previously (1–4). In the previous studies, the pulps of miracle fruit were extracted with carbonate buffer (pH 10.5), which reduced the sweet-inducing activity of miraculin. In addition, the carbonate buffer extracted various substances including deeply colored materials, which were very difficult to remove. On the other hand, the extracted solution obtained by the present method was colorless. The pH of the extracted solution was about 4, indicating that the extraction was carried

TABLE III

Amino-terminal amino acid sequence of purified miraculin

1	5	10	15	20
Asp-Ser-Ala-Pro-Asn-Pro-Val-Leu-Asp-Ile-Asp-Gly-Glu-Lys-Leu-Arg-Thr-Gly-Thr-Asn-				

TABLE IV

Sugar composition of purified miraculin

Sugars	Molar ratio[a]
Glucosamine	3.03
Mannose	3.00
Galactose	0.69
Xylose	0.96
Fucose	2.12

[a] The values represent the molar ratio of sugar residues when the value for mannose is taken as 3.00.

out at acidic pH. In intact miracle fruit, miraculin exists in pulps which contain a rather high concentration of acids and is very stable at acidic pH. For example, the activity of miraculin in acetate buffer of pH 4 was practically unchanged for at least 6 months at 5 °C. The maximum sweetness induced by 0.02 M citric acid was equivalent to the sweetness of 0.5 M sucrose. This value is higher than the value reported previously (10, 11) (the sweetness of 0.4 M sucrose).

The molecular weight of purified miraculin was 28,000, whereas that of miraculin reported was 40,000–48,000. This discrepancy is not due to the difference in the method for determination of molecular weight, since the molecular weight reported in some previous paper (3, 4) was also determined by the SDS-PAGE method. It seems that miraculin in the samples obtained in the previous studies was tightly associated with other compounds. The compounds seem to be non-proteinaceous ones, since the amino acid composition of the miraculin purified in the present study is close to that reported previously (4). The sequence analysis of the purified miraculin indicated that it is composed of a single polypeptide. This also indicates that the miraculin preparation obtained in the present study is highly pure. 20 amino-terminal amino acids were determined in the present study. The molecular weight estimated from the sum of molecular weights of amino acids in peptides obtained by proteolytic cleavage was around 21,200 (data not shown). Hence the total molecular weight of miraculin, which contains as much as 13.9% of sugars, seems to be about 25,000. This value is lower than that determined by SDS-PAGE. Probably SDS-PAGE gave a higher molecular weight than the true one because miraculin is a glycoprotein (13).

The purified miraculin contained five species of sugar components. These results are not identical with those reported in previous papers (1, 2, 4). There is a possibility that the sugar components of miraculin vary depending on cultivating conditions such as the season when the fruit is harvested or the locale where the plant is grown. However, the most probable reason seems to be because miraculin isolated previously was not pure.

Acknowledgments—We are indebted to Professor L. M. Beidler for the supply of seeds of miracle fruit and constant encouragement. We also thank Professor Takeo Aso of Yokohama National University who cultured the miracle fruit. We express our sincere gratitude to Mitajiri Chemical Industry Co. for financial support and encouragement. We are indebted to Drs. Y. Nakamura, K. Nakaya, and S. Nakajo for their invaluable suggestions. We thank Dr. N. Takahashi, who kindly analyzed sugar composition of miraculin. We thank all of our students who participated in this study.

REFERENCES

1. Kurihara, K., and Beidler, L. M. (1968) *Science* **161,** 1241–1243
2. Brouwer, J. N., Van der Wel, H., Francke, A., and Henning, G. J. (1968) *Nature* **220,** 373–374
3. Giroux, E. L., and Henkin, R. I. (1974) *J. Agric. Food Chem.* **22,** 594–601
4. Kurihara, Y., and Terasaki, S. (1982) *Biochim. Biophys. Acta* **719,** 444–449
5. Laemmli, U. K. (1970) *Nature* **227,** 680–685
6. Heinrickson, R. L., and Meridith, S. C. (1984) *Anal. Biochem.* **136,** 65–74
7. Scholze, H. (1985) *J. Chromatogr.* **350,** 453–460
8. Edelhoch, H. (1967) *Biochemistry* **6,** 1948–1954
9. Mikami, H., and Ishida, Y. (1983) *Bunseki Kagaku* **32,** E207
10. Kurihara, K., and Beidler, L. M. (1969) *Nature* **222,** 1176–1179
11. Kurihara, K., Kurihara, Y., and Beidler, L. M. (1969) *Olfaction and Taste III*, pp. 450–469, Rockefeller University Press, New York
12. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
13. Segrest, J. P., and Jackson, R. L. (1972) *Methods Enzymol.* **28,** 54–63