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Functional Expression of Miraculin, a Taste-Modifying Protein in *Escherichia Coli*

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Miraculin isolated from red berries of *Richadella dulcifica*, a native shrub of West Africa, has the unusual property of modifying a sour taste into a sweet one. This homodimer protein consists of two glycosylated polypeptides that are cross-linked by a disulfide bond. Recently, functional expression of miraculin was reported in host cells with the ability to glycosylate proteins, such as lettuce, tomato and the microbe *Aspergillus oryzae*, but not *Escherichia coli*. Thus, a question remains as to whether glycosylation of miraculin is essential for its taste-modifying properties. Here we show that recombinant miraculin expressed in *E. coli* has taste-modifying properties as a homodimer, not as a monomer, indicating that glycosylation is not essential for the taste-modifying property.

Key words: *Escherichia coli*, glycosylation, homodimer, miraculin, taste-modifying protein.

Abbreviations: AAA, ATPase associated with a variety of cellular activities; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

INTRODUCTION

Losses or modifications of taste and smell are common in the elderly and result from aging and environmental stresses, which also represent risk factors for nutritional and immune deficiencies (*1*). Recently, receptors for umami, sweet, bitter and sour tastes have been identified (*2–6*). However, little is known about the molecular mechanism of losses or modifications to these tastes.

Miraculin, isolated from the red berries of *Richadella dulcifica*, a native shrub of West Africa, has a taste-modifying activity that converts a sour taste to a sweet one in primates (*7*). Miraculin consists of 191 amino-acid residues and a carbohydrate content of 13.9%, from which the calculated molecular weight is 24.6 kDa (*8–10*). Five kinds of oligosaccharide structures have been proposed at the two glycosylation sites, Asn-42 and Asn-186 (*9*). Miraculin has seven Cys residues, which form three intra-chain disulfide bridges at Cys-47-Cys-92, Cys-148-Cys-159 and Cys-152-Cys-155, and one inter-chain disulfide bridge at Cys-138 (*11*). Homodimer miraculin, covalently linked at Cys-138, has taste-modifying activity at acidic pH. Moreover, less than 1 μM miraculin induces sweetness corresponding to about 0.3 M sucrose by exposure to 20 mM citrate on the tongue. Although this interesting sensory effect has been previously reported, the molecular mechanism(s) underlying the taste-modifying action remain (s) unknown.

T1R2/T1R3, a heterodimer of members of the G-protein-coupled receptor family, and PKD1L3/PKD2L1, a heterodimer of members of the polycystic-kidney-disease-like ion channel family, have been indicated as receptors for sweet (*3*) and sour (*5*, *6*) tastes, respectively. The molecular interaction between miraculin and the receptor for sweet or sour tastes will be a key event in understanding the mechanism of the sensory effect. It will also be useful to obtain functionally active recombinant miraculin by expression in *Escherichia coli* for such molecular analyses.

Functional expression of recombinant miraculin has reported in hosts with the ability to glycosylate proteins, such as lettuce (*12*) and tomato (*13*) plants, and the microbe *Aspergillus oryzae* (*14*), but not in *E. coli*, which lacks the ability to glycosylate. Thus, a question remains as to whether the glycosylation of miraculin is essential for its taste-modifying property (*9*).

In this study, we attempted to express the taste-modifying protein, miraculin in *E. coli*, incorporating several improvements in expression and purification procedures. To our knowledge, this is the first report on the production of biologically active homodimeric miraculin without glycosylation.

MATERIALS AND METHODS

Construction of Expression Plasmids—Poly(A) RNA was isolated from miracle fruits, the red berries of *R. dulcifica*, using a QuickPrep Micro mRNA Purification Kit (Amersham). cDNA was synthesized from 0.7 μg poly(A) RNA by SuperScript II reverse transcriptase (Invitrogen). The coding region of miraculin, except the

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signal peptide (Accession no D38598) (*10*), was amplified by KOD-Plus DNA polymerase (Toyobo) using a pair of synthetic primers (forward primer: 5′-GCTTAGTCATATGGATTCGGCACCCAATCC-3′, and reverse primer: 5′-CAAGGATCCTTAGAAGTATAGGGTTTTGTTG-3′). The forward primer contained the recognition sequence for *Nde*I, and the reverse primer contained the recognition sequence for *Bam*HI. The amplification reaction consisted of 94°C for 2 min, 30 cycles of amplification (94°C for 15 s, 50–60°C for 30 s and 68°C for 1 min), and a final extension at 68°C for 7 min. The resulting PCR fragment was purified and subcloned into the *Nde*I/*Bam*HI sites of the expression vector pET14b (Novagen). The sequence of this clone was confirmed by DNA sequencing. The resulting vector, named pET14b-miraculin, containing the miraculin coding region with an N-terminal His-tag sequence, was used for further experiments.

Expression and Purification of Miraculin from E. coli—AR797, an *E. coli* strain mutated in *fts*H, which encodes a membrane protein that belongs to the AAA family and over-synthesizes SecY (*15*), was used for expression of the recombinant miraculin after integration of the λDE3 prophage using the λDE3 lysogenization kit (Novagen). These bacterial cells transformed with pET14b-miraculin were grown at 30°C in 4 l (0.5 l × 8) of LB medium, with 100 μg/l ampicillin in shaker flasks to an OD_{600} of ∼0.25–0.35, then grown at 18°C for 16 h after the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested by centrifugation, resuspended in 80 ml of extraction buffer (50 mM Tris–HCl, 0.5 M NaCl, 10% glycerol, pH 8.0, containing 0.8 M arginine, 1 mM PMSF, 0.2 mg/ml lysozyme and one tablet of protease inhibitor cocktail, Complete™ EDTA-free; Roche), and sonicated for 16 min on ice. The lysed cells were centrifuged (12,000 × *g*, 30 min), and the supernatant was filtered using a cell strainer (BD Falcon). This supernatant was dialysed at 4°C against 2 l 50 mM Tris–HCl, 0.5 M NaCl and 0.4 M arginine (pH 8.0) for 6 h, 2 l 50 mM Tris–HCl, 0.5 M NaCl, and 0.2 M arginine (pH 8.0) overnight, 2 l 50 mM Tris–HCl and 0.5 M NaCl (pH 8.0) for 6 h, and then 2 l 50 mM Tris–HCl, 0.5 M NaCl, and 50 mM imidazol (pH 8.0) for 6 h. Aggregates formed in these dialysis steps were removed by centrifugation (39,000 × *g*, 30 min), and a clear supernatant was obtained by filtration through a syringe filter of 0.45-μM pore size (Zartorius). This sample was loaded on a 5 ml Ni-NTA agarose column (Qiagen), equilibrated with 50 mM Tris–HCl, 0.5 M NaCl, and 50 mM imidazole (pH 8.0) at a flow rate of 30 ml/h. The column was washed with 20 volumes of extraction buffer containing 50 mM imidazole, and the protein was eluted with a 100 ml of a 50–350 mM imidazole linear gradient, and collected in 2 ml fractions. The collected fractions containing the recombinant dimeric miraculin were dialysed against 1 l 20 mM phosphate buffer, 0.5 M NaCl, and 0.2 M imidazole (pH 8.0), diluted with five volumes of 20 mM sodium dihydrogen phosphate, and then loaded on a 4 ml CM Sepharose Fast Flow column (Amersham) equilibrated with 20 mM sodium phosphate buffer (pH 6.8) at a flow rate of 30 ml/h. The column was washed with 80 ml of 20 mM phosphate buffer and 100 mM NaCl (pH 6.8), and the miraculin was eluted with 80 ml of a 100–1000 mM NaCl linear gradient. The collected fractions containing miraculin were used for measurement of taste-modifying activities after dilution in water to 170 mM NaCl.

Native miraculin (20 mg) was purified from 50 miracle fruits according to a previously described method (*7*). Protein was quantified using the BCA protein assay kit (Pierce).

Preparation of Anti-Miraculin Antibody and Western Blot Analysis—Bacterial cells transformed with pET14b-miraculin, described above, were harvested by centrifugation, and the recombinant miraculin expressed in inclusion bodies was dissolved in 8 M urea. An antiserum was prepared by immunizing rabbits with adjuvant-conjugated protein, with assistance of Keari Inc.

SDS–PAGE was performed using 15% polyacrylamide gels. After electrophoresis, the proteins were transferred to an Immobilon-P membrane (Millipore) for western blotting using the anti-miraculin antiserum.

Analysis of the Dimerization of Recombinant Miraculin Expressed in E. coli—Dimerization of recombinant miraculin expressed in *E. coli* was confirmed using non-reducing and reducing SDS–PAGE.

Measurement of Taste-Modifying Activity—The taste-modifying activity of miraculin was evaluated as described previously (*16*). Prior to evaluation, subjects (females between 21 and 25 years of age) repeatedly tasted a series of standard sucrose solutions (0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, 0.50 M) and remembered their sweetness intensity. Subsequently, 5 ml of 0.8 μM native or 10 ml of 1.3 μM recombinant purified miraculin was sipped, held in the mouth for 3 min, and spat out. After rinsing their mouth with water, subjects sipped 5 ml of 20 mM citrate and evaluated the sweetness intensity by choosing a standard solution with an equivalent intensity of sweetness. At least 6 h was interposed between each test.

RESULTS AND DISCUSSION

Evaluation of E. coli Strains for Recombinant Miraculin Production—The recombinant miraculin expression plasmid was constructed using pET14b, a protein expression vector carrying an N-terminal His-Tag sequence. We evaluated several *E. coli* strains for transformation with this expression vector and found that AR797 (DE3), an *E. coli* strain mutated in *fts*H that encodes a membrane protein that belongs to the AAA family, was suitable for our study; the amounts of the soluble form of recombinant miraculin were higher than those produced by other *E. coli* strains, such as BL21star (DE3). The *fts*H contributes to quality maintenance of newly synthesized proteins by degradation of misassembled proteins (*17*), which will found in overexpression using the recombinant technique. This will be one of the reasons for the better yield of soluble form of the recombinant miraculin in our study.

Improved Solubilization of Recombinant Miraculin by the Addition of Arginine—Formation of inclusion bodies frequently occurs when heterologous proteins are expressed in *E. coli*, and arginine has been used in

protein refolding (*18*, *19*). We successfully expressed and purified recombinant miraculin in AR797 (DE3); however, less than 1% of the protein was expressed in soluble form in our previous study. Thus, arginine was assessed in our system for both protein solubilization and refolding procedures. In a pilot experiment, the amounts of purified, solubilized miraculin were 1.92 mg and 0.036 mg, in the presence and absence of arginine, respectively, from 4 l of culture medium, indicating an improvement in solubilization of about 50-fold. We also obtained refolded recombinant miraculin from inclusion bodies in the presence of arginine. We chose the purification of the recombinant miraculin from the solubilized fraction because of the better yield of dimeric miraculin, as described below.

Isolation of Recombinant, Dimeric Miraculin—Recombinant miraculin was purified by affinity chromatography using a Ni-NTA agarose column. The elution profile with a 50–350 mM imidazole linear gradient showed two peaks (Fig. 1A), indicating the coexistence of different forms of the protein. Samples of the fractions (*7–33*) were analysed by SDS–PAGE using 15% polyacrylamide gels in the presence (Fig. 1B) or absence of 5% 2-mercaptoethanol (Fig. 1C). CBB staining under reducing conditions showed a single band at 24 kDa (Fig. 1B), consistent with the calculated molecular weight of mature miraculin with the N-terminal His-tag sequence. Western blot analysis using an anti-miraculin antibody under non-reducing conditions showed a single band at 24 kDa in fractions 7–15, and a single band at 40 kDa in fractions 15–31 (Fig. 1C), indicating that the protein fractions from the first and second peaks were derived from monomeric and dimeric miraculins, respectively. Because dimeric miraculin has taste-modifying activity (*11*), the fractions containing the dimeric protein were further purified using a CM Sepharose column as a single peak (Fig. 1D and E), which was then used for measurement of taste-modifying activities.

Purification of Miraculin from Miracle Fruit—Native miraculin was purified from the pulp of *R. dulcifica* according to a previously described method (*7*). The elution profile of native miraculin using a ConA Sepharose 4B column showed a single peak (Fig. 2A). CBB staining of the fractions (*1–10*) under reducing (Fig. 2B) or non-reducing (Fig. 2C) conditions showed double bands and a broad band, respectively, and sizes of these bands are confirmed by western blot analysis using an anti-miraculin antibody (Fig. 2D). These results showed that native miraculin forms a dimer as described (*7*). Since native miraculin contains two N-glycosylation sites (*9*), next, we confirmed whether the native miraculin purified by us is glycosylated. As shown in Fig. 2E, after N-glycosidase A treatment, smaller bands at 20 and 22 kDa were detected. Note that recombinant miraculin expressed in *E. coli* gave a single band on SDS–PAGE (Fig. 1E), while native miraculin (Fig. 2B) (*7*), and recombinant miraculin expressed in plants (*12*, *13*) and *A. oryzae* (*14*), gave *several* bands probably because of the heterogeneity resulting from glycosylation.

Taste-Modifying Activity of Recombinant Miraculin—The taste-modifying activity of native and recombinant unglycosylated miraculin was estimated according to a previously reported method (*16*). As shown in Table 1, after exposure to 0.8 μM purified native miraculin (0.1 mg in 5 ml) or 1.3 μM recombinant miraculin expressed in *E. coli* (0.3 mg in 10 ml), the sweetness induced by 20 mM citrate was equivalent to that of 0.25 M or 0.18 M sucrose, respectively. Although native miraculin is able to dissolve in water, the recombinant miraculin expressed in *E. coli* is able to dissolve not in water but in 170 mM NaCl, which indicates the structural instability of the recombinant because of unglycosylation. However, we observed similar taste-modifying activities of the native miraculin in 170 mM NaCl and water, indicating no effect on the taste-modifying activity of the miraculin in 170 mM NaCl.

The sweetness induced by the 0.8 μM native miraculin was almost the same as that induced by one miracle fruit (Table 1), and recombinant glycosylated miraculin expressed in *Arabidopsis thaliana* (*24*), was also consistent with previous reports of the activity of one miracle fruit and the purified native and recombinant glycosylated miraculins (*12–14*). These results show that unglycosylated miraculin had taste-modifying activity in dimeric form, although its activity was lower than that of native or recombinant, *glycosylated* miraculin. As described previously (*9*), by glycosidase treatment, the native miraculin lost the taste-modifying activity due to denaturalization, which is also confirmed by our preliminary experiment. In our assay, we could not detect the precise dose dependency of the taste-modifying activity although we could obtain the similar activity of miraculin, reported previously in several groups (*12–14*). Therefore, it will be difficult to discuss about accurate quantitative values of the activity between the native and recombinant miraculins. Improvement of the assay system for the taste-modifying activity will be necessary to further biochemical characterizations since the taste-modifying activity of miraculin detect only in primates.

Our experiments showed that the glycosylation of the miraculin is not essential for taste-modifying activity, but is important for protein folding or stability, because unglycosylated dimeric miraculin used in Table 1 was readily precipitated after dialysis against water, a procedure used in the purification of native miraculin.

Curculin, isolated from *Curculigo latifolia*, a plant grown in Malaysia, has both taste-modifying and sweetness-eliciting activities. Recombinant curculin heterodimer, also called neoculin, expressed in *E. coli* has been reported to exhibit both taste-modifying and sweetness-eliciting activities, while recombinant curculin homodimer does *not* (*20*, *21*). Based on a mutational and structural study of recombinant curculin, these activities of the curculin heterodimer have been suggested to be the result of two different modes of interactions with the T1R2–T1R3 heterodimeric sweet taste receptor (*22*).

The inactive homodimer of curculin has high sequence similarity to the mannose-binding lectin from the snow drop (*Galanthus nivalis*), while the active homodimer of miraculin, which has only taste-modifying activity, in this study had high sequence similarity to soy bean trypsin inhibitor (*8*), indicating that these proteins are structurally different.

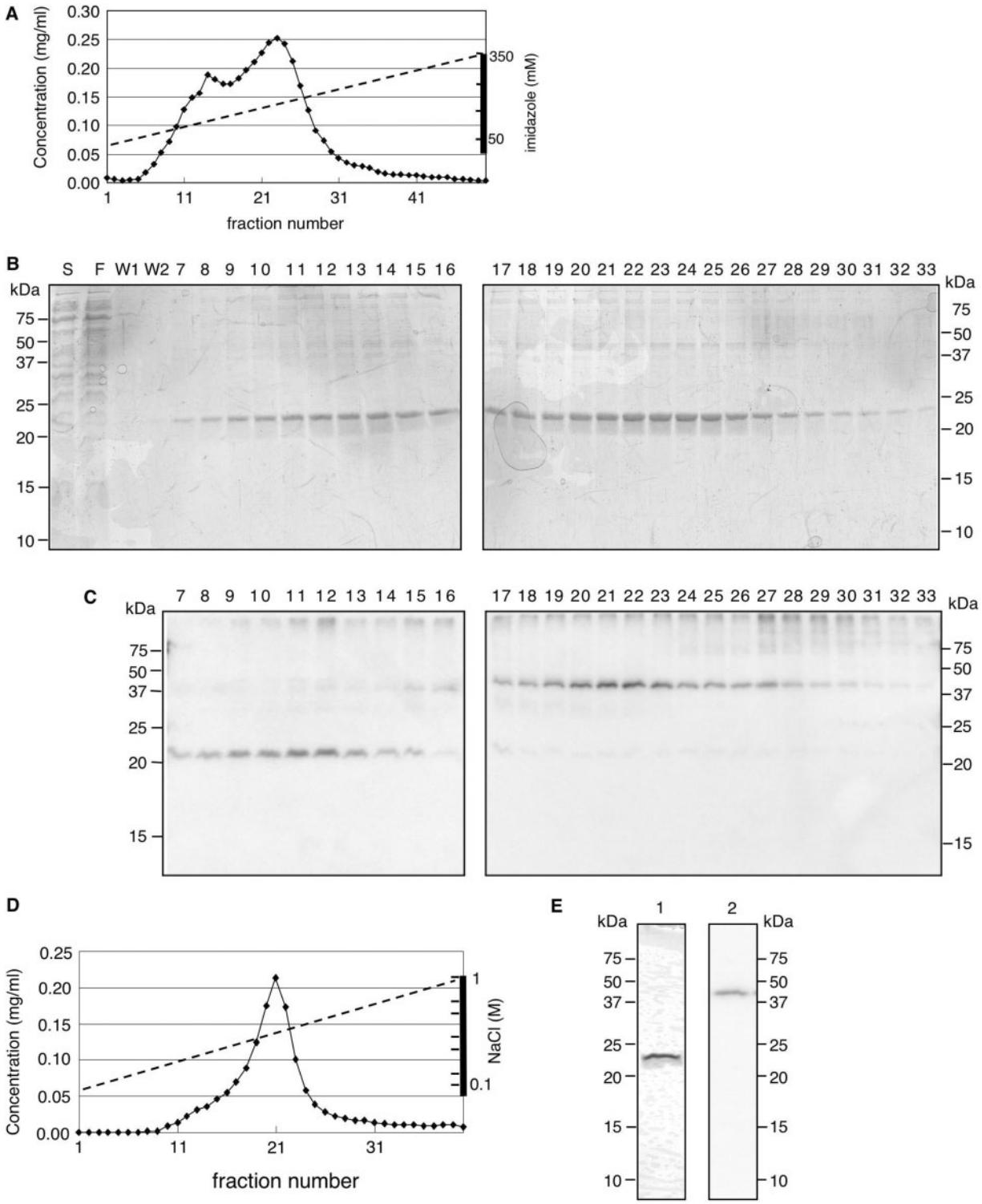

Fig. 1. **Dimer formation of recombinant miraculin expressed in *E. coli*.** (A) Elution profile of recombinant miraculin from a Ni-NTA agarose column. Protein was eluted with 100 ml of a 50–350 mM imidazole linear gradient. The volume of each fraction was 2 ml. Protein was monitored by measuring absorbance at 280 nm. Samples of the fractions (7–33) were analysed by SDS–PAGE using 15% polyacrylamide gels in the presence or absence of 5% 2-mercaptoethanol. (B) CBB staining under reducing conditions. (C) Western blot analyis of miraculin under non-reducing conditions. Lane S, supernatant; lane F, flow through; lanes W1 and W2, wash fractions; lanes 7–33, elution fractions nos 7–33 in (A), respectively. (D) Elution profile of chromatography on a CM Sepharose column. The dimeric miraculin fractions pooled from (A) were applied and eluted with 80 ml of a 100–1,000 mM NaCl linear gradient. The volume of each fraction was 2 ml. Protein was monitored by measuring absorbance at 280 nm. (E) Fraction no. 20 in (D) was analysed by SDS–PAGE using 15% polyacrylamide gel in the presence or absence of 5% 2-mercaptoethanol. Lane 1, silver-staining under reducing conditions; lane 2, western blot analysis of miraculin under non-reducing conditions.

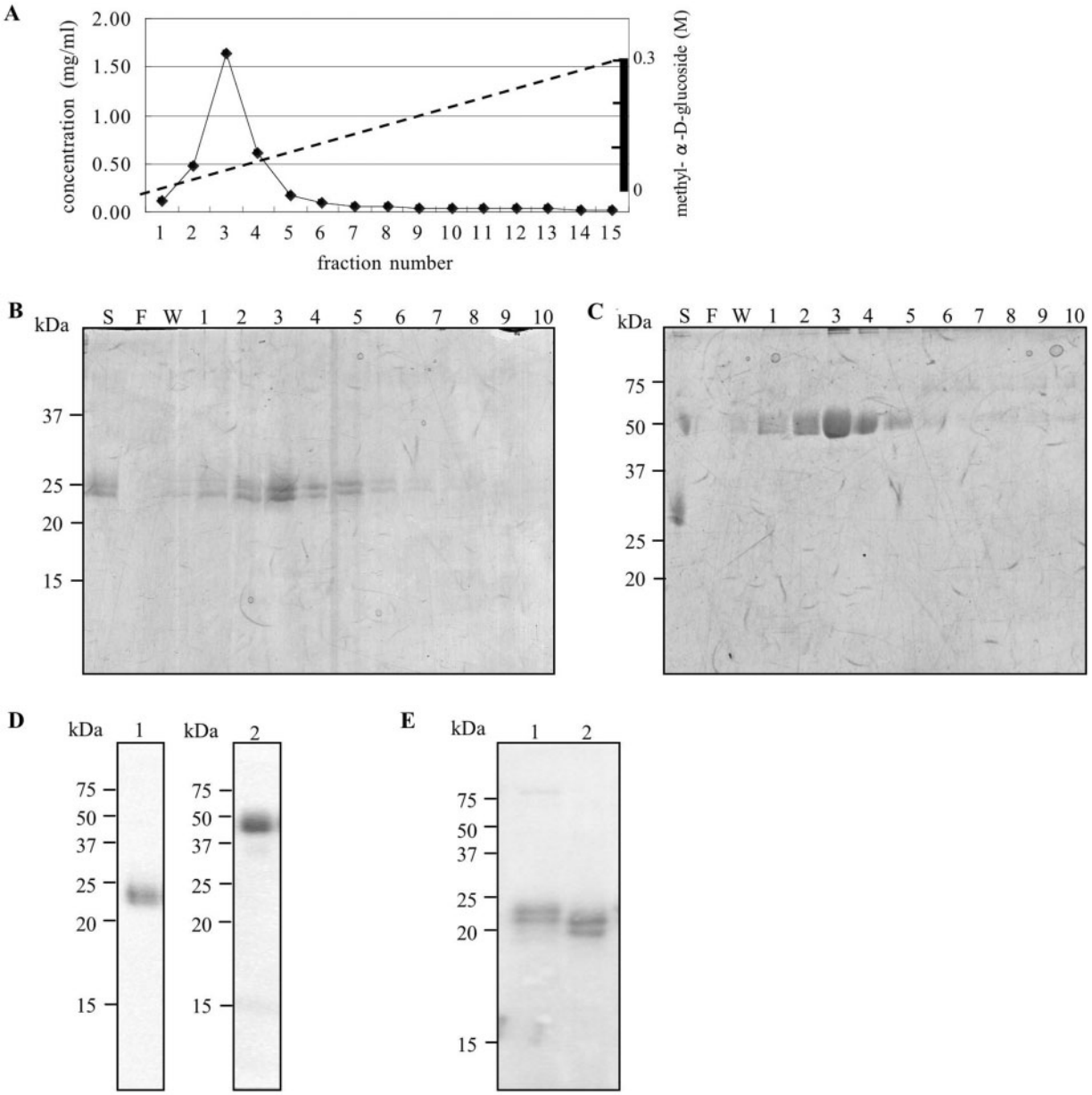

Fig. 2. **Purification of miraculin from miracle fruit.** Native miraculin was purified from the pulp of *Richadella dulcifica* according to a previously described method (*7*). (A) Elution profile of native miraculin from a ConA Sepharose 4B column. Protein was eluted with 60 ml of a 0–300 mM methyl-α-D-glucoside linear gradient. The volume of each fraction was 4 ml. Protein was monitored by measuring absorbance at 280 nm. CBB staining under reducing (B) or non-reducing (C) conditions. Lane S, supernatant; lane F, flow through; lanes W, wash fractions; lanes 1–10, elution fractions nos. 1–10 in (A), respectively. (D) Western blot analysis of the native miraculin. Native miraculin [fraction no. 3 in (A)] was analysed by SDS–PAGE using 15% polyacrylamide gel in the presence (lane 1) or absence (lane 2) of 5% 2-mercaptoethanol. (E) Analysis of the *N*-glycosylation of native miraculin. The purified native miraculin (2 μg) was treated without (lane 1) or with (lane 2) *N*-glycosidase A (0.5 mU) at 37°C for 24 h as described (*12*), which samples (each 50 ng) were subjected to SDS–PAGE in the presence of 5% 2-mercaptoethanol and then to western blot analysis.

Table 1. **Sweetness intensity induced by native and recombinant miraculin.**

Source material	Concentration of miraculin	Induced sweetness (SEV)
Purified native miraculin	0.8 μM (0.1 mg in 5 ml)	0.25 ± 0.04 M (n = 5)
Purified recombinant miraculin from *E. coli*	1.3 μM (0.3 mg in 10 ml)	0.18 ± 0.05 M (n = 5)
Miracle fruit (one fresh fruit)	–	0.31 ± 0.06 M (n = 6)

The sucrose equivalence value (SEV) corresponds to the sweetness intensity induced by 20 mM citric acid evaluated by comparing its sweetness to that of a series of standard sucrose solutions (0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, 0.5 M). Data represent the means of five to six subjects ± S.E.

Recently, studies on miraculin have been reported using mutational analyses (*14*) and molecular modelling (*23*). Functional recombinant miraculin expressed in *E. coli* will be suitable for X-ray crystal structural analysis because of its homogeneity compared to the glycosylated miraculins previously reported (*12–14*), and will aid in studying the molecular mechanisms underlying its taste-modifying activity.

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CONFLICT OF INTEREST

None declared.

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