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ARTICLE

Single-Step Purification of Native Miraculin Using Immobilized Metal-Affinity Chromatography

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Miraculin is a taste-modifying protein that can be isolated from miracle fruit (*Richadella dulcifica*), a shrub native to West Africa. It is able to turn a sour taste into a sweet taste. The commercial exploitation of this sweetness-modifying protein is underway, and a fast and efficient purification method to extract the protein is needed. We succeeded in purifying miraculin from miracle fruit in a single-step purification using immobilized metal-affinity chromatography (IMAC). The purified miraculin exhibited high purity (>95%) in reverse-phase high-performance liquid chromatography. We also demonstrated the necessity of its structure for binding to the nickel-IMAC column.

KEYWORDS: Purification; immobilized metal-affinity chromatography; miraculin; taste-modifying protein

INTRODUCTION

The mass production of sweet-tasting proteins, such as thaumatin, monellin, mabinlin, pentadin, brazzein, and curculin, for commercial use has been investigated for many years (*1*, *2*). Unlike these sweet-tasting proteins, miraculin is unique in that it is not a sweet-tasting protein but rather a taste-modifier (*3*). It can turn a sour taste into a sweet taste by binding into the taste receptor. Sour food tastes sweet when tasted after this protein.

Efforts to make use of miraculin have been successful. Sun et al. (*4*, *5*) have expressed the protein in tomato and lettuce, which are commercial plants with much higher productivity than the native miracle fruits. Sugaya et al. (*6*) reported the production of transgenic strawberry plants expressing miraculin, although the accumulation was less than that in tomato and lettuce. The microbial production of miraculin protein has also been reported using an *Aspergillus oryzae* expression system to produce a recombinant miraculin, which resembles the native miraculin in secondary structure and taste-modifying activity, to generate sweetness at acidic pH (*7*).

Native miraculin is a tetra- and dimer of a 25 kDa peptide. Both forms show the sweet-modifying activity. Miraculin dimer, covalently linked at Cys-138, has taste-modifying activity at acidic pH and is flat at neutral pH (*8*). There is no report of the taste-modifying activity of its monomeric state. All recombinant miraculin produced in tomato, lettuce, strawberry, and *A. oryzae* showed taste-modifying activity in the dimeric form (*4*−*7*).

Miraculin consists of a carbohydrate content of 13.9%. There are at least five types of oligosaccharides at the two glycosylation sites, Asn-42 and Asn-186 (*9*). This sugar was reported not essential to the miraculin sweet-inducing activity but is important for protein folding or stability (*10*).

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Miraculin was first purified from miracle fruit by extraction with carbonate buffer (pH 10.5) (*3*, *11*, *12*) or other highly basic solutions, such as salmine or spermine (*13*). Unfortunately, these extraction methods led to a reduction in the sweetness-inducing activity. The most recent method for purification of miraculin involved extraction with 0.5 M NaCl solution, ammonium sulfate fractionation, CM-Sepharose ion-exchange chromatography, and finally, concavalin A-Sepharose affinity chromatography (*14*). With the necessity of ultrafiltration following ammonium sulfate fractionation and both chromatographic steps, this method was considerably labor-intensive, time-consuming, and costly.

In a recent study, two histidine residues (His-30 and His-60) of miraculin were suggested to be responsible for its taste-modifying activity. Mutations of histidine to alanine, a residue that may exert no particular steric and electronic effect, resulted in the loss of sweetness-inducing activity (*7*). This study suggests that the histidine residue, particularly His-30, is located at the interface of the two monomeric miraculin subunits. In different studies, molecular modeling has predicted higher solvent accessibility of His residues at acidic pH, where miraculin exhibits its sweetness-inducing activity, rather than those in neutral pH (*15*). The same studies showed that a high degree of flexibility was located on these His residues, suggesting that His residues may be important for the miraculin−receptor interaction. Thus, it has a high probability to be located on the surface of the structure to make it possible to bind to the taste receptor.

In the present study, we hypothesized that, to generate sweetness-inducing ability, the two His residues in each monomer, with a total of four histidine residues on its dimeric form, were probably located on the surface of the structure. By exploiting these residues, we have successfully purified miraculin using an immobilized metal-affinity chromatography (IMAC) column loaded with nickel. This 1 day purification method produces a colorless extract in an acidic buffer, in which miraculin has been reported to be very stable. By examining the effect of adding urea,

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a denaturing reagent, we found that the 3D structure of miraculin was essential for the binding of the protein to the IMAC resin. Urea was commonly used to expose His residues of His-tagged protein when His residues was geometrically placed inside the protein structure (*16*, *17*); thus, urea has only a denaturing effect and no direct influence on the nickel−His binding.

MATERIALS AND METHODS

Plant Material. Miracle fruits were obtained from trees growing in a greenhouse at Hayama City, Kanagawa, Japan, and were stored at −30 °C until use.

Extraction. The pulp of miracle fruits, free from skin and seeds, was ground to powder in liquid nitrogen using a pestle. A total of 25 g of the pulp powder was suspended with 100 mL of water and centrifuged at 12000*g* for 20 min. The sediment was suspended and washed again with 100 mL of water. After centrifugation at 12000*g* for 20 min, all of the supernatant was discarded. The sediment was suspended in 50 mL of 20 mM Tris-HCl (pH 7.2) containing 0.5 M NaCl, and the supernatants displaying sweetness-inducing activity were pooled. All procedures were performed below 4 °C.

Affinity Chromatography. The IMAC column (volume, 1 mL; HiTrap IMAC HP, GE Healthcare) was charged with nickel according to the instructions of the manufacturer and equilibrated with binding buffer (20 mM Tris-HCl and 0.5 M NaCl at pH 7.2). A 10 mL sample of the extracted acidic supernatant was adjusted to pH 7.2 using 1 M NaOH and then applied immediately to the column in binding buffer. The column was washed in the same buffer to remove weakly bound proteins and then washed with 20 mL of 0.5 M NaCl/50 mM acetate buffer at pH 6.6. The target protein was eluted with 20 mL of a buffer containing 0.5 M NaCl/50 mM acetate buffer at pH 5.0. Adsorbed proteins remaining on the column were eluted using 20 mL of 0.5 M NaCl/50 mM acetate buffer at pH 4.0. Fractions of 3 mL were collected at a flow rate of 1 mL/min, and fractions were pooled on the basis of their elution pH. To study the importance of the miraculin 3D structure to maintain histidine in an appropriate position during this chromatographic step, we added urea to a sample of purified miraculin and its binding buffer (20 mM Tris-HCl and 0.5 M NaCl at pH 7.2); therefore, the final concentration of urea become 8 M, and it was re-applied to the IMAC column. All experiments were conducted below 4 °C.

Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC). RP-HPLC was carried out on a SOURCE RPC column (0.46 × 15 cm, GE Healthcare) to confirm the purity of the miraculin samples. After dialysis in 50 mM acetate buffer (pH 5), a 1 mL sample was injected onto the column with 1% trifluoroacetic acid at distillated water. Elution was performed using a linear gradient of acetonitrile concentration (20−70%) containing 1% trifluoroacetic acid at a flow rate of 1 mL/min, and absorbance was detected at 280 nm.

Gel Electrophoresis and Protein Analysis. Fractions pooled based on their elution pH was analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) in 12% gels, after boiling the sample for 5 min. SDS−PAGE was performed under nonreducing and reducing conditions by the addition of dithiothreitol. The bands on the gel were visualized by staining with Coomassie Brilliant Blue (Quick-CBB plus, Wako, Japan).

For Western Blot analysis, the proteins separated on the SDS−PAGE gel were transferred to Hybond-P membranes (GE Healthcare). After nonspecific sites were blocked on the membranes overnight at 4 °C in 5% skim milk and TBS−0.05% Tween 20, the membranes were incubated for 30 min at room temperature with peroxidase-conjugated anti-miraculin. The immunoreactive proteins were visualized using a nuclease-tested peroxidase stain kit for immunoblotting (Nacalai Tesque, Inc., Kyoto, Japan). The anti-miraculin antibody was prepared according to a described method (*4*). Peroxidase-conjugated anti-miraculin was prepared using a peroxidase-labeling kit (Dojindo, Japan).

N-Terminal Amino Acid Sequence. To ascertain the identity of the protein, the N-terminal amino acid sequence of the target peak was analyzed by the Edman degradation method. Samples were transferred from SDS−PAGE gels to a Hybond-P membrane (GE Healthcare) and stained with Brilliant Blue R (Sigma, St. Louis, MO). The visible proteins were excised from the blot and analyzed on a ProciseTM 490 sequencer (Applied Biosystems).

Measurement of Induced Sweetness Intensity. Crude samples collected in 20 mM Tris-HCl and 0.5 M NaCl at pH 7.2 extraction and a purified sample collected in an affinity chromatography target peak were diluted so that the miraculin concentrations become 1.3 μM. We considered that this concentration was appropriate to detect slight changes in taste-modifying activity because of the presence of some unactive miraculin. A total of 10 mL of this solution was held in the mouth for 3 min and spat out. After the mouth was rinsed with water, 4 mM citrate were tasted and its sweetness was compared to a series of sucrose solutions (0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, and 0.50 M). At least 12 h was interposed between each test.

RESULTS

Miraculin was extracted from the lyophilized pulp of miracle fruits. The Tris buffer extract from the lyophilized pulp had an acidic pH and did not bind to the nickel-IMAC column when applied to the column because of the high isoelectric point of His residues (data not shown). Therefore, the colorless solution was adjusted to pH 7.2 using 1 M NaOH and applied immediately to the nickel-IMAC column, before the appearance of color caused by the change in pH. This coloring, which was considered caused by dehydrogenization of some polyphenol, bound strongly to the column and made the column regeneration process difficult. The miraculin fraction eluted mainly with 0.5 M NaCl/50 mM acetate buffer at pH 5.0, confirmed by the Western Blot analysis (**Figures 1** and **2**). Although miraculin was not observed in the flow through, Western Blot analysis showed some minor loss in the wash with pH 6.6 and 4.0 buffers (data not shown).

The SDS−PAGE results visualized by Coomassie Brilliant Blue showed the same band as that detected by Western Blot analysis. Miraculin was isolated as a dimer, similar to its natural state. The molecular weights of the miraculin under nonreducing

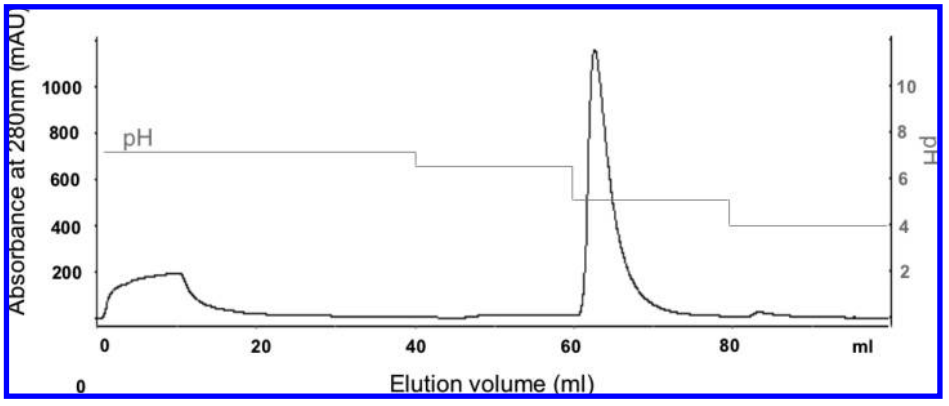

Figure 1. Elution profile of nickel-IMAC of miraculin. The protein was monitored by measuring the absorbance at 280 nm.

and reducing conditions were about 50 and 25 kDa, respectively (**Figure 2**). No monomer was detected under the nonreducing conditions; therefore, it is unlikely that miraculin was in the monomer form during the purification and dimerized after the purification. We considered that the large band on the SDS−PAGE was due to at least five kinds of N-linked oligosaccharides linked to Asn-42 and Asn-186 of each miraculin monomer. The purity of the target fraction was verified by RP-HPLC. As shown in **Figure 3**, the sample gave a sharp single peak, which was shown by analysis of the peak area to be more than 95% pure. At the same amount and concentration, the purified miraculin shows a slightly higher taste-modifying activity compared to miraculin in the Tris-HCl extract before the purification. About 89% of the miraculin in the Tris-HCl extract was collected in the target peak (**Table 1**). The N-terminal amino acid sequence of the purified protein was determined to be Asp-Ser-Ala-Pro, consistent with the expected N-terminal amino acid sequence of miraculin.

To examine the importance of the 3D protein structure in this chromatographic procedure, urea was added to a purified protein sample; therefore, the final concentration become 8 M, before its re-application to the IMAC column. The addition of urea caused miraculin to lose its ability to bind to the nickel-IMAC resin, and it was collected in the column flow through (**Figure 4**).

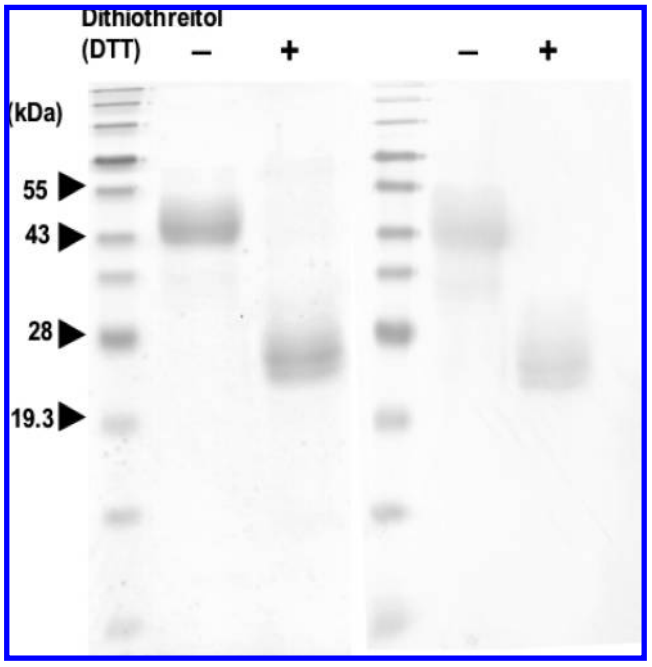

Figure 2. Protein bands from SDS−PAGE visualized by staining with Coomassie Brilliant Blue (left) and Western Blotting (right). SDS−PAGE was performed under nonreducing and reducing condtions. XLmarker was used as a size marker (APRO, Japan). Samples consisted of 2 μg of protein from the target fraction.

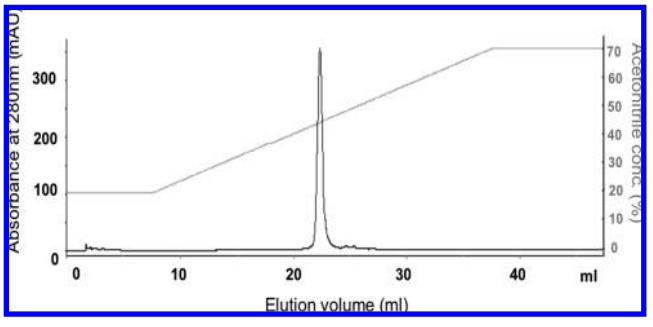

Figure 3. RP-HPLC chromatography of the target fraction. A 1 mL sample of the target fraction obtained from nickel-IMAC was applied.

DISCUSSION

The most recent approach to purifying miraculin was based on a combination of ammonium sulfate fractionation, ion-exchange chromatography, and concavalin A-Sepharose affinity chromatography (*14*). However, these methods are not suitable for routine preparation and mass production. We therefore looked for a simple, easy method of purification that did not compromise the purity and yield of active miraculin. Here, we used nickel-charged IMAC column chromatography to successfully purify miraculin from miracle fruits, obtaining greater than 95% purity without affecting the activity.

The purified miraculin was isolated as a dimer, similar to its natural state (**Figure 2**), and displayed high sweetness-inducing activity, which was higher compared to the crude miraculin extract (**Table 1**). This indicates that the method used outer-part His residues, crucial for the binding of the protein to the tongue receptor, was probably useful to separate active miraculin from the non-active one, which may arise because of denaturation during preservation or during the purification step. In other studies, the presence of Ca^{2+} and/or Mg^{2+}, divalent atoms similar to Ni^{2+}, was reported to interfere with the binding of active miraculin to the sweet receptor and inactivate the sweet-inducing effect (*18*). Histidine residues, reported to be important for the miraculin−receptor interaction, may bind to these divalent atoms and cause the lost of sweet-inducing activity. Therefore, we propose that there are strong connection between divalent atoms and the sweet-inducing activity. Further studies are under development in our group to verify the use of divalent atoms, especially Ni^{2+}, to measure the sweet-inducing activity of the protein.

By adding urea to the protein, we demonstrated that the 3D structure of miraculin is necessary to keep the His residues on the outside of its structure. Urea can affect the molecular structure of proteins (*19*), without influencing the binding of nickel and histidine residues. The addition of urea caused miraculin unable to bind to the nickel-IMAC resin and indicates the importance of the miraculin 3D structure in the binding of His residues to the nickel column.

Nickel-charged IMAC resin binds to proteins with His residues on the outer part of their structures (*16*, *17*). The binding of miraculin to the nickel-charged IMAC column in the present study reconfirmed the presence of His residues on the outer part of the molecular structure. These His residues are likely to play a key role in the binding of the protein to the sweet-taste receptors on the tongue, T1R2−T1R3, which have also been reported to

Table 1. Yield of Protein and Induced Sweetness Intensity[a]

sample	total protein amount (μg)	miraculin purity in the sample (%)	miraculin mass in the sample (μg)	sucrose equivalent value
20 mM Tris-HCl and 0.5 M NaCl extract	3.61 ± 0.05 (n = 2)	60.00 ± 0.56 (n = 2)	2.16 ± 0.15 (n = 2)	0.23 ± 0.06 (N = 5)
target fraction	1.89 ± 0.13 (n = 2)	95.83 ± 0.40 (n = 2)	1.82 ± 0.11 (n = 2)	0.25 ± 0.03 (N = 5)

[a] The amount of miraculin in the sample was estimated by multiplying the purity of miraculin in the sample and the total protein amount. The total protein amount was quantified by the Bradford assay (*21*), and purity was assessed by RP-HPLC. The induced sweetness intensity was shown as a sucrose equivalence value (SEV). A total of 10 mL of 1.3 μM crude and purified miraculin was used for inducing sweetness. n = number of samples, and N = number of tasters.

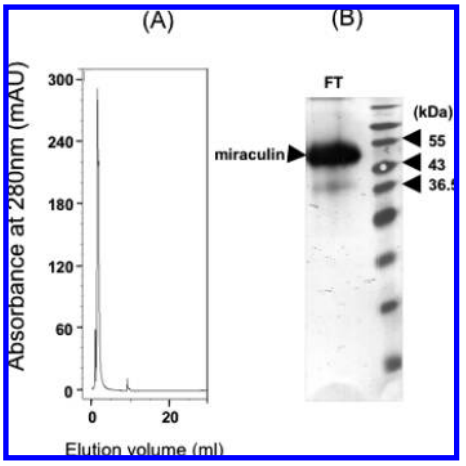

Figure 4. (A) Elution profile of nickel-IMAC of purified miraculin denatured with 8 M urea. Most of the protein was collected in the column flow though. (B) Western Blot of a sample from the collected flow-through volume.

interact with His residues of another sweet-modifying protein, neoculin (*20*).

In conclusion, we have established a protocol for efficiently obtaining pure miraculin from miracle fruits. The novel, rapid, and simple purification method may also be applicable to the purification of miraculin from transgenic plants, thus facilitating its mass production and thereby giving people with diabetes and sugar allergy diseases a brighter and sweeter future. In addition, the availability of the purified protein will allow further basic studies of miraculin. Although miraculin has become familiar as a taste-modifying protein, the reasons for its accumulation at such high levels in miracle fruit and its biological significance are yet to be discovered.

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