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Original Paper

A simple method for purifying undenatured miraculin from transgenic tomato fruit

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Abstract The sweet taste-modifying protein miraculin has been successfully expressed in the tomato, a high-yield commercial plant (Sun et al. 2007). Previously proposed methods of purifying untagged miraculin from transgenic tomato fruit are laborious and time consuming and do not allow the separation of undenatured and denatured miraculin. Therefore, it is necessary to develop an efficient method of purifying undenatured miraculin from transgenic tomato fruit. In this study, we have demonstrated that the combined use of nickel-immobilized affinity chromatography and ion-exchange chromatography readily provides highly purified undenatured miraculin, as demonstrated by the observation of both a single peak from reverse phase high performance liquid chromatography and a single band from SDS-PAGE.

Key words: Miraculin, nickel column, taste-modifying protein, tomato, transgenic plants.

Miracle fruit was first described in the scientific literature in 1852 by F. W. Daniel, who called it 'the miraculous berry of West Africa' (Daniel 1852). The protein in the berry that is responsible for its sweet-inducing effect was isolated and identified as a glycoprotein named miraculin (Kurihara and Beidler 1968). Miraculin is capable of transforming a sour taste into a sweet one. For example, lemons taste like oranges to those who have taken this protein.

Miraculin protein is composed 191 amino acid residues and two sugar chains and the calculated molecular weight is 24.6 kDa as a monomer on the basis of the amino acid sequence and the carbohydrate content (Theerasilp et al. 1989; Theerasilp and Kurihara 1988). The identified nucleotide sequence encoded 220 amino acid residues including 29 amino acids of a signal sequence (Masuda et al. 1995), and it was localized in internal cellular space at miracle fruit (Hirai et al. 2010b). Miraculin has taste-modifying activity at acidic pH when it is dimer or tetramer form not monomer (Igeta et al. 1991; Ito et al. 2007). The homodimer is covalently linked by an inter-chain of disulphide bond.

Previous studies have succeeded in generating transgenic plants expressing miraculin under the control of the 35S promoter (Sugaya et al. 2008; Sun et al. 2006, 2007). These studies have created a path for the mass production of miraculin. Unlike the miraculin that was expressed in *Escherichia coli* (Matsuyama et al. 2009) and *Aspergillus oryzae* (Ito et al. 2007), no reduction in sweet-inducing activity was observed in miraculin expressed in plants. Of the transgenic plants studied, transgenic tomato plants are considered the most suitable background for the mass production of miraculin. Miraculin is produced in relatively large amounts in transgenic tomatoes compared to in transgenic strawberries (Sugaya et al. 2008), and gene silencing did not occur in the next generation as it did in transgenic lettuce (Sun et al. 2006; Yano et al. 2010). Moreover, miraculin expressed in tomato fruit is stable due to the acidic pH inside the tomato fruit (Gancendo and Luh 2006; Theerasilp and Kurihara 1988). Additionally, tomatoes can be easily cultured and harvested, with large production yields.

In addition to economic advantages, the mass production of miraculin in tomatoes may also encourage study of the mysterious protein. Despite the intervening 40 years since the first isolation of miraculin, it is still unclear how this protein induces a sweet taste in acidic pH conditions. Highly-purified miraculin is needed for the detailed characterization and future use of the protein.

To produce intact miraculin, the protein was expressed in tomatoes without a purification tag (Sun et al. 2007). The absence of a purification tag makes the isolation and purification of this protein from protein-rich tomato fruit difficult. Previous study has demonstrated that untagged

Abbreviations: HPLC, high performance liquid chromatography; IMAC, immobilized metal affinity chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, TBS, tris-buffered saline.

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miraculin can be purified from transgenic tomatoes using ammonium sulfate fractionation, ion exchange chromatography, ConA affinity chromatography, and, finally, Sephacryl S-200 HR column chromatography as shown in Figure 1 (Sun et al. 2007). However, this method is laborious and time consuming for daily preparation and mass production. In addition, denatured miraculin that may emerge during cultivation or storage cannot be removed by this method.

There are four histidine residues in each of the miraculin homodimers (His30 and His60). These histidine residues are crucial to the sweet-inducing activity of this protein (Ito et al. 2007; Paladino et al. 2008, 2010). Replacement of these histidine residues by alanine has been shown to result in the loss of sweet-inducing activity (Ito et al. 2007). Other studies have exploited these histidine residues to purify miraculin from miracle fruit using nickel-immobilized affinity chromatography (Duhita et al. 2009). These studies shown that histidine residues are located in the surface of miraculin, probably crucial to bind to the taste receptor, and can be used for purification of miraculin.

In the present study, we purified miraculin from transgenic red tomatoes expressing miraculin by only two-column chromatography, using nickel-immobilized affinity chromatography and ion-exchange chromatography (Figure 1). We have shown that highly purified undenatured miraculin can be obtained using the proposed purification method.

Materials and methods

All experiments were carried out below 4°C.

Extraction of proteins from tomato fruit

Transgenic tomato (*Solanum lycopersicum* cv. Moneymaker) line 56B which was introduced the miraculin gene under the control of the CaMV 35S promoter (Sun et al. 2007) was used in this work. The tomatoes were cultured in a netted greenhouse in the University of Tsukuba, Tsukuba, Japan. Mature red tomato fruit were free of jelly and seed, frozen with liquid nitrogen, and ground into powder. Twenty-five grams of the powder were washed twice with 100 ml of water. With each wash, the homogenate was centrifuged at 12,000 *g* for 20 min, and the supernatant was discarded. The sediment was homogenized in 50 ml of 0.5 M NaCl/20 mM Tris-HCl (pH 7.2), and the supernatant, which demonstrated sweet-inducing activity with previous assay (Sun et al. 2007), was collected.

Nickel-immobilized affinity chromatography

Nickel-immobilized affinity chromatography was performed according to a previously described method, with some modifications (Duhita et al. 2009). The IMAC column (bed volume, 1 ml; HiTrap IMAC HP, GE Healthcare) was charged with nickel according to the manufacturer's instructions and equilibrated with binding buffer [0.5 M NaCl/20 mM Tris-HCl (pH 7.2)]. The pH of the extracted acidic supernatant was adjusted to pH 7.2 using 1 M NaOH and then the supernatant was immediately added to the column with binding buffer. The column was washed with the same buffer to remove weakly bound proteins and then washed with 20 ml of 0.5 M NaCl/50 mM acetate buffer (pH 6.6). The target protein was eluted with 20 ml of 0.5 M NaCl/50 mM acetate buffer (pH 5.0). Adsorbed proteins remaining on the column were eluted using

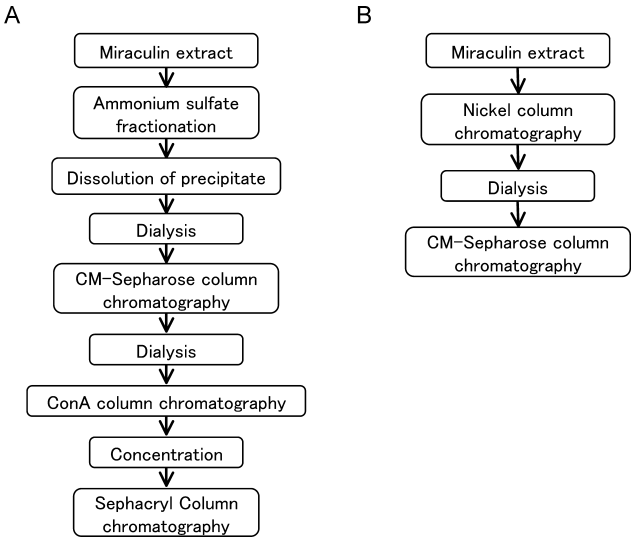

Figure 1. Comparison of purification method in previous work (A) and this work (B).

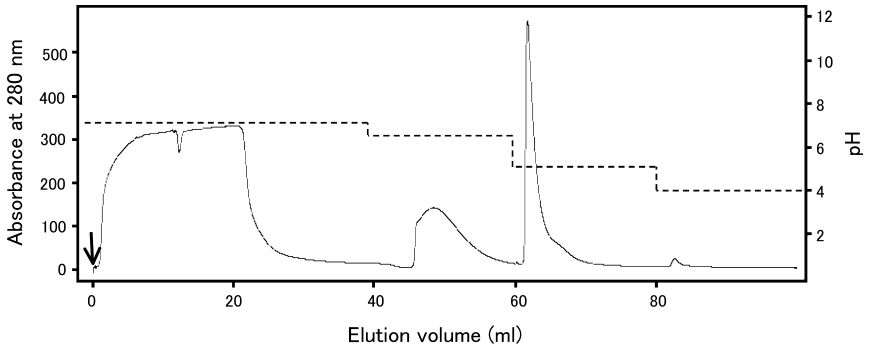

Figure 2. Profile of nickel-immobilized column chromatography. Extractions obtained with 0.5 M NaCl/50 mM Tris-HCl were performed. The amount of protein present was monitored by measuring absorbance at 280 nm. The arrow shows the application of the sample.

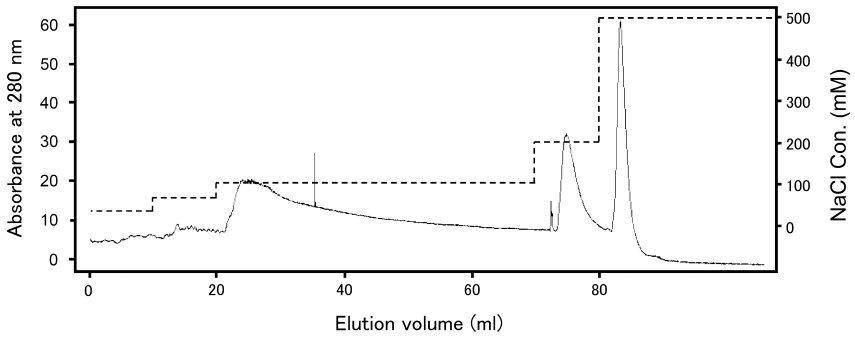

Figure 3. Elution profile of ion-exchange chromatography. The target fraction obtained from nickel-immobilized column chromatography was used. The amount of protein present was monitored by measuring absorbance at 280 nm. Elution of miraculin from the column was performed using 100 mM NaCl/0 mM Tris-HCl (pH 7.2).

20 ml of 0.5 M NaCl/50 mM acetate buffer (pH 4.0). Fractions of 3 ml were collected at a flow rate of 1 ml min^{-1}.

Ion-Exchange Chromatography

Ion-exchange chromatography was carried out on a CM-sepharose column (bed volume, 1 ml; HiTrap CM FF, GE Healthcare). After the fractions containing miraculin were dialyzed over 20 mM Tris-HCl (pH 7.2), the sample was injected into the column and washed with the same buffer [20 mM Tris-HCl (pH 7.2)]. The column was washed again with 10 ml of 25 mM NaCl/20 mM Tris-HCl (pH 7.2) and 10 ml of 50 mM NaCl/20 mM Tris-HCl (pH 7.2). Elution of miraculin from the column was performed using 100 mM NaCl/20 mM Tris-HCl (pH 7.2). Fractions of 5 ml were collected. Adsorbed proteins remaining on the column were eluted using 10 ml of 200 mM NaCl/20 mM Tris-HCl (pH 7.2) and 10 ml of 500 mM NaCl/20 mM Tris-HCl (pH 7.2). Experiments were done at a flow rate of 1 ml min^{-1}, and absorbance was detected at 280 nm.

Reverse-phase HPLC

Reverse-phase HPLC was carried out on a SOURCE RPC column (ϕ0.46×15 cm, GE Healthcare) to confirm the purity of the miraculin samples. After dialysis in 50 mM acetate buffer (pH 5.0), 1 ml of purified sample was injected onto the column with 1% trifluoroacetic acid and distilated water. Elution was performed using a linear gradient of concentrated acetonitrile (20–70%) containing 0.1% trifluoroacetic acid at a flow rate of 1 ml min^{-1}, and absorbance was detected at 280 nm.

Gel electrophoresis and protein analysis

Protein fractions eluted from the ion-exchange chromatography column were boiled for 5 min and then separated by SDS–PAGE in 12% gels. SDS-PAGE was performed under both non-reducing and reducing conditions (with the addition of dithiothreitol). The bands on the gel were visualized with silver staining (Silver Stain Kit, 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 on the membranes were blocked 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 previously described method (Sun et al. 2006). Peroxidase conjugated anti-miraculin was prepared using a peroxidase labeling kit (Dojindo, Japan).

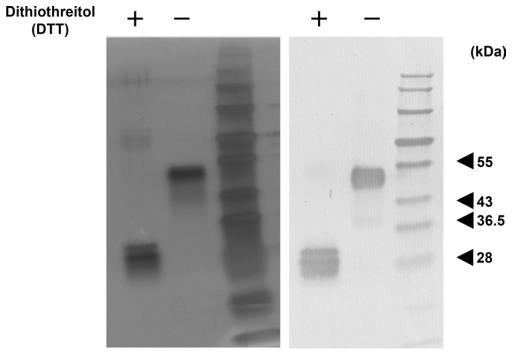

Figure 4. Recombinant miraculin from transgenic tomato fruit after ion-exchange column chromatography. The protein bands from SDS-PAGE gels were visualized by silver staining (left) and western blotting (right). SDS-PAGE was performed under both non-reducing (−) and reducing conditions (+). XLmarker was used as a size marker (APRO, Japan).

Results and discussion

The tomato is a commercial plant with enormous production yield. Unlike miracle fruit, which can only grow in tropical areas, tomatoes can be cultivated in tropical to sub-tropical areas. Moreover, tomatoes can be harvested more than twice a year. Various studies on tomato cultivation have made it easier to cultivate the plant. A closed cultivation system for transgenic tomatoes expressing miraculin was developed recently and has produced fruit with constant levels of miraculin accumulation (Hirai et al. 2010a).

Previous studies have succeeded in generating transgenic tomatoes expressing miraculin under the control of the 35S promoter (Sun et al. 2007). Nevertheless, the purification of miraculin from tomatoes was difficult because there was no purification tag attached to the protein. In the studies, the purification of miraculin from tomatoes was based on the combination of ammonium sulfate fractionation, ion

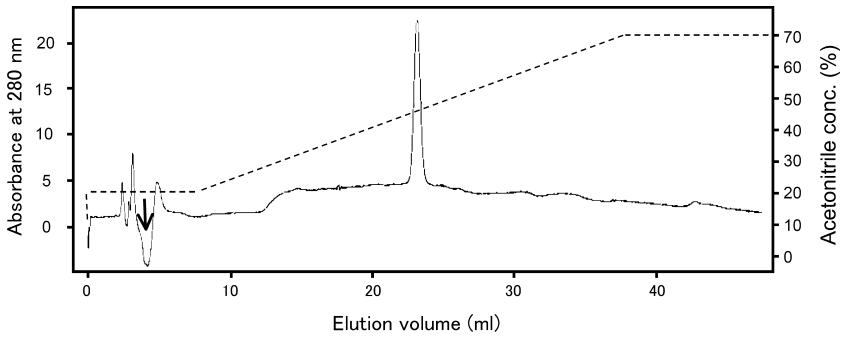

Figure 5. RP-HPLC chromatography of the target fraction. A 1 ml sample of the target fraction obtained from ion-exchange affinity chromatography was applied. The arrow shows the application of the sample.

exchange chromatography, conA Sepharose affinity chromatography and Sephacryl S-200 HR column chromatography (Figure 1). However, these methods are not suitable for the routine preparation and mass production of miraculin because of their time- and labor-intensive steps.

Here we have purified miraculin from transgenic tomato fruit using only two columns, nickel-immobilized affinity and ion-exchange columns (Figure 1). After washing twice with water, we extracted miraculin with 0.5 M NaCl solution. The extracted solution was acidic, indicating the presence of organic acids from the tomato fruit. Miraculin is considered stable in acidic conditions (Gancendo and Luh 2006; Theerasilp and Kurihara 1988). We adjusted the pH of this extract to 7.2 with 1 M NaOH to allow the surface-exposed histidine residues of miraculin to bind to the nickel-immobilized affinity column. If the pH value of the solution is well below the p*Ka* values of these histidine residues, their side chains are completely protonated and the protonation of histidine side chains is thought to prevent binding of the protein to the column. Consequently, by lowering the pH of the elution solution step by step, proteins can be separated on the basis of the p*Ka* values of the surface-exposed histidine residues. Moreover, since the p*Ka* value of the histidine side chain is affected by its micro-environment and by protein structure, nickel-immobilized affinity chromatography also allows the separation of the protein on the basis of its structure. Miraculin produced in tomatoes was bound to a nickel column, washed with Tris buffer at pH 6.6, and eluted with Tris buffer at pH 5.0 (Figure 2). The elution profile in Figure 2 shows that the p*Ka* values of the miraculin histidine residues are between 5.0 and 6.6. The p*Ka* values of the histidine residues of the miraculin purified in this study were similar to those of the protein produced in miracle fruit (Duhita et al. 2009), indicating that the micro-environments of the histidine residues of the miraculin expressed in tomato fruit are identical to those of the authentic protein.

To purify miraculin, miraculin eluted from the nickel column was further purified by ion exchange chromatography (Figure 3). The resultant protein produced a single band on an SDS-PAGE gel at the molecular weight expected for intact miraculin, as shown by western blot analysis (Figure 4). The SDS-PAGE and western blot analysis were performed under non-reducing conditions and reducing conditions and showed molecular weights of about 50 and 28 kDa, respectively. In addition, purified miraculin was not including the monomer of 28 kDa under non-reducing condition. Miraculin was purified in the form of homodimer, the same form where it elicit its sweetness-inducing activity. We considered the large band was due to the variations of N-linked oligosaccharides linked to Asn-42 and Asn-186 of each miraculin monomer. Takahashi et al. (1990) found that miraculin had at least five variations of oligosaccharides. The purity of the obtained protein was also confirmed by reverse phase HPLC, which showed a single sharp peak, indicating that the sample was highly pure (Figure 5).

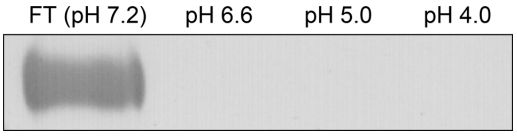

Figure 6. Nickel column binding assay of denatured purified miraculin. Western blot analysis was done to the flow through (FT), pH 6.6, pH 5.0 and pH 4.0 elution.

To study the relationship between the structure of miraculin and its ability to bind to the nickel column used in our purification method, binding assay was carried out on denatured pure miraculin. The assay showed that denatured miraculin was not able to bind to the nickel column at pH 7.2 (Figure 6). Although binding of the histidine residue itself to the nickel column has been shown to be independent of the addition of urea (Li et al. 1999; Lin et al. 2007), the unfolding of miraculin by the addition of 8 M urea as a denaturing reagent makes the protein eluted from the nickel column at pH 7.2. This is because changes in structure of miraculin also change the micro-environment of histidine residues,

thus, led to changes in p*Ka* and the ability of miraculin to bind to nickel column. This result also indicated that the binding affinity of miraculin to the nickel column is affected by protein structure and denatured miraculin can be separated with this nickel column.

In comparison, the purification method in this work is more efficient than the previous method (Figure 1). By the addition of nickel column chromatography, our purification method eliminates ammonium sulfate fractionation which was followed by dissolution of precipitate and dialysis, Con A column chromatography and Sephacryl column chromatography. This nickel column chromatography has also becomes a necessary step to produce a product free from denatured miraculin, which might be economically beneficial for commercial purification. Our purification method required only two days, compared to previous method that required at least four days. Moreover, by reducing purification step we also increased recovery rates from less than 10% to more than 30% (data not shown), although this recovery rate was unstable because of the influence of miraculin content in transgenic tomato fruits and the cultivation environment.

In this study, we have established a protocol for efficiently obtaining pure and untagged recombinant miraculin from transgenic tomato fruits using two-step chromatography methods. Purification of recombinant miraculin in tomato fruits was different from that in miracle fruits, in which another step was necessary because nickel-column bound more non-target proteins than those in purification of miraculin from miracle fruits. However, we had found that utilization of nickel-column chromatography makes the purification of miraculin from transgenic tomato fruits easier and faster compared to previous method (Figure 1). These results indicate that nickel-column chromatography is useful for reduce the steps when recombinant miraculin is purified, and nickel-column chromatography could also be used to purified untagged-miraculin from other transgenic plant.

From the results of nickel-column chromatography, we also found that recombinant miraculin produced in tomato fruits had similar structure compared to those of native miraculin. His residues were located in the surface of miraculin with p*Ka* between 5.0 to 6.6. This also shows the same micro-environment of the His residues of recombinant miraculin. Moreover, similar with the native miraculin, recombinant miraculin produced in tomato fruits was produced as a homodimer and glycosylation also occurred to the recombinant miraculin.

The results obtained from this study might be useful for the development of protocols for the mass production of miraculin and thus promote further studies of the structure and properties of this protein. It remains unclear how the protein produces sweetness in acidic conditions and how it interacts with taste receptors. We hope that the ease of obtaining this protein will both promote and enable its study in the future.

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