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Short Communication

Comparison of the *N*-glycosylation on recombinant miraculin expressed in tomato plants with native miraculin

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Abstract Miraculin is a promising protein with taste-modifying properties. Focusing on the unique function and potential of miraculin, recombinant miraculin production has been explored with the use of heterologous expression systems, but the activities of recombinant miraculins were much lower than those of native miraculin, probably due to the difference in post-translational modification, especially *N*-glycosylation. For practical use therefore, the differences between *N*-glycan of recombinant miraculin compared to that of native miraculin should be minimized. Here, to establish the platform for functional miraculin production, we expressed miraculin in tomato plants with the same taste-modifying activity as native miraculin purified from miracle fruit, and we compared the *N*-glycan structures with those of native miraculin. Our *N*-glycan structural analysis using purified miraculin, followed by hydrazynolysis, 2-pyridylamine (PA)-labeling, high-performance liquid chromatography, and a liquid chromatography tandem-mass spectrometry analysis revealed that both the native and recombinant miraculins carried an M3 structure as a predominant structure and that most of the *N*-glycan structures on the miraculins were pauci-mannosidic structures with a smaller amount of plant-specific α1,3-fucosylated and/or β1,2-xylosylated *N*-glycans and without a Lewis a epitope. These results indicate that the *N*-glycoform of native miraculin from miracle fruit and recombinant miraculin expressed in tomato plants are almost identical to each other with similar ratios and that, therefore, plant-specific *N*-glycans are essential for showing the full taste-modifying activity of miraculin.

Key words: miraculin, *N*-glycan analysis, *N*-glycan diversity, transgenic tomato plants.

Heterologous protein production using plant cells has attracted attention for several years. The plant cell systems have valuable advantages: they are low in cost, they lack animal pathogens, and they provide the potential for post-translational modifications (Basaran and Rodríguez-Cerezo 2008; Fahad et al. 2015; Sabalza et al. 2014). Research focusing on post-translational modifications has revealed that the *N*-glycosylation of protein plays important roles for the full original stability and activity of functional proteins. Interestingly, *N*-glycosylation depends on the host cells, the time and organ of expression, and growth conditions, which lead to the generation of *N*-glycan structural diversity. Therefore, understanding the modification of the *N*-glycan structure on recombinant protein and controlling it is indispensable for practical applications.

Miraculin (MIR), an *N*-glycoprotein produced in red berries known as miracle fruit (*Richadella dulcifica*), has a taste-modifying property that elicits a sweeting effect from a sour taste at acidic pH. Toward the utilization of this unique function for industrial use and/or application, the production of recombinant MIR (rMIR) has been performed using various host cells, such as *Escherichia coli*, yeast, and *Aspergillus oryzae* (Ito et al. 2007, 2011; Paladino et al. 2010), but the activities were far lower than that of native *R. dulcifica* MIR (nMIR) (Hiwasa-Tanase et al. 2012). On the other hand, plant expression systems have successfully produced an active form of rMIR with activity comparable to that of nMIR (Sun et al. 2006, 2007). These results led to the speculation that not only protein *N*-glycosylation but also the plant-specific *N*-glycans carrying β1,2-xylose (Xyl) and α1,3-fucose (Fuc) residue(s) on MIR are critical for protein folding, stability, and therefore the activity. Although the *N*-glycosylation of rMIR was analyzed by an enzymatic approach using peptide *N*-glycanase (Sun

Abbreviations: Fuc, Fucose; GlcNAc, *N*-Acetylglucosamine; HPLC, High performance liquid chromatography; LC-MS/MS, Liquid chromatography-tandem mass spectrometry; nMIR, Native miraculin; Man, Mannose; PA, 2-Pyridylamine; rMIR, Recombinant miraculin; RP, Reverse phase; SF, Size fractionation; Xyl, Xylose.

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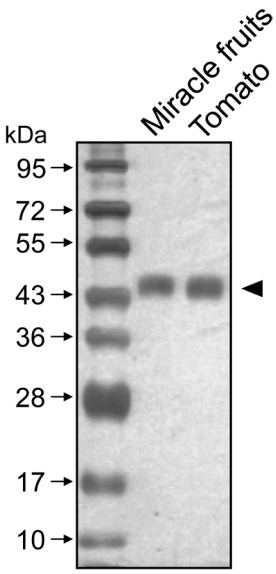


Figure 1. Purification of the native and recombinant MIRs. Purified MIRs from miracle fruits and tomato, i.e., nMIR and rMIR, were separated by SDS-PAGE under non-reducing condition. The purified proteins were visualized by silver staining. Triangle indicates purified nMIR and rMIR.

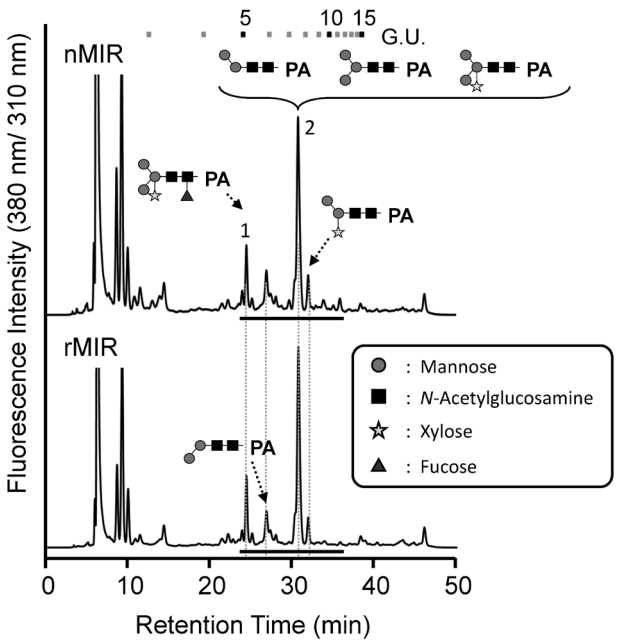


Figure 2. *N*-glycan analysis of MIRs. Total *N*-glycans prepared from MIRs and labeled with PA were analyzed by RP-HPLC with a C18 column. Numbers at the top represent the elution positions of glucose units on the basis of the elution times of PA-isomalto-oligosaccharides with degrees of polymerization from 3 to 15. Under-lining indicates the PA-sugar chain fraction.

et al. 2006, 2007), the details of the *N*-glycan structure(s) remain to be elucidated. Confirmation of the correlations between activities of MIRs and especially their *N*-glycosylation are necessary for the comprehensive utilization of rMIR.

In this study, we produced rMIR in tomato plants using a high-expression system (Sun et al. 2007), and we compared the *N*-glycan structure of rMIR with that of nMIR. Briefly, transgenic tomato (*Solanum lycopersicum* cv. Moneymaker) line 5B, in which the miraculin gene controlled by *CaMV35S* promoter and *NOS* terminator (Sun et al. 2007) was introduced was used for this experiment. After crude miraculin solution was extracted from the tomato fruit as described (Duhita et al. 2011; Sun et al. 2007) with minor modification, the purification of miraculin from the transgenic tomato was achieved with the sequential use of immobilized metal-affinity chromatography, ConA Sepharose affinity chromatography, and ion exchange chromatography. Miraculin from miracle fruit was also purified by the same method (Figure 1). The taste-modifying activities of both MIRs were the same as those reported (Sun et al. 2007). These results indicate that tomato plants are suitable for the production of the active form of rMIR with high expression, and they suggest that rMIR is *N*-glycosylated with the same *N*-glycans as those present in nMIR. For the confirmation of the details of the *N*-glycan structures, *N*-glycans released from MIRs by hydrazinolysis were *N*-acetylated and purified, followed by labeling with 2-pyridylamine (PA) as described (Hase et al. 1978; Kajiura et al. 2013). The excess PA in the reaction product was removed using size-fractionation (SF-) high-performance liquid chromatography (HPLC) with a LaChrom HPLC System (Hitachi High-Technologies, Tokyo). Purified PA-labeled *N*-glycans (PA-glycans) were fractionated by reverse phase (RP)-HPLC using a Cosmosil 5C18-AR column (Nacalai Tesque, Kyoto, Japan) or SF-HPLC using a Shodex Asahipak NH2P-50 column (Showa Denko, Tokyo) (Kajiura et al. 2010). The molecular masses of the PA-glycans and the number of their sugar moieties were estimated by LC-MS/MS using an Agilent Technologies 1200 series (Agilent Technologies, Santa Clara, CA) instrument equipped with high capacity spherical traps (HCT plus; Bruker Daltonics, Bremen, Germany) (Kajiura et al. 2013). On the basis of the structured deduced from the LC-MS/MS analysis, the elution positions of the *N*-glycans were compared with those of authentic PA-glycans (Takara Biomedicals, Shiga, Japan) and those prepared from Arabidopsis plants (Kajiura et al. 2010).

The RP-HPLC analysis of PA-glycans demonstrated that the total *N*-glycans in MIRs were likely to be similar; the *N*-glycans of both the nMIR and rMIR showed a predominant structure and other minor structures (Figure 2). The major peaks detected in nMIR, shown as 1 and 2 in Figure 2, were further separated by SF-HPLC (Supplementary Figure S1). Peak 1 was a single peak, of which the exact molecular weight and sugar composition agreed well with the three hexoses (Hex), two *N*-acetylhexosamines (HexNAc), pentose (Pent), and

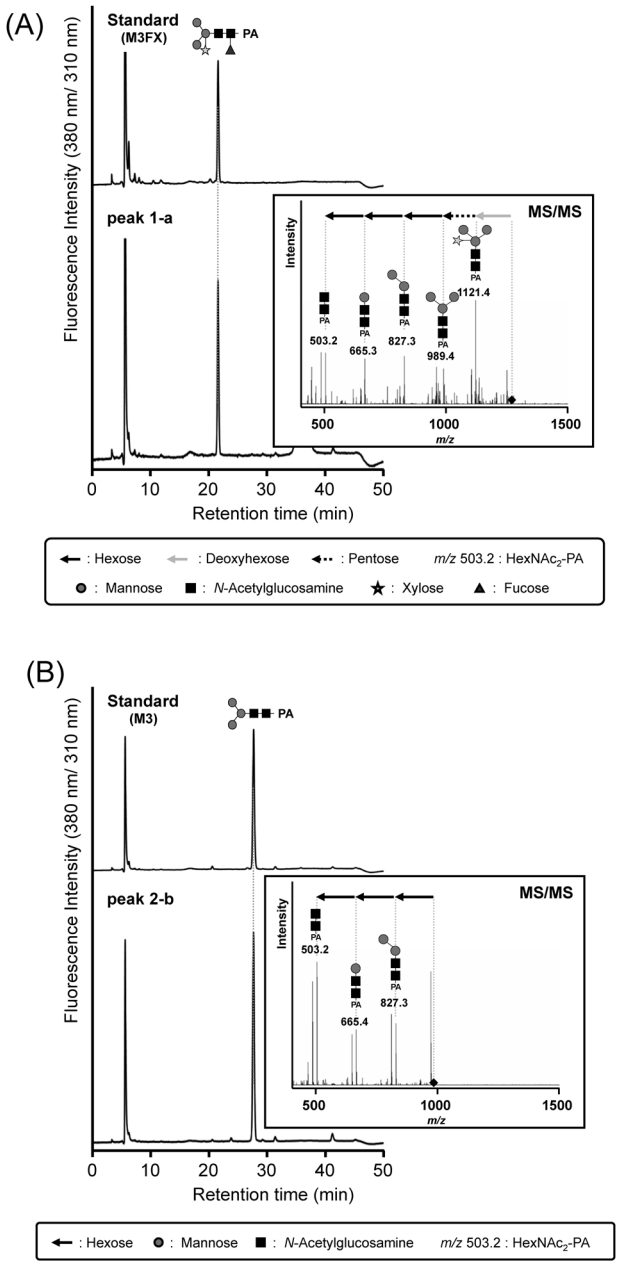


Figure 3. Determination of the PA-glycan structures. Comparison of the predominant structures of (A) peak 1-a and (B) peak 2-b with authentic PA-sugar chains by RP-HPLC and the confirmation of their molecular weight and sugar composition by MS/MS analysis. Small windows show the molecular masses of the peak and the fragmentations of precursors, *m/z* 1267.8 and *m/z* 989.6, by MS/MS analysis. In the MS/MS analysis, the labeled peak represents $(M+H)^+$ ions. In the RP-HPLC analysis, circle: Man residue, square: GlcNAc residue, star: Xyl residue, triangle Fuc residue. The black diamonds in the MS/MS analysis indicate the precursor ions for the MS/MS analysis.

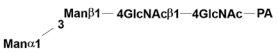
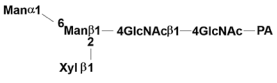
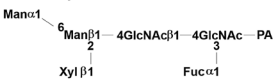
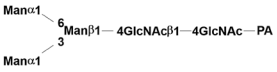
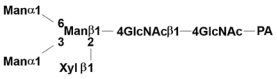
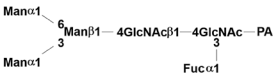
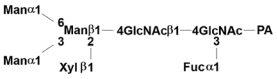
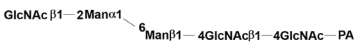
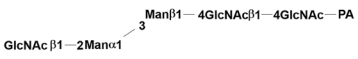
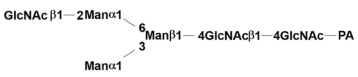
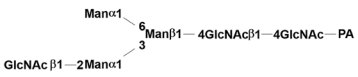
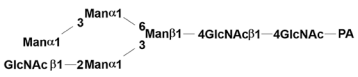
deoxyhexose (DeoxyHex), $Hex_3HexNAc_2Pent_1DeoxyHex_1$-PA (Figure 3A). Moreover, an assignment of the PA-glycan by RP-HPLC demonstrated that the structure was M3FX (Table 1).

Peak 2 in RP-HPLC shown in Figure 1 contained three peaks, and the predominant PA-glycan, assigned as peak 2-b, was $Hex_3HexNAc_2$-PA. The predominant peak corresponded to authentic PA-glycan, M3, in the RP-HPLC analysis (Figure 3B), indicating that the major *N*-glycan of nMIR is M3 and that the ratio is approx. 50% of the total *N*-glycans (Table 1). Interestingly, although MIR is a plant-derived protein, most of the *N*-glycans of nMIR were pauci-mannose type structures with smaller amounts of plant-specific β1,2-Xyl, α1,3-Fuc, and *N*-acetylglucosamine (GlcNAc) residue(s). The second major *N*-glycan of nMIR was ^{M}M with the ratio of 15.4% of total *N*-glycan.

It is noteworthy that the *N*-glycan structures of rMIR and their ratios were almost identical to those of nMIR (Pearson's correlation coefficient calculated from the *N*-glycan ratios of nMIR and rMIR was $r=0.995$, Table 1), suggesting that rMIR is *N*-glycosylated via the same *N*-glycosylation machinery as in *R. dulcifica* and that the N-terminal signal peptide of nMIR, Lys2-Ala29 (Supplementary Figure S2), is also functional in tomato plants. Thus, the expression of MIR in tomato plants is an ideal system for the production of functional recombinant MIR that is identical to nMIR.

To our knowledge, this study is the first report focusing on *N*-glycosylation differences of a plant-derived protein expressed in other plant species. MIR has also been expressed in lettuce and strawberry (Hirai et al. 2011; Sugaya et al. 2008; Sun et al. 2006), but the *N*-glycan structures were not elucidated. To further test the hypothesis that the *N*-glycan structure of plant-produced rMIR is identical to that of nMIR, it is necessary to analyze the *N*-glycan structures of MIRs expressed in lettuce and strawberry. Interestingly, although MIR has three potential *N*-glycosylation sites on the polypeptide sequence, i.e., Asn71, Asn114 and Asn215 (Supplementary Figure S2), only Asn71 and Asn215 are *N*-glycosylated on mature fruit of *R. dulcifica* (Takahashi et al. 1990), suggesting the possibility that depending on the growth condition and/or maturation, Asn114 might be *N*-glycosylated. Indeed, *N*-glycoprotein modeling of MIR using the model constructed by SWISS-MODEL (https://swissmodel.expasy.org/interactive#structure) on the basis of the crystal structure of the homologous *Delonix regia* Kunitz (STI) type inhibitor (PDB code: 1R8N) (Krauchenco et al. 2003) as a query and GlyProt for the in silico glycosylation of protein (http://www.glycosciences.de/modeling/glyprot/php/main.php) demonstrated that Asn114 is more stable and accessible for *N*-glycosylation than Asn41 (data not shown). Thus, for the confirmation of the *N*-glycosylation identities of rMIR and nMIR, temporal and site-specific *N*-glycosylation and their structural analyses are required. We are now conducting advanced spatiotemporal *N*-glycosylation analyses of both native and recombinant MIRs.

Table 1. Relative amount of *N*-glycan structures detected in MIRs.

	Structure	Abbreviations	Ratio (%) nMIR	Ratio (%) rMIR
Mannose type and β1,2-Xylosylated/α1,3-Fucosylated *N*-glycan		M	1.9	1.7
		^{M}M	15.4	18.1
		$_{M}$M	9.1	6.4
		M2X	1.4	1.7
		^{M}MFX	1.6	1.5
		M3	47.0	50.8
		M3X	6.2	7.4
		M3F	2.5	3.3
		M3FX	7.5	8.5
GlcNAc-extended structure		^{GN}M2	1.7	-
		$_{GN}$M2	0.9	-
		^{GN}M3	2.3	0.5
		$_{GN}$M3	1.6	-
		GNM4	0.9	-
Total	Mannose type		73.4	77.0
	β1,2-Xylosylated		16.7	19.1
	α1,3-Fucosylated		11.6	13.3

The relative ratio of the structures was calculated on the basis of the peak area as determined by LC-MS/MS analysis. —: Not detected.

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