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Secretion of miraculin through the function of a signal peptide conserved in the Kunitz-type soybean trypsin inhibitor family

Ayako Takai [a,1], Makiko Satoh [a,1], Tomomi Matsuyama [a], Akane Ito [a], Rieko Nakata [a], Takashi Aoyama [b], Hiroyasu Inoue [a,*]

[a] Department of Food Science and Nutrition, Nara Women's University, Nara 630-8506, Japan
[b] Institute for Chemical Research, Kyoto University, Uji 611-0011, Japan

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ABSTRACT

Miraculin, a glycoprotein that modifies sour tastes into sweet ones, belongs to the Kunitz-type soybean trypsin inhibitor (STI) family. To clarify the functional relation of miraculin with Kunitz-type STIs, we investigated its subcellular localization and trypsin inhibitory activity. In transgenic *Arabidopsis thaliana*, miraculin, fused to yellow fluorescent protein, localized to and outside the plasma membrane depending on the putative secretion signal peptide. When transgenic seedlings were cultured in liquid medium, miraculin was present in the supernatant only after cellulase treatment. No trypsin inhibitory activity was detected in native or recombinant miraculin. In conclusion, miraculin is secreted outside the plasma membrane through the function of a signal peptide, conserved in Kunitz-type STIs, whereas its trypsin inhibitory activity may be lost during its evolution.

1. Introduction

Miraculin, a glycoprotein isolated from the red berries of *Richadella dulcifica*, a native shrub of West Africa, has a taste-modifying activity that converts sour tastes to sweet ones in primates [1]. Because its physiological effect is unique and marked, the berry has been called "miracle fruit," hence, the protein name "miraculin." The acid-induced sweetness of miraculin was predicted by molecular docking techniques [2], and suggested to be involved in human sweet taste receptor by a cell-based assay system [3].

Based on its primary structure, miraculin is classified into the Kunitz-type soybean trypsin inhibitor (STI) family, in which the Kunitz motif, a putative amino-terminal signal peptide, and the positions of several Cys residues are conserved (Fig. 1A). Like other family members, miraculin undergoes several maturation processes, including glycosylation, removal of the signal peptide, and disulfide bond formation. Mature miraculin consists of 191 amino acid residues and oligosaccharides, with a carbohydrate content of 13.9% [4–6]. Glycosylation is observed in recombinant miraculin expressed in lettuce [7] and tomato [8] plants, and in the microbe *Aspergillus oryzae* [9], but it is not essential for the taste-modifying property of miraculin [10]. Mature miraculin contains seven Cys residues forming three intra-chain disulfide bridges and one inter-chain disulfide bridge at Cys-138 [11]. Of these, two of the intra-chain disulfide bridges are largely conserved in the Kunitz-type STI family, but the inter-chain disulfide bridge is found only in miraculin (Fig. 1A). Miraculin forms a homodimer, which is essential for its taste-modifying effect [10], while other members of the Kunitz-type STI family occur as monomers.

The similarity in protein structure between miraculin and other Kunitz-type STI family members [12,13] indicates their common evolutionary origin. Protease inhibitors have been isolated and identified from a broad range of plant species, and seem to be essential for the regulation of seed maturation and germination, and for natural immunity against predators and pathogens [14]. Of these, Kunitz-type STIs have been reported to localize to the apoplast, including the cell wall compartment [15,16], and to act defensively as inhibitors against proteases of predators and pathogens. There are several miraculin-like proteins found in tomato [17], rough lemon [18] and coffee [19], all of which were reported to have defensive function against pathogens and environmental stress. To clarify how miraculin is functionally related to Kunitz-type STIs, we investigated its subcellular localization and trypsin inhibitory activity.

To determine the subcellular localization of miraculin, we introduced a transgene encoding miraculin fused with yellow

Abbreviations: YFP, yellow fluorescent protein; STI, soybean trypsin inhibitor

* Corresponding author. Address: Department of Food Science and Nutrition, Nara Women's University, Kita-Uoya-Nishi-Machi, Nara 630-8506, Japan. Fax: +81 742 203458.
E-mail address: inoue@cc.nara-wu.ac.jp (H. Inoue).

[1] These authors equally contributed to this work.

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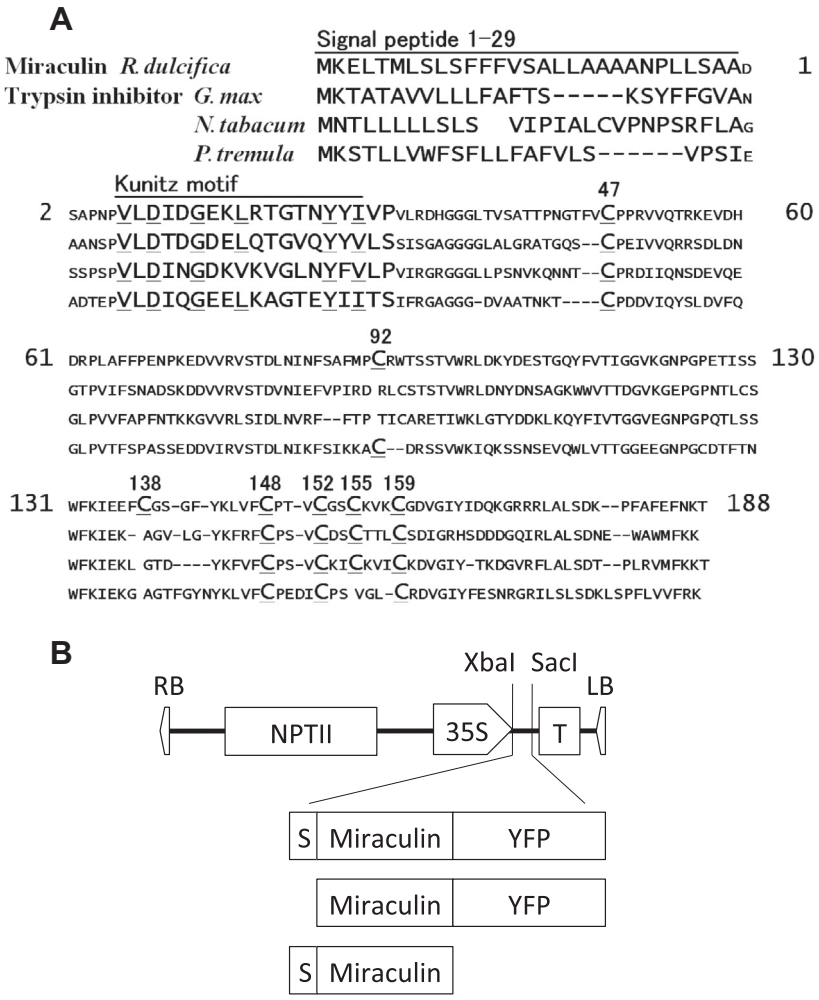

Fig. 1. Amino acid sequences of miraculin and Kunitz-type STIs and structure of the miraculin–YFP fusion protein. (A) Sequence alignment of miraculin (BAA07603) and Kunitz-type STIs from *Glycine max* (AAF87095), *Nicotiana tabacum* (ACL12055), and *Populus tremula* (CAI77898). The miraculin signal sequence (1–29), Kunitz motif, and Cys residues are indicated. (B) The structure of the T-DNA and miraculin transgenes introduced into *A. thaliana* is schematically illustrated. The T-DNA is shown in the upper area as a horizontal line with boxes. RB and LB indicate right and left borders of the pBI121 T-DNA, respectively. NPTII indicates the kanamycin resistance gene for the selection of transgenic plants. 35S and T indicate the 35S promoter and the transcriptional terminator for transgene expression, respectively. Positions of *Xbal* and *Sad* sites used for the cloning of miraculin transgenes are shown as thin vertical lines. Three miraculin transgenes are shown below the T-DNA, in which boxes marked as S, Miraculin, and YFP indicate DNA regions encoding the signal peptide, miraculin, and YFP, respectively.

fluorescent protein (YFP) at its carboxyl terminus into *Arabidopsis thaliana*, and detected YFP fluorescence at the plasma membrane and in the space between the plasma membrane and cell wall. Western blot analysis with a miraculin-specific antibody confirmed that native and recombinant miraculin were secreted outside the plasma membrane, and that recombinant miraculin was retained inside the cell wall in *Arabidopsis*. We also evaluated whether miraculin acted as a trypsin inhibitor, and found that neither native nor recombinant miraculin had trypsin inhibitory activity. Taken together, we conclude that miraculin is secreted by the same mechanism as other Kunitz-type STIs, but that it has lost its trypsin inhibitory activity.

2. Materials and methods

2.1. Construction of transgenes

To overexpress the recombinant miraculin without the signal peptide in *Escherichia coli*, the previously constructed plasmid pET14b-miraculin [8] was used. For further construction, the miraculin cDNA fragment without the signal peptide was re-cloned into the vector plasmid pUC119 (Takara-Bio), resulting in pUC119-miraculin. The upstream and downstream junction sequences of the cDNA are 5′-TCTAGAATGGATTCG-3′ and 5′-TACTTCTAAGGATCC-3′, respectively, in which restriction sites, Xbal at the upstream junction and BamHI at the downstream junction, are underlined. A miraculin cDNA fragment encompassing both the signal peptide and miraculin regions was cloned into the vector plasmid pCR-Blunt-II-TOPO (Invitrogen), and confirmed by sequencing. Sequences of its upstream and downstream junctions are 5′-TCTAGAATGAAGGAA-3′ and 5′-TACTTCTAAGGATCC-3′, respectively, in which restriction sites, Xbal at the upstream junction and BamHI at the downstream junction, are underlined. The resulting plasmid was named as pCR-miraculin. To construct plasmids encoding the miraculin–YFP fusion protein with or without the signal peptide sequence, the stop codon of miraculin in pCR-miraculin or pUC119-miraculin was replaced with a *Bam*HI site, respectively, and then the YFP-coding fragment derived from pEYFP (Clontech) was inserted in frame at the *Bam*HI site. The upstream and downstream junction sequences of the YFP-coding fragment are 5′-GGATCCACCATGGTG-3′ and 5′-TACAAGTAAGAGCTC-3′, respectively, in which restriction sites, BamHI at the upstream junction and Sad at the downstream junction, are underlined. The coding fragments of these recombinant miraculin genes