技術報告

Quantification of protein NP24 in tomato and PR5-like protein in sweet pepper

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Abstract

Protein NP24 found in tomato (*Solanum lycopersicum* L.) and PR5-like protein in sweet pepper (*Capsicum annuum* L.) are homologous proteins of osmotin. Osmotin is known to show adiponectin-like activity, and therefore, protein NP24 and PR5-like protein are expected to show similar activity. In this study, we quantified these proteins contained in the fruits of tomato and sweet pepper cultivars using protein absolute quantification (AQUA) technology employing liquid chromatography/tandem mass spectrometry (LC/MS/MS) and stable isotope-labeled internal standard peptide, GQTWVINAPR[$^{13}C_6$,$^{15}N_4$] (AQUA peptide). The range and average of protein NP24 content in 12 tomato cultivars were 0.43 ± 0.040 (‘Reika’) to 0.83 ± 0.40 (‘Momotaro Fight’) and 0.66 ± 0.14 nmol/g fresh weight, respectively. The PR5-like protein content in 5 sweet pepper cultivars ranged from 0.079 ± 0.034 (‘Ferrari’ harvested in September) to 0.54 ± 0.14 (‘Helsinki’ harvested in December) nmol/g fresh weight. This study could contribute to research on the application of these proteins as adiponectin agonists and the use of tomato and sweet pepper as functional foods.

Key words: protein NP24, PR5-like protein, osmotin-like protein, tomato, sweet pepper

Introduction

Protein NP24[1)] (P12670, accession number: UniProtKB, http://www.uniprot.org/, same hereinafter) in tomato (*Solanum lycopersicum* L.) and PR5-like protein (B2CZJ9) in sweet pepper (*Capsicum annuum* L.) are homologous proteins of osmotin[2)] (P14170), a protein in tobacco (*Nicotiana tabacum* L.) induced by osmotic stress (Fig. 1). Osmotin and osmotin-like proteins reside in family 5 of pathogenesis-related proteins (PR5), and members of this family are called thaumatin-like proteins, which possess sequence similarity with thaumatin, a sweet-tasting protein contained in the fruit of the West African rain forest shrub *Thaumatococcus daniellii* (Benth.)[3, 4)] . The amino acid sequences of protein NP24 and PR5-like protein are very similar to that of osmotin (91.5% identities).

Adiponectin, a mammalian hormone, has anti-hyperglycemic, anti-atherogenic, and anti-inflammatory effects, and hence, is expected to show significant benefits for the prevention of obesity and obesity-related diseases[5)] . Osmotin is reported to behave as adiponectin in various *in vitro* and *in vivo* models[6, 7)] . Osmotin is known to be a structural and functional homolog of adiponectin, and domain I of this protein can be overlapped with the β-barrel domain of adiponectin. Moreover, osmotin stimulates AMP activated protein kinase α (AMPKα) phosphorylation in an AdipoR (major receptor for adiponectin)-dependent fashion in murine skeletal muscle cells. Therefore, osmotin or its derivative, e.g., osmotin-like proteins, can have potential

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use as adiponectin agonist[8, 9] , and hence, protein NP24 and PR5-like protein are expected to act as adiponectin agonists.

We have developed a quantification method for protein NP24 in tomato and PR5-like protein in sweet pepper using the protein absolute quantification (AQUA) technology employing liquid chromatography/tandem mass spectrometry (LC/MS/MS) and stable isotope-labeled internal standard peptide, GQTWVINAPR[$^{13}C_6$,$^{15}N_4$] (AQUA peptide, Fig. 1)[10, 11] . In order to clarify the differences in the content of protein NP24 among tomato cultivars and the influence of harvesting time on PR5-like protein in sweet pepper cultivars, we quantified these osmotin-like proteins by using the AQUA-based method. In this paper, we report their contents in cultivars of these fruits.

Materials and methods

1. Materials

Ammonium bicarbonate, iodoacetamide, and trichloroacetic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan). Dithiothreitol was purchased from Nacalai Tesque (Kyoto, Japan) and Wako Pure Chemical Industries. GQTWVINAPR[$^{13}C_6$,$^{15}N_4$] and trypsin were purchased from Thermo Fisher Scientific (MA, USA). Guanidinium chloride and β-mercaptoethanol were purchased from Sigma-Aldrich (MO, USA). LC/MS grade solvent acetonitrile, formic acid, and water were used for the LC/MS/MS analysis.

2. Tomato and sweet pepper

Tomatoes cv. 'CF Momotaro Haruka', 'House Momotaro', 'Momotaro', 'Momotaro Fight', and 'Momotaro 8' (Takii Seed Co., Ltd., Kyoto, Japan); 'Gyokko-Derishasu' (Musashi Plant Breeding Corp., Tokyo, Japan); 'Kanpuku F1' (Kaneko Seeds Co., Ltd., Gunma, Japan); 'Paruto', 'Reika', 'Rinka 409', and 'Zuiei' (Sakata Seed Corporation, Kanagawa, Japan); and 'Tomimaru Mucho' (Monsanto, MO, USA) were seeded on March 5 and transplanted on April 26. They were cultivated in an open field culture, and harvested from June to July in 2013 at Institute of Vegetable and Floriculture Science, NARO (Mie, Japan).

The fruits of sweet peppers cv. 'Coletti' and 'Ferrari' (Enza Zaden, Enkhuizen, Netherlands), 'Helsinki' and 'Sapporo' (Rijk Zwaan, De Lier, Netherlands), and 'Orange Glory' (Monsanto) harvested from September 2014 to January 2015 were purchased from VEGi-Dream Kurihara Corporation (Miyagi, Japan).

3. Sample preparation

Each fruit of two to five tomatoes or five sweet peppers was cut into four pieces along the longitudinal axis. Furthermore, two opposite pieces of one fruit were cut into 1 cm cubes for sample reduction. After mixing these cubes, 200 g of them were frozen in liquid nitrogen, freeze-dried, and ground into powder using GRINDOMIX GM 200 (Retsch, Haan, Germany). The powder prepared by this procedure was considered as one sample and stored at −30°C until protein extraction.

4. Trichloroacetic acid/acetone extraction

Tomato and sweet pepper protein extractions were undertaken as follows[10, 11] . Trichloroacetic acid extraction buffer was prepared by mixing 10% (w/v) trichloroacetic acid in acetone with 2% (v/v) β-mercaptoethanol. The powders (10 mg) obtained from the abovementioned preparation were suspended in 1 mL of trichloroacetic acid extraction buffer plus 90 μL of water, and stored at −20°C overnight. The samples were centrifuged at 5,000 × g for 30 min at 4°C, and the supernatants were discarded. Cold acetone (1 mL) was added to the pellets, and the mixtures were centrifuged at 5,000 × g for 10 min at 4°C; then, the supernatants were discarded. This washing procedure with acetone was repeated twice, and then the pellets were naturally dried.

5. Trypsin digestion

Tomato and sweet pepper protein extracts prepared by trichloroacetic acid/acetone treatment were digested by trypsin[10, 11]. These extracts were resuspended in 100 μL of 6 M guanidinium chloride in 50 mM ammonium bicarbonate, and added with 5 μL of 200 mM dithiothreitol in 50 mM ammonium bicarbonate. The mixtures were boiled for 10 min. The samples were mixed with 4 μL of 1 M iodoacetamide in 50 mM ammonium bicarbonate and incubated under shading for 1 h at room temperature. Then, the mixtures were added with 40 μL of 200 mM dithiothreitol in 50 mM ammonium bicarbonate and incubated for 1 h at room temperature. These samples were diluted with 50 mM ammonium bicarbonate (851 μL), and applied to the measurement of protein concentration by using

Quick Start Bradford Protein Assay (Bio-Rad Laboratories, CA, USA) and γ-globulin (Bio-Rad Laboratories) as a calibrator. Aliquots (50 μL) of the diluted solutions with 50 mM ammonium bicarbonate were mixed with 25 μL of 4, 10, or 20 μg/mL trypsin in 50 mM ammonium bicarbonate, which were determined according to the protein amount of these aliquots. The concentrations of trypsin conformed to manufacturer's protocol. Then, these samples were added with 25 μL of 80 fmol/μL GQTWVINAPR[$^{13}C_6$,$^{15}N_4$] (stable isotope-labeled internal standard peptide) in 50 mM ammonium bicarbonate, and incubated overnight at 37°C.

6. LC/MS/MS analysis

LC/MS/MS analysis was performed by using an ACQUITY UPLC connected with a XEVO TQD equipped with a Zspray ion source (Waters, MA, USA)[10, 11]. Capillary voltage (3 kV), collision energy (20 V), cone gas flow (50 L/h), cone voltage (36 V), desolvation gas flow (1000 L/h), desolvation temperature (500°C), and source temperature (150°C) were set. Trypsin digestion solutions (100 μL) were mixed with 100 μL of acetonitrile with 0.2% formic acid and centrifuged at 16,000 × g for 10 min at 4°C. The supernatants (5 μL) were injected to a SeQuant ZIC-HILIC guard column (20 mm × 2.1 mm, Merck, Darmstadt, Germany) followed by a SeQuant ZIC-HILIC column (150 mm × 2.1 mm, Merck) at 30°C. The analyte was eluted at a flow rate of 0.1 mL/min with an isocratic mobile phase (water with 0.1% formic acid/acetonitrile with 0.1% formic acid, 60:40). The mass spectrometer monitored multiple reaction monitoring (MRM) transitions as follows: dwell time for transitions 571.3 to 669.4 and 576.3 to 679.4 for quantification set to 210 ms, and transitions 571.3 to 855.5, 571.3 to 956.5, 576.3 to 865.5, and 576.3 to 966.5 for identification set to 20 ms. The analysis of data was undertaken using MassLynx software (v. 4.1, Waters).

Results and Discussion

1. Protein NP24 content in tomato cultivars

Protein NP24 content in tomato fruits of 12 cultivars were quantified using the AQUA-based assay (Fig. 2). The range of all cultivars was 0.43 ± 0.040 ('Reika') to 0.83 ± 0.40 ('Momotaro Fight') nmol/g fresh weight, and the average was 0.66 ± 0.14 nmol/g fresh weight. The contents

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        10         20         30         40         50         60
----MGYLTS SFVLFFLLCV TYTYAATIEV RNNCPYTVWA ASTPIGGGRR LNRGQTWVIN
MSPNMGYYLS SFVFFLLAFV TYTYAATIEV RNNCPYTVWA ASTPIGGGRR LNRGQTWVIN
----MGNLRS SFVFFLLALV TYTYAATIEV RNNCPYTVWA ASTPIGGGRR LDRGQTWVIN

APRGTKMARI WGRTGCNFNA AGRGTCQTGD CGGVLQCTGW GKPPNTLAEY ALDQFSNLDF
APRGTKMARI WGRTGCNFNA AGRGSCQTGD CGGVLQCTGW GKPPNTLAEY ALDQFSNLDF
APRGTKMARV WGRTNCNFNA AGRGTCQTGD CGGVLQCTGW GKPPNTLAEY ALDQFSGLDF

WDISLVDGFN IPMTFAPTKP SGGKCHAIHC TANINGECPR ALKVPGGCNN PCTTFGGQQY
WDISLVDGFN IPMTFAPTKP SGGKCHAIHC TANINGECPR ALKVPGGCNN PCTTFGGQQY
WDISLVDGFN IPMTFAPTNP SGGKCHAIHC TANINGECPR ELRVPGGCNN PCTTFGGQQY

CCTQGPCGPT ELSKFFKKRC PDAYSYPQDD PTSTFTCPGG STNYRVVFCP NGVADPNFPL
CCTQGPCGPT ELSKFFKKRC PNAYSYPQDD PTSTFTCPGG STNYRVVFCP NGVADPNFPL
CCTQGPCGPT FFSKFFKQRC PDAYSYPQDD PTSTFTCPGG STNYRVIFCP NGQAHPNFPL

EMPASTDEVA K   Protein NP24 (tomato)
EMPTSTDEVA K   PR5-like protein (sweet pepper)
EMPGSDEVAK -   Osmotin (tobacco)
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Fig. 1 Amino acid sequences of protein NP24, PR5-like protein, and osmotin.

Red amino acid = AQUA peptide; green amino acid = identical amino acid among all proteins. Variant amino acids exist in amino acid number 28 and 42 in the sequence of protein NP24 (I and S, NP24 I; F and F, NP24 II, respectively).

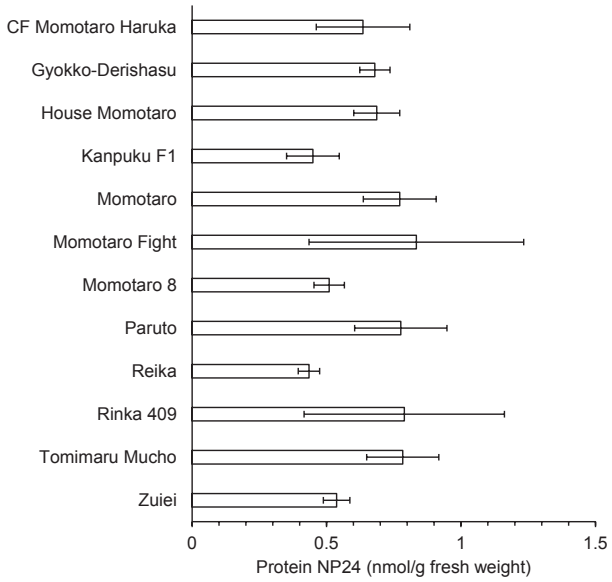

Fig. 2 Content of protein NP24 in various cultivars of tomato fruit.

The numbers of analytical samples derived from one cultivar for LC/MS/MS measurement were three. Data are expressed as average ± standard deviation.

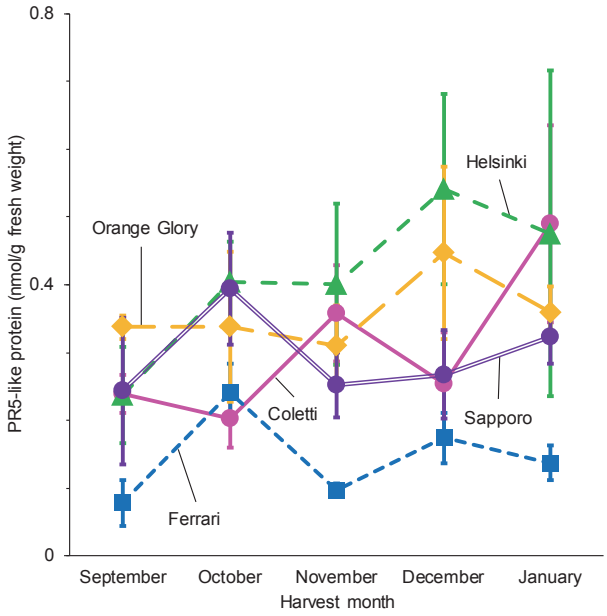

Fig. 3 Content of PR5-like protein in some cultivars of sweet pepper fruit harvested at different months.

The numbers of analytical samples derived from one cultivar harvested in the same month for LC/MS/MS measurement were three. Data are expressed as average ± standard deviation.

of 'Momotaro Fight', 'Rinka 409', 'Tomimaru Mucho', 'Paruto', and 'Momotaro' were greater than 0.7 nmol/g fresh weight. However, no significant differences among all of the cultivars were found.

The contents of NP24 I and NP24 II, which are two isoforms of protein NP24 (Fig. 1)[10], in tomato cv. 'Better Boy' at the pink stage determined by fast protein liquid chromatography were reported to be approximately 4 and 14 μg/g fresh weight, respectively[1]. Their sum was calculated to be approximately 0.74 nmol/g fresh weight, which was close to the average of all cultivars (0.66 ± 0.14 nmol/g fresh weight, Fig. 2) assayed using the AQUA-based method.

2. Effect of harvesting time on PR5-like protein in sweet pepper

We surveyed the content of PR5-like protein in 5 sweet pepper cultivars harvested between September and January. The contents in 'Coletti', 'Ferrari', 'Helsinki', 'Orange Glory', and 'Sapporo' ranged from 0.20 ± 0.043 (October) to 0.49 ± 0.15 (January), 0.079 ± 0.034 (September) to 0.24 ± 0.041 (October), 0.24 ± 0.072 (September) to 0.54 ± 0.14 (December), 0.31 ± 0.059 (November) to 0.45 ± 0.13 (December), and 0.24 ± 0.11 (September) to 0.39 ± 0.083 (October) nmol/g fresh weight, respectively (Fig. 3). There was no characteristic relationship between the harvesting time and the content of PR5-like protein. One of the reasons could be that these sweet peppers were cultivated under controlled conditions in an advanced green house, and thus, were hardly influenced by the seasonal change of environmental factors observed in an open field, including microbial and pathogenic conditions.

PR5 gene expression is affected by various factors such as microbial infection, pathogenic elicitors, osmotic stress, wounding, and plant hormones, abscisic acid, ethylene, salicylate, and methyl jasmonate[4]. Therefore, the amounts of protein NP24 and PR5-like protein are thought to be influenced by the cultivation conditions of tomato and sweet pepper. Hence, the optimization of the cultivation conditions could lead to the increase of the content of these osmotin-like proteins. For example, the optimal osmotic pressure of medium in soilless culture presumably increases the content of protein NP24 and PR5-like protein affected by osmotic stress.

Conclusion

To our knowledge, this is the first report on the quantitative determination of protein NP24 and PR5-like protein contained in various cultivars of tomato and sweet pepper, respectively. If these osmotin-like proteins are demonstrated to exert an adiponectin-like activity, this investigation may contribute to research involving the use of tomato and sweet pepper as functional foods.

Acknowledgments

This work was supported in part by a grant from the research project "A Scheme to Revitalize Agriculture and Fisheries in Disaster Area through Deploying Highly Advanced Technology (NouMiya 2-02)" from the Ministry of Agriculture, Forestry and Fisheries of Japan. We thank Ms. Motoe Sasanuma for her technical assistance.

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トマトのタンパク質 NP24 およびパプリカの PR5 様タンパク質の定量

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要旨

トマト（*Solanum lycopersicum* L.）のタンパク質 NP24 およびパプリカ（*Capsicum annuum* L.）の PR5 様タンパク質は，オスモチンの相同タンパク質である．オスモチンは，アディポネクチン様活性を示すことが知られており，それ故に，タンパク質 NP24 および PR5 様タンパク質は，同様の活性を示すことが期待されている．この研究において，我々は，液体クロマトグラフィー／タンデムマススペクトロメトリー（LC/MS/MS）と安定同位体標識内部標準ペプチド GQTWVINAPR[$^{13}C_6$,$^{15}N_4$] を用いたタンパク質絶対定量法（AQUA）により，トマトおよびパプリカの果実中のタンパク質 NP24 および PR5 様タンパク質を定量した．トマト 12 品種を測定したところ，0.43 ± 0.040（‘麗夏’）～ 0.83 ± 0.40（‘桃太郎ファイト’）nmol/g 新鮮重で，平均値は，0.66 ± 0.14nmol/g 新鮮重であった．パプリカ 5 品種を測定したところ，0.079 ± 0.034（9 月収穫の‘フェラーリ’）～ 0.54 ± 0.14（12 月収穫の‘ヘルシンキ’）nmol/g 新鮮重であった．この研究は，これらタンパク質のアディポネクチンアゴニストおよびトマトやパプリカの機能性食品としての利用に関する研究に貢献するかもしれない．

キーワード：タンパク質 NP24，PR5 様タンパク質，オスモチン様タンパク質，トマト，パプリカ