



Absolute quantification of protein NP24 in tomato fruit by liquid chromatography/tandem mass spectrometry using stable isotope-labelled tryptic peptide standard



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ABSTRACT

Protein NP24 is a thaumatin-like protein contained in tomato (*Lycopersicon esculentum* Mill.). This protein is reported to be a putative tomato allergen and is listed as a food allergen in Structural Database of Allergenic Proteins (SDAP). In this research, we developed the quantitative analysis of NP24 by employing the protein absolute quantification (AQUA) technology composed of stable isotope-labelled internal standard (SIIS) peptide (GQTWVINAPR[¹³C₆, ¹⁵N₄]) and liquid chromatography/tandem mass spectrometry (LC/MS/MS). A linear relationship ($r^2 > 0.99$) was found throughout the concentration range (2.0–500 fmol/μL). The coefficients of variation (CVs) measured on each of the five days when NP24 contained in the tomato skin was analysed did not exceed 13%. Our developed assay of NP24 will contribute to the allergological examination of tomato and its derived products.

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1. Introduction

Protein NP24, a thaumatin-like protein contained in the tomato fruit (*Lycopersicon esculentum* Mill.), has two isoforms (NP24 I and NP24 II) (Fig. 1). NP24 has been reported to be a putative tomato allergen (Kondo, Urisu, & Tokuda, 2001), and is listed as a food allergen in the Structural Database of Allergenic Proteins (SDAP, <http://fermi.utmb.edu/SDAP/>; Sharma, Gaur, & Arora, 2013). In the report regarding the allergenic activity of tomato cultivars against subjects with a tomato allergy, four of 12 subjects showed the binding of immunoglobulin E (IgE) to NP24 (Dölle et al., 2011).

NP24 I increases considerably during ripening, and exhibits the largest content when the tomato fruit is red, whereas NP24 II increases slightly as the fruit begins to change colour, and then remains unchanged. These proteins possess antifungal activity against *Phoma betae* and *Verticillium dahliae*, and are thought to be related to disease resistance in tomatoes (Pressey, 1997). According to another report (King, Turner, Hussey, Wurtele, & Lee, 1988), NP24 is induced by osmotic stress. The amount of mRNA for NP24 is at least 100-fold larger in tomato suspension

cells grown in a medium with NaCl as compared to a control. Moreover, the mRNA is induced earlier and at a greater level in the roots of tomatoes grown hydroponically with 0.171 M NaCl as compared to a control. This protein is localised in the cytoplasm or the soluble fraction of the nucleus, vacuole, or microbodies, and is not secreted.

The amino acid sequence of NP24 is very similar to that of osmotin (91% identity to osmotin), which is a protein in tobacco (*Nicotiana tabacum* L.) (King et al., 1988; Singh et al., 1987). Osmotin also exhibited IgE binding with 22 of 117 patients' sera sensitised to tomato and apple (Sharma, Singh, Singh, Gaur, & Arora, 2011). Because osmotin and NP24 have substantial similarity of their amino acid sequences and exhibited IgE binding, osmotin-like proteins are thought to be potential allergens. The risk of tomato allergy increased among certain people with the popularisation of tomato, which has become widely consumed throughout the world (Welter et al., 2013).

The protein absolute quantification (AQUA) technology is based on stable isotope-labelled internal standard (SIIS) peptide and liquid chromatography/tandem mass spectrometry (LC/MS/MS). This technique enables a sensitive and accurate quantification of an aimed protein and post-translational modification in a complex mixture containing various proteins (Kamii et al., 2008;

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      10      20      30      40      50      60
MGYLTSSSEVL FLLLCVTTY AATIEVRNNC PYTVWAASTP IGGGRRLNRG QTWVINAPRG
TKMARIWGRT GCNFNAAGRG TCQTGDCGGV LQCTGWGKPP NTLAEYALDQ FSNLDFWDIS
LVDGFNIPMT FAPTKPSGGK CHAIHCTANI NGECPRALKV PGGCNPCTT FGGQQYCCTQ
GPCGPTELSK FFKKRCPCDAY SYPQDDPTST FTCPGGSTNY RVVFCPNGVA DPNFPLEMPA
STDEVAK

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Fig. 1. Amino acid sequence of protein NP24; italicised/bolded/underlined = selected peptide for AQUA; bolded/underlined = variant of amino acid (I and S in NP24 I → F and F in NP24 II); italicised/underlined = signal peptide.

Kettenbach, Rush, & Gerber, 2011). An AQUA experiment is undertaken as follows; the peptide suitable for this experiment is selected among peptides digested by enzymes (e.g. trypsin) or chemicals *in silico*, and synthesised with stable isotopes (^{13}C , ^2H , or ^{15}N) as an internal standard in LC/MS/MS analysis. The amount of the synthetic peptide and its native counterpart derived from a targeted protein is determined by mass spectrometry (MS) in the multiple reaction monitoring (MRM) mode.

In this study, we developed an AQUA-based method to quantify the sum of NP24 I and II contained in tomato fruit. The method was assessed for the clarity of MRM chromatogram, the assay linearity, and precision. Since there is no paper dealing with the enzyme-linked immunosorbent assay (ELISA) approach for NP24, this is the first report describing the method to accurately quantify NP24 for the evaluation of a tomato allergen. Traditionally, ELISA has been the principal quantification technique for a target protein. However, the ELISA method is restricted by the absence of antibodies with high specificity (Pan et al., 2009). Compared to ELISA, one of AQUA's benefits is that a quantitative method may be developed for a targeted protein without an antibody. Though ELISA is sensitive, easy, and exhibits high-throughput analysis, it requires the use of a well-characterised antibody (Hale, 2013).

2. Materials and methods

2.1. Materials

Ammonium bicarbonate, iodoacetamide, and trichloroacetic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan). Dithiothreitol was purchased from Nacalai Tesque (Kyoto, Japan). Guanidinium chloride and β -mercaptoethanol were purchased from Sigma–Aldrich (St Louis, MO). Trypsin was purchased from Thermo Fisher Scientific (Waltham, MA). LC/MS grade solvents acetonitrile, formic acid, and water were used for LC/MS/MS analysis. Ripe red tomatoes were purchased from a local store.

2.2. Protein NP24

A sequence of NP24 (UniProtKB P12670) was retrieved from UniProtKB (protein knowledgebase, <http://www.uniprot.org/>). This sequence was digested *in silico* and used to generate a list of tryptic peptides from NP24 using PeptideCutter (web-based software, http://web.expasy.org/peptide_cutter/). The syntheses of two peptides, GQTWVINAPR (native) selected among the list of tryptic peptides and GQTWVINAPR[$^{13}\text{C}_6$, $^{15}\text{N}_4$] (SIIS), were ordered from Thermo Fisher Scientific. Before the synthesis, GQTWVINAPR was not checked to be easily detected in a tryptic digest of tomato proteins.

2.3. Trichloroacetic acid/acetone extraction

Tomato protein extraction was performed as follows (Isaacson et al., 2006). The tomato fruit (93 g) was symmetrically cut into eight pieces and, furthermore, one (12 g) of them was cut into 1–2 cm cubes. These cubes were ground into fine powder in liquid nitrogen with a mortar and pestle. The powder (100 mg) was

suspended in 1 mL of 10% (w/v) trichloroacetic acid in acetone mixed with 2% (v/v) β -mercaptoethanol, and stored at -20°C overnight. The suspension was centrifuged at 5000g for 30 min at 4°C , and the supernatant was discarded. Cold acetone (1 mL) was added to the pellet, centrifuged at 5000g for 10 min at 4°C , and the supernatant was discarded. This washing procedure with acetone was repeated twice. The pellet was gently dried and stored at -80°C until trypsin digestion.

2.4. Trypsin digestion

The tomato protein extract obtained using trichloroacetic acid/acetone treatment was digested by trypsin (Simpson, 2006; Yeats et al., 2010). This extract was resuspended in 100 μL of 50 mM ammonium bicarbonate, 6 M guanidinium chloride, mixed with 5 μL of 200 mM dithiothreitol in 50 mM ammonium bicarbonate, and boiled for 10 min. For protein alkylation, the mixture was added with 4 μL of 1 M iodoacetamide in 50 mM ammonium bicarbonate and incubated under shading for 1 h at room temperature. Then, the sample was added with 40 μL of 200 mM dithiothreitol in 50 mM ammonium bicarbonate and incubated for 1 h at room temperature. This mixture was diluted with 50 mM ammonium bicarbonate (851 μL), and checked for protein content by using Quick Start Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA) and γ -globulin (Bio-Rad Laboratories) as a protein standard. An aliquot (50 μL) of dilute solution was added with 25 μL of 2 $\mu\text{g}/\text{mL}$ trypsin in 50 mM ammonium bicarbonate and 25 μL of 80 fmol/ μL SIIS in 50 mM ammonium bicarbonate, and incubated overnight at 37°C . The addition of trypsin followed the manufacturer's instruction. After incubation, the sample was stored at -20°C until LC/MS/MS analysis.

2.5. LC/MS/MS optimisation

LC/MS/MS apparatus used for this study was an ACQUITY UPLC connected with a XEVO TQD equipped with a Zspray ion source (Waters, Milford, MA). Instrumental optimisation of mass signal, a precursor scan, and a product ion scan for GQTWVINAPR in the mass spectrometer were performed by combined infusion. The peptide solution used for infusion was prepared by mixing 40 μL of 5 pmol/ μL stock solution in 5% acetonitrile, 576 μL of water with 0.1% formic acid (solvent A), and 384 μL of acetonitrile with 0.1% formic acid (solvent B). The peptide was delivered to the electrospray ionisation (ESI) probe through combined infusion of the peptide solution (10 $\mu\text{L}/\text{min}$) with solvent A/solvent B (60:40, 90 $\mu\text{L}/\text{min}$). Capillary voltage (3 kV), collision energy (20 V), cone gas flow (50 L/h), cone voltage (36 V), desolvation gas flow (1000 L/h), and desolvation temperature (500°C) were optimised for MRM intensity derived from GQTWVINAPR. Skyline software (v. 1.4, <https://skyline.gs.washington.edu/>) was used to create the MRM transition list (Table 1).

2.6. LC/MS/MS analysis

Trypsin digestion solution (100 μL) was added with 100 μL of acetonitrile with 0.2% formic acid and centrifuged at 16,000g for

Table 1
MRM transitions for GQTWVINAPR and GQTWVINAPR*.

Peptide	<i>m/z</i>	
	Precursor ion (<i>z</i> = +2)	Product ion (<i>z</i> = +1)
GQTWVINAPR (native)	571.3	669.4 (y₆)
	571.3	855.5 (y ₇)
	571.3	956.5 (y ₈)
GQTWVINAPR* (SIIS)	576.3	679.4 (y₆)
	576.3	865.5 (y ₇)
	576.3	966.5 (y ₈)

R* indicates the heavy isotope-labelled R. The *m/z* values of precursor and product ions were obtained through the calculation of Skyline software. The italicised and bolded transitions were used to quantify the peptides, and other transitions were used to identify them.

10 min at 4 °C. The supernatant (5 µL) was loaded onto 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 (solvent A/solvent B, 60:40). The mass spectrometer monitored MRM transitions shown in Table 1. Dwell time for each transition (571.3⁺² to 669.4⁺¹ [y₆] and 576.3⁺² to 679.4⁺¹ [y₆]) was set to 210 ms, and the other was set to 20 ms. In addition to the MRM experiment, total ion scan of mass range *m/z* 50–2000 with scan time duration of 0.4 s/scan was carried out. The acquisition and analysis of data was performed by MassLynx software (v. 4.1, Waters).

2.7. Linearity of LC/MS/MS analysis

An aliquot (450 µL) of the dilute solution after addition of 50 mM ammonium bicarbonate (851 µL) described in Section 2.4. was mixed with 225 µL of 2 µg/mL trypsin in 50 mM ammonium bicarbonate, and incubated overnight at 37 °C. Five aliquots (75 µL) of this mixture were added with 25 µL of SIIS peptide solutions (4000, 1000, 250, 63, or 16 fmol/µL), and then mixed with 100 µL of acetonitrile with 0.2% formic acid. These mixtures were centrifuged at 16,000g for 10 min at 4 °C, and the supernatants (5 µL) were applied to LC/MRM analysis.

2.8. Intra- and inter-day variability

The tomato skin (1.5 g) was ground into fine powder in liquid nitrogen with a mortar and pestle, and treated by the abovementioned procedure (Sections 2.3. and 2.4.) before the addition of trypsin. An aliquot (50 µL) of dilute solution derived from the tomato skin was added with 25 µL of 10 µg/mL trypsin in 50 mM ammonium bicarbonate, and 25 µL of 80 fmol/µL SIIS in 50 mM ammonium bicarbonate, and incubated overnight at 37 °C. The additive content of trypsin was determined according to manufacturer's instruction. This sample was applied to LC/MS/MS analysis. The experiments on and after the step of trypsin addition were repeated five times on different days.

3. Results and discussion

3.1. Selection of AQUA peptide

The selection criteria (Kamiie et al., 2008) for AQUA peptide used in this work are as follows; length between 8 and 10 amino acids, no posttranslational modification and no single nucleotide polymorphism for detection by MS, no continuous sequence of R or K in the digestion region for complete digestion by trypsin, and no M and no C for stability of peptide. Considering these criteria, we selected GQTWVINAPR (amino acid number 50–59, Fig. 1)

as an AQUA peptide among peptides generated from NP24 by trypsin digestion *in silico*. The use of this peptide can quantify a total of NP24 I and II, because its sequence is not located in the amino acid variant site of NP24. GQTWVINAPR and this peptide with the C-terminal residue substituted by R [¹³C₆, ¹⁵N₄] were synthesised as native and SIIS peptides for AQUA, respectively.

3.2. Creation of MRM transition

The selection of the MRM transitions is important for the responsiveness and selectivity of AQUA analysis as well as the inherent capability of the apparatus (Fortin et al., 2009). Fig. 2A shows the MS spectrum of GQTWVINAPR in positive ion mode. The doubly protonated ion of this peptide was observed with high intensity. Measuring the MS/MS spectrum of this precursor ion, C-terminal y-series ions were prominently detected (Fig. 2B) due to basic R residue on the C-terminus yielding higher signal intensities (Sturm et al., 2012). Though the intensity of the y₅ ion was highest among the y ions observed, this ion is not suitable for MRM transition; since the *m/z* difference between the precursor ion and y₅ ion calculated by Skyline software is <1, it is difficult for the y₅ ion to be distinguished from the precursor ion that passes through a collision cell with no fragmentation. Therefore, the y₆ ion, which has the second highest intensity, was selected and the MRM transitions were determined (Table 1). The ion transitions, *m/z* 571.3⁺² to 669.4⁺¹ and 576.3⁺² to 679.4⁺¹, were exploited in quantification of native and SIIS peptides, respectively, and other transitions were used for their identification.

3.3. Development of LC method

Since an LC method providing adequate retention of GQTWVINAPR on reverse-phase LC (RPLC) columns such as C₁₈ could not be found, a hydrophilic interaction LC (HILIC)-mode column (Kawachi et al., 2011) was applied using an isocratic mobile phase consisting of water, acetonitrile and formic acid to separate this peptide. HILIC is a complementary LC to RPLC, and presents an elution order opposite to that of RPLC. In most cases, HILIC gives the best selection for the separation of compounds which are not satisfactorily

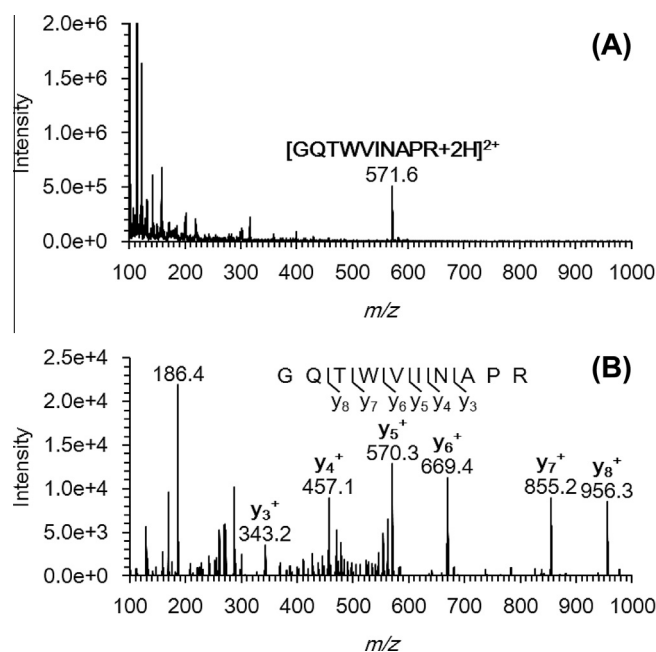


Fig. 2. Full-scan (A) and product ion (B) spectra of the doubly-protonated peptide [GQTWVINAPR+2H]²⁺.

retained on RPLC columns (Greco & Letzel, 2013). Fig. 3A and B show total ion and MRM chromatograms of a tryptic digest of the tomato protein extract containing SIIS peptide, respectively. The peaks corresponding to native and SIIS peptides were clearly and sharply observed at the retention time of 5.6 min on the MRM chromatogram (Fig. 3B).

3.4. Linearity of LC/MRM measurement

The linearity of the developed LC/MRM method for GQTWVINAPR within a concentration range of 2.0–500 fmol/ μ L in the matrix of a tryptic digest of the tomato extract was evaluated by spiking with this SIIS peptide (Fig. 4). All values of peak area were linear ($r^2 > 0.99$) in the examined concentration range, and the coefficients of variation (CVs) at 7.8–500 fmol/ μ L were <20%.

3.5. Intra- and inter-day variability

Intra-day variability of the LC/MRM analysis for GQTWVINAPR, and inter-day variability of trypsin digestion plus the LC/MRM analysis were evaluated by quantifying NP24 present in the tomato skin (Table 2). An aliquot of the denatured protein solution derived from the tomato skin was digested by trypsin and the digestive product was applied five times to the LC/MRM analysis. These experiments were carried out on five different days. The CVs on each day indicating intra-day precision of the LC/MRM analysis were <13%. The CV (10%) through five days was below the CVs on each day, excluding day 5 (5.2%); because this value shows the reproducibility of trypsin digestion of NP24 plus inter-day precision of the LC/MRM analysis through five days, trypsin used in these experiments is thought to digest NP24 reproducibly.

According to a previous report (Pressey, 1997), the contents of NP24 I and II in the tomato skin determined by extraction with cold water and fast protein liquid chromatography (FPLC) were

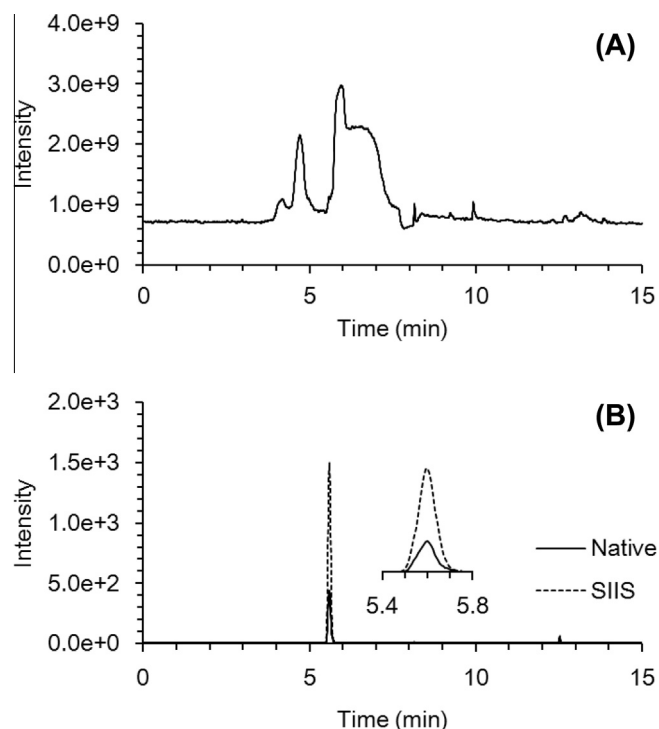


Fig. 3. Total ion (A) and MRM (B) chromatograms of tryptic digestion products from the tomato protein extract. (B) Solid (native) and dotted (SIIS) lines indicate data monitored with MRM transitions for quantification shown in Table 1. The enlarged area of chromatogram (5.4–5.8 min) corresponding to GQTWVINAPR and GQTWVINAPR* is shown.

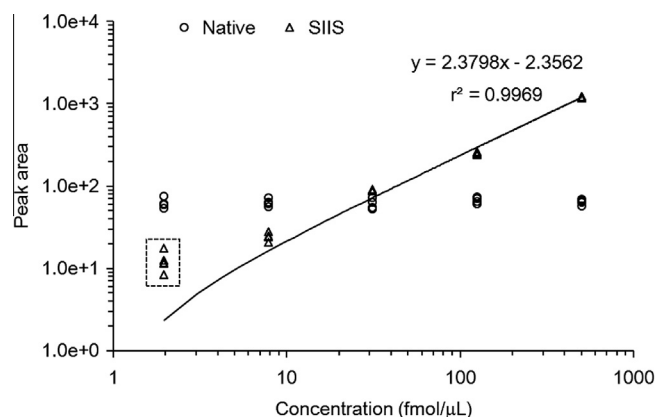


Fig. 4. Linearity of LC/MRM measurement evaluated by using SIIS peptide (triangles) spiked into tomato digest at different concentrations (2.0–500 fmol/ μ L). The measured level of native peptide derived from tomato digestion is shown as circles. The triangles surrounded by dotted square indicate CV > 20%. $n = 5$.

Table 2

Intra- and inter-day variability for quantitative determination of protein NP24 existing in tomato skin.

Day	Concentration (nmol/g fresh weight)	% CV	<i>n</i>
1	2.9	11.4	5
2	3.0	10.7	5
3	2.8	12.7	5
4	3.1	10.5	5
5	3.0	5.2	5
1–5	3.0	10.0	25

7.1 and 11.2 μ g/g fresh weight, respectively. Because the molecular weights of NP24 I and II without signal peptide calculated by Compute pI/Mw (a tool that computes molecular weight, http://web.expasy.org/compute_pi/) are 24,224.32 and 24,318.43 Da, respectively, the sum of NP24 I (7.1 μ g/g fresh weight) and II (11.2 μ g/g fresh weight) in the tomato skin is 0.75 nmol/g fresh weight, which is a quarter of the content determined in this study (3.0 nmol/g fresh weight). The divergence of data between the previous study (Pressey, 1997) and work reported herein is thought to arise from the difference of the extraction and analytical methods for NP24 determination and, of course, the tomato skins used (e.g., ripeness degree).

4. Conclusions

In this work, an LC/MS/MS analysis using AQUA peptide was developed for accurate quantitation of the sum of NP24 I and II, two isoforms of protein NP24 contained in tomato fruit. The peak of GQTWVINAPR was clearly detected on the MRM chromatogram, easily enabling distinction of other constituents in tryptic digestion of the tomato protein extract. To our knowledge, this is the first report to accurately analyse the absolute content of NP24 in tomato, because the ELISA method for the quantification of NP24 has never been reported until now. It is suggested that the method reported herein will contribute to the allergological examination of tomato and its derived products.

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