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Research article

Analysis of allergenic proteins in sweet cherry by liquid chromatography/tandem mass spectrometry using stable isotope-labeled peptides

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Abstract: Pru av 1, Pru av 3, and Pru av 4 are main allergenic proteins present in sweet cherry (*Prunus avium* L.). In this study, an analytical method was developed for these proteins based on a protein absolute quantification (AQUA) strategy. This method involves performing liquid chromatography/tandem mass spectrometry (LC/MS/MS) using stable isotope-labeled internal standard (SIIS) peptides, LVASPSGGSIIK[$^{13}C_6$,$^{15}N_2$], ISPSTNC[CAM]ATVK[$^{13}C_6$,$^{15}N_2$] (C[CAM]: carbamidomethyl-modified C), and LGDYLIEQGL[$^{13}C_6$,^{15}N], for the analysis of Pru av 1, Pru av 3, and Pru av 4, respectively. This method showed linear relationships ($r^2 > 0.99$) in concentration ranges of 0.4–200, 0.2–200, and 1.6–200 fmol/μL of LVASPSGGSIIK[$^{13}C_6$,$^{15}N_2$], ISPSTNC[CAM]ATVK[$^{13}C_6$,$^{15}N_2$], and LGDYLIEQGL[$^{13}C_6$,^{15}N], respectively, spiked into the matrix of a tryptic digest from sweet cherry. However, the overall coefficients of variation for inter-day tests were unsatisfactory. Pru av 1, Pru av 3, and Pru av 4 present in four cultivars of sweet cherry could be detected using this method. This method is expected to detect and semi-quantify allergenic proteins in sweet cherry fruits as an alternative to western blotting.

Keywords: Pru av 1; Pru av 3; Pru av 4; sweet cherry; *Prunus avium* L.; targeted proteomics; AQUA; liquid chromatography/tandem mass spectrometry; stable isotope-labeled peptide

1. Introduction

Oral allergy syndrome (OAS) or pollen-food allergy syndrome (PFS) is a hypersensitive reaction to a variety of fruits, nuts, vegetables, flowers, and spices and is commonly associated with itching of the lips, tongue, and mouth. Approximately 20%–70% of patients sensitized to pollen allergens experience OAS symptoms after eating the above-mentioned foods. The frequency of sensitization to plant foods is highest in those with birch pollen allergies [1].

The World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-Committee officially listed allergens contained in *Rosaceae* fruits, including sweet cherries (*Prunus avium* L.), as these fruits often cause allergies [2]. The key allergenic proteins in sweet cherries have been identified and named Pru av 1, Pru av 2, Pru av 3, and Pru av 4 [3]. Pru av 1, a primary sweet cherry allergen, shares an IgE epitope with Bet v 1, a major birch pollen allergen, and possesses a tertiary structure virtually identical to that of Bet v 1 [4]. Pru av 2, a thaumatin-like protein, is a major cause of OAS and shares considerable sequence identity with Jun a 3, a pollen allergen of mountain cedar (*Juniperus ashei*) [5]. Pru av 3 is a lipid transfer protein (LTP), and monosensitization to Pru av 3 causes severe systemic reactions such as urticaria and angioedema in cherry-allergic patients [6]. Finally, Pru av 4 is a profilin that presumably acts as an important mediator of membrane-cytoskeleton communication and has a similar sequence identity (76%) to Bet v 2, a birch pollen allergen [7].

In a previous study [8], a quantitative assay for Pru av 2 was developed using the protein absolute quantification (AQUA) method. This method involves liquid chromatography/tandem mass spectrometry (LC/MS/MS) and a stable isotope-labeled internal standard (SIIS) peptide, TGC[CAM]STDASGK[$^{13}C_6$,$^{15}N_2$] (C[CAM]: carbamidomethyl-modified C). Because TGC[CAM]STDASGK was assumed to be hydrophilic, a hydrophilic interaction LC (HILIC) mode column was used in this assay. In the AQUA method, a SIIS peptide (AQUA peptide) is added to a sample. A comparison of the labeled peak area derived from the SIIS peptide with the unlabeled peak area derived from the counterpart peptide (native peptide) in a multiple reaction monitoring (MRM, also designated as selected reaction monitoring) chromatogram measured by a quadrupole mass spectrometer enables accurate determination of the native peptide produced from the target protein via tryptic digestion [9,10].

To analyze Pru av 1, Pru av 3, and Pru av 4 contained in sweet cherry fruits by using AQUA technology, an AQUA assay has been developed with an ultra-performance liquid chromatography (UPLC) C_{18} column. The linearity, limits of detection and quantification, and precision of this assay were evaluated.

2. Materials and methods

2.1. Materials

Ammonium bicarbonate, dithiothreitol, iodoacetamide, and trichloroacetic acid were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Guanidinium chloride and β-mercaptoethanol were purchased from Sigma-Aldrich (St. Louis, USA). Trypsin was purchased from Thermo Fisher Scientific (Waltham, USA). All LC/MS/MS solvents were of LC/MS grade. Sweet cherry cultivars “Benisayaka”, “Benisyuho”, “Satonishiki”, and “Takasago” were purchased from a local store (Tsukuba, Japan).

2.2. Pru av 1, Pru av 3, and Pru av 4

The amino acid sequences of Pru av 1 (O24248, UniProtKB accession number), Pru av 3 (Q9M5X8), and Pru av 4 (Q9XF39) were obtained from UniProtKB. To create lists of tryptic peptides of Pru av 1, Pru av 3, and Pru av 4, they were cleaved in silico using PeptideCutter, a web-based software (http://web.expasy.org/peptide_cutter/). Suitable peptides were chosen from these lists to analyze Pru av 1, Pru av 3, and Pru av 4 via the AQUA technology. Stable isotope-labeled peptides, ISPSTNC[CAM]ATVK[$^{13}C_6$,$^{15}N_2$] (C[CAM]: carbamidomethyl-modified C) and LGDYLIEQGL[$^{13}C_6$,^{15}N], were synthesized by peptide synthesis services at Sigma-Aldrich. LVASPSGGSIIK[$^{13}C_6$,$^{15}N_2$] for Pru av 1 was prepared from a stock [11].

2.3. Trichloroacetic acid/acetone extraction and trypsin digestion

Sweet cherry fruits (Benisayaka, Benisyuho, Satonishiki, and Takasago) were cut using a kitchen knife to remove the seeds (5.1, 6.5, 4.5, and 5.9 g, respectively, one fruit per cultivar). Samples were ground to a fine powder in liquid nitrogen using a mortar and pestle. Sweet cherry protein extracts were prepared using a trichloroacetic acid/acetone method [8]. The protein extract was resuspended in 100 μL of a mixture of 6 M guanidinium chloride and 50 mM ammonium bicarbonate [8]. After mixing with 5 μL of a mixture of 200 mM dithiothreitol and 50 mM ammonium bicarbonate, the sample was heated for 10 min in boiling water on an electric stove (SURE SK-1200, Ishizaki Electric MFG, Tokyo, Japan). Then, 4 μL of a 1 M iodoacetamide and 50 mM ammonium bicarbonate mixture was added to the sample and incubated in the dark for 1 h at room temperature. Thereafter, the sample was mixed with 40 μL of the 200 mM dithiothreitol and 50 mM ammonium bicarbonate mixture and incubated for 1 h at room temperature. The mixture was diluted with 50 mM ammonium bicarbonate (851 μL), and protein concentration was determined using the Quick Start Bradford Protein Assay (Bio-Rad Laboratories, Hercules, USA) with γ-globulin (Bio-Rad Laboratories) as a standard. An aliquot (50 μL) of the diluted solution was mixed with 25 μL of 4 or 10 μg/mL trypsin in 50 mM ammonium bicarbonate and 25 μL of 100 fmol/μL LVASPSGGSIIK[$^{13}C_6$,$^{15}N_2$], ISPSTNC[CAM]ATVK[$^{13}C_6$,$^{15}N_2$], and LGDYLIEQGL[$^{13}C_6$,^{15}N] in 50 mM ammonium bicarbonate. The required trypsin concentration for the protein concentration was adjusted according to the manufacturer's instructions. Samples were incubated overnight at 37 °C in an incubator (Synthetech Oven SO-1G, Nippon Genetics, Tokyo, Japan). After incubation, samples were stored at -20 °C until LC/MS/MS measurement.

2.4. LC/MS/MS optimization

The LC/MS/MS instrument used was an ACQUITY UPLC instrument linked to an XEVO TQD using a Zspray ion source (Waters, Milford, USA). The optimization of the mass signals and precursor and product ion scans of ISPSTNC[CAM]ATVK[$^{13}C_6$,$^{15}N_2$] and LGDYLIEQGL[$^{13}C_6$,^{15}N] was performed by combined infusion. These peptide solutions were prepared by mixing 20 μL of a 5 pmol/μL stock solution in 5% acetonitrile with 131 μL of water with 0.1% formic acid (solvent A) and 349 μL of acetonitrile with 0.1% formic acid (solvent B). The peptides were sent to an electrospray ionization (ESI) probe by the combined infusion of these peptide solutions (10 μL/min) with solvent A/solvent B (30:70, 60 μL/min). The capillary voltage, cone gas flow, desolvation gas flow,

desolvation temperature, and source temperature were set at 3 kV, 50 L/h, 800 L/h, 300 °C, and 150 °C, respectively. The MRM transition values calculated using Skyline software (v. 3.5, https://skyline.ms/project/home/begin.view), cone voltages, and collision energies are listed in Table 1.

Table 1. MRM transition, cone voltage, and collision energy of LVASPSGGSIIK, ISPSTNC[CAM]ATVK, LGDYLIEQGL, and their stable isotope-labeled counterparts.

Peptide	*m/z* Precursor ion (z = +2)	Product ion	Cone voltage (V)	Collision energy (V)
LVASPSGGSIIK	***564.8***	***916.5 (y_{10}^{+})***	38	26
(native)	564.8	758.4 (y_8^{+})		
	564.8	845.5 (y_9^{+})		
LVASPSGGSIIK[$^{13}C_6$,$^{15}N_2$]	***568.8***	***924.5 (y_{10}^{+})***	38	26
(SIIS)	568.8	766.5 (y_8^{+})		
	568.8	853.5 (y_9^{+})		
ISPSTNC[CAM]ATVK	***589.3***	***489.2 (y_9^{2+})***	34	24
(native)	589.3	532.8 (y_{10}^{2+})		
	589.3	977.5 (y_9^{+})		
ISPSTNC[CAM]ATVK[$^{13}C_6$,$^{15}N_2$]	***593.3***	***493.2 (y_9^{2+})***	34	24
(SIIS)	593.3	536.8 (y_{10}^{2+})		
	593.3	985.5 (y_9^{+})		
LGDYLIEQGL	***560.8***	***446.2 (y_4^{+})***	24	16
(native)	560.8	189.1 (y_2^{+})		
	560.8	675.4 (b_6^{+})		
LGDYLIEQGL[$^{13}C_6$,^{15}N]	***564.3***	***453.2 (y_4^{+})***	24	16
(SIIS)	564.3	196.1 (y_2^{+})		
	564.3	675.4 (b_6^{+})		

The *m/z* values of the precursor and product ions of ISPSTNC[CAM]ATVK and LGDYLIEQGL and their stable isotope-labeled counterparts were calculated using Skyline software. The values of LVASPSGGSIIK and its stable isotope-labeled counterpart were set according to a previous report [11]. Italicized and bold transitions were used to quantify the peptides, and other transitions were used to identify them.

2.5. LC/MS/MS analysis

The trypsin digestion solution (100 μL) was centrifuged at 16,000 × *g* for 10 min at 4 °C in a centrifuge (MX-307, Tomy Seiko, Tokyo, Japan). The supernatant (5 μL) was injected into the ACQUITY UPLC CSH130 C_{18} column (150 mm × 1 mm, 1.7 μm, Waters) at 40 °C. The analyte was eluted at a flow rate of 70 μL/min using a gradient mobile phase consisting of solvents A and B as follows: 2% B (0 min), 2% B (0.5 min), 55% B (2 min), 70% B (9 min), 98% B (9.2 min), 98% B (9.5 min), and 2% B (10 min). MRM (Table 1) was performed using a mass spectrometer. Dwell times for each quantification and identification transition were 100 and 10 ms, respectively. Data acquisition and analysis were performed using MassLynx software (v. 4.1, Waters).

2.6. Linearity of LC/MS/MS measurement

An aliquot (700 μL) of the solution, derived from Satonishiki fruit and diluted by 50 mM ammonium bicarbonate (851 μL) (as described in section 2.3), was mixed with 350 μL of 4 μg/mL trypsin in 50 mM ammonium bicarbonate. This solution was incubated overnight at 37 °C in an incubator (Synthetech Oven SO-1G). Eleven aliquots (75 μL) of the sample were mixed with 25 μL of the SIIS peptide solutions (800, 400, 200, 100, 50, 25, 13, 6.3, 3.1, 1.6, or 0.78 fmol/μL). These solutions were centrifuged at 16,000 × *g* for 10 min at 4 °C in a centrifuge (MX-307), and the supernatants (5 μL) were subjected to LC/MRM measurements.

2.7. Intra- and inter-day variability

An aliquot (50 μL) of the solution, derived from Satonishiki fruit and diluted with 50 mM ammonium bicarbonate (851 μL) (as described in section 2.3), was mixed with 25 μL of 4 μg/mL trypsin in 50 mM ammonium bicarbonate and 25 μL of 100 fmol/μL SIIS peptides in 50 mM ammonium bicarbonate. Subsequently, the solution was incubated overnight at 37 °C in an incubator (Synthetech Oven SO-1G). This sample was analyzed by LC/MS/MS. The procedure during and after the trypsin treatment was repeated seven times on different days.

3. Results and discussion

3.1. Selection of AQUA peptide

Among all tryptic peptides produced from these allergenic proteins, LVASPSGGSIIK (present in amino acids 105–116 of Pru av 1), ISPSTNC[CAM]ATVK originating from ISPSTNCATVK (present in amino acids 107–117 of Pru av 3), and LGDYLIEQGL (present in amino acids 122–131 of Pru av 4) were selected as the AQUA peptides (Figure 1).

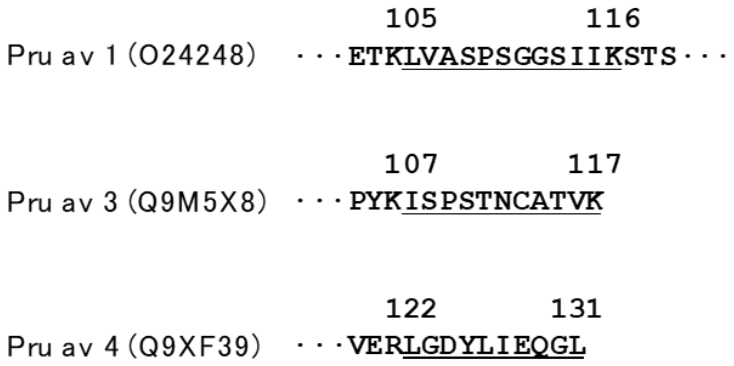

Figure 1. Amino acid sequences of Pru av 1, Pru av 3, and Pru av 4. Underlined: chosen peptides for the AQUA method.

These peptides obey the following rules of in silico peptide choice for AQUA peptides: a length of 6–16 amino acids for analysis by a triple quadrupole mass spectrometer, no posttranslational

modification, and no consecutive sequence of R or K (RR, KK, RK, KR) at the digestive site by trypsin for effective cleavage [10]. Pru av 3 contained in the sample is reduced by dithiothreitol, and its free cysteine thiol groups are carbamidomethylated with iodoacetamide through the trypsin digestion procedure [12]. As a result of these reactions, ISPSTNC[CAM]ATVK is generated from Pru av 3. Iodoacetamide is a strong alkylating agent, and its carbamidomethylation of cysteine under appropriate conditions is highly specific [13]. Peptides containing the C-terminal K[$^{13}C_6$,$^{15}N_2$] or L[$^{13}C_6$,^{15}N] were synthesized as SIIS peptides. LVASPSGGSIIK for the AQUA assay of Pru av 1 exists in the sequence of Pru p 1 (Q2I6V8, UniProtKB accession number), which is an allergenic protein present in peaches [*Prunus persica* (L.) Batsch] that shares 98.1% identity with Pru av 1. This peptide was used for the quantitative determination of Pru p 1 based on the AQUA technology in a previous paper [11].

3.2. Setting of MRM transition

The product ion spectra of ISPSTNC[CAM]ATVK[$^{13}C_6$,$^{15}N_2$] and LGDYLIEQGL[$^{13}C_6$,^{15}N] are shown in Figure 2A and B, respectively.

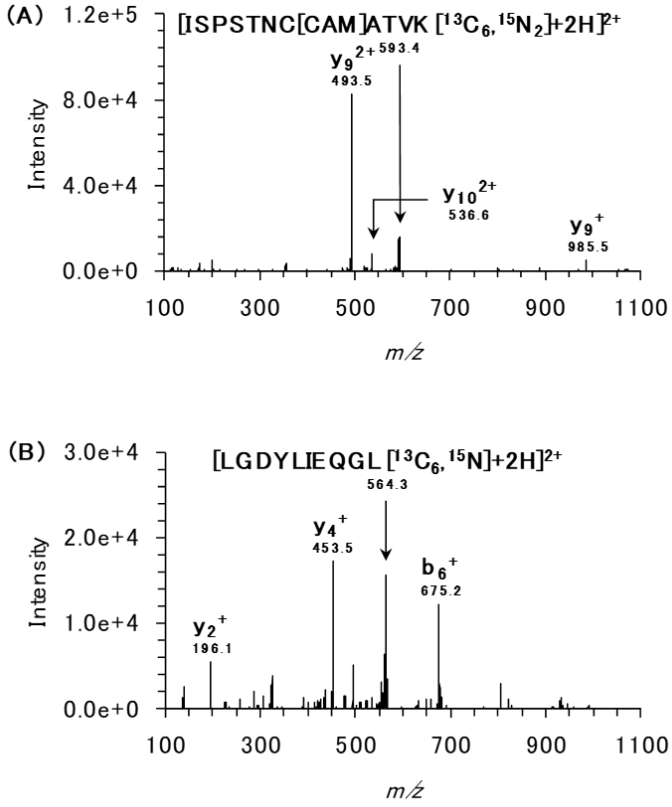

Figure 2. Product ion spectra of the doubly protonated peptides: (A) [ISPSTNC[CAM]ATVK[$^{13}C_6$,$^{15}N_2$]+2H]$^{2+}$ and (B) [LGDYLIEQGL[$^{13}C_6$,^{15}N]+2H]$^{2+}$.

A doubly charged y_9 ion, fragmented from the doubly protonated precursor ion with high intensity in the positive ion mode MS measurement of ISPSTNC[CAM]ATVK[$^{13}C_6$,$^{15}N_2$], exhibited the highest intensity among the y-series ions (Figure 2A). This was thought to be due to the promotion of selective

cleavage by proline at its N-terminal side, which resulted in a strong y ion at this position [14,15]. Therefore, this double-charged y_9 ion was selected as the quantified target of native and SIIS ISPSTNC[CAM]ATVK; the double-charged y_{10} and single-charged y_9 ions, showing the second and third highest intensities, respectively, were used for identification. In case of LGDYLIEQGL[$^{13}C_6$,^{15}N], the single-charged y_4, b_6, and y_2 ions fragmented from the doubly protonated precursor ion yielded the first, second, and third strongest signals, respectively (Figure 2B). Hence, the y_4 and b_6 and y_2 ions were employed for the quantification (y_4 ion) and identification (b_6 and y_2 ions) of native and SIIS LGDYLIEQGL. The MRM transitions of native and SIIS LVASPSGGSIIK were set to the transition values used in a previous study [11], which reported the quantitative determination of Pru p 1 (a peach allergenic protein) using LVASPSGGSIIK[$^{13}C_6$,$^{15}N_2$] as the AQUA peptide. Finally, the MRM transitions for Pru av 1, Pru av 3, and Pru av 4 assays were determined, as shown in Table 1.

3.3. Determination of LC condition

The LC method employed a C_{18} microbore column containing sub-2-micron particles and a gradient mobile phase consisting of water, acetonitrile, and formic acid. Figure 3 shows the MRM chromatogram of a trypsin digest originating from a protein extract of the sweet cherry cultivar Satonishiki.

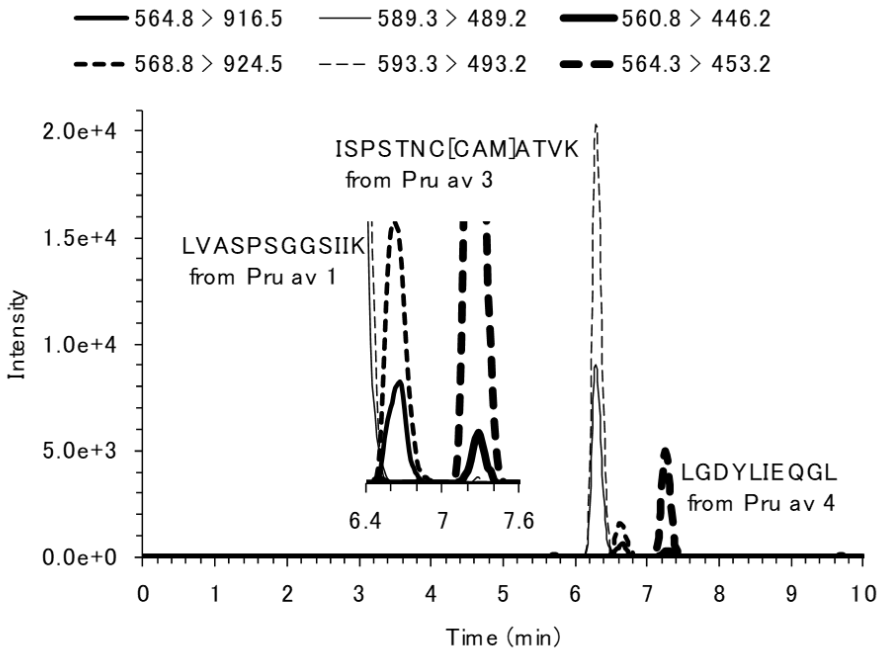

Figure 3. MRM chromatogram of the trypsin digestion solution derived from the Satonishiki fruit. The solid (native) and dotted (SIIS) lines show the data obtained from the MRM transitions (Table 1) for the quantification of LVASPSGGSIIK (intermediate lines), ISPSTNC[CAM]ATVK (thin lines), and LGDYLIEQGL (bold lines).

Peaks of the native and SIIS peptides, LVASPSGGSIIK, ISPSTNC[CAM]ATVK, and LGDYLIEQGL, were distinctly detected at retention times of 6.7, 6.3, and 7.3 min, respectively.

However, the LC conditions for the C_{18} column for the appropriate retention of native and SIIS TGC[CAM]STDASGK used for the analysis of Pru av 2 [8] could not be identified. It is necessary to evaluate different types of columns, including the amide column [16,17], in order to achieve their appropriate retention. Hence, a method for the simultaneous analysis of Pru av 1, Pru av 2, Pru av 3, and Pru av 4 could not be developed.

3.4. Linearity of LC/MS/MS analysis

The linearity of the LC/MS/MS analysis of LVASPSGGSIIK, ISPSTNC[CAM]ATVK, and LGDYLIEQGL was evaluated by spiking their SIIS peptides (0.2–200 fmol/μL) into the matrix of a tryptic digest from Satonishiki sweet cherry extract (Figure 4). The results of this evaluation are summarized in Table 2. ISPSTNC[CAM]ATVK[$^{13}C_6$,$^{15}N_2$] showed the widest linearity range and lowest limits of detection and quantification among the three SIIS peptides, owing to the significantly high intensity of the double-charged y_9 ion of ISPSTNC[CAM]ATVK[$^{13}C_6$,$^{15}N_2$] (Figure 2A).

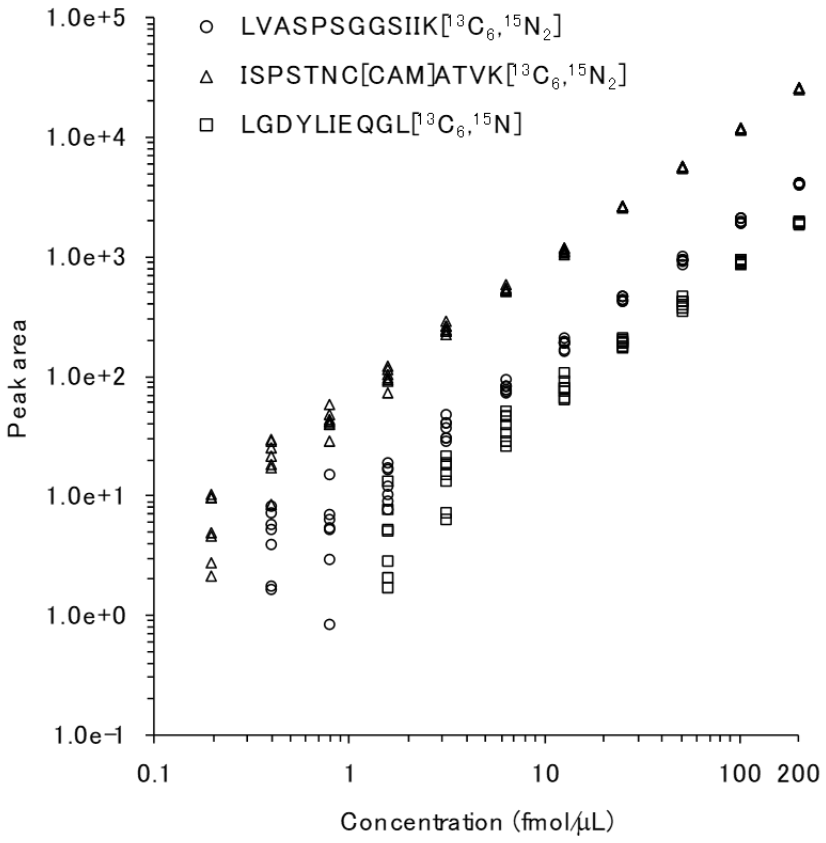

Figure 4. Linearity of the LC/MS/MS assay evaluated by spiking the SIIS peptides into a sweet cherry digest at various concentrations (0.2–200 fmol/μL). n = 7.

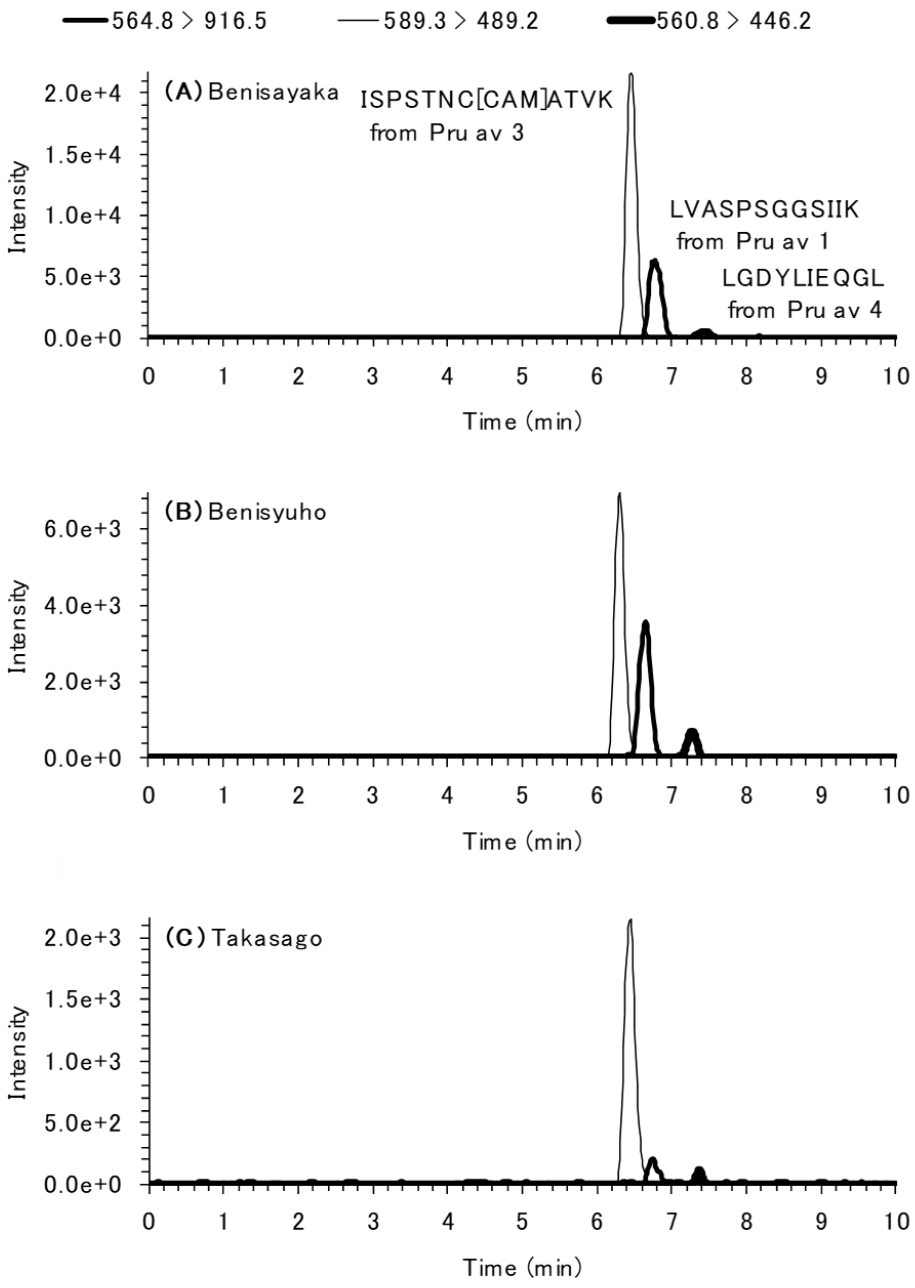

Figure 5. MRM chromatograms of the trypsin digestion solution derived from (A) Benisayaka, (B) Benisyuho, and (C) Takasago. Intermediate, thin, and bold lines indicate the data obtained from the MRM transitions (Table 1) for the quantification of LVASPSGGSIIK, ISPSTNC[CAM]ATVK, and LGDYLIEQGL, respectively.

Table 2. Linearity and limits of detection and quantification of the SIIS peptides.

SIIS peptide	Linearity range (fmol/μL)	r^2	Limit of detection (fmol/μL)	Limit of quantification (fmol/μL)
LVASPSGGSIIK[$^{13}C_6$,$^{15}N_2$]	0.4–200	0.999	0.1	3
ISPSTNC[CAM]ATVK[$^{13}C_6$,$^{15}N_2$]	0.2–200	0.998	0.04	0.9
LGDYLIEQGL[$^{13}C_6$,^{15}N]	1.6–200	0.997	0.5	9

The limit of detection was established at a signal-to-noise ratio of 3. The limit of quantification was estimated as the concentration detected with a coefficient of variation of 20%.

3.5. Intra- and inter-day variability

Intra-day variability of the LC/MS/MS analysis of LVASPSGGSIIK, ISPSTNC[CAM]ATVK, and LGDYLIEQGL and inter-day variability of the process of trypsin digestion plus LC/MS/MS analysis were evaluated by the quantitative measurement of Pru av 1, Pru av 3, and Pru av 4 in the sweet cherry Satonishiki (Table 3).

Table 3. Intra- and inter-day variability in the Pru av 1, Pru av 3, and Pru av 4 assay in the Satonishiki fruit.

Day	Concentration (nmol/g fresh weight)			Coefficient of variation (%)			*n*
	Pru av 1	Pru av 3	Pru av 4	Pru av 1	Pru av 3	Pru av 4	
1	0.21	0.23	0.040	9.4	3.7	17.3	7
2	0.14	0.17	0.031	15.4	1.8	22.3	7
3	0.094	0.14	0.033	11.8	5.3	15.3	7
4	0.048	0.10	0.020	15.9	7.1	26.2	7
5	0.048	0.092	0.023	19.4	5.7	13.2	7
6	0.026	0.072	0.018	42.4	7.6	25.2	7
7	0.17	0.20	0.044	11.8	4.1	15.1	7
1–7	0.11	0.14	0.030	63.5	38.3	36.9	49

The tryptic digest derived from an aliquot of the denatured cherry protein sample was analyzed seven times using LC/MS/MS. The analyses were conducted on seven different days. The coefficient of variation on each day (intra-day precision of the LC/MS/MS analysis) of Pru av 1, Pru av 3, and Pru av 4 ranged from 9.4% to 42.4%, 1.8% to 7.6%, and 13.2% to 26.2%, respectively. ISPSTNC[CAM]ATVK[$^{13}C_6$,$^{15}N_2$], the SIIS peptide for Pru av 3, showed the lowest limit of quantification among the three SIIS peptides (Table 2). Because the SIIS peptides and their native counterparts share the same physicochemical properties, except for their molecular weight, Table 2 shows that ISPSTNC[CAM]ATVK produced from Pru av 3 by trypsinization has the lowest limit of quantification among the three native peptides. This is thought to lead to the lowest coefficient of variation of Pru av 3 among the three allergenic proteins. The entire coefficients of variation of Pru av 1, Pru av 3, and Pru av 4, indicating the reproducibility of trypsin digestion plus inter-day precision of the LC/MS/MS analysis during the seven days, were 63.5%, 38.3%, and 36.9%, respectively. Because

these values are not satisfactory for the precise quantification of Pru av 1, Pru av 3, and Pru av 4, the developed assay is judged to be an analytical method that is restricted for the detection and semi-quantification of these allergenic proteins. These unsatisfactory values are thought to be due to uneven spiking of SIIS peptides, inconsistencies in trypsin digestion caused by substances present in the matrix, and instrumental drift in the mass spectrometer.

3.6. Analysis of other sweet cherry cultivars

Next, the detection of Pru av 1, Pru av 3, and Pru av 4 contained in other sweet cherry cultivars was investigated. Figure 5 shows the MRM chromatograms of the sweet cherry cultivars Benisayaka (A), Benisyuho (B), and Takasago (C). The peaks of LVASPSGGSIIK, ISPSTNC[CAM]ATVK, and LGDYLIEQGL derived from Pru av 1, Pru av 3, and Pru av 4, respectively, were observed in all MRM chromatograms of these three cultivars as well as Satonishiki. Therefore, the developed method is thought to be available for detecting Pru av 1, Pru av 3, and Pru av 4 in sweet cherry fruits.

4. Conclusions

In this study, an analytical assay based on an AQUA method using LVASPSGGSIIK[$^{13}C_6$,$^{15}N_2$], ISPSTNC[CAM]ATVK[$^{13}C_6$,$^{15}N_2$], and LGDYLIEQGL[$^{13}C_6$,^{15}N] was developed for the analysis of Pru av 1, Pru av 3, and Pru av 4 present in sweet cherry fruits. Peaks of the native peptides were clearly detected in the MRM chromatograms of the four sweet cherry cultivars, which led to an easy identification of these peptides in a complicated trypsin-digestion produced from protein extracts of sweet cherries. The developed method is expected to detect and semi-quantify Pru av 1, Pru av 3, and Pru av 4 as an alternative to western blotting. This analytical method is thought to be used for screening sweet cherry cultivars that lack or contain low levels of these allergenic proteins, and to contribute to the development of allergen-free or hypoallergenic sweet cherry cultivars.

Use of AI tools declaration

The author used ChatGPT in order to improve this article. After using this service, the author reviewed and edited the content as needed and takes full responsibility for the content of the published article.

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Conflict of interest

The author declares no conflict of interest in this paper.

Author contributions

Katsunari Ippoushi: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing.

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