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Analytical Methods

Absolute quantification of Pru av 2 in sweet cherry fruit by liquid chromatography/tandem mass spectrometry with the use of a stable isotope-labelled peptide

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ABSTRACT

Pru av 2, a pathogenesis-related (PR) protein present in the sweet cherry (*Prunus avium* L.) fruit, is the principal allergen of cherry and one of the chief causes of pollen food syndrome (oral allergy syndrome). In this study, a quantitative assay for this protein was developed with the use of the protein absolute quantification (AQUA) method, which consists of liquid chromatography/tandem mass spectrometry (LC/MS/MS) employing TGC[CAM]STDASGK[$^{13}C_6$,$^{15}N_2$], a stable isotope-labelled internal standard (SIIS) peptide. This assay gave a linear relationship ($r^2 > 0.99$) in a concentration range (2.3–600 fmol/μL), and the overall coefficient of variation (CV) for multiple tests was 14.6%. Thus, the contents of this allergenic protein in sweet cherry products could be determined using this assay. This assay should be valuable for allergological investigations of Pru av 2 in sweet cherry and detection of protein contamination in foods.

1. Introduction

The number of people suffering from allergies to fruits or vegetables has increased along with the increase in the incidence of pollinosis (Inschlag et al., 1998). Pollen food syndrome, also known as oral allergy syndrome (Rotiroti, Roberts, & Scadding, 2015), is a term explaining the relation between inhalant pollen allergies and symptoms caused by eating particular fruits and vegetables. This syndrome is ascribed to cross-reactivity between pollen-specific immunoglobulin E (IgE) and homologous proteins contained in fruits and vegetables. The percentage of patients with pollen allergy who also suffer from pollen food syndrome is deduced to vary from 47% to 70%. It has been pointed out that one of the plant protein families involved in pollen food syndrome is pathogenesis-related (PR) proteins (Hofmann & Burks, 2008).

The expression of PR proteins is commonly induced in plants by various pathogens such as viruses, bacteria, and fungi, but some PR proteins are constitutively expressed in some organs or during certain developmental stages. The PR proteins are composed of 17 families (PR-1–17) defined by their amino acid sequence similarities, enzymatic activities, or other biological features. The PR-5 proteins, which constitute one of the families, are known as thaumatin-like proteins, and they possess high amino acid sequence homology to thaumatin, a sweet-tasting protein found in the South African berry bush *Thaumatococcus daniellii* (Sinha et al., 2014).

Pru av 2 (glucan endo-1,3-beta-glucosidase, Fig. 1A) from sweet cherry (*Prunus avium* L.) is a food allergenic protein that belongs to the PR-5 class (Ivanciuc et al., 2003). Food allergens in this class besides Pru av 2 include Cap a 1 from sweet pepper (*Capsicum annuum* L.), Mal d 2 from apple (*Malus pumila* Mill.), Act d 2 from kiwi fruit (*Actinidia deliciosa* (A. Chev.) C.F. Liang & A.R. Ferguson), and Pru p 2 from peach (*Prunus persica* (L.) Batsch). Pru av 2 is the major allergen of sweet cherry and one of the main causes of pollen food syndrome. In immunoblotting investigations, IgE of a large proportion of cherry-allergic patients was shown to respond intensely to Pru av 2 among other cherry proteins (Sinha et al., 2014).

Sweet cherry is one of several fruits belonging to the Rosaceae botanical family, which also includes common foods such as almond (*Prunus dulcis* (Mill.) D.A. Webb.), apricot (*Prunus armeniaca* L.), peach—Amygdaloideae subfamily, including sweet cherry—, apple, pear (*Pyrus communis* L.)—Maloideae subfamily—, blackberry (*Rubus fruticosus* L.), and strawberry

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(A)

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        10         20         30         40         50         60
MMKTLVVVLS LSLTILSFGG AHAATISFKN NCPYMVWPGT LTSDQKPQLS TTGFELASQA
SFQLDTPVPW NGRFWARTGC STDASGKFVC ATADCASGQV MCNGNGAIPP ATLAEFNIPA
GGGQDFYDVS LVDGFNLPMS VTPQGGTGDC KTASCPANVN AVCPSELQKK GSDGSVVACL
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ITFCP
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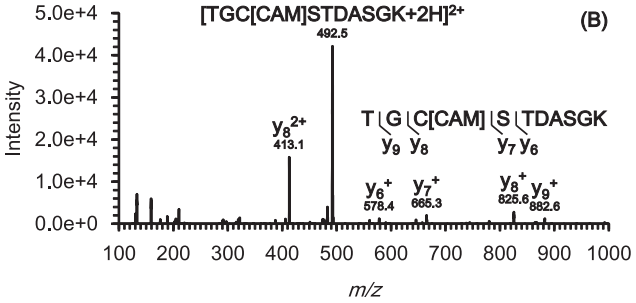

Fig. 1. (A) Amino acid sequence of Pru av 2; bolded/underlined = selected peptide for AQUA; italicised/underlined = signal peptide. (B) Product ion spectrum of the doubly protonated peptide [TGC[CAM]STDASGK+2H]$^{2+}$.

(*Fragaria* × *ananassa* Duchesne)—Rosoideae subfamily—. Fruits of the Rosaceae family often cause allergies, particularly in adults with pollinosis. The Allergen Nomenclature Sub-Committee officially listed the protein families of Rosaceae fruits as allergens. This list describes Pru av 2 as an allergenic protein of sweet cherry (Andersen, Hall, & Dragsted, 2011; Rodriguez et al., 2000).

Pru av 2 is the most plentiful soluble protein contained in the ripe fruit of sweet cherry, but it does not produce a sweet taste like thaumatin does. This protein hydrolyses (1 → 3)-beta-D-glucosidic linkages in (1 → 3)-beta-D-glucans. Pru av 2 starts to accumulate at the beginning of ripening when the fruit changes from yellow to red (Fuchs et al., 2006). The mRNA of Pru av 2 is reported to be one of the 40 most abundant mRNA transcripts in the transcriptome of developing sweet cherry fruits (Alkio, Jonas, Declercq, Van Nocker, & Knoche, 2014).

Targeted proteomics is increasingly contributing to the accurate quantification of key proteins in biological samples. Multiple reaction monitoring (MRM) mass spectrometry (MS) when utilised for targeted proteomics can determine the content of multiple proteins with higher sensitivity and throughput than that achieved with shotgun proteomics. The MRM-MS method is generally carried out on a triple quadrupole (QQQ) mass spectrometer, which produces unique fragment ions from their corresponding precursor ions to quantify the objects in a very complex matrix (Boja & Rodriguez, 2012).

The protein absolute quantification (AQUA) method is commonly performed with a QQQ mass spectrometer equipped with a liquid chromatograph, and it uses stable isotope-labelled internal standard (SIIS) peptides. This method provides the sensitive and accurate determination of targeted proteins in complex samples (Kamiie et al., 2008; Kettenbach, Rush, & Gerber, 2011). In an AQUA experiment, an adequate peptide for the analysis is chosen from among proteases (e.g. trypsin) or chemical substances—digested peptides of a targeted protein *in silico*, and then, the selected peptide is synthesised with stable isotopes (e.g. ^{13}C and ^{15}N). The targeted protein is quantified by comparing the amounts of the synthetic peptide (internal standard) to its native counterpart from the digested protein via measurements performed by liquid chromatography (LC)-MRM-MS.

To quantify Pru av 2 in sweet cherry fruit and detect allergen contamination in foods, we developed an assay based on AQUA technology. The assay was evaluated based on the clarity of the MRM chromatogram and the linearity, quantification limit, and precision of the analyses. This is the first paper reporting on the AQUA method to quantify Pru av 2 for the assessment of this major allergen in sweet cherry.

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) and Wako Pure Chemical Industries. Guanidinium chloride and β-mercaptoethanol were purchased from Sigma–Aldrich (St. Louis, MO). Trypsin was purchased from Thermo Fisher Scientific (Waltham, MA). High quality LC/MS grade solvents including acetonitrile, formic acid, and water were used for LC/MS/MS analyses. Fresh, frozen, and dried sweet cherries, cherry jelly, and cherry jam were purchased from stores.

2.2. Pru av 2

The sequence for Pru av 2 (UniProtKB P50694) was retrieved from UniProtKB (protein knowledgebase, http://www.uniprot.org/). This protein was digested *in silico* by PeptideCutter, web-based software (http://web.expasy.org/peptide_cutter/), to make a list of tryptic peptides. The peptide TGC[CAM]STDASGK (native, C[CAM]: carbamidomethyl-modified C) was chosen from among the list of tryptic peptides, and the native peptide along with TGC[CAM]STDASGK[$^{13}C_6$,$^{15}N_2$] (SIIS) were ordered from Thermo Fisher Scientific, which provides custom peptide synthesis services.

2.3. Trichloroacetic acid/acetone extraction

The extraction of cherry protein was undertaken as follows (Ippoushi, Sasanuma, Oike, Kobori, & Maeda-Yamamoto, 2015). A fresh cherry fruit (7.7 g, edible part) was cut into small pieces and ground into a 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 the sample was stored at −20 °C overnight.