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Identification of a freshness marker metabolite in stored soybean sprouts by comprehensive mass-spectrometric analysis of carbonyl compounds

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Abscisic Acid (PubChem CID5375199)
Acetonitrile (PubChem CID6342)
Butylated Hydroxytoluene (PubChem CID31404)
Chloroform (PubChem CID6212)
Dansyl hydrazine (PubChem CID94442)
Formic acid (PubChem CID284)
Methanol (PubChem CID887)
p-Benzyloxybenzaldehyde (PubChem CID78109)
p-Toluenesulfonic acid (PubChem CID6101)

ABSTRACT

The objective of this study was to identify metabolites that quantitatively indicate degrees of freshness of soybean sprouts. Self-cultivated soybean sprouts were stored at 5 °C, 10 °C or 20 °C, and respiratory CO_2 production rates were monitored using gas chromatography during storage. Carbonyl compounds (CCs) were analyzed comprehensively using mass-spectroscopic metabolomics analyses. CCs were derivatized using dansyl hydrazine (DH) and were then analyzed using high performance liquid chromatography-electrospray ionization tandem mass spectrometry (HPLC-MS/MS) with multiplexed multiple reaction monitoring (MRM). In the MRM chromatogram, 171 to 358 peaks were observed from stored soybean sprouts. Principle component analysis and discriminant analysis (PCA-DA) selected the CC-DH derivative ion with a m/z 512 at a retention time of 9.34 min as the most significant metabolite. Searching online metabolomics databases and matching fragment patterns of product ion mass spectra of an authentic standard revealed abscisic acid is a freshness marker of soybean sprouts.

1. Introduction

Fresh fruits and vegetables are critical components of human diets and provide many health benefits (Slavin & Lioyd, 2012). Although freshness is associated with attractiveness and nutritional benefit, most consumers do not have direct access to fresh fruits and vegetables, and it can take several days for distribution of commercial harvests to consumers. Because substantial losses of nutritional value can occur during distribution, freshness of fruit and vegetables may facilitate consumers' decisions to purchase produce, and could also be used to suggest appropriate postharvest techniques for farmers, distributors and retailers who intend to maintain freshness from farm to table.

Although freshness of fresh fruits and vegetables is widely assessed using observations of the colour change and degree of wilting of produces, validated assessments of freshness are often required to avoid purchasing substandard produce, because visual assessment is limited to the skill of person. In particular, early reductions in freshness are often not visible (Nilsson, 2000).

Fruit and vegetable deterioration reflects increased prevalence of senescence (Paliyath & Droillard, 1992). Plant senescence is a complex and highly regulated process that is characterized by the degradation of chlorophyll, carotenoids, protein, and the cell membrane as well as loss of moisture (Biswal, 1995; Zhou & Gan, 2009). Among these, cellular membrane integrity is an important indicator (Dörnenburg & Davies, 1999). In most cases, cell membrane degradation follows lipid decomposition and is indicated by increasing peroxidized lipid contents (Paliyath & Droillard, 1992), decreasing phospholipids (Lester & Whitaker, 1996) and unsaturated fatty acid levels (Lester, 2003). Numerous peroxidized lipids, including subsequent production of carbonyl compounds (CCs), such as aldehydes, ketones, and carboxylic acids

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(Wills, 1980), are formed during lipid degradation. Therefore, specific CCs accumulate during membrane lipid degradation as well as in senescence and could be a potential biomarker for quantitative freshness assessment of fruits and vegetables.

Recently, metabolomics using analytical instrumentations such as gas chromatography (GC) and liquid chromatography (LC) coupled with mass spectrometry (MS) has been introduced as a powerful approach to identify biomarkers in postharvest science (Singh, 2015). For instance, by means of GC–MS based metabolomics, 10 volatile compounds have been identified as potential markers of chilling injury of basil leaves (Cozzolino et al., 2016). Pentane and 2-ethylfuran have been detected as markers of quality changes of stored wild rocket (Luca, Kjær & Edelenbos, 2017). In addition, Rudell, Mattheis and Hertog (2009) employed untargeted metabolic profiling to characterize metabolomic changes associated with superficial scald development in "Granny Smith" apple following 1-methylcyclopropene or diphenylamine treatment. In case of tomato fruits, LC-MS based metabolomics has been introduced to characterize metabolic changes during ripening (Moco et al., 2006). However, in the case of freshness biomarker identification, LC-MS based metabolomics has not yet been done. Since, Tomono, Miyoshi and Ohshima (2015) developed and validated a method for detecting trace levels of CCs in mice plasma using HPLC/ESI-MS/MS with multiplexed multiple reactions monitoring after derivatization by dansyl hydrazine (DH), we applied this method for the establishment of CC profiles in fruits and vegetables to identify the freshness marker.

Soybean sprouts are a popular vegetable globally, and especially in Japan, Korea, China, and other Southeast Asian countries (Huang, Cai & Xu, 2014). Soybean sprouts are rich in nutrients and are available all year round, but are highly perishable due to high respiration rate (Snowdon, 2010). Therefore, a quantitative freshness assessment is necessary for postharvest management of soybean sprouts.

Brash, Charles, Wright and Bycroft (1995) indicated that the rate of fruit and vegetable perishability is related to the cumulative respiratory CO_2 production during postharvest. Therefore, the cumulative postharvest CO_2 production can be used as a reference indicator for the degree of freshness. Hence, we compared CCs contents with cumulative CO_2 production during storage of soybean sprouts under various temperature conditions, and selected specific CC as a freshness biomarker. Subsequently, we identified the structure of the specific CC by using online metabolomics databases and confirmed by comparing fragmentation of mass spectra with the authentic standard.

2. Materials and methods

2.1. Plant material and storage conditions

Soybean seeds (*Glycine* max, cultivar 'BS5012') were provided by Saladacosmo Co., Ltd., a sprouts manufacturer in Japan, and used to produce soybean sprouts according to the manufacturer's instructions. Briefly, 40 g of soybean seeds were sterilized by dipping into water at 70 °C for 10 s and were then incubated (MIR-154-PJ Panasonic, Gunma, Japan) for 8 h in water at 20 °C to induce the germination. After soaking, the seeds were separated and placed in 4, 250 ml, plastic cups, which were used as cultivation chambers, and were incubated in the dark at 20 °C with 70–80% relative humidity (RH). The seeds were watered with 100 ml of tap water twice daily at 10 am and 4 pm. After 4 days, cultivated sprouts were harvested and selected on the basis of uniform hypocotyl lengths of 10 ± 2 cm. Samples were divided into three groups and each was stored in incubators set at 5 °C, 10 °C and 20 °C with 70–80% RH and were collected periodically for measurements.

2.2. Reagents

Abscisic Acid (ABA) (98%), DH (98%) and *p*-Toluenesulfonic acid (*p*-TsOH) (98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). *p*-Benzyloxy-benzaldehyde (*p*-BOBA) (98%), LCMS-grade acetonitrile and methanol, and HPLC-grade chloroform and formic acid were obtained from Wako Pure Chemical Industries (Osaka, Japan). Butylated Hydroxytoluene (BHT) (≥98%) was purchased from Nacalai Tesque (Kyoto, Japan).

2.3. Measurement of respiration rate by a flow-through method

Rates of respiratory CO_2 production of stored soybean sprouts were measured by a flow-through method using on-line gas chromatography (GC) as described in Fahmy and Nakano (2014) with some modifications. Briefly, 40 g of soybean sprouts were placed into an acrylic chamber (2 L) equipped with gas inlet and outlet tubes. The chambers were closed and fresh air flowed into the chamber from an air compressor through the inlet tubes at the flow rate of 6 L h^{-1}. The chambers were then placed in incubators at 5 °C, 10 °C and 20 °C. Inlet and outlet gas samples were injected automatically into a GC (GC-14A Shimadzu, Kyoto, Japan) alternately via a 0.5 ml sampling loop attached to a rotating stepping valve. CO_2 was separated using a Porapak Q column and detected by a thermal conductivity detector. Helium gas was used as a carrier gas. The chromatograms were analyzed using an integrator (C-R7A plus Shimadzu, Kyoto, Japan) based on a CO_2 standard curve. The results were expressed as percentage of total gas volume. The rate of CO_2 production was calculated from the differences in gas concentration between the inlet and outlet using Eq. (1) (Fonseca, Oliveira, & Brecht, 2002).

$$R_{CO_2} = (y^{out}_{CO_2} - y^{in}_{CO_2})/100 \times F/W \times P/RT \times 10^3 \quad (1)$$

where $R_{CO_2}$is the respiration rate for CO_2 production (mmol $kg^{-1}h^{-1}$), $y^{out}_{CO_2}$ and $y^{in}_{CO_2}$ are the volumetric concentration of CO_2 in inlet and outlet gas samples, respectively (%), W is the weight of the sample (kg), F is flow rate (L h^{-1}), P is the atmospheric pressure (=101.3 kPa), R is the universal gas constant (=8.314 l kPa K^{-1} mol^{-1}) and T is the absolute temperature (K).

CO_2 production rates of soybean sprouts were monitored every hour during storage and cumulative CO_2 production after harvest was calculated by integrating respiration rates throughout the storage duration using the trapezoidal rule. The experiment was repeated three times.

2.4. Sample preparation and CCs extraction

Freshly harvested soybean sprouts and those stored for 4, 8 and 12 d at 5 °C, 2, 4 and 6 d at 10 °C, and 0.5, 1 and 2 d at 20 °C were collected for determination of CCs contents. Sampling times were designed based on the pattern of the cumulative CO_2 production during storage at different temperatures observed in a preliminary experiment. For example, the cumulative CO_2 production for 4 d at 5 °C was approximately similar to that for 2 d at 10 °C and for 0.5 d at 20 °C.

Collected sprouts were then divided into cotyledon and hypocotyl parts and about 100 mg samples of precisely weighted cotyledon and hypocotyl were placed in 2 mL self-standing screw cap microtubes (Watson, Kobe, Japan) containing a single zirconia ball of 5 mm diameter. Samples were soaked in liquid nitrogen for 2 min and were finally stored at –80 °C for further analysis.

Frozen sprout samples were crushed in 200 μL aliquots of methanol containing 0.05% BHT using a bead crusher (Shake Master Neo BMS, Tokyo, Japan) at 1500 rpm for 180 s. Subsequently, 100 μL aliquots of 0.1 μmol mL^{-1} *p*-BOBA were added as an internal standard. After adding 400-μL aliquots of chloroform, mixtures were homogenized again using the bead crusher for 120 s, and mixtures were then centrifuged at 12,000 rpm for 10 min at 10 °C (1720 Kubota, Osaka, Japan). Finally, organic phases were collected and were derivatized with DH.

2.5. DH derivatization

DH derivatization was performed as described by Tomono et al. (2015) with some modifications. Briefly, 200 μL organic phases were