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Comparative Localization Analysis of Immature Soybean (*Edamame*) Content via Mass Spectrometry Imaging

Shu Taira,[1*] Daisaku Kaneko,[2**] Hitomi Shikano,[1] Yoko Miyama,[1]
Toshimitsu Kurosawa,[3] Yuya Kuwaba,[1] Ayano Miyabayashi,[1]
Hideki Terasawa,[4] Shoko Kobayahi,[5] and Kosuke Okajima[4]

[1]Faculty of Food and Agricultural Sciences, Fukushima University, Kanayagawa, Fukushima 960-1248, Japan
[2]The Key Laboratory of Synthetic and Biological Colloids, Ministry of Education, School of Chemical and Material Engineering, Jiangnan University, Wuxi, Jiangsu 214122, China
[3]Agricultural Products Producers and Shippers Association, Mori Town, Mori, Hokkaido 049-2313, Japan
[4]Agriculture and Forestry Division, Mori Town office, Mori, Hokkaido 049-2393, Japan
[5]Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

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We compared the the nutrient and functional contents of two immature soybean (*edamame*) varieties, "*Tamafukura*" and "*Yuagarimusume*" (as the control), using matrix-assisted laser desorption/ionization (MALDI) quadrupole (Q) time-of-flight (TOF) mass spectrometry imaging (MSI). From soybean cross sections, we detected vitamin B_1 (VB_1; *m/z* 265.123) and vitamin B_2 (VB_2; *m/z* 377.146), and daidzein 7-O-glycoside as an isoflavone precursor (*m/z* 417.118) by tandem MS analysis. Results from MALDI-Q-TOF MSI indicated VB_1 and VB_2, and daidzein 7-O-glycoside as functional components localized in the cotyledon and hilum, respectively. The relative amounts of VB_1 and daidzein 7-O-glycoside were 1.13–1.15-fold higher in *Tamafukura* than in *Yuagarimusume*. There was no difference in the relative amount of VB_2 between the two types of *edamame*. These findings highlight the importance of not only determining the difference between soybean varieties, but also the value of visualizing the localization of their content.

1. Introduction

Edamame are immature soybeans that have been harvested before they have ripened. Although *edamame* are immature soybeans, breeding has led to the development of specialized species suitable for eating. The variety commonly marketed as *edamame* is called the white-haired variety, which is characterized by mushrooms with white hairs covering the pods.(1)

Edamame are widely adaptable in terms of soil properties, and can be grown in almost any soil. However, fields that are prone to dryness will reduce the shelling rate, so soils that are rich in moisture and somewhat heavy are preferable. The harvest of *edamame* in Japan is 73100 tons

*Corresponding author: e-mail: staira@agri.fukushima-u.ac.jp
**Corresponding author: e-mail: daisaku@jiangnan.edu.cn
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(in 2004). In major production areas, shipments of *edamame* begin around May to meet the summer demand. Tunnel-grown *edamame* are shipped first, followed by open-air *edamame* in the latter half of the season. *Edamame* are relatively resistant to low temperatures, germinating at 8 ℃ and growing at 10 ℃, but the optimum temperature for growth is 20–25 ℃.

Since *edamame* are immature soybeans, they can be considered a vegetable with the nutritional characteristics of both a bean and a vegetable. First, like soybeans, which are known as the "meat of the field", *edamame* are rich in energy, fat, and high-quality protein, with a protein content of 11% per 100 g, which is close to the 12% contained in eggs. *Edamame* are also unique in that they contain vitamins A and C, which are not found in mature soybeans. Other vitamins such as those in the vitamin B family are also abundant in *edamame*. Vitamin B_1 (VB_1), also called thiamine, metabolizes and converts energy sources (sugar) such as those found in white rice, bread, meat, and fish into energy. A recent study found that VB_1 stimulates voluntary activity through dopamine production in the brain.(2) Vitamin B2 (VB_2), also called riboflavin, is converted to flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD) within an organism to metabolize proteins and lipids, correlates energy production as a coenzyme, and also exhibits antioxidant activity.(3) Daidzein (MW: 254.2), an isoflavone that is typically present in a glycoside form in food, is usually cleaved into isoflavones during fermentation or in the body.(4) In addition, recently, equol (4',7-isoflavandiol) has been noted because it functions like estrogen, is a metabolite of daidzein, and improves our physical condition.(5) Thus, in this study, we measured daidzein 7-O-glycoside (MW: 416.1) as an isoflavone precursor. The chemical structures of the target compounds investigated in this study are shown in Fig. 1. "*Tamafukura*" is an extra-large-grain *edamame*,(6) created by crossing the premium soybean "Shin-Tambakuro" with the extra-large white-eyed "Tsurumusume", and is

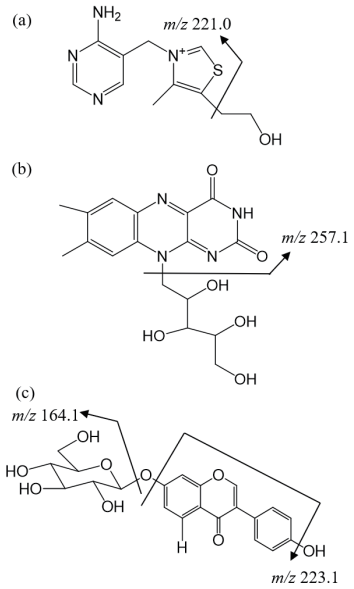

Fig. 1. Chemical structures of target molecules and product ions. (a) Vitamin B1 (thiamine, MW: 265.1), (b) vitamin B2 (riboflavin, MW: 376.1), and (c) daidzein 7-O-glycoside (isoflavone, MW: 416.1).

grown in southern Hokkaido, where the weather is mild and frost is slower to form than in other regions in Hokkaido. The functional components of *edamame* may vary among varieties. However, to the best of our knowledge, there have been no comparative analyses among *edamame* varieties, nor have there been any visual analyses. Conventional imaging methods include labeling-based staining methods, such as immunostaining, histochemical assays, and gene expression induction methods, which are often used to detect and image the distribution of target molecules. However, these indirect approaches are not capable of high throughput and have limited spatial resolution. Moreover, it is difficult to construct antibodies for small molecules such as vitamins and isoflavones. Thus, a more effective imaging technique is needed. Mass spectrometry imaging (MSI) was developed as an expandable MS technique in which MS spectra are reconstructed as ion images to show the target molecule's distribution in the absence of specific markers such as antibodies and fluorescent dyes in a single experiment.[7,8] MSI has been used in a wide range of fields, including biology, pharmacy,[9] food chemistry,[10–12] plant science,[13] and neuroscience.[14,15] In this study, we visualized vitamins and an isoflavone as functional components of *edamame* (*Tamafukura*). We selected *Yuagarimusume*[16] as the control *edamame* because it is a commonly available commercial product.

2. Data, Materials, and Methods

2.1 Sample harvest and preparation

Tamafukura were sown outdoors in May and harvested in September. *Yuagarimusume*, which were also cultivated outdoors, were purchased from a local supermarket. The *Tamafukura* and *Yuagarimusume* were dissected using surgical scissors and tweezers at room temperature to separate the beans from the pod. The peeled beans were embedded into a super cryo-embedding medium (Section Lab Co., Ltd., Hiroshima, Japan), flash-frozen in liquid nitrogen, and stored at −80 °C until use. The frozen beans were cut into serial sagittal sections (10 μm) using a cryostat (NX70, PHC, Tokyo, Japan).

2.2 Protein assay

Coomassie brilliant blue (CBB) G250 dye was used for the protein quantification assay (Bradford protein assay). Equal amounts of *Tamafukura* and *Yuagarimusume* (100 mg) were separately crushed and immersed in methanol (5 mL) to remove fat-soluble molecules. The immersed samples were centrifuged at 10,000 g for 10 min, and the pellet was recovered and immersed in water (40 °C) for 10 min to dissolve the protein. Protein in the recovered supernatant was quantified using the Bradford protein assay. The binding of the CBB dye used in the assay to protein causes a shift in its maximum absorbance from 470 nm to 590 nm. A standard for the linear calibration assay was created using bovine serum albumin (BSA).

2.3 MSI

2.3.1 Preparation of soybean sections for MSI

A 10 mg/ml solution of α-cyano-4-hydroxycinnamic acid (CHCA, Nacalai Tesque, Japan) was suspended in 6 ml of a mixture of acetonitrile/water/trifluoro acetic acid (50/49/1 v/v) and sprayed onto two immature soybean tissue sections placed on ITO-coated glass slides (Bruker Daltonics) using an automated pneumatic sprayer (TM-Sprayer, HTX Tech., Chapel Hill, NC, USA). The soybeans were sprayed 10 times under the following conditions: flow rate 120 µl/min, air flow 10 psi, and nozzle speed 1,100 mm/min.

Before MSI, we confirmed whether the detected *m*/*z* correlated with VB_1, VB_2, and daidzein 7-O-glycoside as an isoflavone by tandem MS. All analyses were performed five times to confirm the reproducibility of the technique.

Optical images of bean sections were obtained using a scanner (GT-X830, Epson, Tokyo, Japan), followed by MALDI-Q-TOF MSI (tims-TOF, Bruker Daltonics Bremen, GmbH) of the sections. Ionization and imaging of the target molecules were confirmed. To detect the laser spot area, each section was scanned, and laser spot areas (200 shots) were detected with a spot-to-spot center distance of 100 µm. Signals between *m*/*z* 100–1000 were collected. The surface of each section was irradiated with smart beam 3D laser shots in positive ion mode. The laser power was optimized to minimize the in-source decay of the targets. The obtained MS spectra were reconstructed into mass images with a mass bin width of m/z ± 0.02 from the exact mass using FlexImaging 5.0 software (Bruker Daltonics). The peak intensity values of the spectra were normalized by dividing by the total ion current to evaluate the semi-quantitative analysis between *Tamafukura* and *Yuagarimusume*.

Measurements are reported as mean ± standard deviation [SD]. We performed unpaired two-tailed Student's *t*-test for single comparisons. Significance was considered at $*P < 0.05$ and $**P < 0.01$. All analyses were performed using Excel 2013 software (Microsoft, Redmond, WA).

3. Results and Discussion

3.1 Confirmation of target molecule chemical structures by tandem MS

In positive ion mode, measurements of VB_1 (MW: 265.1), VB_2 (MW: 376.1), and daidzein 7-O-glycoside (MW: 416.1) in *edamame* sections were achieved by MALDI-Q-TOF-MS/MS analysis to confirm the chemical structures of the target molecules (Table 1). All precursor and fragment ions of the targets are shown in Table 1. The *m*/*z* values of the precursor ions of VB_1 at *m*/*z* 265.123 as open as protonated ions were detected. The protonated VB_1 ion as a precursor was cleaved at the ethyl hydroxy group, yielding ions with *m*/*z* 221.086, corresponding to the pyrimidine and thiazole moieties [Fig. 1(a)]. The protonated VB_2 ion as a precursor was cleaved at the ribitol group, yielding ions with *m*/*z* 257.103, corresponding to the isoalloxazine ring [Fig. 1(b)]. Daidzein 7-O-glucoside as an isoflavone was cleaved at the glycoside group and the 3-phenylchromone skeleton, yielding ions with *m*/*z* 164.076 and *m*/*z* 223.075, corresponding to

Table 1
Precursor ions and product ions for targets by tandem MS analysis

Target	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)
Vitamin B_1	265.123	221.086
Vitamin B_2	377.146	257.103
GirT-labeled γ-decalactone (standard)	417.118	164.076, 223.075

the glucose and aglycone skeleton form [Fig. 1(c)], respectively.-shell ions ($[M]^+$), VB_2 at *m*/*z* 377.146 as protonated ions ($[M+H]^+$), and daidzein 7-O-glycoside at *m*/*z* 417.118.

3.2 Protein assay of *edamame* samples

Comparisons of protein yields between *Tamafukura* (10.2 ± 0.45 g/100 g: as mean ± SD) and *Yuagarimusume* (9.79 ± 0.38 g/100 g) did not reveal any significant differences, but *Tamafukura* tended to have slightly more protein per sample than *Yuagarimusume* (Table 2). However, regarding the average weight per grain, *Tamafukura* (1.43 ± 0.27 g/grain) was significantly heavier than *Yuagarimusume* (0.59 ± 0.09 g/grain; $^{**}p < 0.01$, $n = 8$). Thus, *Tamafukura* had approximately 2.4-fold more nutrients per grain than the control.

3.3 MSI for *edamame* contents

Figure 2 shows the MALDI-Q-TOF-MSI data for VB_1, VB_2, and daidzein 7-O-glycoside in *Tamafukura* and *Yuagarimusume*. The optical images provided information on the morphology of the beans. The seed coat, hypocotyl, and hilum could be observed in raw *Tamafukura* and *Yuagarimusume* [Fig. 2(a)]. The *in situ* localization analysis of bean cross sections using MSI confirmed the presence of signals correlating with the target VB_1, VB_2, and daidzein 7-O-glycoside in the raw *edamame* samples [Fig. 2(b)]). VB_1 was mainly located in the inner region at the cotyledon zone of both beans [Fig. 2(c)] and was more abundant at the hilum in *Tamafukura* than in *Yuagarimusume*.

We semiquantitatively analyzed the trends in VB_1 distribution by correlating the normalized MS intensities in whole bean sections and the hilum area in the image [Figs. 2(d)–2(h)]. The total intensity of the whole section per unit area of *Tamafukura* was approximately 1.15-fold higher than that of *Yuagarimusume* [Fig. 2(d) left]. For the total intensity per hilum region, *Tamafukura* had a 1.2-fold higher intensity than *Yuagarimusume* [Fig. 2(d) right]. The total intensities of the whole section per unit area and per hilum region between *Tamafukura* and *Yuagarimusume* were significantly different ($^{**}p < 0.01$, $n = 4$).

VB_2 was localized throughout the cotyledon region [Fig. 2(e)], and no differences in the total intensity per unit area of the whole section or the hilum region were observed between the samples. However, there tended to be more VB_2 in *Tamafukura* than in *Yuagarimusume* [Fig. 2(f) left and right].

Daidzein 7-O-glucoside was densely localized throughout the whole bean in both samples, and was particularly concentrated in the hilum region [Fig. 2(g)]. Semiquantitative results for the hilum region indicated that *Tamafukura* had a significantly higher daidzein 7-O-glucoside

Table 2
Amount of protein in young soy beans (g) ± S.D. /100 (g)

Tamafukura	*Yuagarimusume*
10.2 ± 0.45	9.79 ± 0.38

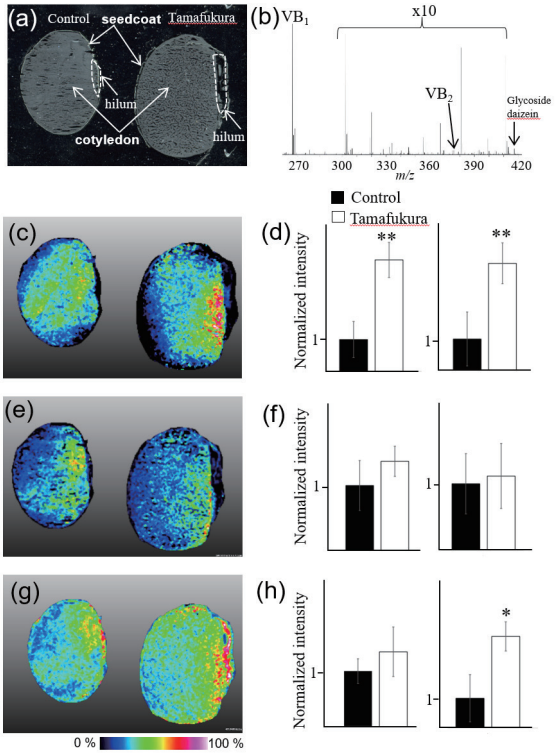

Fig. 2. (Color online) Imaging mass spectrometry of functional components in immature soybeans. Optical image of immature soybean sections (left, *Yuagarimusume* as control; right, *Tamafukura*) (a). MS spectrum of *Tamafukura* section (b). MS spectra reconstructed image. Vitamin B1 (c) and normalized ion intensity of whole bean (left) and hilum region (right) (d), Vitamin B2 (e) and normalized ion intensity of whole bean (left) and hilum region (right) (f), and daidzein 7-O-glycoside as an isoflavone (g) and normalized ion intensity of whole bean (left) and hilum region (right) (h). $*p < 0.05$ and $**p < 0.01$; Student's *t*-test, $n = 4$.

content (1.13-fold higher; $*p < 0.05$, $n = 4$) than *Yuagarimusume* [Fig. 2(h) right], although that for the whole region was not significantly different between the samples [Fig. 2(h) left].

These findings suggested that target molecules, as secondary metabolites, were produced in the leaves and preferentially transported to the beans for storage. Thus, we were able to visualize the phenomenon of vitamin and isoflavone storage in the hilum of immature soybeans.

VB_1 plays a critical role in energy metabolism and prevents beriberi[17] and brain dysfunction.[18] VB_2 correlates energy production as a coenzyme and exhibits antioxidant activity. Daidzein 7-O-glycoside is degraded by bacterial flora and converted to an equol molecule that shows high affinity towards estrogen receptors. Several healthcare applications utilizing equol's anti-cancer, cardioprotective, antidiabetic, antiosteoporosis, anti-ageing, and neuroprotective effects are anticipated.[19]

To the best of our knowledge, this is the first study to simultaneously visualize major vitamins and an isoflavone in immature soybeans. While our images are only semiquantitative, they make it possible to compare the relative concentrations of a single analyte in one image. From our results, we hypothesize and expect that multiple vitamins and an isoflavone can be ingested by eating *Tamafukura* rather than *Yuagarimusume* due to the amount of nutrients and size per grain. MSI is a powerful tool for the direct visualization of fruit compounds and biomolecules in biological tissues, and may have applications in agriculture relevant to commercial food products.

5. Conclusions

We confirmed the chemical structures of target molecules such as VB_1, VB_2, and the isoflavone precursor daidzein 7-O-glycoside in immature soybeans by tandem MS. The amount of protein tended to be slightly higher in *Tamafukura* than in *Yuagarimusume*. *Tamafukura* is an extra-large-grain *edamame*, so the amount of protein contained per grain is expected to be higher than that in the smaller grain *edamame*. Visualizing the localizations of VB_1, VB_2, and an isoflavone precursor revealed differences between the tested *edamame*, suggesting that MSI could be used to separate agricultural products on the basis of visualized nutrient content. From IMS data, we hypothesize that the hilum in *edamame* was the primary region for the storage of secondary metabolites such as vitamins and isoflavones. Because the *edamame* fruit is encased in a pod, the pod protects the fruit and also performs photosynthesis to fatten the fruit.[20] The fruit is bound to the pod through the hilum and grows by receiving nutrients from the hilum. In addition, secondary metabolites are also produced in the leaves or roots of soybean plants.[21] The hilum region first receives these secondary metabolites anatomically. Although further investigation is needed to clarify the physiological mechanisms of the transport and storage of nutrient and functional contents. MSI has the potential to be a valuable food science tool.

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