Visualization of Asparaptine in Asparagus (*Asparagus officinalis*) using MALDI-IMS

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Abstract

Asparaptine, an inhibitor of angiotensin-converting enzyme (ACE) is a newly discovered compound in asparagus. Asparaptine is a conjugate of arginine and asparagusic acid; this compound is suggested to be effective in preventing high blood pressure. For this reason, asparaptine has attracted remarkable attention in recent years and it was therefore necessary to carry out further research. No studies to date have investigated the localization of asparaptine in asparagus. In this study, the localization of asparaptine in asparagus was clarified using matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS), a powerful method to visualize molecules.

Keywords: Imaging mass spectrometry, Plant, MALDI, Asparaptine, Asparagus
Introduction

Asparagus (Asparagus officinalis) is a plant of the family liliaceae, originating from southern Europe. Asparagus has been the subject of interest for a long time due to its noted effectiveness in lowering blood pressure.\textsuperscript{1-3} A reason for this effect was suggested to be related to the active nitrogen-containing compounds contained in asparagus.\textsuperscript{4} However, the existence of other active molecules has also been suggested. Attention has begun to focus on the presence of possible sulfur-containing compounds within asparagus, due to the frequent comparisons with allium species (Alliaceae),\textsuperscript{5} which contain many such compounds.\textsuperscript{6,7}

In 2015, a newly discovered compound, asparaptine, composed of arginine and asparagus acid (Fig.1A) was identified in asparagus by Nakabayashi et al.\textsuperscript{8} It has been suggested that asparaptine is an inhibitor of angiotensin-converting enzyme (ACE),\textsuperscript{9} and therefore reduces blood pressure elevation. Thus, asparaptine has attracted remarkable attention in recent years and it was necessary for more detailed research to be carried out in this area. However, no studies to date had reported the localization of asparaptine in asparagus.

In recent years, imaging mass spectrometry (IMS) has attracted interest as an analysis method for examining the localization of such a compound in plant tissues as well as animal or human tissues.\textsuperscript{10} IMS is an analysis technique which identifies molecular species and locations of compounds by performing mass spectrometry on the tissue surface, while maintaining positional information of a sample, alike observing a sample through a microscope. In particular, matrix assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS) is used for localized visualization of various biomolecules because of its scope to analyze molecules of a wide mass range.\textsuperscript{11,12} MALDI-IMS has many advantages, such as analyzing without labeling, acquiring a wide variety of molecular distributions with one measurement, and distinguishing between metabolites. Because of these advantages, it has been applied not only to medical research, but also to the fields of food and plant in recent years.\textsuperscript{13,14} Thus, it is considered that
MALDI-IMS is also useful for the visualization of asparaptine in asparagus.

In this study we examined the difference of asparaptine localization in asparagus parts (top, middle and bottom) using MALDI-IMS. Further, we also confirmed ion intensities of asparaptine across asparagus sections using liquid chromatography mass spectrometry (LC-MS/MS) to support imaging results.
Experimental

Chemicals and plant material

α-cyano-4-hydroxycinnamic acid (α-CHCA) was purchased from Sigma-Aldrich (MD, US) as a matrix for MALDI-IMS. Methanol, ethanol, acetonitrile, and formic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan) for matrix solution preparation. Ultrapure water was obtained using GENPURE (Thermo Scientific, Osaka, Japan). Chloroform was purchased from Kishida Chemical (Osaka, Japan). All reagents except for ethanol were LC/MS grade.

In this study, the plant material used was green asparagus (A. officinalis) from Mexico, sold in supermarkets. The whole asparagus was cut into thirds, with an upper, middle and lower parts. In addition, a triangular portion (asparagus scale) at the stalk of one asparagus in Fig. 1(B) arrowheads was collected for LC-MS analysis.

Tissue sectioning

For sectioning, cryostat microtome (CM 1950, Leica, Wetzlar, Germany) was used. Once the sample was frozen, asparagus was cut into thirds and frozen. In this study, we performed two methods for freezing. In the first method, the asparagus parts were rapidly frozen with a pack made of aluminum foil to avoid contacting the generated bubbles around asparagus and cracking, using liquid nitrogen, (a common method for sample freezing),15 and then stored in a freezer at -80°C. In the second method, asparagus was packed using a vacuum pack (Sinkuupakkunn, Wide system, Yamaguchi, Japan), then slowly frozen in a freezer at -80°C and stored. We observed tissue sections using toluidine blue staining to compare these two methods. The asparagus preserved at -80 °C were placed in a microtome cabinet for one hour at -20°C and sections were prepared. Three parts were sectioned at 20 μm and thaw-mounted onto ITO (indium-tin-oxide)-coated glass slides (100 Ω/m² without anti-peeling coating, Matsunami Glass,
Osaka, Japan). During thaw-mounting, the sections were fixed onto glass slides by warming them with a fingertip from the opposite sides of the glass slides. For toluidine blue staining, samples from the three sections were attached to a glass slide (PLATINUM PRO, Matsunami Glass Industry, Osaka, Japan).

**Toluidine blue staining**

We stained sample sections using toluidine blue to compare the difference in state due to the differences in freezing methods. First, a glass slide with sample sections was immersed in toluidine blue solution for one minute before being immersed in water for another minute. Thereafter, three consecutive one-minute immersions in ethanol were carried out, followed by two consecutive one-minute immersions in xylene. Finally, cover glasses were placed on sample sections using a soft mount (Wako, Osaka, Japan). For obtaining toluidine blue stained images, we used an all-in-one optical microscope BZ-X 710 (Keyence, Osaka, Japan), BZ-X Viewer (version 01.03.01.01) and BZ-X Analyzer (version 1.3.1.1, Keyence, Osaka, Japan) was used.

**Matrix application and imaging mass spectrometry**

α-CHCA was chosen as a matrix to visualize asparaptine. The matrix solution was prepared at a concentration of 10 mg/mL (30% acetonitrile, 10% 2-propanol, 0.1% formic acid). For spraying, 400 µL matrix solution was sprayed on the sample section using airbrush (PS-270, GSI Creos, Tokyo, Japan). The distance between the tip of the airbrush and the tissue surface was kept at 10 cm.

MALDI-IMS was performed using iMScope TRIO (Shimadzu, Kyoto, Japan). The instrument has a sample chamber for microscopic observation and MALDI ion source with Nd: YAG laser (λ = 355 nm and 1 kHz) under atmospheric pressure. Therefore, it is possible to
acquire optical images and ion distribution images in the same instrument. All mass spectra were measured in the positive ion detection mode in the range of $m/z$ 100-350. Laser irradiation was performed on the tissue surface with 100 shots for each data point. The laser irradiation diameter was set to 2 (arbitrary unit of iMScope, approximately 25 µm). In MALDI-IMS experiments, constant voltages of the sample stage and the detector were applied at 3.50 kV and 2.1 kV, respectively. The laser power was maintained at a constant of 47 (arbitrary unit of iMScope). MS/MS analysis was performed for a selected precursor ion of asparaptine ([M+H]$^+$ 307.09) to obtain an asparaptine signal.

**LC-MS/MS analysis**

We prepared three asparagus and cut into three parts (top, middle and bottom). For an asparagus scale, we collected all scales from one asparagus. After packaging them into a vacuum pack, they were frozen slowly in a freezer at -80°C. Thereafter, each sample was freeze-dried overnight. After freeze-drying, each sample was put into a crushing tube (YASUI KIKAI, Osaka, Japan) together with a crushing metal (YASUI KIKAI, Osaka, Japan). After sufficiently cooling with liquid nitrogen, the samples were crushed using a multi beads shocker (2000 rpm, 60 s) (YASUI KIKAI, Osaka, Japan) and made into fine powder form. Powdered samples were weighed at 10 mg and transferred from the freezing crushing tube to a 1.5 mL microtube. Three 1.5 mL microtubes containing 10 mg samples were prepared for all samples ($n$ = 9). For the asparagus scale, 3 mg samples were collected in each microtube, due to difficulty in obtaining 10 mg samples ($n$ = 3).

Asparaptine was extracted using an extraction solvent (methanol: chloroform: water = 2 : 2 : 1). First, 1 mL of extraction solvent was added to a 1.5 mL microtube containing the sample. At the same time, a 1.5 mL microtube with no sample was prepared as a control and the same operation was performed. After thorough stirring with a vortex mixer (10 s), centrifugation (4
°C, 10000 rpm, 10 min) was carried out and the solid component of the sample was settled. We transferred 100 µL of the supernatant to a new 1.5 mL microtube and added 400 µL of 0.1% formic acid solution. After this, the solvent was mixed again using a vortex mixer (10 s) and centrifuged (4 °C, 10000 rpm, 10 min) again. Finally, 100 µL of the supernatant was transferred to LC vials.

LC-MS/MS analysis was performed using Nexera UHPLC System (Shimadzu, Kyoto, Japan) connected to LCMS-8050 (Shimadzu, Kyoto, Japan). For mass spectrometry, multiple reaction monitoring was used with the transition of \( m/z \ 307.10 > 248.05 \) for quantification and \( m/z \ 307.10 > 170.08 \) for confirmation. A Mastro HPLC column (2.1 mm i.d. × 100 mm, 3 µm) (Shimadzu GLC Ltd., Tokyo, Japan) was used at 40 °C. Mobile phase A consisted of water with 0.1% formic acid and B consisted of acetonitrile with 0.1% formic acid. The flow rate was 0.3 mL/min and the injection volume was 1 µL. The gradient started with solvent B at 2% until four minutes. The proportion was changed linearly so that solvent B became 80% after six minutes and 100% after eight minutes. The analysis was terminated after 12 min.
Results and Discussion

Optimization of sample freezing

In this experiment, we made a comparison between two freezing methods using liquid nitrogen and a vacuum pack, respectively. Samples frozen by each method were sectioned using a cryostat microtome. Each sample section was observed using an all-in-one optical microscope BZ-X 710 (Fig. 2). According to Fig. 2A, it was possible to make sections whilst keeping the shape intact for samples frozen using a vacuum pack. In contrast, samples frozen using liquid nitrogen failed to make shapely sample sections, as cracks occurred during freezing and the outside of the sections became rough. In sum, samples frozen in a vacuum pack maintained the shape of sample tissue cells. Conversely, the sample tissue cell frozen using liquid nitrogen collapsed and the tissue had cracks (Fig. 2B).

One of the reasons why samples frozen using a vacuum pack made sections which maintained the sample tissue cell is due to the principles behind the high-pressure freezing method. Usually, when water freezes, it crystallizes to form ice crystals inside the cell. In the high-pressure freezing method, the melting point of water decreases and the viscosity rises by applying a pressure of about 2000 atmospheres when the sample freezes. Therefore, it is possible to suppress the formation of ice crystals that cause the destruction of the tissue cell. Conversely, when using liquid nitrogen with aluminum pack, a sample is immersed in a liquid, it boils, and a nitrogen gas layer is formed around the sample. Thus, liquid nitrogen results in slow freezing with poor cooling efficiency. Further, in freezing using liquid nitrogen, the depth to be frozen in non-crystalline is less than 5-20 µm, whereas in the high-pressure freezing method it is ~200 µm. This phenomenon appears remarkably in a large sample containing high amounts of water, such as asparagus. Therefore, it is concluded that freezing using the vacuum pack is a superior method that results in plant sections maintaining the sample tissue cell. In addition, when a vacuum pack was used, the sample could withstand expansion due to the
process of water becoming ice, as the sample was pressurized from the surroundings. When using liquid nitrogen, it is assumed that the cracks form due to the expansion of water with nothing to suppress it.

**Visualization of asparaptine in asparagus**

To observe the localization of asparaptine, we first performed MS analysis on sample sections. We observed asparaptine at \( m/z \) 307.09 (Fig. S1(A)), as shown in the previous study,\(^8\) therefore the obtained \( m/z \) 307.09 ion was selected as the precursor ion and MS/MS analysis was carried out (Fig. S1(B)). A product ion with \( m/z \) 248.05±0.05 was used for asparaptine visualization.

The results of visualization of asparaptine localization in the three parts and asparagus scale using MALDI-IMS are shown in Fig. 3. It was found that many asparaptine compounds were visualized on the outside of the stem in the top section and many were visualized in the center of the bottom section. According to the enlarged overlay image, asparaptine was localized around vascular bundles as shown in Fig. S2(A)-(C). In addition, asparaptine was localized throughout the whole of the middle section. Asparaptine was localized more so in the asparagus scale than the stem part.

We speculated the reason for these localization patterns as follows: Asparaptine is a sulfur-containing compounds; sulfur is an essential nutrient for plants and is known as the secondary macronutrient. Plants ingest sulfur by selective absorption of sulfate ions (SO\(_4^{2-}\)) in the soil.\(^20\) This absorption is carried out by sulfate ion transporter. Sulfur-containing compounds absorbed by root epidermal cells are transported to the conduit of the central cylinder. Thereafter, the sulfur-containing compound is distributed to where sulfur is most required (e.g., young leaves).\(^21\) Consequently, asparaptine was localized on the outside of the stem at the top part of the asparagus as this is where sulfur is most required for growth. Conversely, at the
bottom section of asparagus, asparaptine is localized more so in the center (where the conduit passes) as opposed to the outside of the stem, as it is not needed for further growth.

The reason for a predominant localization of asparaptine in the asparagus scale is as follows: The asparagus scale is a leaf of asparagus and plays a role in protecting pseudo leaves which are a part of the stem. The top section of asparagus is where the leaves overlap and plant leaves can incorporate sulfate ions. Therefore, it is assumed that sulfate ions were incorporated from the leaves and produced asparaptine.

Ion intensity confirmation by parts using LC-MS/MS

Results of ion intensity (peak area) comparison using LC-MS/MS are shown in Fig. 4. These experiments were done with three spears of asparagus. In addition, we performed triplicated measurements for each part. Although individual differences exist, the peak area of asparaptine gradually increased in order from the bottom to the top part. Furthermore, the peak area of asparaptine contained in the asparagus scale was almost equal to the top part. From these results, we considered that there were two types of asparaptine production. First, asparaptine is produced using the sulfur from the soil. Second, it is produced using sulfur from asparagus scales. Additionally, it is concluded that asparaptine is largely involved in the growth of asparagus, as asparaptine accumulates at the outer layer of the top part of asparagus.
Conclusions

In this study, the localization of asparaptine in asparagus was clarified using MALDI-IMS. In addition, freezing at -80°C freezer using a vacuum pack was shown to have greater usefulness than the commonly used method of freezing using liquid nitrogen for plant tissue sectioning.

It was found that the distribution of asparaptine spreads outward from the bottom section to the top. This is because more amount of sulfur is required in the middle of the top section for growth, and the asparagus scale incorporates sulfur ions. The result of LC-MS/MS support this reason.

Through this study, we could obtain new findings by clarifying the localization of the substance called asparatine. In the future, it is expected that the results from this study could be applied to functional foods and the elucidation of asparaptine synthesis mechanisms.
Supporting Information: Enlarged images of asapartine distribution in asparagus.

This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

References

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Figure Captions

Fig. 1  (A) Structure of asparaptine. Product ions of m/z 248 and 170 were derived from illustrated cleavage of asparaptine (m/z 307.09). (B) Picture of asparagus. Arrowheads indicate asparagus scales.

Fig. 2  Images of asparagus stained with toluidine blue. Asparagus was frozen using liquid nitrogen (upper) and vacuum pack (lower). (A) Overall images of sections. Arrow heads show cracks. Freezing in liquid nitrogen led to damaged sections. Scale bars: 1000 µm (B) Enlarged images of sections. Arrow heads show damaged tissue parts. Freezing in liquid nitrogen led to collapsed and tissue cracks. Scale bars: 100 µm (Top), 400 µm (middle), 150 µm (bottom)

Fig. 3  Distribution of asparaptine in asparagus. Figures show the distribution of asparaptine (m/z 307.09 > 248.05) in the top, middle and bottom sections and the asparagus scale. Red arrows show scales. The distribution of asparaptine spreads outward from the bottom to the top. Much asparaptine was localized in the asparagus scale. The color bar reflects the concentration of asparaptine. Pitch: 100 µm, Scale bars: 1 mm

Fig. 4 Dot plots to compare peak area of asparaptine in the top, middle and bottom parts and the asparagus scale (averages and standard deviations are illustrated). These experiments were performed using three spears of asparagus with triplicate except for asparagus scale, n = 9 for top, middle and bottom, n = 3 for scale.
Fig. 1(A) Structure of asparagine. \( m/z \) 248.05 and 170.08 were product ions from the precursor ion of asparagine (\( m/z \) 307.09). (B) Picture of asparagus. Arrow heads show asparagus scale.
Fig. 2 Images of asparagus stained with toluidine blue. Asparagus was frozen using liquid nitrogen (upper) and vacuum pack (lower). (A) Overall images of sections. Arrow heads show cracks. Freezing in liquid nitrogen led to damaged sections. Scale bars: 1000 µm. (B) Enlarged images of sections. Arrow heads show damaged tissue parts. Freezing in liquid nitrogen led to collapsed and tissue cracks. Scale bars: 100 µm (Top), 400 µm (middle), 150 µm (bottom)
Fig. 3 Distribution of asparagine in asparagus. Figures show the distribution of asparagine ($m/z$ 307.09 > 248.05) in the top, middle and bottom sections and the asparagus scale. Red arrows show scales. The distribution of asparagine spreads outward from the bottom to the top. Much asparagine was localized in the asparagus scale. The color bar reflects the concentration of asparagine. Pitch: 100 µm, Scale bars: 1 mm
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