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Journal of Chromatography A, 822 (1998) 310–315

JOURNAL OF CHROMATOGRAPHY A

Short communication

Quantification of amino acid conjugates of jasmonic acid in rice leaves by high-performance liquid chromatography–turboionspray tandem mass spectrometry

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Received 20 May 1998; received in revised form 15 July 1998; accepted 16 July 1998

Abstract

HPLC–turboionspray MS–MS (TIS-MS–MS) was shown to be useful in the quantification of the amino acid conjugates of jasmonic acid (JA) as well as free JA in rice leaves. With the TIS-MS–MS system, high sensitivity was obtained under multiple reaction monitoring conditions. Using this quantification method, rapid increase and subsequent decrease in the leucine conjugate and valine conjugate of JA were observed in rice leaves stressed by wounding. These amino acid conjugates of JA may work as endogenous signalling compounds as well as free JA.

Keywords: Ionspray ionization; Turboionspray ionization; Interfaces, LC–MS; Plant materials; Mass spectrometry; Amino acids; Jasmonic acid

1. Introduction

Plants have developed highly effective and complex chemical systems to protect themselves from herbivores and microbial pathogens. For example, antifungal compounds are well known to be produced by plants just after an attack by microbial pathogens. These antifungal compounds are secondary metabolites called phytoalexins [1]. The production of phytoalexins contributes to the self-defense systems of plants, because the compounds show high antifungal activity and accumulate around the infection sites soon after the infection of pathogen. A large number of phytoalexins have been isolated from various kinds of plants and their importance in self-defensive systems has been discussed. In rice plants, we have identified oryzalexins and a flavonoid compound, sakuranetin as the rice phytoalexins [2,3].

Jasmonic acid (JA, Fig. 1) has been suggested to play an important role in activating these self-defensive systems, such as the rice phytoalexin production, because exogenously applied JA significantly elicits phytoalexin production in rice suspension-cultured cells and endogenous JA rapidly increases under stressed conditions [4]. Recently, we found that exogenously applied amino acid conjugates of JA (Fig. 1) are also active in phytoalexin production

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0021-9673/98/$ – see front matter
PII: S0021-9673(98)00599-8

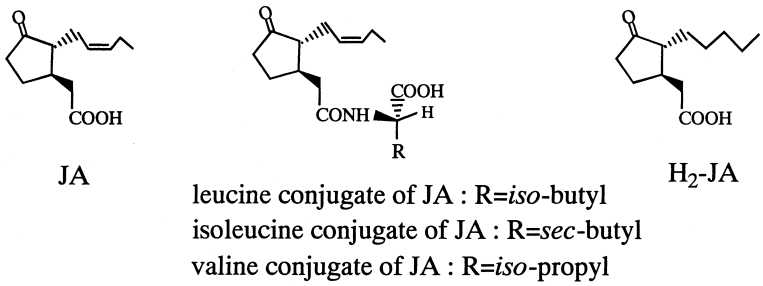

Fig. 1. Structures of JA, amino acid conjugates of JA and H_2-JA.

as well as the free JA in rice leaves [5]. To confirm the role of the amino acid conjugates of JA in phytoalexin production, it is necessary to quantify the endogenous content of the compounds in rice leaves.

GC–MS (gas–liquid chromatography–mass spectrometry) and radioimmunoassay methods have been developed for the purpose of analyzing JA and jasmonates in plants [6,7]. Although these methods have a high level of sensitivity, they require a complicated purification process before analysis; therefore, a more practical and simple method for the detection of the amino acid conjugates of JA must is needed. The use of ionspray (IS) with a tandem mass spectrometric (MS–MS) method has been increasing due to its significantly high sensitivity and selectivity. For example, reversed-phase capillary liquid chromatography (LC) which is interfaced with an IS-MS–MS system has been shown to be effective in the quantification of JA and salicylic acid [8]. The recently turboionspray (TIS) inlet system can obtain more efficient and sensitive quantification as compared to the conventional IS inlet system. Utilizing this TIS as an ion source, we established a simple and useful method to quantify the amino acid conjugates of JA including free JA. Using this method, a usual ODS reversed-phase column can be used at a flow-rate of 0.5 ml/min. We found a rapid increase in the amino acid conjugates of JA in stressed rice leaves using the above technique. This study describes high-performance liquid chromatography–turboionspray tandem mass spectrometry (HPLC–TIS-MS–MS) and its application to the quantification of the amino acid conjugates of JA in plants.

2. Experimental

2.1. Chemicals

Racemic JA was prepared according to the previous method [9]. Racemic dihydrojasmonic acid (H_2-JA) was prepared by the hydrogenation of racemic JA with H_2 in the presence of Pd–C. Amino acid conjugates of JA; *N*-[(−)-jasmonoyl]-*S*-isoleucine, *N*-[(−)-jasmonoyl]-*S*-leucine, *N*-[(−)-jasmonoyl]-*S*-valine were synthesized according to a previous method [10].

2.2. High-performance liquid chromatography

A HP 1100 HPLC Series (Hewlett-Packard, Waldbronn, Germany) equipped with an Inertsil ODS-2 column (150 mm×4.6 mm I.D., 5 μm, GL Sciences, Japan) was used. Elution with 80% aqueous acetonitrile (containing 0.1% formic acid) was carried out at a flow-rate of 0.5 ml/min.

2.3. Mass spectrometry

A Sciex API-300 (Perkin-Elmer SCIEX Instruments, Foster City, CA, USA) equipped with a TIS inlet system, was used for the HPLC–MS–MS system. All the tested compounds were analyzed in a negative-ion mode. Nitrogen was used as the collision gas. State files are as follows; NEB=11, CUR=9, CAD=3, IS=−3800, TEM=425, OR=−25, RNG=−375, QO=10, IQ1=11, ST=15 RO1=12, IQ2=40, RO2=30, IQ3=60, RO3=43, DF=100, CHM=1900. Quad 1: 10 (0.05), 100 (0.11), 1000 (0.473), 10000 (0.875). Quad 2: 10 (0.006), 100

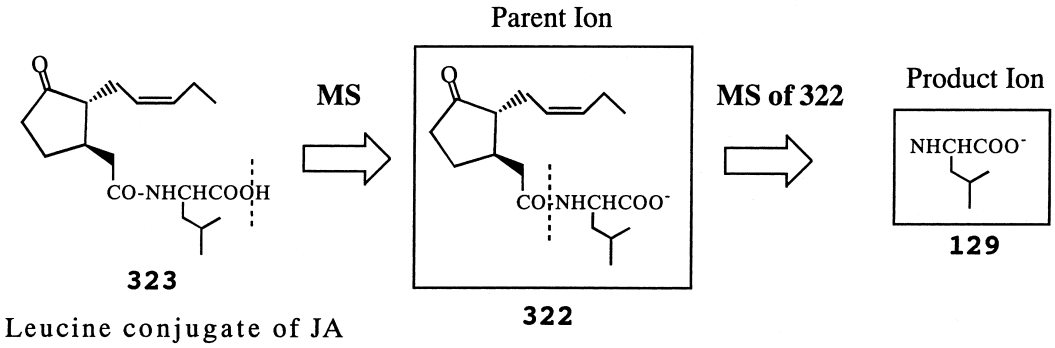

Fig. 2. Electrospray MS–MS of the leucine conjugate of JA.

(0.040), 1000 (0.060), 10000 (0.080). The multiple reaction monitoring (MRM) mode was used to monitor the parent and product ions. The dwell time was set at 500 ms and the duration time was 10 min. The pause time was 5.0 ms.

2.4. Plant material and sample preparation

Rice plants (*Oryza sativa* L. Nipponbare) were cultivated and used as described previously [11]. One g of rice leaves was cut into small pieces (5 mm), and 50 ml of acetone and 100 ng of H_2-JA (as an internal standard) were added. After being kept overnight at ambient temperature, acetone was concentrated and the residue was dissolved in 3.0 ml of 75% aqueous MeOH. The MeOH solution was passed through a Sep-Pak Light C_{18} cartridge (Waters) to prepare the partially purified sample. Five μl of the solution was injected into the HPLC–TIS-MS–MS system. The recovery yield with H_2-JA (50 ng) by this extraction and purification procedure was more than 75%.

When the endogenous content of the amino acid conjugates of JA and free JA were analyzed in stressed rice leaves, the rice leaves were punctured by needles and kept for at an appropriate period of time under high humidity.

3. Results and discussion

3.1. Ionspray MS–MS conditions for amino acid conjugates of JA

IS ionization with MS–MS enables the quantification of compounds at an ultratrace level. The parent ion in IS ionization is usually given as its molecular ion without fragmentation. The fragmentation of the parent ion by N_2 gas takes place in the second process to give the product ions. The target compound can be monitored at more than two points (parent ion and product ions) with MS–MS. Thus this method is called MRM. Amino acid conjugates of JA gave their parent ions as deprotonated negative ions, and gave their product ions as amino acid fragments in the negative-ion mode experiment. In the case of the leucine conjugate of JA, the parent ion appeared at m/z 322, and its product ion appeared at m/z 129 from leucine moiety by MS–MS as shown in Fig. 2. Thus, the leucine conjugate of JA can be detected with a combination of m/z 322/129 in MRM. The MS–MS experiments to determine the MRM conditions for other compounds including isoleucine conjugate of JA were done in the same way.

3.2. Turboionspray ion source

TIS consists of an ionspray inlet that is used in co-injection with a heated turboprobe. The turboprobe directs a jet of heated dry nitrogen at the spray which is produced by the ionspray. The difference between the IS and the TIS is that the latter is the ionization of a sample with additional heated gas (nitrogen) to desolvate the spray. The MRM conditions determined by conventional IS can be used in the TIS inlet system. With TIS, higher HPLC flow-rates of HPLC (at 0.5 ml/min) are accepted than with IS, with improved sensitivity; thus, the TIS is

appropriate for quantitative analysis. All the standard and extracted samples were ionized at 425°C.

3.3. Quantification of amino acid conjugates of JA in rice leaves

Standard and extracted samples were injected into the TIS-MS–MS system under MRM analytical conditions via HPLC. Using these MRM conditions (Table 1), background noise is minimized, and therefore sensitivity was very high. The detection limit of these amino acid conjugates of JA is approximately between 1 pg to 500 fg. The retention times of the standard compounds are as follows; a valine conjugate of JA=3.47 min, a leucine conjugate of JA=4.0 min, a isoleucine conjugate of JA=4.0 min, free JA=3.50 min and H_2-JA=4.08 min at a flow-rate of 0.5 ml/min. The contents of the amino acid conjugates of JA and free JA in the sample solutions that were prepared from rice leaves were determined by calibration curves with H_2-JA as an internal standard. H_2-JA is a precise internal standard for the amino acid conjugates of JA, as well as for free JA [8]. Fig. 3 shows the calibration curve for the leucine conjugate of JA with H_2-JA as an internal standard. In order to obtain the standard curves, seven standards of the leucine conjugates of JA (25 pg to 2.5 ng) were combined with 20 ng of H_2-JA (internal standard), injected into the TIS-MS–MS system, and monitored by MRM. Calibration curves with other standard compounds including the valine conjugate of JA and free JA were obtained in the same manner (results not shown). The analyte standard curves were calculated using the SCIEX MacQuan 1.5 program. Analyte ions in the samples were monitored by MRM, and the concentration of the analyte was determined in relation to the internal standard. This HPLC–TIS-MS–MS quantification method was effective for the amino acid conjugates of JA and free JA, because the analytical procedure consisted of (i) simple and efficient purification procedure without any loss, (ii) highly sensitive and reproducible turboionspray inlet system, (iii) highly selective analytical method MRM, and (iv) high-resolution reversed-phase HPLC.

Using the HPLC–TIS-MS–MS method described above, rice leaves are shown to contain the amino acid conjugates of JA as well as free JA. Fig. 3 shows the MRM spectrum of rice leaf extract in comparison to that of the synthetic standards of the amino acid conjugates of JA. The peaks of the leucine conjugate of JA and the valine conjugate of JA can be identified in rice leaves by comparing them to those of the synthetic standard under the above HPLC–TIS-MS–MS conditions. Although the leucine conjugate of JA and the isoleucine conjugate of JA show the same fragmentation patterns in MRM (Table 1), they gave different retention times at a low flow-rate in the HPLC analysis. At a flow-rate of 0.2 ml/min with 50% aqueous acetonitrile containing 0.1% formic acid, the retention times of the leucine conjugate of JA and the isoleucine conjugate of JA were 22.73 min and 22.07 min, respectively. The retention time of the analyte in natural rice leaves was 22.65 min. When the synthetic isoleucine conjugate of JA was co-injected with the natural leucine conjugate of JA in rice leaves, these two conjugates appeared at different retention times. These results showed that the analyte in rice leaves was the leucine conjugate of JA.

In order to confirm the importance of the amino acid conjugates of JA in rice leaves, the time dependent endogenous content of these compounds under stressed conditions were analyzed. Fig. 4 shows the increase and decrease of the endogenous amino acid conjugates of JA and free JA in rice leaves stressed by wounding. One hour after treatment, a rapid increase and subsequent decrease in the leucine conjugates of JA was observed in the stressed rice leaves as well as free JA. The valine conjugate of JA showed the same appearance, but the amount was far less than that of the leucine conjugate of JA. The increase in the amino acid conjugates of JA in the stressed rice leaves was rapid and sharp, therefore, these compounds can be used as endogenous signaling compounds.

Previous result has shown the importance of JA as a endogenous signaling compound in plant cell cultures [6], and the amino acid conjugates of JA are reported to be naturally occurring jasmonates and increase in sorbitol-stressed barley leaves [12]. The present results in this study suggest that the amino acid conjugates of JA including the leucine and valine conjugates might also play the important roles as well as free JA in stressed rice leaves.

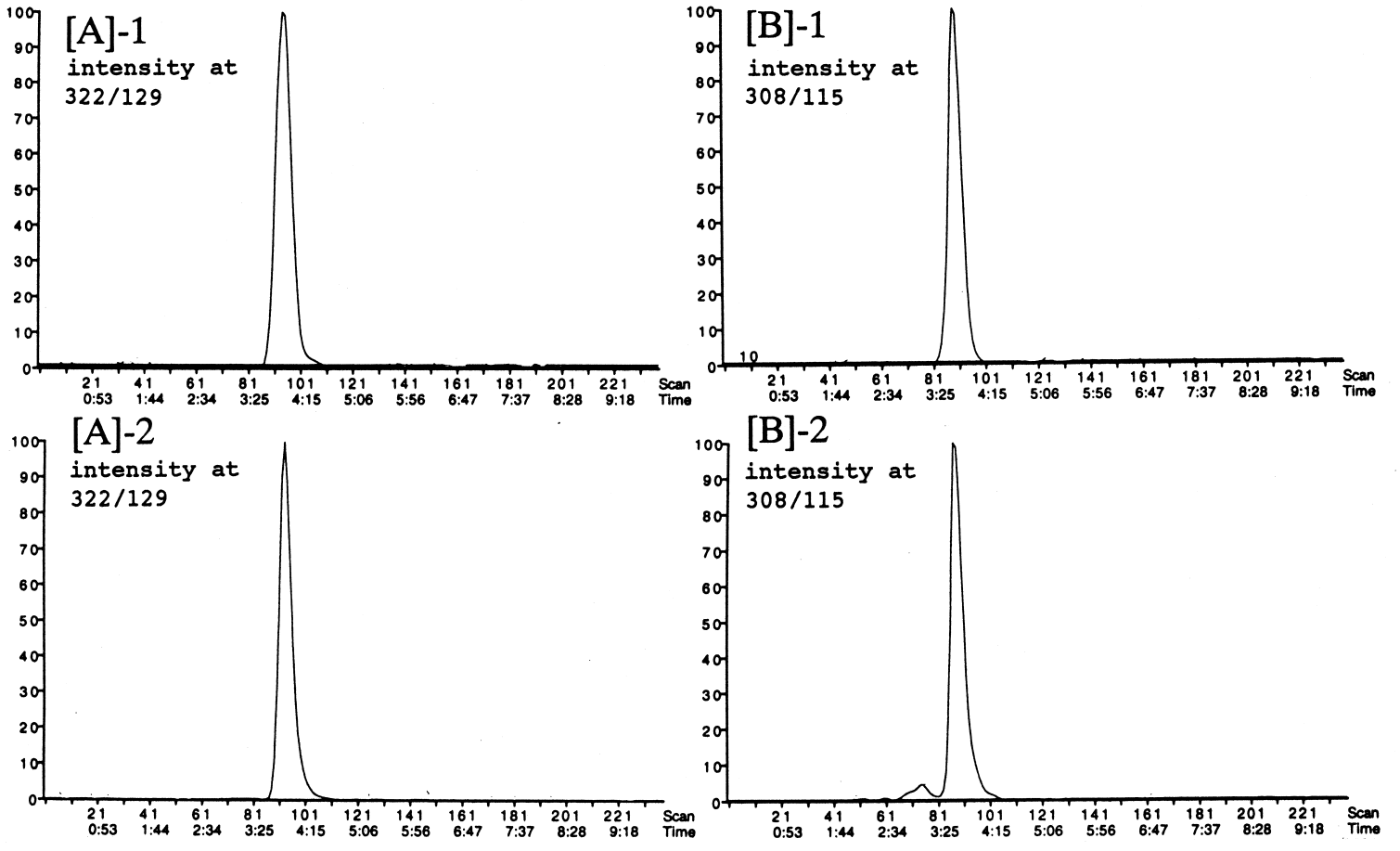

Fig. 3. MRM spectrum of rice leaf extract in comparison to the synthetic amino acid conjugate of JA. [A]-1=MRM spectrum of the rice leaf extract monitored at m/z 322/129. [A]-2=MRM spectrum of the synthetic leucine conjugate of JA monitored at m/z 322/129. [B]-1=MRM spectrum of the rice leaf extract monitored at m/z 308/115. [B]-2=MRM spectrum of the synthetic valine conjugate of JA monitored at m/z 308/115. Scan numbers (above) and retention time in min (below).

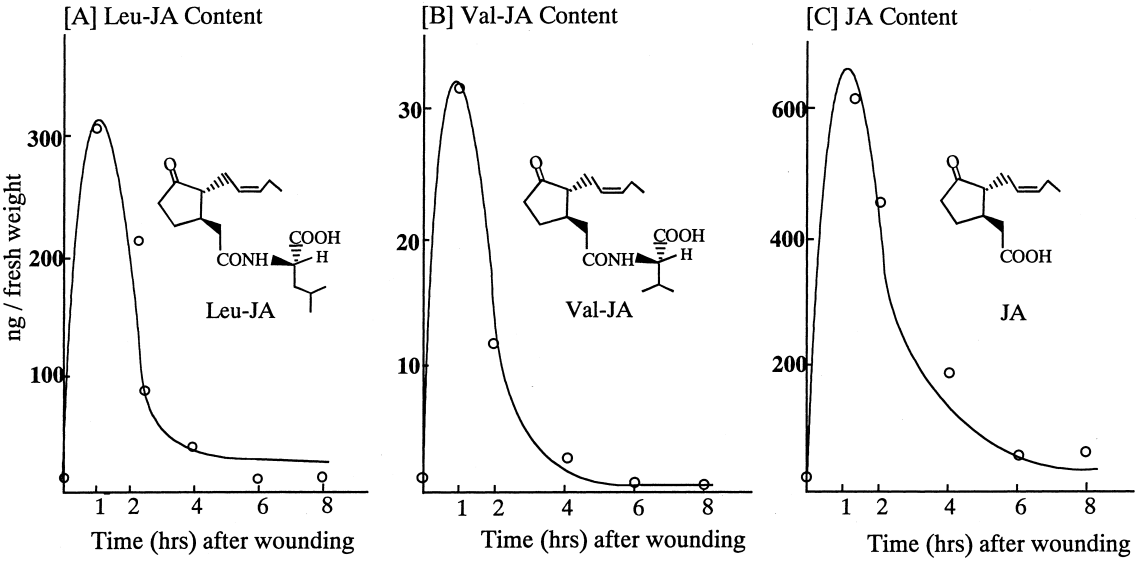

Fig. 4. Increase and decrease of endogenous amino acid conjugates of JA (Leu-JA and Val-JA) and free JA in rice leaves stressed by wounding.

Acknowledgements

This study has been supported by CREST (Core Research for Evolutional Science and Technology) of Japan Science and Technology Corporation.

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