Quantification of Cytokinin O-Glucosides by Negative Ion Mass Spectrometry

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ABSTRACT

Pulsed positive ion-negative ion chemical ionization mass spectra of O-glucosyl-zeatin and 2-dihydrozeatin, their ribosides, and their N-9 2-cyanoethyl and 2-chloro-2-cyanoethyl derivatives are reported. By methods based on these spectra, the levels of the glucosides were determined in soybean (Glycine max) leaves.

The first mass spectrometric quantifications of the endogenous O-glucosides, OGZ, OGDZ, OGZR, and OGDZR, were based on EI mass spectrometry of TMS derivatives of the intact glucosides, the samples being introduced into the mass spectrometer via the direct inlet probe (8). Subsequently, a number of workers have purified an O-glucoside fraction, hydrolyzed this with β-glucosidase, and quantified the released bases and ribosides by GC-MS. The assumption has been made that these are derived from the known glucosides OGZ, OGDZ, OGZR, and OGDZR (3, 9). However, for reasons already presented (6) this assumption is not valid. It is important to quantify these glucosides per se, and not as aglycones formed by enzymic cleavage. The procedures used to achieve this with soybean leaf extracts are detailed herein.

The techniques employed are based on negative ion CI mass spectrometry. To facilitate determination of OGZ and OGDZ, a new selective derivatization procedure was developed which yields a stable chlorine-containing derivative of these glucosides. The chlorine atom greatly facilitates detection of the glucosides by mass spectrometry. The derivative has other advantages over per-TMS and permethyl derivatives for direct inlet probe analysis. With cytokinin glucosides, multiple product formation tends to accompany preparation of per-TMS derivatives (1, 4). Per-TMS derivatives are also very rapidly hydrolyzed by traces of water. While permethyl derivatives of OGZ and OGDZ can be subjected to GC (5) and are stable, the preparation of the necessary reagents and the derivatization procedure are inconvenient and the concentration of the methylsulfanyl carbanion must be controlled carefully (1, 4). Because derivatized cytokinin glucosides yield complex EI spectra with weak molecular ions, CI mass spectrometry has clear advantages for quantification of these compounds.

MATERIALS AND METHODS

Mass Spectrometry

All spectra were determined with a Finnigan 4530 mass spectrometer. PPINICI spectra were determined at 140 eV (source temperature of 120°C) by the desorption method. The sample was applied to a rhenium wire mounted at the end of the direct inlet probe which was inserted into the plasma of the CI source. Unless stated otherwise, the wire was heated at the rate of 20 mA s⁻¹. The reagent gas was ammonia at 1 torr. EI spectra were determined at 70 eV and a source temperature of 150°C. Samples were applied to a rhenium wire at the probe tip.

Preparation of Derivatives

N-9 CCE derivatives of OGZ, OGDZ, iP, and DZ were prepared by heating the sample with anhydrous n-butanol (50 μL), triethylamine (3 μL), and 2-chloroacrylonitrile (10 μL; source: Fluka AG, Switzerland) for 30 min at 80°C. To prepare the N-9 CyE derivatives, the 2-chloroacrylonitrile was replaced with acrylonitrile. The reaction solutions were evaporated under vacuum at 40°C, and the residues were chromatographed on Merck HPTLC reverse phase plates (RP-2, 10 × 10 cm) using 40% (v/v) ethanol as solvent. Prior to use, the plates were washed with 80% (v/v) ethanol, and after chromatography, the spots of derivatives were eluted by stirring with this solvent (60 μL). An aliquot of the supernatant obtained by centrifugation was used for mass spectrometry.

The CCE derivative of OGDZ was hydrolyzed with β-glucosidase (6) and the aglycone was partitioned into n-butanol and purified by HPTLC as above.

Purification of Glucoside Fraction for Mass Spectrometry

To the glucoside fraction prepared previously from soybean (Glycine max) leaf blades (6), D5 OGZ, OGDZ, OGZR, and OGDZR (5 μg of each) were added. These D5 glucosides (labeled in the methyl and adjacent methylene group of the isoprenoid sidechain) were prepared according to Summons et al. (7). A solution of the glucoside fraction in acetic acid (1%, v/v) was passed through a Baker 10 SPE C18 column (0.5 g packing; J. T. Baker, Phillipsburg, NJ) which was washed with 1% acetic acid and then eluted with 25% (v/v) acetic acid at 5 ml/min. The eluate was collected, concentrated to dryness, and the residue was rechromatographed to give purified D5 OGZ, OGDZ, OGZR, and OGDZR.
methanol containing acetic acid (1%, v/v). The eluate was subjected to TLC on cellulose (6; layer washed with 65% v/v methanol) using n-butanol-14 N ammonia-water (6:1:2, upper phase) as solvent. Two UV-absorbing zones were clearly evident on the layer at Rf 0.15 (OGZ plus OGZR) and Rf 0.22 (OGDZ plus OGDZR). These were eluted with 65% (v/v) methanol and rechromatographed on Merck HPTLC reverse phase plates (RP-2) as described above for derivatives. Elution with 80% (v/v) ethanol (50–70 uL) yielded the four O-glucosides for PPINICI mass spectra; Rf values observed were: OGZ, 0.71; OGDZ, 0.62; OGZR, 0.87; OGDZR, 0.82.

**RESULTS AND DISCUSSION**

PPINICI mass spectra were first determined for D3 OGZ, OGDZ, OGZR, and OGDZR (Table I). The heating rate of the probe tip greatly influenced the spectra of OGZR and OGDZR. A rapid rate of heating favored quasi-molecular and molecular ions (MH* and M+), while a slow rate of heating enhanced the intensity of the fragment ions caused by dehydrobolsylation (−R ions). This is illustrated by the negative spectra of OGDZR. Thus, when the rhenium wire at the tip of the probe was heated at the rate of 5 and 20 mamp s−1, the intensity of the −R ion (m/z 387) was 1.3 and 0.28 times, respectively, that due to the M+ ion (m/z 520). However, at 100 mamp s−1, only the M+ ion was definitely evident. In the spectra given (Table I), a heating rate of 20 mamp s−1 was used which corresponds approximately to 20* s−1.

In the positive ion spectra of OGZ and OGDZ, the MH*
ions predominated, while [M-H]− ions were dominant in the negative ion spectra. The ratio of summed ion currents for [M-H]−/MH+ was 6 and 8 for OGZ and OGDZ, respectively. In the positive ion spectra of OGZR and OGDZR, the base peak was due to the −R ions (m/z 387 and 389, respectively), while M+ ions predominated in the negative ion spectra. As in the cases of OGZ and OGDZ, the summed ion currents due to the base peaks of the negative ion spectra greatly exceeded those of the positive spectra. Thus, the current ratios for m/z 518 (negative)/m/z 387 (positive) of OGZR, and m/z 520 (negative)/m/z 389 (positive) of OGDZR, were 35 and 21, respectively. Because of this great difference in sensitivity, negative ion mass spectrometry has clear advantages over positive ion spectrometry for detection and quantification of cytokinin O-glucosides. This was further substantiated in work with actual samples from soybean leaves.

Quantification of organic compounds by mass spectrometry using stable isotope labeled internal standards should be based on at least two ion pairs. By selection of a suitable heating rate, it is possible to fragment OGZR and OGDZR to obtain prominent −R fragment ions as well as MH+ and M− ions. Hence, suitable ion pairs can be readily obtained for quantification. In contrast, the O-glucoside bases, OGZ and OGDZ, yield pronounced quasi-molecular ions with little fragmentation. Hence, for quantification of these compounds by CI mass spectrometry, formation of stable derivatives suitable for liquid chromatography is desirable. These glucosides could then be quantified per se and again after derivatization and chromatography of the remaining sample. Agreement between the two analyses would validate the results. A derivatization procedure which would exhibit specificity for O-glucoside bases was developed and involved Michael-type additions of acrylonitrile or 2-chloroacrylonitrile to the N-9 position of the purine ring to yield compounds with CyE (Scheme I, I) or CCE (Scheme I, II) moieties. BAP has been converted to a CyE derivative for use as a synthetic intermediate (2); however, the reaction method was not adaptable to the µg level required in the present study. Under the conditions now used, cyanoethylation of the glucose hydroxyl groups did not occur. PPINICI spectra of these derivatives are given in Table I.

All spectra of the derivatives showed intense molecular ions or quasi-molecular ions with two exceptions. In the negative ion spectra of the CyE derivatives of OGZ and OGDZ, M− and [M-H]− ions were not detected, and intense fragment}

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**Table III. Levels of Cytokinlin O-glucosides in Soybean Leaf Blades Determined by Negative Ion MS and by RIA**

<table>
<thead>
<tr>
<th>Method of Analysis</th>
<th>Glucoside</th>
<th>OGZ</th>
<th>OGDZ</th>
<th>OGZR</th>
<th>OGDZR</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS of underivatized glucoside</td>
<td></td>
<td>19.8</td>
<td>40.1</td>
<td>&lt;2.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>MS of CCE derivative</td>
<td></td>
<td>19.0</td>
<td>37.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS of CCE aglycone</td>
<td></td>
<td>13*</td>
<td>38.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIA</td>
<td></td>
<td>22.4</td>
<td>44.1</td>
<td>0.56</td>
<td>0.53</td>
</tr>
</tbody>
</table>

*Not determined.

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**Figure 1. Negative ion mass spectra used for the quantification of OGDZ in soybean leaf blades.** The spectra were scanned over a m/z range of 30. A, Underivatized D5 OGDZ; B, underivatized mixture of D5 OGDZ and endogenous (D3) OGDZ purified from blade extract; C, CCE derivative of D5 OGDZ; D, CCE derivative of D5 plus D3 mixture purified from blade extract.
ions formed by cleavage of the CyE group were the ions of highest mass. The intensities of the positive and negative ion spectra were compared. Ion current for the most intense ion suitable for quantification in the negative spectrum was always greater than that in the positive spectrum, and this was especially so with CCE derivatives. Ion current ratios were as follows for D₃ glucosides:

\[
\begin{align*}
\text{CyE OGZ} & : 385 (-)/440 (+) = 15 \\
\text{CyE OGDZ} & : 387 (-)/442 (+) = 7.1 \\
\text{CCE OGZ} & : 473 (-)/474 (+) > 300 \\
\text{CCE OGDZ} & : 475 (-)/442 (+) > 100
\end{align*}
\]

The above ratios for CCE derivatives were determined with 50 to 100 ng of sample. However, when the amount of CCE-OGDZ was reduced to below 10 ng, no ion peaks were detectable in the positive spectrum (scanned m/z 150–650), but the M⁻ ion was still pronounced in the negative spectrum. Hence, the negative ion spectra are clearly preferable for quantification of these derivatives, especially CCE derivatives, because of the much greater sensitivity. The negative ion spectra of OGZ and CyE-OGZ exhibit prominent ions at m/z 179 attributable to a glucosolxy fragment ion.

Three types of evidence indicated that the CyE and CCE moieties were attached to the purine ring at N-9 and not to the sugar moiety. First, the UV spectra of the derivatives differed from those of N⁹-monosubstituted adenines and were characteristic of N⁹,9-disubstituted adenines (Table II). Second, ions in the mass spectra were indicative of cleavage to a CyE-adenine or CCE-adenine, fragment ion. Thus, in the CI spectra of CyE OGZ and CyE OGDZ, ions are present at m/z 189 (positive) and 187 (negative). The positive spectrum of CCE OGZ shows a chlorine-containing fragment ion at m/z 223 (Table I). Third, when the CCE derivative of OGDZ was treated with β-glucosidase (6), it yielded an aglycone which cochromatographed with the CCE derivative prepared directly from DZ. This was identified as 9-CCE-DZ (see below). Both compounds exhibited the same CI mass spectrum, which was as follows: negative ion—308* (100, M⁻), 272 (30), 271 (25), and 220 (11); positive ion—309* (100, MH⁺); an asterisk denotes the presence of a chlorine isotope peak. The ion current for the M⁻ ion was 87 times that of the MH⁺ ion.

The CCE derivative of DZ exhibited the following EI mass spectrum: m/z 308 (M⁺), 291 (10), 277 (10), 249 (53), 235 (100), 222 (35), 199 (13), 186 (8), 179 (12), 161 (18), 160 (20), 149 (35), 148 (21), 135 (49). This establishes that the CCE group is attached to the purine ring, and the UV spectra indicate that this was N-9 (Table II).

Soybean leaf extracts were purified as a described before (6) to yield an O-glucoside fraction. An aliquot (10%) was treated with β-glucosidase and the released Z, DZ, ZR, and DZR were quantified by RIA (6). D₃ O-glucosides were added to the remaining 90% of the sample which was then further purified (see "Materials and Methods") to separate the four O-glucosides. By methods based on the mass spectra detailed above, the endogenous intact O-glucosides were determined by short scan PPINICI mass spectrometry using the negative scans. OGDZ was quantified by three methods: directly, as the CCE derivative, and as the CCE aglycone derived by β-glucosidase hydrolysis (Table III; Fig. 1). The contents of OGZ and OGDZ were very similar to those determined by RIA. However, the levels of OGZR and OGDZR were too low to permit an accurate comparison of the two methods (Table III).

Negative ion mass spectrometry is a highly sensitive technique for quantification of cytokinin O-glucosides. For quantification of the O-glucoside bases, OGZ and OGDZ, two derivatives have been devised, namely CyE and CCE derivatives. The latter with a chlorine atom is preferable as it yields, by resonance capture ionization, an extremely intense molecular ion allowing ready quantification of ng amounts by short scan methods.

ACKNOWLEDGMENT

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LITERATURE CITED