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Isolation of novel indole-3-acetic acid conjugates by immunoaffinity extraction

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ABSTRACT

An analytical protocol for the isolation and quantification of indole-3-acetic acid (IAA) and its amino acid conjugates was developed. IAA is an important phytohormone and formation of its conjugates plays a crucial role in regulating IAA levels in plants. The developed protocol combines a highly specific immunoaffinity extraction with a sensitive and selective LC–MS/MS analysis. By using internal standards for each of the studied compounds, IAA and seven amino acid conjugates were analyzed in quantities of fresh plant material as low as 30 mg. In seeds of *Helleborus niger*, physiological levels of these compounds were found to range from 7.5 nmol g^{-1} fresh weight (IAA) to 0.44 pmol g^{-1} fresh weight (conjugate with Ala). To our knowledge, the identification of IAA conjugates with Gly, Phe and Val from higher plants is reported here for the first time.

1. Introduction

Indole-3-acetic acid (IAA), referred to as auxin, is an important phytohormone. It plays crucial roles in many aspects of the regulation of plant growth and development, including cell elongation [1], tropisms [2] and the establishment of apical-basal polarity in both individual cells and the whole plant [3]. Such wide-ranging regulation of developmental processes by IAA requires that its concentration in cells and tissues is rapidly and sensitively regulated in both space and time. One of the mechanisms by which IAA level in plants is regulated is by conjugation of free IAA with amino acids, giving rise to either biologically inactive, but hydrolyzable, conjugates with alanine (IAAla) and leucine (IALeu) or to unhydrolyzable conjugates with aspartic acid (IAAsp) and glutamic acid (IAGlu). These two groups of conjugates are believed to have a storage function or to precede degradation of excessive IAA, respectively [4].

Although plant materials consist of a very complex matrix containing large amounts of ballast compounds, quantification of IAA – the quantities of which per gram of fresh weight (FW) vary typically between tens and hundreds of pmol – is not too difficult to perform. In current practice, raw extract is usually purified by solid-phase extraction [5] and analyzed by GC–MS [6] following methylation [7], trimethylsilylation [6] or other kind of derivatization [8,9]. Analysis by HPLC coupled to tandem mass detection is also an option, both with [10] or without [11] prior derivatization.

Compared with free IAA analysis, the quantification of IAA amino acid conjugates is much more elaborate due to the significantly lower levels of the analytes present in plant material. Thus in *Arabidopsis thaliana*, a model organism often used in plant biology, Kowalczyk and Sandberg [12] have described, together with free IAA (the amounts of which ranged between 40 and 130 pmol per gram of fresh material) conjugates with the following four amino acids: Asp, Glu (both present at picomolar level), Leu (hundreds fmol g^{-1} FW) and Ala, with quantities as low as 20 fmol g^{-1} FW. The extracts from *A. thaliana* were subjected to solid-phase (SPE) extraction and after methylation analyzed by HPLC linked to tandem mass detection. The protocol appeared to be notably simpler than that of Tam and coworkers [13] who used preparative HPLC before quantification of IAAsp and IAGlu by GC–MS.

Recently, an analytical protocol for the quantification of IAA and its conjugates in rice (*Oryza sativa*) has been described [14], which consists of SPE and subsequent HPLC–MS/MS analysis without prior derivatization. The protocol allows quantification of IAA conjugates with these amino acids: Ala, Asp, Glu, Leu, Phe and Val. It was applied to rice samples of 20–100 mg in fresh weight.

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Table 1
Selected parameters of HPLC–MS/MS analysis of methyl esters of IAA and seven IAA amino acid conjugates. In parentheses, transitions of ^{15}N- and/or $^{2}H_5$-labeled standards are described.

RT (min)	Analyte	Transition	CV (V)	CE (eV)	LOD (fmol)	Dynamic range (pmol)	Calibration curve	R^2
8.60	IAGly-Me	247.2 > 130.1 (253.2 > 134.1)	19	22	0.2	0.004–2.5	$y = 0.9744x + 0.3592$	0.9998
11.41	IAAla-Me	261.2 > 130.1 (267.2 > 134.1)	21	19	0.7	0.004–2.5	$y = 0.9853x + 0.4162$	0.9995
12.53	IAAsp-Me_2	319.2 > 130.1 (325.2 > 134.1)	21	25	0.7	0.2–125	$y = 0.9829x + 0.3599$	0.9993
14.19	IAGlu-Me_2	333.2 > 130.1 (339.2 > 134.1)	21	35	0.6	0.04–25	$y = 1.0132x + 0.2683$	0.9998
16.19	IAA-Me	190.2 > 130.1 (195.2 > 134.1)	21	13	1.2	0.2–125	$y = 0.9693x + 0.1860$	0.9996
17.42	IAVal-Me	289.2 > 130.0 (295.2 > 134.1)	23	22	0.3	0.004–2.5	$y = 0.9962x + 0.4024$	0.9998
19.86	IALeu-Me	303.2 > 130.1 (309.2 > 134.0)	25	24	1.0	0.004–2.5	$y = 0.9888x + 0.3500$	0.9992
20.36	IAPhe-Me	337.2 > 130.0 (343.1 > 134.1)	16	27	0.6	0.004–2.5	$y = 0.9680x + 0.3064$	0.9998

However, only conjugates with Ala, Asp and Glu were detected in the selected plant material.

In this paper we introduce a complex analytical protocol suitable for isolation and quantification of IAA and a broad range of its amino acid conjugates. Based on a polyspecific anti-IAA immunoaffinity purification, it may be used to study novel (previously undescribed) naturally occurring IAA conjugates. Since we used internal standards for each of the analyzed compounds, we were able to quantify four known and three novel derivatives over a wide range of concentrations in which they are present in plant material.

2. Experimental

2.1. Reagents and materials

Indole-3-acetic acid and other indole compounds were obtained from OlChemIm (Olomouc, Czech Republic). ^{15}N- and/or $^{2}H_5$-labeled internal standards were prepared according to Ilić et al. [15]. Affi-Gel 10 was ordered from Bio-Rad (Hercules, CA, USA). Other reagents and solvents were provided by Sigma–Aldrich (St. Louis, MO, USA). Water was purified by the Simplicity 185 water purification system (Millipore, Bedford, MA, USA).

2.2. Preparation of antibodies and their use in immunoaffinity extraction

Polyspecific polyclonal antibodies against IAA and its conjugates were obtained by immunizing rabbits with an IAA–protein conjugate. To couple IAA to bovine serum albumin (BSA) through its carboxyl group, the procedure described by Weiler [16] was used, resulting in a hapten:protein molar ratio of 23. Purification of the antibodies and the preparation of immunoaffinity columns and their use were essentially as described earlier [17]. Capacity and recovery of the columns estimated by application of various amounts of indole-3-acetamide was about 3 nmol and 95–100%, respectively.

2.3. Sample processing and LC–MS/MS analysis

The extraction and subsequent SPE were similar to those used by Karin Ljung (personal communication). Approximately 30 mg (fresh weight) of plant material frozen in liquid nitrogen was ground with pestle and mortar and extracted for 5 min with 1 ml of cold phosphate buffer (50 mM; pH 7.0) containing 0.02% sodium diethyldithiocarbamate and the following ^{15}N- and/or $^{2}H_5$-labeled internal standards: [$^{2}H_5$]IAA, [^{15}N,$^{2}H_5$]IAAla, [^{15}N,$^{2}H_5$]IAAsp, [^{15}N,$^{2}H_5$]IAGlu, [^{15}N,$^{2}H_5$]IAGly, [^{15}N,$^{2}H_5$]IALeu, [^{15}N,$^{2}H_5$]IAPhe, and [^{15}N,$^{2}H_5$]IAVal.

After centrifugation (36,000 × *g*; 10 min; +4 °C), each sample was transferred into an eppendorf tube, acidified with 1 M HCl to pH 2.7 and applied on a C8 column (Bond Elut, 500 mg, 3 ml; Varian) pre-washed with 2 ml of methanol and equilibrated with 2 ml of formic acid (1%; v/v). The column was washed with 2 ml of methanol (10%; v/v) acidified with formic acid (1%; v/v) and retained analyte was eluted with 2 ml of methanol (70%; v/v) acidified with formic acid (1%; v/v). The eluate was evaporated to dryness *in vacuo*.

Samples were reconstituted in 100 μl of methanol acidified with concentrated hydrochloric acid (1 μl per ml of methanol) and methylated with 300 μl of ethereal solution of diazomethane. After 10 min the reaction mixture was evaporated under a stream of gaseous nitrogen (40 °C).

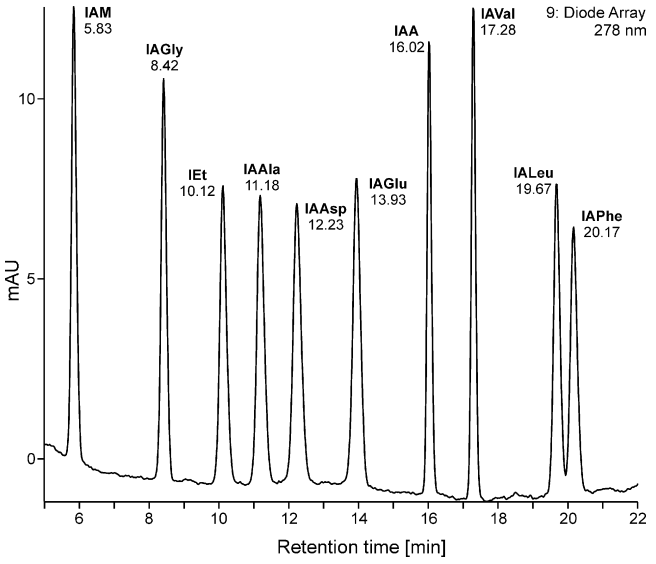

Fig. 1. HPLC–UV chromatogram (278 nm) of methyl esters of IAA, seven IAA conjugates and two other indole compounds retainable by a polyspecific anti-IAA immunoaffinity extraction: Indole-3-acetamide (IAM) and indole-3-ethanol (IEt).

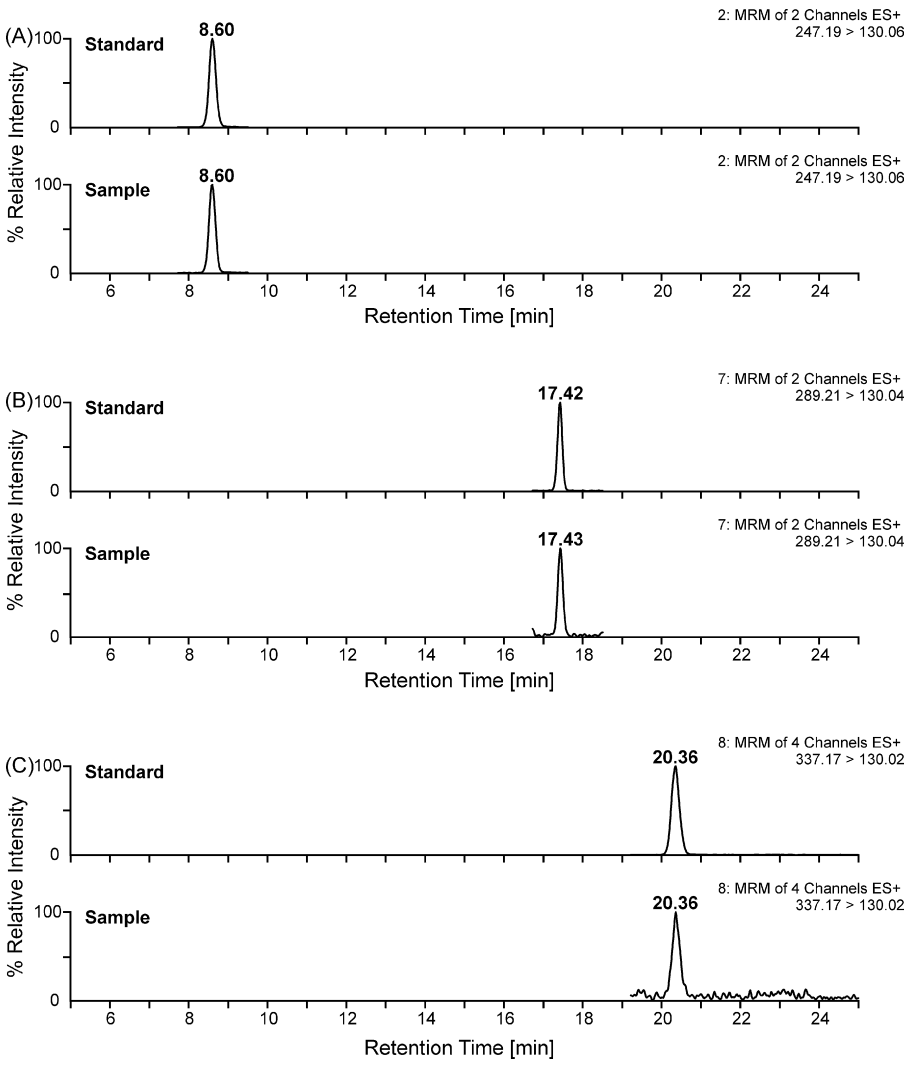

Fig. 2. Chromatograms of the newly discovered IAA amino acid conjugates compared with chromatograms of the corresponding authentic compounds: IAGly (A), IAVal (B) and IAPhe (C).

Prior to immunoaffinity purification, the sample was dissolved in 50 μl of ethanol (70%; v/v) and 450 μl of phosphate buffer (20 mM NaH_2PO_4; 15 mM NaCl; pH 7.2) and passed through a pre-column containing gel with immobilized BSA. The pre-column was then washed with 0.5 ml phosphate buffer and pooled effluent was applied on an immunoaffinity column with immobilized polyspecific rabbit polyclonal antibody against IAA. The application of the solution was repeated five-times. The immunoaffinity column was then washed three-times with 3 ml of H_2O and the analyte was subsequently eluted with 3 ml of methanol (−20 °C). The eluate was evaporated to dryness under nitrogen (40 °C).

Final analysis was done by HPLC coupled to tandem MS/MS detection with the use of a triple-quadrupole mass spectrometer. Separation was performed on an Acquity UPLC System (Waters) equipped with a Symmetry C18 column (5 μm, 2.1 mm × 150 mm; Waters) at 30 °C by gradient elution with a flow-rate of 250 μl min^{-1}. The mobile phase consisted of 10 mM aqueous formic acid and methanol containing 10 mM formic acid. The content of methanol kept at 25% during the first minute was then increased linearly to 38% (at 7 min), 40% (12 min), 58% (15 min), and 60% (26 min). The effluent was introduced into the ion source of a Quatro micro API tandem quadrupole mass spectrometer (Waters). The capillary voltage was set to +500 V, desolvation gas flow was 500 l h^{-1}, desolvation temperature was 350 °C and source block temperature was 100 °C. Other settings of the instruments, together with multiple reaction monitoring (MRM) transitions of individual compounds, are listed in Table 1.

3. Results and discussion

3.1. Anti-auxin polyspecific immunoaffinity purification

Immuno-based sample preparation techniques have extraordinary potential for trace analysis [18] and have been successfully utilized in analytical protocols used for quantification of various phytohormones including IAA [19,20], cytokinins [10,21–25] and abscisic acid [20,26,27].

In our study of IAA and its conjugates, we exploited the polyspecificity of anti-IAA antibodies. These were obtained by immunizing rabbits with a BSA conjugate in which IAA was linked to the protein through its carboxyl group (IAA-C1′-BSA). Such antibodies are capable of interacting specifically not only with IAA itself but also with other indole compounds substituted in position 3, such as indole-3-acetamide and indole-3-acetonitrile [28]. Furthermore, such antibodies have been reported [16] to cross-react in radioimmunoassays with the IAA homologues indole-3-propionic and indole-3-butyric acid and with IAA conjugated with aspartic

acid. However, this relatively high cross-reactivity is contingent on the free acids being transformed into their methyl esters. Hence, it is necessary to treat samples with diazomethane prior to analysis by an immunomethod [16,29].

An extensive study of our rabbit anti-IAA antibodies, performed by ELISA, has corroborated their polyspecificity. Compared to methylated IAA, cross-reactivities of 3-substituted indoles, including indole-3-acetamide, indole-3-acetonitrile, indole-3-ethanol and methyl esters of IAA conjugates, range typically between 85% and 210%. This allows the antibodies to be used in specific immunoaffinity purification of diverse IAA derivatives. We successfully used immunoaffinity extraction based on these antibodies to isolate and subsequently quantify IAA conjugates with Ala, Asp, Glu and Leu. Furthermore, we succeeded in isolating from a vascular plant and identifying novel naturally occurring IAA conjugates with the three amino acids: Gly, Val and Phe.

3.2. HPLC–MS/MS analysis

Using a linear methanol gradient acidified with formic acid, we achieved base-line separation (see Fig. 1) of a mixture containing methyl esters of IAA and seven IAA conjugates and also two other indole derivatives retainable by the developed immunoaffinity extraction: indole-3-acetamide and indole-3-ethanol (data not shown). In Table 1 the methyl esters are listed in order of their retention time.

Positive ESI tandem mass detection was run in multiple reaction monitoring (MRM) mode. Diagnostic transitions were similar to those used by Kowalczyk and Sandberg [12]: The parent ion of the protonated molecule $[M+H]^+$ fragments to a quinolinium ion of m/z 130.

To cover various recoveries of individual analytes, which may vary depending on the plant material being analyzed, we used internal standards corresponding to each of the studied compounds. All the standards comprised the indole skeleton labeled with five deuterium atoms; additionally, in standards of the conjugates the amino acid was ^{15}N-labeled. Thus values of m/z of internal standard parent ion differed by six units (or by five in the case of IAA) from those of the corresponding unlabeled compounds. Collision-induced dissociation yielded fragments of m/z = 135, 134 and 133. We used the fragment of m/z 134, the most intensive one, to perform MRM detection of labeled IAA and its conjugates. We achieved limits of detection (for signal-to-noise ratio of 3) around 1 fmol per injection. Detailed parameters together with other MS settings are listed in Table 1.

3.3. Identification of novel IAA conjugates

A recent study by Staswick et al. [30] has revealed the capability of *Arabidopsis* GH3 enzymes to catalyze synthesis of IAA conjugates with a wide range of amino acids, thus raising the question of whether IAA conjugates other than those with Ala, Asp, Glu and Leu exist. The question gains significance as Ludwig-Müller et al. [31] have very recently identified IAA conjugate with Val in non-vascular plant (moss *Physcomitrella patens*) grown on medium supplemented with IAA.

In our screening for putative IAA conjugates we concentrated on immature seeds of the Christmas rose (*Helleborus niger* L.), in which we had previously detected very high levels of free IAA (around 10 nmol g^{-1} FW). Alongside IAA and the conjugates described earlier [12] we identified novel IAA conjugates with Gly, Phe and Val.

In Fig. 2, chromatograms of newly discovered IAA conjugates are compared with chromatograms of the corresponding authentic compounds. Each pair of chromatograms is characterized by practically identical retention times of peaks for the standard and for the putative compound isolated from plant material. Each pair of chromatograms was obtained in MRM mode where the m/z of the parent ion corresponds to the m/z of the pseudomolecular ion $[M+H]^+$ of the respective IAA conjugate, while an m/z equal to 130 for the daughter ion corresponds to an m/z of the quinolinium ion, which is typical of fragmentation of IAA and its conjugates.

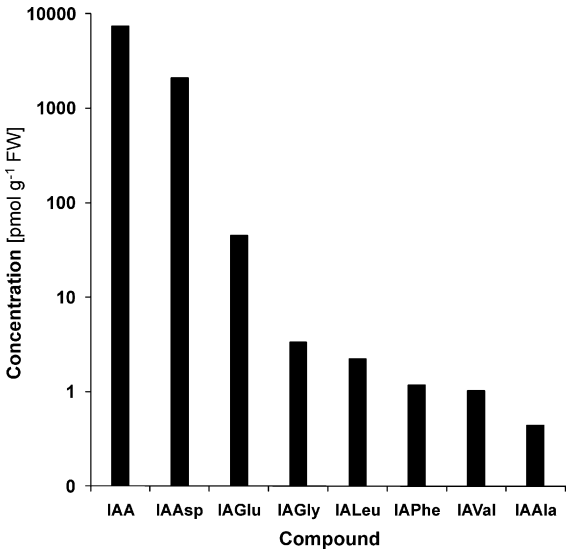

Fig. 3. Semi-logarithmic overview of IAA and seven IAA amino acid conjugates quantified in immature seeds of the Christmas rose (*Helleborus niger* L.).

The identity of the conjugates analyzed in *H. niger* seeds seems to be further verified by the fact that they passed through a preceding immunoaffinity purification designed to interact specifically with (3-substituted) indole skeleton. Thus, to our knowledge, for the first time naturally occurring IAA conjugates with Gly, Phe and Val have been identified in vascular plant.

3.4. Quantification of IAA and its conjugates in milligram amounts of plant material

Combination of the highly specific immunoaffinity extraction described above and a sensitive and selective LC–MS/MS – together with the application of internal standards for each of the studied compounds at the initial phase of sample processing – allowed us to reliably quantify IAA and a wide spectrum of its amino acid conjugates. Amounts of fresh plant material required for such an analysis typically lie between 25 mg and 40 mg.

On the basis of our previous experience, we supply the analyzed samples with amounts of internal standards corresponding to expected levels of respective native compounds. In a parallel manner we construct calibration curves which cover typical physiological levels of individual analytes in plant material. The curves

Table 2
Indole-3-acetic acid and its amino acid conjugates as quantified by HPLC–MS/MS in immature seeds and pericarp of *Helleborus niger*.

Analyte	Content in plant tissue (pmol g^{-1} FW)[a]	
	Immature seeds	Pericarp
IAA	7378 ± 17	313 ± 16
IAAsp	2089 ± 79	62.5 ± 3.7
IAGlu	44.9 ± 1.2	2.86 ± 0.05
IAGly	3.35 ± 0.19	n.d.[b]
IALeu	2.24 ± 0.11	1.60 ± 0.14
IAPhe	1.17 ± 0.17	n.d.
IAVal	1.02 ± 0.13	n.d.
IAAla	0.44 ± 0.02	n.d.

[a] Mean value of three independent analyses ± standard deviation.
[b] n.d.: not detected.

extend to a little under three orders of magnitude, with good linearity and correlation coefficients (R^2) between 0.9992 and 0.9998 (see Table 1).

Fig. 3 shows the results of an analysis of IAA and its conjugates performed on immature seeds of *H. niger*. The semi-logarithmic plot of the results demonstrates clearly the vast difference (of about four orders of magnitude) between the contents of free IAA (7.5 nmol g^{-1} fresh weight) and IAAla, the least abundant of quantified conjugates (0.44 pmol g^{-1} fresh weight). The value found for IAA itself is comparable to that estimated by Matsuda et al. [14], who analyzed auxins in seeds of rice (*O. sativa*).

Detailed results of our quantification are listed in Table 2 together with the results obtained by analysis of *H. niger* pericarp (husk). Levels of IAA and its conjugates with Asp, Glu and Leu in the pericarp were remarkably lower than those found in seeds, while conjugates with Ala, Gly, Phe and Val were below their limits of detection.

4. Conclusion

We developed a highly specific protocol for analysis of IAA and its amino acid conjugates in small quantities (about 30 mg) of fresh plant material. The protocol is based on a combination of polyspecific anti-IAA immunoaffinity extraction and a sensitive LC–MS/MS method. We successfully used the protocol for quantification of IAA and its conjugates in immature seeds and in the pericarp of *H. niger*. Furthermore, we isolated three novel naturally occurring conjugates of IAA with Gly, Phe and Val and confirmed their identity by analysis of corresponding synthesized standards.

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