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Purification and determination of plant hormones auxin and abscisic acid using solid phase extraction and two-dimensional high performance liquid chromatography☆

P.I. Dobrev*, L. Havlíček, M. Vágner, J. Malbeck, M. Kamínek

Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Rozvojová 135, 16502 Praha 6, Czech Republic

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Abstract

A method for separation and purification of plant hormones auxin and abscisic acid based on mixed mode reversed-phase anion-exchange solid phase extraction and two-dimensional HPLC was developed. Two-dimensional HPLC in "heart cutting" mode was very efficient in the purification of these two hormones. Its purification power is high enough to allow reliable on-line quantification of both hormones even with non-selective detectors.

Keywords: Auxin; Abscisic acid; Solid phase extraction; 2D-HPLC

1. Introduction

Plant hormones are of vital importance for the normal functioning of plants. Their minute quantities trigger basic developmental processes such as cell division, enlargement and differentiation, organ formation, seed dormancy and germination, leaf and organ senescence and abscission [1]. Plant hormones are difficult to analyze because they occur in very low amounts in plant extracts which are very rich in interfering substances, especially secondary metabolites. To cope with this problem the plant extract must undergo several purification steps using unrelated separation mechanisms in order to increase orthogonality and purification efficiency. Common purification procedures such as column chromatography, solid phase extraction (SPE), liquid–liquid extraction, etc. are employed for plant hormone purification. However, these procedures usually require significant amounts of solvent, time and labor. Furthermore, they all are "off-line" procedures often requiring sample pre-treatment (e.g. preconcentration) when used in series.

"On-line" purification methods encompassing multidimensional HPLC have become increasingly popular. Separation of peptides by comprehensive two-dimensional high performance liquid chromatography (2D-HPLC) has appeared to be complementary to the traditional 2D-gel electrophoresis in the proteome analysis [2–4]. So called "Heart-cutting" 2D-HPLC, in which only a part of the first dimension run is "heart-cut" and introduced into the second dimension, is a very suitable purification technique when a limited number of substances have to be purified [5,6]. Features contributing to 2D-HPLC popularity are high purification potential, reproducibility, robustness, high throughput and unattended operation.

Auxin (indole-3-acetic acid, IAA) and abscisic acid (ABA) are plant hormones with contrasting biological functions. Whereas IAA stimulates growing processes such as cell elongation and division, ABA controls plant senescence and responses to stress [1]. However, IAA and ABA exhibit many similar chemical properties which can be exploited for their chromatographic purification. Both IAA and ABA are relatively hydrophobic compounds containing a carboxylic group

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* Corresponding author. Tel.: +420 2 20390444; fax: +420 2 20390446.
E-mail address: dobrev@ueb.cas.cz (P.I. Dobrev).

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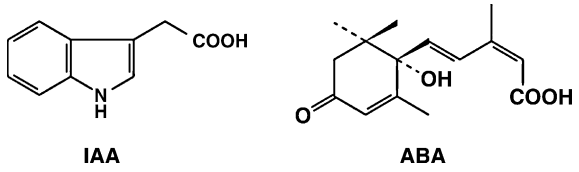

Fig. 1. Chemical structures of indole-3-acetic acid (IAA) and abscisic acid (ABA).

(Fig. 1). Therefore, when IAA and ABA are extracted and purified from plant material by common chromatographic techniques they very often end up in the same fraction.

IAA and ABA determination is usually preceded by extensive purification involving, for example liquid–liquid extraction [7–9], solid phase extraction [10], or HPLC purification [11]. Pre-purification of IAA and ABA on immuno-affinity columns was also reported [12]. Sample purification time can be reduced by application of a very specific separation and quantification technique that is able to distinguish the analyte of interest from the matrix. In the past, immunoassays as specific quantification methods were popular [13]. They excel in specificity due to the unique ligand–antibody binding. However, when applied to low purity extracts, quantification using immunoassays can be misleading due to the cross-reactivity of antibodies or their inhibition (or activation) by interfering substances. Capillary electrophoresis (CE) was also applied for the determination of IAA and ABA [8,14,15]. This technique is promising due to its high resolving power, excellent sensitivity and low sample consumption. Although good CE methods were developed for pure hormones, the complex nature of plant extracts presents the main challenge for CE. Mass spectrometric (MS) detection is another very specific technique for analyte determination. GC–MS is a well recognized technique for determination of IAA and ABA [10,16,17]. However, it also requires considerable sample purification as well as derivatization to run on GC. There have been reports on utilizing the selectivity of MS detection to measure hormones in crude extracts. Gómez-Cadenas et al. [18] analyzed ABA in crude citrus extracts using LC–MS. However, as the authors pointed out, the technique is reliable only when tandem MS (MS/MS) in selected reaction monitoring mode and internal standardization with heavy isotope labeled ABA are used. They were not successful in ABA quantification in single stage MS (selected ion monitoring mode) due to the high background. There are several other reports where brief sample pre-purification is followed by tandem MS. For example, several acidic phytohormones, including IAA and ABA, were analyzed on GC–MS/MS, preceded by purification on one or two microscale SPE columns [10]. Similarly, rapid extraction of ABA and its metabolites and purification through single polymeric SPE column followed by quantification on LC–MS/MS was reported [19]. In conclusion, there is a compromise between thorough pre-purification followed by lower specificity detection and brief pre-purification followed by very selective but expensive detection.

Recently we developed a procedure for fast and efficient extraction and purification of three groups of plant hormones, namely IAA, ABA and cytokinins [20]. It allowed us to prepare a very pure fraction of cytokinins. However, the fraction of co-eluting IAA and ABA contained relatively high amounts of UV-absorbing and fluorescing contaminants. This shortcoming complicated their quantification using HPLC coupled to fluorescence detection (IAA) and/or mass spectrometry (IAA and ABA). To overcome this problem we developed an additional SPE purification step, as well as a 2D-HPLC system that allowed us to obtain very pure separate fractions of IAA and ABA and to quantify these compounds with much higher reliability.

2. Experimental

2.1. Chemicals and materials

Unlabeled IAA and ABA were from Sigma, St. Louis, MO, USA. Radioactive [5-^{3}H]IAA (0.74 TBq mmol^{-1}) was from ARC Inc., St. Louis, MO, USA, and [G-^{3}H]ABA (1.74 TBq mmol^{-1}) was from Amersham Biosciences UK Ltd., Little Chalfont, UK. $^{13}C_6$-IAA was from CIL, Andover, MA, USA. Deuterated ABA ($^{2}H_6$-ABA) was synthesized by a modified procedure of Rivier et al. [21] which is based on base-induced hydrogen exchange as follows: A solution of NaO^{2}H was prepared by reaction of Na with $^{2}H_2O$ (99.9%, IsotopTech, Russia) under an argon atmosphere. ABA was dissolved into 0.3 N NaO^{2}H and kept at 65 °C for 1 h. After cooling, the solution was acidified to pH 2 with $^{2}H_3PO_4$ (prepared by reaction of P_2O_5 with $^{2}H_2O$). The resulting $^{2}H_8$-ABA was filtered, converted to $^{2}H_6$-ABA by washing with ice cold $^{1}H_2O$, dried and re-crystallized from a mixture of ethyl acetate and diethyl ether. The synthesized $^{2}H_6$-ABA had identical melting point and infra-red spectrum as the parent ABA. Mass spectrometric analysis (electro-spray, negative mode) revealed a molecular ion at *m*/*z* 269 with no detectable *m*/*z* 263 from the molecular ion of parent ABA confirming complete exchange of six deuterium atoms in the molecule. The synthesized $^{2}H_6$-ABA is stable, provided that pH is kept lower than pH ∼ 8.

1-Methyl-3-nitro-1-nitrosoguanidine (MNNG, 97%) was from Aldrich, Milwaukee, WI, USA. HPLC gradient grade methanol and acetonitrile were obtained from Merck KGaA, Darmstadt, Germany. Formic acid and ammonium hydroxide both of p.a. grade were from Lachema a.s., Neratovice, Czech Republic. Oasis MAX columns (150 mg/6 cc) were obtained from Waters, Milford, MA, USA.

2.2. Recoveries of standards of IAA and ABA on Oasis MAX columns

Tested compounds were dissolved in 5 ml of 1 M formic acid to give 0.5–1 AU and the actual absorbances of solutions were measured. The standard solution of a single compound