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Immunoaffinity chromatography combined with tandem mass spectrometry: A new tool for the selective capture and analysis of brassinosteroid plant hormones

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ABSTRACT

Brassinosteroids (BRs) are plant-specific steroid hormones that play essential roles in the regulation of many important physiological processes in plant life. Their extremely low concentrations (~pmoles/g FW) in plant tissue and huge differences in polarity of individual members within the BR family hamper their detection and quantification. To address this problem, an immunoaffinity sorbent with broad specificity and high capacity for different BR metabolites containing a monoclonal antibody (mAb) against a BR spacer (20S)–2α,3α-dihydroxy-7-oxa-7α-homo-5α-pregnane-6-one-20 carboxylic acid (BR4812) was used for the rapid and highly selective isolation of endogenous BRs containing a 2*α*,3*α*-diol in ring A from minute plant samples. This enrichment procedure was successfully applied as a sample preparation method prior to quantitative analysis of BRs in real plant tissues by ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). Use of immunoaffinity chromatography (IAC) increased the sensitivity of the UHPLC-MS/MS analysis owing to improvements in the BR signal-to-noise ratio (S/N) and matrix factor (MF). Although MF values of BRs analyzed in classical samples ranged from 8.9% to 47.4%, MF values for the IAC purified samples reached 44.5–96.6%. Thus, the developed IAC-UHPLC-MS/MS approach was shown to be a simple, robust, effective and extremely fast procedure requiring minute amounts of plant samples suitable for the quantitative profiling of many BR metabolites, helping to overcome the major problems associated with their determination in very complex plant matrices.

1. Introduction

Brassinosteroids (BRs) are steroidal plant hormones that affect many physiological processes throughout the plant's life cycle, including vascular differentiation, male fertility, flowering senescence and photomorphogenesis as well as plant architecture [1]. These hormones are also known to modulate the response of plants to abiotic and biotic stress [2,3]. To date, more than 70 BRs have been isolated from plants [4,5] and they have been detected in almost every part of the plant, such as in pollen, flower, buds, fruits, seeds, leaves, shoots, roots and also insect and crown galls [6,7]. The physiological concentrations of BRs in plants are extremely low. Compared to reproductive tissues, such as pollen and immature seeds, where micrograms of BRs are found (1–100 μg/kg fresh weight, FW), other plant parts contain BRs

Abbreviations: Ab, antibody; BRs, brassinosteroids; HRP, horseradish peroxidase; IAC, immunoaffinity chromatography; IAG, immunoaffinity gel; ELISA, enzyme-linked immunosorbent assay; EtOAc, ethyl acetate; Et_3N, triethylamine; mAb, monoclonal antibody; SPE, solid phase extraction; UHPLC-MS/MS, ultra-high performance liquid chromatography-tandem mass spectrometry; *nor*BL, 28-*nor*brassinolide; DL, dolicholide; *nor*CS, 28-*nor*castasterone; *homo*DL, homodolicholide; DS, dolichosterone; BL, brassinolide; *epi*BL, 24-*epi*brassinolide; *homo*DS, homodolichosterone; CS, castasterone; *epi*CS, 24-*epi*castasterone; *nor*TE, 28-*nor*teasterone; *homo*BL, homobrassinolide; *homo*CS, homocastasterone; TE, teasterone; TY, typhasterol

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only at nanograms per kilogram FW levels [5]. These low concentrations of BRs are problematic when attempting to quantify their levels in plant samples [8]. A wide range of methods are currently employed for their determination in plants, including bioassays, diverse chromatographic procedures and immunoassays (radioimmunoassay-RIA, enzyme-linked immunosorbent assay-ELISA). The most widely used bioassays are the second bean internode bioassay and rice-lamina inclination test [9,10]. Interestingly, these bioassays were used to isolate the first BRs identified in nature, i.e., brassinolide (BL) (Fig. S1) [11]; and castasterone (CS) (Fig. S1); [12]. The detection limits of the above bioassays vary from $2{\cdot}10^{-11}$ to $1{\cdot}10^{-13}$ mol. Their main disadvantage is they are only able to detect BRs with certain biological activities: non-active biosynthetic precursors and metabolites cannot be detected using this approach. To address this problem with relatively straightforward analytical instrumentation, immunoassays were developed from the 1990s onwards. The first assay was performed in RIA format [13]. Antibodies against CS have been generated in rabbits and shown to recognize a number of natural BRs with different specificities but with only low levels of cross-reactivity toward brassinosteroid 6-deoxoderivatives. This method has been applied for the quantitation of BRs in bean seeds and stems and BR localization in cellular organelles of the rye-grass *Lolium perenne* [14]. The weakness of this methodology is that there is little or no cross-reactivity with BRs bearing substituents at position C-24 with different structure and side chain conformation compared to that of CS. Hence, an immunogenic conjugate synthesized from 24-*epi*castasterone (*epi*CS; Fig. S1) and 24-*epi*brassinolide (*epi*BL; Fig. S1) has been prepared and used to quantify these BRs and their C-24 analogues in different biological samples by means of ELISA [15–17]. Among the available hyphenated techniques, gas chromatography-mass spectrometry (GC-MS) is the most widely used approach for the instrumental analysis of BRs. However, because BRs are non-volatile, they must be derivatized prior to analysis by GC. Methaneboronic acid and trimethylsilane are frequently used as derivatization agents [18–20].

Recently, liquid chromatography-mass spectrometry (LC-MS) has become the most effective detection platform for the quantitative analysis of BRs. Several efforts have been made to improve this analytical approach, either by optimizing the BR isolation procedure or by focusing on analyte structure modification before MS detection [21–24]. Xin and co-workers [25] have reported a highly sensitive analytical method using purification of plant extracts based on solid phase extraction (SPE) with a mixed-mode anion exchange (MAX) and cation exchange (MCX) sorbent followed by a BR vicinal diol derivatization step before using ultra-high performance LC-tandem MS (UHPLC-MS/MS) detection in multiple reaction monitoring mode (MRM). Unfortunately, this method has only been applied for the quantification of four bioactive BRs (BL, CS, typhasterol-TY and teasterone-TE) in Arabidopsis and rice plants. Deng and co-workers quantified endogenous BRs in sub-milligram fresh plant sample by pipette-tip solid-phase extraction coupled with ultra-performance liquid chromatography tandem mass spectrometry (PT-SPE-UPLC-MS/MS), in which a quaternary ammoniumphenyl boronic acid was used for chemical derivatization of BRs [26]. Ding and co-workers [27,28] improved the methodology for assaying BRs in plant tissues by using magnetic SPE combined with in situ derivatization and UHPLC-ESI-MS/MS analysis. This method largely simplified the sample preparation procedure. The assay can be accomplished within 1 h and the amount of sample can be reduced to 100 mg FW. However, the approach has limitations of reproducibility and robustness. Furthermore, in many cases, laborious purification and derivatization steps are required prior to the chromatographic separation and final BR analyses.

To improve the rapidity, throughput and convenience of BR analyses, we have developed an immunoaffinity chromatography (IAC) method for the fast and selective purification of 2*α*,3*α*-diol-containing BRs. This approach is based on a unique monoclonal antibody (mAb) that is able to bind almost all natural and bioactive BRs. As reported here, highly purified BR preparations containing only traces of other UV- and MS-absorbing material can be obtained in this way. We also report the successful combination of IAC with a sensitive UHPLC-MS/MS step for determining endogenous BR levels in plant tissues. To demonstrate the potential of the technique, we isolated several BRs from oil seed rape (*Brassica napus* L.) flower tissue prior to their quantitation by UHPLC-MS/MS in MRM mode and validated the method accordingly.

2. Materials and methods

2.1. Chemicals

Brassinosteroids (brassinolide, 24-*epi*brassinolide, 28-*nor*brassinolide, castasterone, 24-*epi*castasterone, 28-*nor*castasterone, 28-homocastasterone, 28-*nor*teasterone, campesterol, campestanol and stigmasterol) were obtained from OlChemIm Ltd. (Olomouc, Czech Republic). Deuterium-labelled BRs ([26-$^2H_3$]brassinolide, [26-$^2H_3$]castasterone, [26-$^2H_3$]*epi*brassinolide, [26-$^2H_3$]*epi*castasterone, [26-$^2H_3$]28-*nor*brassinolide and [26-$^2H_3$]28-*nor*castasterone) were synthesized in accordance with [51–54]. Other unlabelled BR standards (28-homobrassinolide, dolicholide, 28-homodolicholide, dolichosterone, 28-homodolichosterone, teasterone and typhasterol) were purchased from Chemiclones Inc. (Waterloo, Canada). Formic acid (FA), methanol (MeOH, HPLC grade) and acetonitrile (ACN, HPLC grade) were purchased from Merck (Darmstadt, Germany). Deionized (Milli-Q) water obtained from a Simplicity 185 water system (Millipore, Bedford, MA, USA) was used to prepare all aqueous solutions. All other chemicals (analytical grade or higher purity) were from Sigma-Aldrich (St. Luis, MO, USA).

2.2. General procedures used for the synthesis of tritium-labelled BRs

^3H and ^1H NMR spectra were acquired on a Bruker Avance II 50 MHz spectrometer (Bruker BioSpin Corp.). For ^3H spectra, the signal of water at 4.7 ppm was taken as an external standard. Radio-HPLC was performed using a system consisting of a Waters Delta 600 pump and controller, Waters 2487 UV detector (all Waters, Milford, MA, USA), RAMONA radio chromatographic detector (Raytest, Germany) with interchangeable fluid cells (for preparative runs, a cell with a single small crystal of solid scintillator was used; for analytical runs, the column effluent was mixed with a Zinsser Quickszint Flow 302 cocktail in 1:3 ratio) and Waters Fraction Collector III. Water–organic solvent gradients were used as mobile phases for radio-HPLC (organic solvents are specified in each experiment). Data were collected and processed using Empower software (version 2.0, Waters, Manchester, UK). Radioactivity was measured using a Perkin-Elmer Tri-Carb 2900TR liquid scintillation counter (LSC) in a Zinsser Quicksafe A cocktail. Evaporations were carried out using a CentriVap® benchtop concentrator (Labconco Corp., MO, USA). Ethyl acetate (EtOAC), which was distilled from P_2O_5, the 5% PdO on $CaCO_3$ catalyst and triethylamine (Et_3N) were all purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Solvents used for radio-HPLC were of "for gradient HPLC" purity. Other solvents were used as purchased.

2.3. Synthesis of 24-[3*β*-^3H]epicastasterone

The 3*β*-chlorocarbonate [29] (3.7 mg, 6.5 μmol) and 5% PdO/$CaCO_3$ (37.2 mg, 12.4 μmol) were placed in a 1 mL flask containing a magnetic stir bar and dry EtOAc (0.7 mL) and Et_3N (5 μL, 31 μmol) were added. The flask was connected to a tritiation manifold, the reaction mixture was degassed by three successive freeze-thaw cycles under vacuum and then 5 Ci of carrier-free tritium gas was transferred. The reaction mixture was vigorously stirred under tritium gas (starting pressure≈600 mBar) at room temperature for 24 h. Afterwards, the