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A new ELISA for quantification of brassinosteroids in plants

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ABSTRACT

Starting from (22*R*,23*R*)-2α,3α,22,23,26-pentahydroxy-5α-cholestan-6-one 26-hemisuccinate, conjugates of 28-norcastasterone with horse radish peroxidase and bovine serum albumin were prepared. The latter conjugate was injected into rabbits; produced polyclonal antibodies were used to quantitate 6-keto-brassinosteroids. The newly developed analytical system was used in combination with two other immunoenzymatic assays for brassinosteroids to determine individual compounds of this series. In addition, a direct method of brassinosteroid analysis was proposed. It has the advantage of requiring no sample pretreatment steps such as extraction with organic solvents and chromatography.

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1. Introduction

Brassinosteroids (BS) are low molecular bioregulators playing an important role in plant growth and development [1]. Content of natural BS in plant sources is very low (less than 10^{-5}%) [2], and to achieve notable biological effects from their application, only a minute amount of exogenous BS is required. A pronounced plant growth stimulating and adaptogenic effect was observed on BS treatment of various plant species at a dose 5–20 mg/ha resulting in a positive effect on the quantity and quality of crops and tolerance against various biotic and abiotic stresses [3]. These features of BS have made them attractive as active ingredients for agrochemicals [4].

The most active and the most important BS from both scientific and practical point of view are those containing 6-oxo-7-oxa- or a 6-ketone function in the B-cycle [5–8] (Fig. 1). 24-Epibrassinolide **2** and 28-homobrassinolide **3** bearing a lactone moiety in the B-cycle have already found a practical use in agriculture [9,10].

It is accepted that compounds **5–8**, which belong to 6-keto-BS subgroup, are not only biosynthetic precursors of the corresponding lactones **1–4**, but they also play an independent role in plant growth and development [11]. There are reasons to believe that these highly active and easily accessible BS could also find their practical applications in agriculture. One of the preconditions to achieve their massive use is a simple, selective and highly sensitive method for their analysis. Based on our and others experience in BS research [12–20], the ELISA could be considered as a method of choice for the routine analyses.

In this respect, the first task of the present investigation was to develop an ELISA for 6-keto-BS. A certain disadvantage of immunochemical methods is that they are group specific ones and cannot be applied for analysis of an individual compound. The obvious solution of the problem is a combined application of several immunoassays separately quantifying the group components and/or immunoassaying of chromatographic fractions. In this connection, the present study proposes quantification of individual BS of 28-homo-series (28-homocastasterone and 28-homobrassinolide) by a joint utilization of the assay for 6-keto-BS (**IA1**) elaborated in this study with previously developed test systems for B-lactone-BS (**IA2**) [21] and 28-homo-BS (**IA3**) [22] (Fig. 2). Although each of these three assays is a group specific, their groups are different, and this allows differentiation of the hormones that are interfering when the only one group is analyzed with a single ELISA.

Abbreviations: BS, brassinosteroids; BSA, bovine serum albumin; C.V., coefficient of variation; LOD, limit of detection; DCC, *N,N′*-dicyclohexylcarbodiimide; DW, dry weight; HRP, horse radish peroxidase; NHS, *N*-hydroxysuccinimide; PBS, phosphate buffered saline; SD, standard deviation; SIM, selective ion monitoring; TMB, 3,3′,5,5′-tetramethylbenzidine; Tris, tris(hydroxymethyl)aminomethane.

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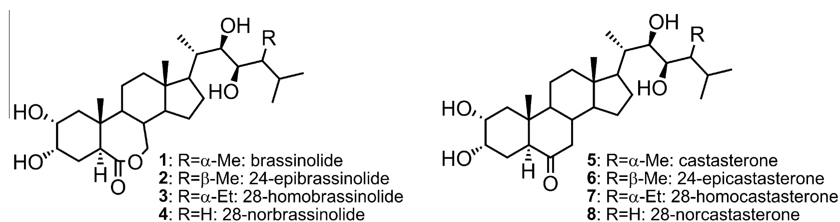

Fig. 1. Structures of the most important natural brassinosteroids.

2. Experimental

2.1. Reagents and materials

The chemicals and materials used in this study were purchased from following sources: DCC, NHS, HRP, dioxane, and Sephadex G-25 from Sigma–Aldrich (USA); BSA from Acros Organics (Germany); chloroform, cyclohexane from ECOS-1 (Russia); complete Freund's adjuvant from ICN Biochemicals (France); methanol from Fisher Scientific UK Ltd. (UK); silica gel from Macherey–Nagel GmbH & Co KG (Germany). High binding capacity 96 flat-bottom wells microtiter plates (Maxisorp™) used acquired from Nalgene Nunc (Thermo Fisher Scientific, Inc., USA). The following buffers were used for the immunoassay: coating buffer 1 (0.05 M PBS, pH 7.4, containing 0.9% NaCl), coating buffer 2 (0.025 M PBS, pH 7.4, containing 0.1% BSA, 0.02% Tween™ 20), blocking buffer (0.025 PBS, pH 7.4, containing 0.1% BSA, 2% sucrose, 5% sorbitol), assay buffer (0.05 M Tris, pH 7.4, containing 0.9% NaCl, 0.1% BSA, 0.02% Tween™ 20) and washing buffer (1% NaCl, containing 0.02% Tween™ 20). The ready solution of TMB in the substrate buffer containing H_2O_2 (Enhanced K-Blue TMB Substrate, Neogen, USA) was used for staining in assay, 5% H_2SO_4 was used as a stopping reagent for quenching the color reaction.

BS and their derivatives were synthesized in the Laboratory of Steroid Chemistry (Institute of Bioorganic Chemistry, NAS of Belarus); other steroids were purchased from Steraloids Inc. (USA).

2.2. HPLC

Preparative high-performance liquid chromatography was carried out on a HPLC–Hitachi (UV-VIS Detector L-4250, Intelligent Pump L-6200A) with reversed phase using column RP18 (LiChroCART 250 mm × 10 mm × 10 μm). 70% Aqueous acetonitrile was used as mobile phase with flow rate 1.5 mL/min. UV detection was performed at a wavelength of 204 nm.

2.3. HPLC–MS

The HPLC–MS experiments were carried out on a Accela chromatograph (Thermo Electron Corp., USA) linked to a LCQ Fleet 3D ion trap mass spectrometer equipped with an electrospray interface (Thermo Fisher Scientific, USA) with reversed-phase column C18 HYPERSIL Gold (50 mm × 2.1 mm × 1.9 μm). The column thermostat was set at 25 °C and 75% acetonitrile in water was used as a mobile phase for 15 min at a flow rate of 100 μL/min. The column was equilibrated to initial conditions for 5 min. The ESI-MS parameters (positive mode) were as follows: spray voltage (4.5 kV), capillary temperature (325 °C), and capillary voltage 36 V. Nitrogen was used as sheath, auxiliary and sweep gas. The sheath, auxiliary and sweep gas flow rates were 30, 0 and 0 arbitrary units, respectively. Data were processed by Xcalibur software (version 2.0.6, Thermo Electron Corp., USA).

2.4. Synthesis of the immunogenic conjugate of 28-norcastasterone with BSA

A solution of (22*R*,23*R*)-2α,3α,22,23-tetrahydroxy-5α-cholestan-6-one 26-hemisuccinate **9** (60 mg, 107 μmol, prepared according to [21]) and NHS (16 mg, 139 μmol) in anhydrous dioxane (10 mL) was added sequentially to a solution of DCC (26 mg, 126 μmol) in anhydrous dioxane (10 mL) at 8–10 °C. The reaction mixture was stirred for 30 min at this temperature and then for 6 h at room temperature. The precipitated dicyclohexylurea was removed by filtration, and obtained solution of ester **10** was added to a solution of BSA (120 mg in 20 mL of 0.1 M $NaHCO_3$, pH 8.35). The mixture was kept for 20 h at room temperature. Then an excess of activated ester and dioxane was removed by dialysis against 0.05 M NaCl to give, after lyophilization, 160 mg of the conjugate **11**, which was frozen and stored at −18 °C.

2.5. Synthesis of labeled antigen

A solution of active *N*-hydroxysuccinimide ester **10** (4.5 mg, 6.6 μmol), prepared as described above, was added to a solution of HRP (9 mg) in distilled water (900 μL) with 0.1 M $NaHCO_3$ (2–3 drops, pH 8.35). The reaction mixture was stirred at 10 °C for 1.5 h and purified on a column with Sephadex eluting with distilled water. The yellow–brown fraction containing conjugate **12** was collected, diluted with glycerol (1:2), and stored in a freezer at −18 °C.

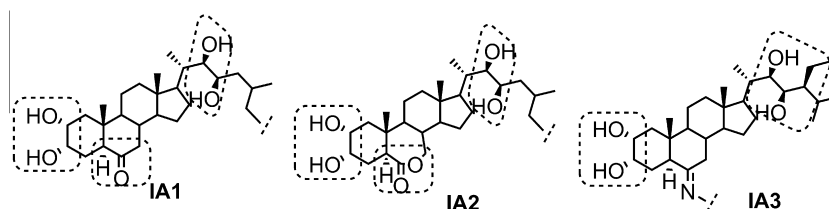

Fig. 2. Immunodominant fragments of BS-molecules specific for recognition by **IA1**, **IA2**, and **IA3**.

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