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Liquid chromatography–electrospray ionization-mass spectrometric quantitation of juvenile hormone III in whole body extracts of the Formosan subterranean termite

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ABSTRACT

Juvenile hormone (JH) III is responsible for control of a variety of insect physiological and developmental states, including caste differentiation of the Formosan subterranean termite (*Coptotermes formosanus* Shiraki). We report here a simplified, efficient sample preparation and an optimized LC–ESI-MS method for quantifying JH III in whole body extracts. Sample preparation comprises hexane extraction (from termite whole bodies) and C18 cartridge purification. Previous LC–ESI-MS protocols exhibited the following two problems: (1) ion fragmentation differed when comparing spectra from insect samples and authentic JH III and (2) a JH III monitoring ion was not resolved from other unknown compounds in whole body samples from termites. To overcome these problems, we used a pentafluorophenyl LC column and water/acetonitrile containing ammonium acetate as solvent. In a mass chromatogram (*m*/*z* 235) of termite samples, a peak was detected at the retention time of authentic JH III, and MS^2 of this peak confirmed that the ion is a fragment of JH III, $[M–CH_3OH+H]^+$, being the base peak in both termites and authentic JH III samples. The protocol enables quantification of JH III in a single termite with signal/noise >10:1 and the limit of quantification is 21 pg.

1. Introduction

Juvenile hormones (JH) are a group of structurally related sesquiterpenes that regulate many aspects of physiology in insects, including development, reproduction, polymorphism, diapause, and pheromone production [1,2]. There are six known JHs in insects, however, many insect species contain only JH III, which has the structure: methyl 10*R*,11-epoxy-3,7,11-trimethyl-2*E*,6*E*-dodecadienoate (Fig. 1).

Quantification of JH III is important to ascertain its physiological roles in insects. Previous techniques included bioassays, immunological analysis, and radiochemical methods [3–5]. Gas chromatography–mass spectrometry (GC/MS) has been used as an accurate method for quantification of JH III. Bergot et al. used GC–electron impact (EI)-MS and selective ion monitoring (SIM) [6]. A required step for this method was to convert the monoepoxide JH III to its corresponding methoxy-hydrin derivative. In 2001, Teal et al. were able to avoid this step in hemolymph samples by using capillary GC–chemical ionization (CI)-MS [7]. They directly analyzed hexane extracts of Caribbean fruit fly hemolymph by GC–CI-MS, and could detect as little as 21.1 pg of JH III in the samples. However, their work comprised extraction of hemolymph, which is a much less complex sample than insect whole body extracts and does not sample glandular material or cuticle for JH III.

Recently, in addition to GC–MS methods, liquid chromatography (LC)–MS has also been applied to quantify JH III titer in insects, first by Westerlund and Hoffmann [8]. They chromatographed samples prepared from hemolymph on a reverse-phase C18 HPLC column using a water/methanol solvent system and the effluent from the HPLC was detected by electrospray ionization (ESI)-MS in the positive mode. This LC–ESI-MS protocol has been used in several other reports, e.g. Refs. [9–12]. However, in their report, ion fragmentation patterns of JH III were different when comparing samples of insect hemolymph and authentic JH III [8]. In mass spectra of hemolymph samples, the JH III sodium adduct ion (*m*/*z* 289) was the base peak [8,10,11]. In contrast, for their authentic JH III, a fragment ion of JH III (*m*/*z* 235), $[M–CH_3OH+H]^+$, was the base peak [9,10]. Westerlund and Hoffmann suggested that JH III sodium adduct ion

Abbreviations: JH, juvenile hormone; PFP, pentafluorophenyl.

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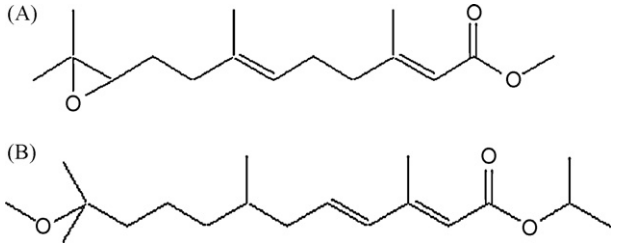

Fig. 1. Chemical structures of JH III ((A) $C_{16}H_{26}O_3$; MW: 266.38) and methoprene ((B) $C_{19}H_{34}O_3$; MW: 310.47).

is detected as a base peak due to the high abundance of sodium ion in insect hemolymph, and Ichikawa et al. reported the addition of a trace amount of sodium salt to the mobile solvent could enhance intensity of the JH III sodium adduct ion in LC–ESI-MS [13]. It is our opinion that to optimally quantify JH III by LC–ESI-MS, it is necessary to find a method in which the ion fragmentation pattern of JH III is the same when comparing samples from insects and authentic JH III, and that samples and standards should be analyzed under similar ionic conditions so that an ion detected at either *m*/*z* 289 or 235 should be extant in both samples as a major peak exhibiting the same patterns of ion fragmentation. An obvious difference in the mass spectra between authentic JH III used for a standard curve and from complex insect samples was a nettlesome problem in displaying accurate quantification of JH III.

The aim of this study was to develop an optimized LC–ESI-MS protocol which would allow precise quantification of JH III in whole body extracts of Formosan subterranean termites (*Coptotermes formosanus* Shiraki). Previous methods for LC–ESI-MS were performed with much simpler insect samples, e.g. hemolymph [8–13]. In this study, we examined JH III from the entire insect which constitutes the most complex possible insect sample for any assay, the whole body extract. In termites, JH III is a candidate hormone for an important role in controlling caste differentiation [14,15]. Previously, GC–EI-MS methods were used in our laboratory for the quantification of JH III in Formosan termite samples which required >50 termite workers to obtain sufficient signal-to-noise to quantify JH III titer in whole body extracts [15,16]. A method for quantification of JH III in one or small numbers of individuals will allow us to examine individual variation in JH III titers and will help to understand control of caste differentiation and colony organization. Preparation of JH III samples using C18 cartridges for crude samples of whole body extracts of Formosan termite workers was followed by an LC–ESI-MS protocol using a pentafluorophenyl (PFP) LC column and a water/acetonitrile LC solvent system containing 1 mM ammonium acetate.

2. Experimental

2.1. Materials

Formosan subterranean termite workers were obtained from a colony collected in New Orleans on November 7, 2008. Synthetic JH III (purity 75% by HPLC), synthetic methoprene (for analytical standard), and acetonitrile (LC–MS grade), were purchased from Sigma–Aldrich (St. Louis, MO, USA). Methanol (LC–MS grade) and chloroform (HPLC grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA). *n*-Hexane was purchased from Baker Chemical Co. (Phillipsburg, NJ, USA). Reverse-phase C18 cartridges (UNIBOND C18 SPICE Sample Preparation Cartridges) were purchased from ANALTECH, Inc. (Newark, DE, USA). HPTLC plates were purchased from E. Merck KgaA (Darmstadt, Germany). A reverse-phase PFP LC column (Pursuit® PFP, 2.0 mm i.d. × 250 mm) and a guard column (MetaGuard 2.0 mm Pursuit PFP) were purchased from Varian, Inc. (Palo Alto, CA, USA). All other chemicals were of analytical grade. Water of the required purity for the sample preparation and LC–MS was obtained using a Corning Mega Pure automatic water purification system.

2.2. Preparation of JH III from termite workers

2.2.1. Lipid extraction

According to the extraction methods from previous reports [16], Formosan subterranean termite workers were homogenized in a mixture of 1 ml of *n*-hexane, 0.5 ml of acetonitrile, and 0.5 ml of 2% NaCl with or without 40 ng of methoprene as an internal standard by using a W-385 sonicator (Heat Systems Ultrasonics Inc., Farmingdale, NY, USA) for 5 s three times on ice. The homogenates were centrifuged at 4900 × *g* for 2 min in a model IEC-MULTI RF centrifuge (Thermo Fisher Scientific Inc., MA, USA), and the hexane upper phase was collected. Two additional extractions with 1 ml of *n*-hexane were performed and the *n*-hexane upper phases were combined, after which the sample was dried under a stream of nitrogen.

2.2.2. C18 cartridge procedure

The dried samples were redissolved in 100 μl chloroform–methanol (1:1, v/v). This solution was applied to a C18 cartridge that had been prewashed with 4 ml of methanol. Then 2.5 ml of methanol was added to elute JH III and methoprene (Eluate 1). To confirm the recovery of the two compounds, 1.5 ml of methanol, followed by chloroform–methanol (1:1, v/v) were applied to the cartridge sequentially and the bound lipids were designated Eluates 2 and 3, respectively. After drying Eluate 1 under a stream of nitrogen, 16 μl of methanol was added to the tube and the methanol-soluble phase was used for LC–MS analysis.

2.3. High performance thin-layer chromatography (HPTLC)

Eluates 1, 2, and 3 were prepared from hexane extraction of JH III (50 μg) spiked methoprene (50 μg) and 200 Formosan termite workers. After drying the eluates under a stream of nitrogen, they were completely redissolved in 100 μl of chloroform–methanol (2:1, v/v). Then, 10 μl of standards and a 5 μl sample of termite whole body extracts were spotted on a silica gel HPTLC plate, which was developed in a glass chamber with a solvent system of *n*-hexane:ethyl ether:acetic acid (70:30:1, v/v/v). Spots were detected by spraying 3% copper II acetate containing 8% phosphoric acid and heating at 120 °C on a hot plate.

2.4. Liquid chromatography–mass spectrometry (LC–MS)

Samples were chromatographed on a reverse-phase PFP LC column (2.0 mm i.d. × 250 mm) with a guard column using VARIAN 212-LC pumps (Varian, Inc.). Each sample (aliquot 10 μl) was injected by an autosampler (Model 430, VARIAN Inc.) using μl pickup mode. The separations were performed using solvent A (0.2% acetonitrile in water containing 1 mM ammonium acetate) and solvent B (95% acetonitrile in water containing 1 mM ammonium acetate) on a programmed gradient (50% B for 8 min, 50–100% B for 15 min, 100% B for 5 min, and 50% B for 7 min) at a flow rate of 200 μl/min and a LC temperature of 40 °C.

The effluent from the HPLC was introduced on-line into an ion trap mass spectrometer (Model 500-MS LC Ion Trap, VARIAN Inc.). MS and MS^2 analyses were accomplished by using electrospray ionization (ESI) in the positive mode under the following conditions: needle voltage, 5000 V; spray shield voltage, 600 V, and capillary voltage, 60.0 V in the standard mode. The spray was stabilized with nitrogen gas at 50.0 psi, and the drying gas pressure was 30 psi heated to 320 °C. Mass ranges of *m*/*z* 150–350 and 230–290 were