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Short communication

Separation of juvenile hormone metabolites with a silica-based gel permeation column

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Abstract

Metabolites of juvenile hormone (JH) III, JH acid, JH diol and JH acid diol in an aqueous solution were separated by gel permeation chromatography using an UltraSpherogel SEC 2000 column. Each metabolite was eluted in an inclusion volume in the order JH acid diol, JH acid and JH diol. Although JH was the last compound eluted, it was co-eluted with a JH-binding protein (JHBP) when JHBP was present in the solution. Using this method, in vivo and in vitro JH catabolism studies were performed in the fifth stadium larvae of *Bombyx mori*.

1. Introduction

Juvenile hormones (JHs) help to regulate the development and reproduction of insects. Some JH analogues have been used as insect pest control agents [1–3] and as stimulating silk-producing agents in sericulture [4].

The major routes of JH metabolism are ester cleavage to JH acid, epoxide hydration to JH diol and/or a combination of these pathways to JH acid diol [5]. In order to understand the endocrine control of insect development and silk production, it is desirable to investigate the effect of JH and its analogues and the activity of JH-specific esterase and epoxide hydrolase.

A few methods have been reported for separating JH metabolites in an aqueous solution or in an organic solvent, including gel permeation chromatography [6,7], thin-layer chromatography [5,6], reversed-phase liquid chromatography [8–10] and a partition method in which JH partitions into an isooctane phase and metabolites into an aqueous methanol phase [11,12].

In the gel permeation method, ^{14}C- or ^{3}H-labelled *Hyalophora cecropia* JH and its metabolite, the epoxy acid, are separated with a Sephadex G-100 column. The intact JH is eluted in the exclusion volume to form a complex with JH carrier protein, and JH freed from a carried protein and the epoxy acid are in the inclusion volume [6,7]. This paper deals with the high-performance liquid chromatographic (HPLC)

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separation of JH metabolites in an aqueous solution with a silica-based gel permeation column.

2. Experimental

2.1. Reagents and chemicals

All reagents were GC grade. Radio-inert juvenile hormone III (JH III) standard [methyl (2*E*,6*E*)-3,7,11-trimethyl-*cis*-10,11-epoxytrideca-2,6-dienoate] was obtained from Sigma (St. Louis, MO, USA). Radiolabelled JH III (15.5 Ci/mmol) was obtained from New England Nuclear (Boston, MA, USA). Juvenile hormone esterase inhibitor, octyl-1,1,1-trifluoro-2-propanone (OTFP) was denoted by Dr. Shiotsuki (Department of Insect Physiology and Behaviour, National Institute of Sericultural and Entomological Science, Tsukuba, Ibaraki, Japan).

2.2. Partially purified juvenile hormone-binding protein (JHBP)

Partially purified JHBP was prepared by a two-step purification of the haemolymph of 1-day-old larvae of the fifth instar silkworm *Bombyx mori*. A haemolymph sample obtained by ammonium sulfate precipitation (50–75% saturation precipitation) was passed through a Sephacryl S 200 HR column. The fractions were assayed as described by Kurata et al. [13] and the JHBP peak was pooled.

2.3. Standard JH derivatives

Standard derivatives, [^{3}H]JH III acid (JHA) and [^{3}H]JH III diol (JHD), were prepared from [^{3}H]JH III according to Goodman and Adams [8] and Strambi et al. [14], respectively. For the preparation of [^{3}H]JH III acid diol (JHAD), the JHA obtained by the above method was treated according to Strambi et al. [14].

2.4. Metabolite JHA produced in vitro

An aliquot of the haemolymph collected from 5-day-old larvae of the fifth instar *B. mori* was diluted to 1:400 with 10 m*M* phosphate buffer (pH 7.4) containing 1 m*M* phenylthiourea (PTU) and 1 m*M* *p*-amidinophenylmethansulfonyl fluoride (*p*-APMSF). [^{3}H]JH III (0.25 pmol) and cold JH III (50 pmol) were incubated in the diluted haemolymph (200 μl) at 25°C for 20 min. The enzymatic reaction was stopped by the addition of both OTFP (final concentration 10^{-5} *M*) and an aliquot (about 20 μg) of the partially purified JHPB and stored for 20 min in an ice-box.

2.5. Metabolite JHD produced in vitro

JHD was prepared enzymatically by incubating 1 pmol of [^{3}H]JH with a supernatant of homogenate of *B. mori* eggs for 30 min at 30°C. The freshly laid eggs were homogenized at the rate of 1 g wet mass per 100 ml of 10 m*M* phosphate buffer (pH 7.4) with a glass homogenizer and centrifuged at 50 000 *g* for 30 min as described by Share and Roe [15]. The supernatant was treated with OTFP at a final concentration of $5 \cdot 10^{-5}$ *M* and 1 m*M* diisopropyl fluorophosphate (DFP) for 10 min at 0°C.

2.6. Metabolites produced in vivo

Silkworms were reared on artificial diets in the laboratory at 25°C. One-day-old larvae of the fifth instar were applied topically with about 12 pmol of [^{3}H]JH III and then 30 min after the application the haemolymph was gathered with a glass capillary from a cut caudal horn. Immediately after collection, the haemolymph was diluted 50% with 10 m*M* phosphate buffer (pH 7.4) containing 1 m*M* PTU, 1 m*M* *p*-APMSF and $2 \cdot 10^{-5}$ *M* OTFP and stored for 20 min in an ice-box.

All the haemolymph samples used here were centrifuged for 30 min at 50 000 *g* at 4°C and the supernatant was filtered through a 0.45-μm filter and subjected to experiments.

2.7. Procedure

Separations of standard JH derivatives and JH metabolites in the *B. mori* haemolymph were performed by gel permeation chromatography

on an UltraSpherogel SEC 2000 column (300 × 7.5 mm I.D.) (Beckman, Fullerton, CA, USA). The volumes of samples applied to the column ranged from 100 to 200 μl. Chromatography was carried out isocratically at ambient temperature using a Beckman System Gold radiochromatography system with a Model 171 radioisotope flow detector. The elution buffer was 10 m*M* phosphate buffer (pH 7.4) containing 100 m*M* sodium sulfate at a flow-rate of 0.5 ml/min. The flow cell of the radioisotope detector was 1 ml in volume and a scintillation cocktail was supplied at 1.5 ml/min. Except for the samples monitored with the radioisotope detector, radioassays were performed with a Beckman Model 882 liquid scintillation counter.

3. Results and discussion

3.1. Standard JH derivatives

A standard derivative solution containing JHAD, JHA, JHD and JH was applied to the column. Fig. 1A shows the separation of the JH derivatives and JH. The radioactive profile showed four peaks: a JHAD peak at retention time of 21 min, a JHA peak at 24 min, a JHD peak at 41 min and a JH peak at 56 min. To confirm the effect of the haemolymph on the separation of the standard derivatives, a mixed solution of the derivatives and haemolymph (diluted to one tenth) was loaded and then eluted. As shown in Fig. 1B, JHAD, JHA and JHD were separated.

When the partially purified JHBP (ca. 20 μg) containing $2 \cdot 10^{-5}$ *M* OTFP and 1 m*M* DFP was applied to the solution, the JH III was eluted together with JHBP at a retention time of 15 min, corresponding to purified JHBP [13] (Fig. 1C). When absorption of proteins on the column is negligible, omitting sodium sulfate from the elution buffer shortened the retention times of both JHD and JH, from 41 to 32 min and from 56 to 46 min, respectively. Further, a new column gave a longer retention time of JH than a used column (data not showed). Under the same conditions, the runs ($n > 5$) were reproducible both within day and day-to-day.

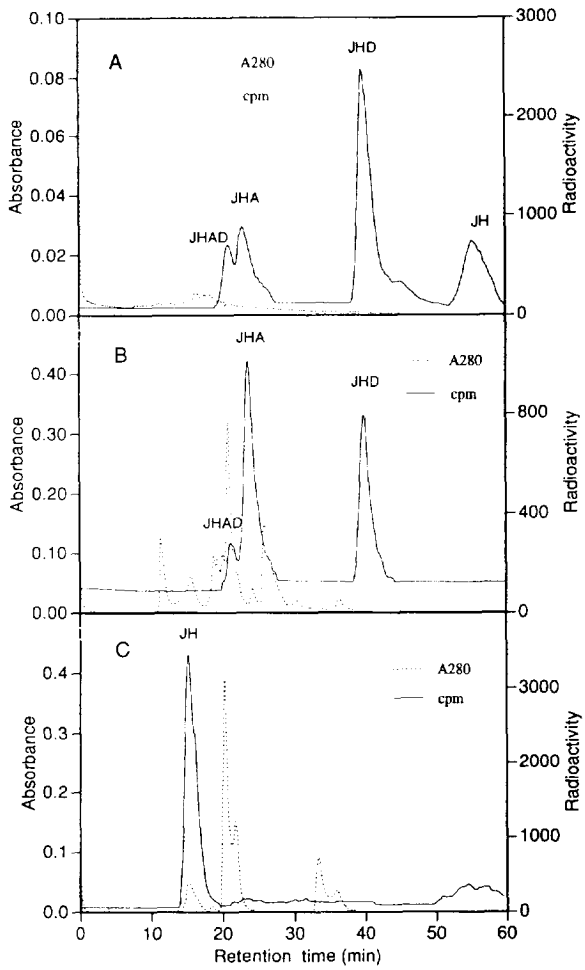

Fig. 1. Separation of JH standard derivatives and JH by HPLC equipped with a radioisotope detector. (A) JHA, JHD, JHAD and JH in 100 μl of phosphate buffer (pH 7.4); (B) JHA, JHD and JHAD in 100 μl of *B. mori* haemolymph containing 0.1% DFP and 10^{-5} *M* OTFP; (C) elution of JH with partially purified JHBP.

3.2. JHA produced in vitro

For the separation of JHA produced in vitro, [^{3}H]JH III metabolized in diluted haemolymph was subjected to separation after the addition of OTFP and partially purified JHBP. As shown in Fig. 2A, two peaks of radioactivity appeared at 15.2 and 23.4 min, corresponding to JH and JHA, respectively. Acetonitrile can be used to minimize the time required for the separation of JH and JHA without the use of JHBP. When the

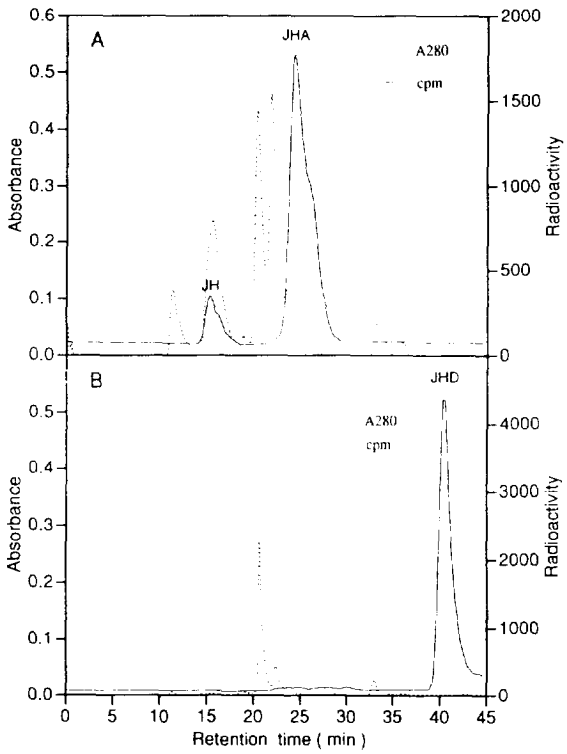

Fig. 2. Separation of JH metabolites produced in haemolymph in vitro and in egg homogenate in vitro. (A) Separation of JHA; (B) separation of JHD.

elution buffer was replaced with acetonitrile 15 min after the separation had started, the retention time of JH was 20 min shorter (data not shown).

3.3. JHD produced in vitro

[^{3}H]JH III metabolized with the homogenate of eggs was chromatographed and the radioactive profile showed one peak at a retention time of 41 min, identical with that of JHD (Fig. 2B).

3.4. JH metabolites produced in vivo

In vivo metabolites of JH in 200 μl of a haemolymph were analysed by HPLC using the elution buffer containing 10^{-5} *M* OTFP. Three peaks of radioactivity appeared and were identified as JH, JHAD and JHA, with retention

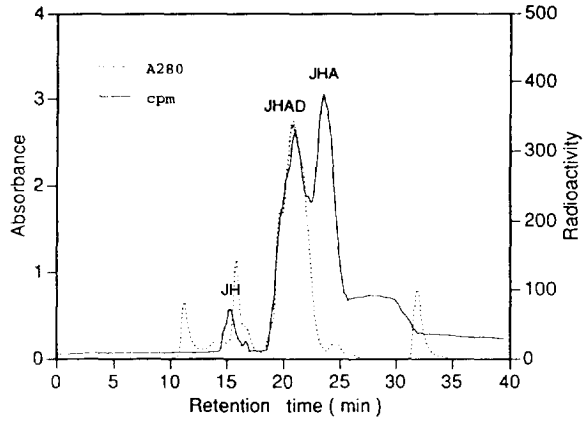

Fig. 3. Separation of JH metabolites catalysed by endogenous enzymes in *B. mori* haemolymph in vivo. A 200-μl volume of haemolymph was loaded on the column.

times corresponding well with those of the standard peaks shown in Fig. 1B and C (Fig. 3). In the in vitro study, haemolymph that had been diluted 1:400 metabolized JH into JHA alone (Fig. 2A), whereas in the in vivo study, haemolymph metabolized JH into JHA and JHAD. It is thought that this indicates that JH esterase activity in the haemolymph of the 5-day-old larvae is much higher than epoxide hydrolase activity. Incidentally, measuring the peak at 15 min presumes a certain amount of a complex with JH and JHBP in the haemolymph containing enzymes to metabolize JH.

The UltraSpherogel column separated a mixture of JH and its metabolites into each metabolite as described above. The separation of JH and its metabolites by the column was not based on a size separation because their molecular sizes were smaller than the exclusion limit of the gel. A Sephadex G-100 column could not separate a mixture of JHA and free JH [6,7]. This difference may be attributed to the different abilities of the raw materials comprising the gels to adsorb JH and its metabolites; Sephadex G-100 gel is a dextran whereas UltraSpherogel is a silica.

A silica gel TLC method for the separation of JH and its metabolites has been reported [5]. Reversed-phase liquid chromatography can also separate the JH metabolites well [10]. As the

present method allows aqueous solutions to be subjected to chromatography without extraction (with organic solvents), it is convenient for application in JH metabolism experiments.

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