Determination by LC-MS of Juvenile Hormone Titers in Hemolymph of the Silkworm, *Bombyx mori*

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Juvenile hormone (JH) I, II and III in the hemolymph of the silkworm, *Bombyx mori* were quantified by liquid chromatography-mass spectrometry (LC-MS). JHs were treated with methanol and trifluoroacetic acid to convert into JH methoxyhydrines (JH-MHs). The key to the analytical condition for JH-MHs was the addition of 5 μM sodium acetate to the eluting solution. Each JH-MH was observed as the sodium adduct ion with good sensitivity. This improved method enabled the titration of JH I, II and III in hemolymph of the silkworm to be monitored from the 3rd instar through to the early pupal stage. A peak of JH I was observed immediately after eclosion in the 3rd and 4th instar stages. The JH I titer sharply decreased on day 1 and reached the lowest level before eclosion, but there was no peak at the beginning of the 5th stadium, and no apparent increase was observed until pupation.

**Materials and Methods**

**Insects and samples.** Bombyx mori silkworms of the Kinshu x Showa strain were reared at 25 °C on an artificial diet (Nihon Nosan Kogyo, Yokohama, Japan) under a 12 h light/12 h dark photoperiod. Hemolymph was collected in microtubes containing approx. 5% (w/v) phenylthiourea crystals and 1 mM EDTA by cutting an abdominal leg. Samples were stored at -80 °C until needed.

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*Abbreviations:* JH, juvenile hormone; JH-MH, JH methoxyhydrine; LC-MS, liquid chromatography-mass spectrometry; ELISA, enzyme-linked immunosorbent assay; RIA, radioimmunoassay; GC-MS, gas chromatography-mass spectrometry
**Reagents and glassware.** JH I and II were both purchased from SciTech (Prague, Czech Republic), and JH III from Sigma-Aldrich (St. Louis, MO, USA). JH III-d3 was prepared according to the method described in a previous report. Each JH was purified by HPLC in a silica gel column (Shiseido SG-80, 10 × 250 mm; Tokyo, Japan), using diethyl ether/hexane (1:9, v/v) as the normal phase, and then stored in toluene at −20 C. All solvents were of residual pesticide analysis grade (5000-fold concentration guaranteed; Wako Pure Chemicals, Osaka, Japan). Trifluoroacetic acid of HPLC grade was purchased from Nacalai Tesque (Kyoto, Japan). Alumina N Activity I (MP Biomedicals, CA, USA) was used with 6% distilled water to prepare activity grade III. All glassware was successively rinsed with distilled water, acetone and hexane, and then baked overnight at 50 C before being used.

**Purification of JHs from hemolymph and preparation for the LC-MS analysis.** Extraction and derivatization were carried out by the method described for JH quantification by GC-MS, but with a slight modification. Ten microliters of JH III-d3 in toluene (67.1 pg/µL) as an internal standard was transferred into a clean glass tube, and 0.5 mL of methanol was added. Hemolymph of the silkworm (50 or 100 µL) was then added and mixed vigorously, before 1.5 mL of a 2% NaCl solution was added to the sample solution. JHS were extracted by partitioning with 0.5 mL of hexane. After centrifuging at 3200 rpm for 5 min, the hexane phase was collected. This extraction with hexane was conducted three times. The combined solvent containing JHs (1.5 mL) was evaporated under a stream of nitrogen. Methanol (100 µL) and 2 µL of trifluoroacetic acid were then added to the crude JH extract which was reacted at 60 C for 30 min to convert into the methoxyhydrine derivatives of JHS (JH-MHs). After removing the solvent by evaporation, the JH-MHs were purified in a Pasteur pipette packed with 1.0 g of aluminum oxide (activity III) that had been prewashed with 1 mL of hexane. After loading the crude reactant and washing with 2 mL of diethyl ether/hexane (3:7, v/v), the JH-MHs were eluted with 2 mL of ethyl acetate/hexane (1:1, v/v) and then dried under a stream of nitrogen. The residue was dissolved in 25 µL of acetonitrile/distilled water (4:1, v/v) containing 5 µM sodium acetate.

**Analytical conditions for LC-MS.** The HP1100 MSD system was equipped with a 150 × 3 mm UG80 C18 reversed-phase column (Shiseido, Tokyo, Japan) protected by a guard column and eluted with acetonitrile/distilled water (7.5, v/v) supplemented with 5 µM sodium acetate as the mobile phase at a flow rate of 0.4 mL/min. Electrospray ionization (ESI) in the positive mode was used for the MS analysis with a drying gas temperature of 320°C at a 10 L/min flow rate, with an ionization voltage of 70 V. The selected ion monitoring (SIM) groups were changed three times according to the retention times of JH-MHs. The time sequences and monitored ions were as follows: 4–6 min, m/z 321 (JH-III-MH) and 324 (JH-III-d3-MH); 6–8 min, m/z 335 (JH-II-MH); 8–10 min, m/z 349 (JH-I-MH).

**Results and Discussion**

**Effect of supplementing sodium salt on the LC-MS detection**

Each JH-MH showed in the ESI positive mode an intense peak of the sodium adduct ion with weak peaks of the proton adduct ion and its fragment. However, the quantification of JH-MHs was unstable in terms of their sensitivity under conditions containing no sodium salt in the eluent. We therefore examined the supplementing effect of sodium salt as an ion source. To optimize the salt condition, 671 pg of JH III-d3 was converted into methoxyhydrin for an LC-MS analysis under various conditions (Fig. 2). The addition of sodium salt resulted in improved sensitivity and reproducibility. Sodium acetate had a better effect than sodium chloride because higher sensitivity could be achieved with a lower salt concentration. After further optimization, the addition of 5 µM sodium acetate showed approximately 4-fold higher sensitivity than with no salt addition. All JH-MHs were observed in this condition as only sodium adduct ions with some fragments (data not shown). The relative limits of detection (LOD) and quantification (LOQ) of JHs were approximately 5 and 15 pg, showing equivalent sensitivity to that in a recent report using LC-MS/MS. When known amounts of JH III-d3 and the JH I standard mixture were converted and then analyzed, linear standard curves for JH III-d3-MH and JH I-MH were respectively obtained in the range of 50–800 pg and 20–350 pg (Fig. 3). More importantly, JH III-d3-MH could be detected with the same sensitivity as that for JH I-MH. This result indicates that JH III-d3 would be an appropriate internal standard in LC-MS measurements to correct not only the extraction efficiency, but also the conversion ratio for methoxyhydrin and the variation of sensitivity.

**Analysis of JH concentration in the silkworm hemolymph under the optimized condition for LC-MS**

High sensitivity and reproducibility were achieved under the optimized conditions for the instrumental analysis just described. Hemolymph of the silkworm (Kinshu × Showa) was then applied for a quantitative
analysis. Figure 4 shows a chromatogram of the hemolymph sample from newly molted 3rd-instar larvae (day 0 of the 3rd stadium, L3D0). JH I-MH (m/z 349) and JH II-MH (m/z 335) were detected with good sensitivity, but no JH III-MH (m/z 321) was apparent. Applying a correction based on the peak area ratio of each JH for JH III-MH gave respective concentrations of JH I and JH II in the hemolymph of L4D0 larvae of 1.37 and 0.56 ng/mL.

JHs were further quantified from the 3rd instar through to the early pupal stage (Fig. 5). The titer of JH I in the hemolymph had peaks (1.5–2.0 ng/mL) on day 0 of the 3rd (L3D0) and 4th (L4D0) instar immediately after ecdysis, and decreased to the lowest level before ecdysis. The titer of JH II changed similarly to JH I, but the concentration was lower at L3D0 and L4D0. JH III was not detected during the same period. The amount of JH I determined by LC-MS was four times lower than that reported by RIA, but the developmental pattern was similar. In contrast, the titration of JH after final ecdysis was completely different from previous reports. No pronounced peak of JH could be detected at the beginning of the 5th stadium (L5D0). Furthermore, only a slight increase of the JH III titer was found on day 7 of the 5th instar (L5D7), and an apparent increase of JH I was observed just after pupation. The measurement by RIA showed that the JH I titer increased in the latter stage of 5th-instar larvae, and then decreased after pupation; hemolymph samples of L5D0 and L5D7 larvae were therefore collected at intervals of 6h during these periods, JH I then being quantified by the non-derivatization method. The measurements were conducted at least five times, but the JH I titers of all samples were almost the same as those by the JH-MH conversion method (data not shown). Kayukawa et al. have reported that the expression level of Kru¨ppel-homolog 1 (Kr-h1), an early JH-response gene in the epidermis of the silkworm, was low at the beginning of the 5th stadium.

The expression in prepupa of JH acid methyl transferase (JHAMT), a key enzyme in JH biosynthesis, declined in the corpora allata (CA), although low JHAMT expression was apparent in such peripheral tissues as the ovaries and testes. These results suggest that the concentration of JH was not high at L5D0, and that JH was directly synthesized in each tissue, not being released into the hemolymph in prepupa. However, the reason for the increase of JH I after pupation remains unclear, because no expression of JHAMT at CA was observed.

Fig. 4. Chromatogram for a Hemolymph Sample on Day 0 of 4th-Instar Larvae Monitoring JH-MHs as a Sodium Adduct.
A, JH III-MH (m/z 321); B, JH III-d3-MH (m/z 324); C, JH II-MH (m/z 335); D, JH I-MH (m/z 349).

Fig. 5. Developmental Changes in JH Concentration in Hemolymph of the Silkworm, Bombyx mori from the 3rd Instar through to the Early Pupal Stage.
Filled circle, JH I; open circle, JH II; filled triangle, JH III. Data are presented as the mean ± SD (n = 4). E, larval ecdysis; GP, gut purge; P, pupation.
In conclusion, a quantitative simultaneous analysis of JH I, II, and III was achieved by the combination of conversion to methoxyhydrin, use of JH III-$d_3$ as an internal standard, and supplementation of sodium acetate in the mobile phase. This improved LC-MS method enabled JH in hemolymph of the silkworm containing a low level of JHs to be detected with good sensitivity and specificity. Moreover, JH titers from the 3rd instar through to the early pupal stage could be accurately determined, indicating that the method developed in the present study was sufficiently reliable for JH quantification. This analytical method will be beneficial and applicable to other insect species for a better understanding of insect physiology related to JH.

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