Note

Quantitative Determination Method for a Juvenile Hormone III Titer in Honeybee Haemolymph by High-Performance Liquid Chromatography

Hiromi SASAGAWA
and Yasumasa KUWAHARA*

Institute of Agriculture and Forestry,
University of Tsukuba, Tsukuba,
Ibaraki 305, Japan
*Institute of Applied Biochemistry,
University of Tsukuba, Tsukuba,
Ibaraki 305, Japan
Received October 29, 1987

Juvenile hormones (JHs) are among the most important insect hormones, and regulate insect metamorphosis with ecdysone. They also contribute to many physiological and behavioral aspects of insects development, such as ovarian development in adults, phase differentiation, diapause and so on. Four compounds are known as juvenile hormones (JH-0, JH-I, JH-II and JH-III), among which methyl 10-epoxy-3,7,11-trimethyl dodeca-2,6-dienoate (JH-III) is known as the only hormone found in the worker honeybee, Apis mellifera L.1 A convenient and sensitive method is necessary for measuring a JH titer to study the physiological and behavioral effects of the hormone in the honeybee. Several sophisticated bioassays involving chemical and spectral methods are available2; however, these methods do not seem to be readily applicable for measuring at the individual hormone level of the honeybee.

The JH-III hormone possesses a strong UV-absorption band at \( \lambda_{\text{max}} \) (hexane) 217 nm (\( \epsilon \)) of 16,600, and can indicate a 0.1 absorbance if we could fill a flow cell (1 cm light path) with 1.6 ng/μl of a JH-III solution. On the other hand, the natural quantity of the hormone in an adult worker honeybee haemolymph has been suggested to be within a range from 2 ng/ml to 30.56 ng/ml,3 0.7 ng/g of fresh weight,4 37.5 pmol/g of fresh weight,5 or 0.48 ~ 6 ng/bee when calculated from the reported value.6 This order just matches the workable range for UV-monitoring in HPLC. Therefore, we realized that we could determine an individual JH titer of worker honeybees if a suitable HPLC column was selected with operating conditions to separate JH from other biogenetic materials.

We chose the following equipment and operating conditions: a Micropak column (1.5 mm i.d. × 250 mm in length) paired with μs-Finepak SIL CN (JASCO); a JASCO 880-PO HPLC pump with an ML-425 micro-injection system (with a 1 μl sampling loop); a JASCO UVIDEC-100-V UV-monitor (8 μl flow cell volume) with peak monitoring at 217 nm and recording at a 1 mV full scale; an n-hexane- n-butanol mixture (100:0.6) was used as the developing solvent at a 0.1 ml/min flow rate and 6 ~ 7 kg/cm² pressure.

As shown in Fig. 1, the μs-Finepak SIL CN column could produce a modest peak corresponding to JH-III (t_R 8.8 ~ 9.2 min), when part of a 200 μl total hexane extract from individual worker honeybee haemolymph (0.1 ~ 21.1 μl per bee) was concentrated to 2 ~ 3 μl and submitted to HPLC. The JH-III peak disappeared completely in the chromatograms of all extracts when exposed to the JH-III decomposing reactions involving LAH reduction, hydrolysis with TsOH and catalytic hydrogenation. The same phenomena were observed with the reaction products of authentic JH-III. Therefore, the peak appearing on the chromatogram of the honeybee extracts was concluded to consist solely of JH-III. This fact was further reinforced by a multiple-ion detection (MID) experiment, using a Hitachi M-80B GC-MS instrument, which supported the presence of JH-III in the eluate corresponding to the HPLC peak. The addition of 30 ng of thymol to each extract was a satisfactory quantity and was usable as an internal standard to determine the JH titer in individual adult bee haemolymph (Fig. 1). The peak areas of JH-III and of standard thymol were calculated by hand, measuring each peak height and peak width at its half-peak height. Using authentic JH-III (Sigma; 0.5, 1, 2.5 and 5 ng) and thymol (Wako Chem.; 30 ng), a standard curve was prepared. Relationships between the peak-area ratio

![Fig. 1. Typical HPLC of JH-III and of a Hexane Extract of the Haemolymph from an Adult Worker Honeybee, Apis mellifera L.](A), JH-III standard; (B), haemolymph; H, JH-III; S, internal standard thymol.)
and amount of JH (ng) gave the following equation with a correlation coefficient of \( R = 0.9997 \):

\[
Y (\text{ng of JH-III in the extract}) = 19.7291 X (\text{JH-III peak area/thymol peak area}) + 0.0107
\]

Using this equation, recovery tests (5 replications) were conducted by adding JH-III (2 ng) to half of a worker honeybee haemolymph, using the other half as a control. The results shown in Table I indicated the feasibility of the proposed method for JH-III analysis.

One result of the application of this method is shown in Fig. 2, and clearly indicates that the JH titer per haemolymph volume changed with the age of the worker honeybee. A day 0 worker possessed 0.05 ng/\( \mu \)l of haemolymph, and a day 26 (honey-collecting) worker, 7.64 ng/\( \mu \)l. These results provide evidence that the well-known progressive change of labour of worker honeybees with age\(^5\) is also correlated to the JH-III titer\(^3\) in individual haemolymph; however, these JH-III values turned out to be at least 200 times larger than has previously been indicated.\(^3\)\(^-\)\(^6\) More data accumulated for the JH titer under various conditions, and a discussion on their biological significances will be summarized elsewhere.

It has now become possible to determine individually the unexpectedly large quantity of JH-III titer present in worker honeybees with a simple and convenient method. We believe the proposed method will be applicable not only to the honeybee but also to other insect species and their organs under various physiological conditions.

Acknowledgments. The authors wish to thank Mr. T. Nire of JASCO, Dr. M. Sasaki of Tamagawa University, and Professor T. Kusano and Dr. S. Tatsuki of the University of Tsukuba for their valuable advice and encouragement.

### REFERENCES

Quantitative Determination of JH-III by HPLC