Review Article

Structure Determination of a Natural Juvenile Hormone Isolated from a Heteropteran Insect

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Juvenile hormone (JH), which occurs in several forms in different insects, is one of the most important insect hormones. The structure of JH in Heteroptera has not been elucidated until recently, although insects in this suborder have long been used as experimental animals for JH research. Here we review the structure determination of a novel JH in a stink bug, *Plautia stali*, which was named juvenile hormone III skipped bisepoxide [JHSB₃: methyl (2R,3S,10R)-2,3;10,11-bisepoxyfarnesoate], based on the arrangement of two epoxides at C2,3 and C10,11 with a skipped double bond at C6,7.

1. Introduction

Juvenile hormone (JH) is an important regulator of many functions in all insects (Figure 1) [1, 2]. It controls various aspects of development including metamorphosis, reproduction, and polyphenism. This hormone was discovered in a blood-sucking bug, *Rhodnius prolixus*, by Wigglesworth in 1934 [3]. He demonstrated a hormone, produced by a gland behind the brain, the corpus allatum (CA), that was responsible for the maintenance of juvenile characters as the insect grew, hence the name juvenile hormone [3]. He also found that the gland became inactive to allow metamorphosis to the adult and active again in the adult to support reproductive functions such as deposition of yolk in eggs and production of secretions of accessory reproductive glands [4, 5]. Since the chemical structure of JH isolated from a moth, *Hyalophora cecropia*, was elucidated in 1967 [6], several forms of JH were also determined [1, 2]. JH III is the most common among various insect orders while JH 0, JH I, JH II, and 4-methyl JH I were found in Lepidoptera. JHB₃ was identified as a JH specific to higher Diptera (Cyclorhapha) [1, 2]. However, the structure of JH in the suborder Heteroptera has remained uncertain in spite of attempts to identify it [7–13]. Recently, we identified the structure of JH in a stink bug, *Plautia stali*, and named it juvenile

hormone III skipped bisepoxide (JHSB₃) and demonstrated its biological function as the JH in this stink bug [14, 15]. Here we review the process of structure determination of JHSB₃ and its biological function as JH in *P. stali*.

2. Heteropteran Insects in JH Research

In his pioneering work, Wigglesworth found a humoral factor controlling metamorphosis and reproduction in *R. prolixus*, which he first referred to as “inhibitory hormone” [3, 5] and later as JH [16]. Since then heteropterans have been employed for evaluation of the biological efficacy of synthetic derivatives of natural JHs and anti-JH compounds, which lead to the discovery of a JH analogue specific to *Pyrrhocoris apterus*, juvabione and anti-JH, precocenes [17–20].

Heteropteran insects are used for studies to elucidate the role of JH in various aspects of insect development. For example, in *Oncopeltus fasciatus*, JH was demonstrated to control reproduction, adult diapauses, and diapause-associated migration [21, 22]. In diapausing adults of *P. apterus*, the activity of CA was inhibited by the brain via nervous connections [23, 24]. The production of methyl farnesoate [25] and JH III [26] by the CA *in vitro* was reported in *Dysdercus fasciatus*, *O. fasciatus*, and *Nezara viridula*.

FIGURE 1: Structure of known JHs.

FIGURE 2: Radiochemical assay for JH biosynthesis. Incubation of CA from *Plautia stali* adults in a medium containing radiolabeled methionine revealed the presence of an unknown product with an R_f value different from those of JH I, JH III, or JHB₃ in TLC analysis.

However, Baker et al. failed to detect any form of known JHs in *O. fasciatus* [12].

3. Evidence for a New JH in Stink Bugs

In 1989, we reported that the CA product was responsible for control of adult diapause in *P. stali* [27]. This was shown by surgical operations such as CA extirpation and implantation. To gain insight into the CA product, the *in vitro* radiochemical assay [28, 29] was adopted to this bug (Figure 2). Assuming that the CA product was a methyl ester as with known JHs, the tritium-labeled CA product would be obtained by the incubation experiments in the presence of *L*-[methyl-³H]methionine in the medium. After the incubation period, the product was extracted with hexane and analyzed by thin-layer chromatography (TLC) using a liquid scintillation counter to detect radioactivity of each part of TLC plate. The CA of *P. stali* and three other heteropteran species were subjected to this assay. The R_f values for the CA product of four species were almost identical with each other, whereas

they were different from those for JH I, JH III, and JHB₃ [8, 9]. When precursors of JH III, *E,E*-farnesol or farnesoic acid were added to the medium, the biosynthesis of CA product was enhanced. This suggested that the product possessed the same sesquiterpenoid skeleton as did JH III [8]. Because radiolabeled JH III added to the incubation medium was not converted to the “CA product,” the possibility that the CA released JH III but the latter was degraded or metabolized to the “CA product” in the incubation medium was ruled out [9].

Thus far, at least 7 species from 4 families of Heteroptera seem to share this product [7, 9, 10, 30]. Although the CA of *O. fasciatus*, *D. fasciatus*, and *N. viridula* were reported to produce JH III and/or methyl farnesoate *in vitro* [25, 26] and JH I was found in the hemolymph of *R. pedestris* (formerly *R. clavatus*) [31], the production of JH III and JH I in *N. viridula* and *R. pedestris*, respectively, was not confirmed [9].

To assess the JH activity of the CA product, we developed a bioassay method using *P. stali*. The CA product, collected as hexane extracts of medium for CA incubation, was typically

FIGURE 3: Bioassay for juvenilizing activity in *Plautia stali*. A last instar nymph (a), an adult (b), and a nymph-adult intermediate (c) obtained as a result of application of JH-active sample. Scale bar: 5 mm. Arrows with labels W and S indicate forewing and scutellum lengths and pronotum width, respectively.

applied to last instar nymphs, and following the final ecdysis, relative lengths of forewings and scutellum were determined [8]. Adults have fully developed long forewings and scutellum (Figure 3(b)) while in last instar nymphs and metamorphosis-inhibited insects by JH application, forewings and scutellum were not developed into the adult form yet but remained in buds or partially developed, short form (Figures 3(a) and 3(c)). The more the CA product was applied to test insects, the shorter their wings and scutellum were. This result indicated that the CA product had the activity to inhibit metamorphosis of the test insects, hence JH activity. Therefore, JH in *P. stali* was very likely to be a new JH that is different from any known JH.

4. Structure Determination of Stink Bug JH

To elucidate the structure of stink bug JH, we first examined the molecular weight of CA product using high resolution fast atom bombardment ionization (FAB) mass spectrometry to estimate its compositional formula. The mass of protonated molecule $[M+H]^+$ was estimated to be 283.1885, leading to a compositional formula, $C_{16}H_{26}O_4$, for the CA product. This formula is identical to that for dipteran JHB₃. However, previous studies clearly showed that the R_f values of the CA product and JHB₃ were not the same despite the supposition that the CA product and JHB₃ shared a sesquiterpenoid skeleton in common [8, 9]. Based on these observations, we proposed that the structure of JH in *P. stali* would be a regio- or geometric isomer of JHB₃.

To test this hypothesis, we synthesized a compound mixture consisting of 32 isomers of bisepoxides of methyl farnesoate with 2*E* or 2*Z* double bond, epoxides at C2,3 and at either C6,7 or C10,11 by two steps starting from a mixture of *E*- and *Z*-geranylacetone. The bioassay for JH activity indicated that the mixture was JH active. In a GC-MS

analysis using a DB-35MS column the mixture gave several peaks. Cochromatography of the mixture and the CA product indicated that one of peaks of the mixture coincided with the CA product in retention time and mass spectrum.

Using a normal phase chiral column, Chiralpak IA (DAICEL Co., Ltd) on an HPLC system, the bisepoxide mixture was separated into 21 fractions and each of them was subjected to the bioassay for JH activity. Two fractions (retention time: 28 min. and 38 min.) applied topically to last instar nymphs showed a juvenilizing effect at a dose of 0.1 $\mu\text{g}/\text{insect}$. These biologically active fractions were subjected to one and two-dimensional ^1H NMR analyses. Data obtained indicated that each fraction consisted of a single stereoisomer of a novel JH structure, methyl *E,E*-2,3;10,11-bisepoxyfarnesoate (Figure 4). Although stereoisomers of this bisepoxide had distinctly different retention time when separated by the chiral HPLC, their ^1H NMR data were not distinguishable from one another. Probably the positions of two epoxides were too distant to give different signal patterns. Therefore, we synthesized four possible stereoisomers 1–4 in an optically pure form (Figure 4), and these were compared with the natural CA product to determine the relative and absolute structure. Each stereoisomer was obtained starting from *E,E*-farnesol by asymmetric Katsuki-Sharpless epoxidation and Sharpless dihydroxylation reactions in which stereochemical outcomes were reliably controlled by ingeniously designed chiral reagent systems [32]. Bioassay indicated that isomers 1 and 2 showed more JH activity than the other two isomers. The chiral HPLC analysis of these stereoisomers gave four peaks. Elution time for isomers 1 and 2 were almost the same as that for the JH-active fractions obtained by the chiral HPLC separation of the bisepoxide mixture. In consideration of the detection limit of HPLC analysis using a UV detector, the stereoisomers and CA product were subjected to more sensitive GC-MS (chemical ionization, CI with NH_3 as

FIGURE 4: Four stereoisomers of JHSB₃. FIGURE 5: Chiral GC-MS(CI) analysis of synthetic standards of JHSB₃ (1) and isomer 2 and natural product by the CA of *Plautia stali* using an Rt- β DEXcst column. Vertical axis indicates signal intensity for m/z 300, $[M+NH_4]^+$. Black, blue, and red lines indicate analysis of JHSB₃ (1) and isomer 2 (10 ng each), CA product, and cochromatography of these two samples, respectively [15].

a reagent gas) using a chiral column, Rt- β DEXcst. With this column, isomers 2, 3, and 4 showed almost the same retention time of 44.7 min while isomer 1, with retention time of 45.7 min, was distinctly separated from the others (Figure 5). The CA product produced one main peak at 45.8 min in this system. The mass spectra for isomer 1 and CA product were identical (Figure 6 insets). Cochromatography of isomer 1 and CA product indicated that the peaks for these two overlapped completely with an increase in peak height in an additive fashion (Figure 5). These results demonstrate that isomer 1, a novel form of JH, is the natural JH in *P. stali*. We named it juvenile hormone III skipped bisepoxide [JHSB₃: methyl (2R,3S,10R)-2,3,10,11-bisepoxyfarnesoate], based on the arrangement of two epoxides at C2,3 and C10,11 with a skipped double bond at C6,7.

5. Biological Function of JHSB₃

Although, in the process of JHSB₃ structure determination, a juveniling JH activity was indicated in JHSB₃ and one of its stereoisomers, how active the remaining two stereoisomers at higher doses were was not explored. JH activity of the four isomers were, therefore, compared using last instar nymphs as well as adult females of *P. stali* kept under reproduction-promoting, long-day conditions whose CA were surgically removed. The latter test examined the activity to stimulate reproduction, another function of JH.

Topical application of JHSB₃ to last instar nymphs inhibited their metamorphosis in a dose-dependent fashion (Figure 7). Nymphs treated with 0.001 μ g of JHSB₃ molted to

normal-looking adults. With an increase in the dose, the relative lengths of forewings and scutellum decreased. At a dose of 0.1 μ g or higher, nymphs molted to intermediates with wings and a scutellum reduced to a similar extent to those of normal last instar nymphs. A diastereomer of JHSB₃, isomer 2, revealed a similar dose-response curve. On the other hand, isomers 3 and 4 were less active than JHSB₃ and isomer 2. A dose of 1 μ g of isomers 3 and 4 showed little effect on the metamorphosis of bugs. At a dose of 5 μ g, bugs molted to an intermediate, but their wings and scutellum were still slightly longer than those of bugs treated with 0.1 μ g or 1 μ g of JHSB₃. Application of 10R-JH III showed a similar dose response to those of isomers 3 and 4, but even at the highest dose of 10 μ g, its effect was not so evident as that of isomers 3 and 4 at 5 μ g.

Extirpation of the CA from females reared under long-day conditions inhibited the development of ovaries. More than half (10 of 14) of allatectomized hexane-treated females underwent oosorption when they were dissected 4 days after allatectomy. Topical application of JHSB₃ rescued those adults from the inhibitory effect of CA removal in a dose-dependent fashion (Figure 8). Isomer 2 seemed as potent as JHSB₃ whereas isomers 3 and 4 showed virtually no stimulatory effect on oocyte development even at the highest dose of 5 μ g. These results indicated that JHSB₃ and isomer 2 were highly JH active while the other two were about 1,000 times less active. The downward epoxide configuration at C2,3, shared by JHSB₃ and isomer 2, seems important for manifestation of JH activity.

JHSB₃ structure determination was accomplished by analyzing the product of CA *in vitro*. The biosynthesis of JHSB₃ by the CA, however, does not automatically imply its presence in the hemolymph *in vivo*. It must be experimentally verified by detecting JHSB₃ in the circulating hemolymph. We collected hemolymph samples from reproductively active and diapause females. According to the previous studies [27, 33], in the former the concentration of JH in the hemolymph is expected to be high while in the latter low. These two samples were analyzed using LC-MS. In the hemolymph sample from reproductively active females, a peak was observed at the same retention time accompanied by the same mass spectrum as synthetic JHSB₃ standard. No significant peaks of characteristic fragments corresponding to JH I, II, nor III were observed in the hemolymph samples. This indicated the presence of JHSB₃ alone in the hemolymph from reproductively active females, and on the other hand, virtually no peak corresponding to JHSB₃ or any other

FIGURE 6: Detection of JHSB₃ (1) from the hemolymph of *Plautia stali* females. Hemolymph samples collected from reproductively active (a) and diapausing females (b), and 10 ng of synthetic standard of JHSB₃ (c) were analyzed on GC-MS using a DB-35MS column. Vertical axis indicates signal intensity for m/z 283, $[M+H]^+$ (solid line) and m/z 300, $[M+NH_4]^+$ (dotted line). Insets in a and c indicate a mass spectrum of the peak at 16.2 min [14].

FIGURE 7: Juveniling activity of JHSB₃ (1), its stereoisomers and 10R-JH III on *Plautia stali*. Last instar nymphs were treated with a test compound. Following the final molt, lengths of forewing (a) and scutellum (b) relative to the width of pronotum were determined. S on the horizontal axis indicates solvent control. Each datum point and error bar represents average value $\pm$ SD ($n = 8-18$). Asterisks indicate that the average value was significantly different from that of the solvent control (Steel's test, $P < 0.05$) [14].

FIGURE 8: Reproduction-stimulating effect of JHSB₃ (1) and its stereoisomers on allatectomized females of *Plautia stali* reared under long-day conditions. Females were allatectomized and treated with a test compound on day 4 of adult life, and oocyte diameter was determined on day 8. Solid diamonds labeled with I and S on the horizontal axis indicate results of day 8 untreated and solvent-treated adults, respectively. Each datum point and error bar represents average value $\pm$ SD ($n = 8-12$). Asterisks indicate that the average value was significantly different from that of the solvent control (Steel's test, $P < 0.05$) [14].

JHs was detected in the sample from diapause females. JHSB₃ was, therefore, the only molecule found in the hemolymph and its concentration was likely to fluctuate as expected by the previous studies.

Dahm et al. [34] pointed out three criteria that have to be fulfilled to chemically identify JH. These points, derived from the classical definition of the hormone, are as follows: (1) production by the CA; (2) titer fluctuation in synchrony with the processes controlled by JH; (3) rescue effect in JH-deprived insects. As indicated above, these three criteria were met for JHSB₃ in *P. stali*. JHSB₃, therefore, functioned as the JH in this species. It was likely that other heteropteran insects share this new JH in common because the CA of at least seven heteropterans other than *P. stali* also produced the products *in vitro* that behaved similarly to that of *P. stali* on the TLC plate [7, 9, 10, 30].

6. Conclusion

JH in Heteroptera has been a long-lasting enigma in spite of that JH research began with morphological studies in *R. prolixus* [3, 4]. We have successfully determined the structure of novel, Heteroptera-specific JH, JHSB₃. Because JHSB₃ is the only JH with a C2,3 epoxide, heteropterans using this molecule likely possess an enzyme responsible for conversion of the C2,3 double bond to epoxide. The presence of specific JH suggests underlying specific pathways for not only biosynthesis but also degradation. A JHSB₃-specific receptor should also play a role in heteropterans. Our discovery has provided a basis for all these suppositions and will enhance further studies on heteropteran JH. An attempt to elucidate

the enzymes involved in JHSB₃ biosynthesis is in progress. How far JHSB₃ is shared among the suborder Heteroptera or the order Hemiptera is a question to be answered in the future in the viewpoint of insect endocrinology and practical insect control, as well. Structure-activity relation study is underway to gain insight into specificity of JHSB₃ receptor and development of JHSB₃-based control agents.

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