RH-5992—An ecdysone agonist on model system of the silkworm
*Bombyx mori*

V S Kumar1, M Santhi2 & M Krishnan1

1Department of Biotechnology, Bharathidasan University, Tiruchirappalli 620 024, India
2Department of Biochemistry, Seethalakshmi Ramaswamy college, Tiruchirappalli 620 002, India

Received 4 June 1998; revised 6 September 1999

RH-5992 is a novel synthetic non-steroidal ecdysteroid agonist with a high selectivity towards Lepidopteran species. The effect of ecdysone agonist RH-5992 on larval period, larval weight, silk gland weight and haemolymph protein profile were examined in the model organism, the larvae of silkworm, *Bombyx mori*. The LD50 values were found to be 16.21 and 12.01 μg/larva for 72 and 96 hr respectively. In the present study, three sublethal concentrations of 1/5th, 1/10th and 1/20th of LD50 at 72 hr were selected and applied on the mid-dorsal line of the silkworm *B. mori*. The maximum mortality of 35% was observed in the group which received the highest (3.2 μg/larva) concentration of RH-5992. The mortality rate was found to be dose dependent as well as time dependent. Interesting results were observed in haemolymph profile of the RH-5992 treated larvae as staining intensity of 30kDa protein decreased significantly whereas the effect was not marked on other major proteins like storage proteins and vitellogenin polypeptides. From the results, it is confirmed that RH-5992 causes changes in larval characters and protein profile of silkworm *B. mori*. It is proposed that RH-5992 may cause negative effect specifically on reproductive characters like development of ovary and egg production due to decrease in 30kDa protein.

The indiscriminate use of pesticides have caused irreparable damage to the ecosystem in many places of the world. The wide applicability of pesticides threatens the survival of non-targeted beneficial organisms. During the past few decades organic biocides have been found to be increasingly important in controlling pests in and around mulberry fields. By feeding the silkworm with such contaminated mulberry leaves, various effects such as premature molting, deteriorated cocoon quality, reduced cocoon yield and eventual death often occur; therefore, this situation augments the study of the effects of pesticides in the model organism with special reference to silkworm growth and physiology of the silk production.

The non-steroidal ecdysteroid agonist, RH-5992, has been shown to act similar to ecdysteroids in initiating precocious molting in Lepidopterans. RH-5992 mimics the molting hormone 20-hydroxyecdysone and compete effectively for binding to ecdysteroid receptors and is 10 times more active than the natural hormone and appears to persist in the epidermis much longer than 20-hydroxyecdysone.

Because of their physiological significance, the ecdysteroid-endocrine system, offers a complete panel of potential targets for pest management strategies which also have the additional advantage of being unlikely to affect vertebrates. RH-5992, a closely related compound, has undergone considerable field evaluation and has proven to be a very efficient control of Lepidopterans in orchards, vineyards, vegetable fields and forests.

The effect of such analogues on insects is varied. Wing et al., were able to induce premature initiation of molting at all larval stages in tobacco hornworm, *Manduca sexta* with RH-5992. They have pointed out that the effects can be characterized by pathophysiological symptoms of hyperecdysionism. It has been shown to cause neurotoxic side effects.

Here we extend the study on the effect of ecdysone agonist RH-5992 on the physiology and biochemistry of the economically important, non-target Lepidopteran, silkworm, *Bombyx mori*.

Materials and Methods

Experimental animals—Silkworm larvae, *B. mori* (cross breed: L. a multivoltine, X NB, D. a bivoltine) were reared on mulberry leaves at 25°C, 65 ± 5% relative humidity and a photoperiod of 12:12 hr light and darkness. Newly ecysed 5th instar larvae were used for the present study.

**In vivo assay for the effects of RH-5992**

Application of RH-5992—From the stock solution of RH-5992 (1mg/2ml of acetone), various concen-
tations were prepared and applied topically along the mid-dorsal line of the larvae. Mortality percentage included both dead and affected larvae.

Medial lethal doses were assessed by the Finney’s method of probit analysis. Out of the three groups of larvae, group I were treated with 1/5th (3.2 µg/larvae), group II with 1/10th (1.6 µg/larvae) and group III with 1/20 (0.8 µg/larvae) of the LD50 concentration at 72 hr respectively. Positive control group received 10 µl of acetone and negative control group was maintained without any treatment. Both treated and control larvae were maintained at rearing conditions described earlier. The effects were observed daily after the treatment during the fifth instar development period (day 2-6).

Haemolymph proteins—Collection of haemolymph was done by injuring the abdominal legs of B. mori larvae and haemolymph was bled from the wound directly into the eppendorf tubes coated with thiourea, an antioxidant agent, and centrifuged at a low speed to remove haemoctyes. The supernatant was stored in -70°C until further analysis.

Electrophoresis of samples—Polyacrylamide gel electrophoresis was carried out to study the protein profile of haemolymph during development of both control and treated larvae, in the presence of Sodium Dodecyl Sulphate (10% SDS-PAGE), according to the method of Laemmli. Densities of protein bands were measured with a microdensitometer (Hoefer GS 300). The 30KDa proteins were identified by their positions.

Results

In vivo effects of RH-5992 on larval duration—In order to study the developing larval characters, sub-lethal concentrations were chosen from LD50 values which allows the larvae to survive for 6 days. The LD50 values determined for 72 and 96 hr were 16.22 and 12.01 µg/larva respectively. For the present study, 1/5, 1/10 and 1/20 of LD50 at 72 hr was chosen.

It was found that the percentage of mortality increased in all the treated groups as the time of exposure increased. It was also observed that none of the larvae died in the test groups before 48 hr. After 72 hr, the percentage of mortality was 10% & 35% in the group that received lowest (0.8 µg/larva) and highest (3.2 µg/larva) concentration of RH-5992 respectively (Fig. 1). The percentage mortality also increased with increasing concentration of RH-5992. Although the larvae of treated groups survived till 6th day, none of the larvae lived to spin the cocoon.

Typical phenotypic effects induced by RH-5992 was observed in the present study. The RH-5992 treated larva started showing effects 12 hr after treatment when they become quiescent and stopped feeding. Within 24 hr of treatment, the head capsule started slipping for the moult. Unlike in normal larvae where head capsule slippage is followed by molting, treated larvae remained arrested at this stage without further development and ecysis. The treated larvae were similar in size and showed lethargic movement. After 24 hr the colour of the skin changed to brown and excreted a semisolid faecal matter. The RH-5992 treated larvae died after 6 days without proceeding to larval-pupal transformation, unlike the control which completed cocoon spinning in 7 days.

Effects of RH-5992 on larval and silk gland weight—It was observed that the larval and silk gland weight decreased with increasing concentration of RH-5992. Larval weight decreased dramatically in the treated larvae from 2nd day of treatment at high concentrations. Figs 2 and 3 show the observed larval and silk gland weights of the control and treated larvae at various RH-5992 concentrations. Larval and silk gland weights showed an increase at low concentration while they decreased at higher concentrations of RH-5992. It was found that a negative linear relationship existed between the applied RH-5992 and the silk gland weight.

Effects of RH-5992 on haemolymph protein profile—To find out the variations in haemolymph proteins during the larval period of the experiment, analysis of the protein profile was carried out daily during the development of 5th instar larva. In control

![Fig. 1—Larval mortality in control (a: positive control, b: negative control) and larvae treated with different sublethal concentrations of RH-5992 during fifth instar of silkworm, Bombyx mori.](image-url)
larvae, 14 coomassie blue stained bands of molecular weight ranging from 194-14 kDa were resolved by SDS-PAGE (10%). The haemolymph protein profile of the control larvae showed increasing staining intensity of 30 kDa and 80 kDa proteins from day 1-5 of 5th instar. It was found that the haemolymph protein profile of the RH-5992 treated larvae showed significant decrease in 30 kDa protein during 5th instar development. The intensity of 30 kDa protein decreased with increasing concentration of RH-5992. Quantitative changes in the protein profile was observed in the RH-5992 treated groups by Densitometric scanning. The staining intensity of 30 kDa protein in the haemolymph significantly decreased as correlated by the percentage decrease in the scanning area. Maximal percentage decrease was observed at high concentration of RH-5992 (Figs 4-8).

Discussion

The studies reported here show that the substituted dibenzoyl hydrazine, RH-5992, induces an incomplete moult in the B. mori larvae, causing the immediate onset of a moult with the slippage of the head capsule within 24 hr and died without further development and ecdysis. When penultimate instar larvae of Choristoneura fumiferana were treated with RH-5992, a new larval epicuticle was formed but no larval late endocuticle was deposited and the new head capsule remained thin and untanned. Similar effects were seen in the B. mori larvae, treated in this study, except that the head capsule and mandibles often showed marginal sclerotization.

The morphological deformities of cuticle formation are a result of the effects on these compounds on the expression of the genes for endocuticular proteins such as LCP-14 and for Dopa DeCarboxylase (DDC), the enzyme required for sclerotization. RH-5992 prevented release of eclosion hormone (EH) (A. Retnakaran et al., unpublished). Injections of ecdysteroids into larvae cause an immediate interruption of feeding. Gut purge in the last instar larvae of M. sexta is preceded by two small peaks and associated with a distinct commitment peak of endogenous ecdysteroids. Termination of feeding is an important preparatory step of moulting and its control by an increased level of ecdysone is probably common but
Fig. 5—Densitometric scanning of haemolymph polypeptides of control (a: positive control, b: negative control) and larvae treated with different sublethal concentrations of RH-5992 (c: 1/20, d:1/10, e: 1/5, of LD₅₀) on day 2 of fifth instar silkworm, *Bombyx mori*.

Fig. 6—Densitometric scanning of haemolymph polypeptides of control (a: positive control, b: negative control) and larvae treated with different sublethal concentrations of RH-5992 (c: 1/20, d:1/10, e: 1/5, of LD₅₀) on day 3 of fifth instar silkworm, *Bombyx mori*.
not general. High concentration of 20-hydroxyecdysone (HE) have been suggested to cause mortality due to excessive haemolymph ecdysteroid levels which cannot be metabolized or excreted rapidly (hyper ecdysionism)\(^1\).

The central role of ecdysteroids in coordinating growth and development during insect postembryonic development has been well documented\(^1\). Artificial elevation of ecdysteroid during early stages by treatment with 20-hydroxyecdysone, causes CA to elevate JH and an additional larval-larval moulting was induced instead of metamorphosis\(^14\). We suppose that when RH 5992 is applied at high concentration, during early last instar, the CA stimulating factors (neural or hormonal) are continually present and effect direct stimulation of CA-activity. The observed mortality can be correlated to this hormonal imbalance.

An induction of silk gland degradation by high 20-hydroxyecdysone doses was shown both \textit{in situ} and \textit{in vitro}\(^15,16\). The decline of RNA and the functional regression of silk glands are primarily due to the cessation of transcription that is caused either by lack of nutrients or by high ecdysteroid titre. This is linked to degradation of cellular organelles of proteosynthesis that also ensures both in the starving and pupating larva\(^17\). On the other hand, the involvment of silk gland is a specific response to high ecdysteroid concentration acting in the absence of the JH. Possible role of high concentration of RH-5992 in the decline of total RNA synthesis and degradation of tissues have been thought to decrease the larval and silk gland weights of \textit{B. mori}.

In the present study major haemolymph proteins, 30 kDa decreased with increasing concentration of RH-5992. It has been shown that 30 kDa proteins are the other most abundant proteins in the haemolymph.

---

Fig. 7—Densitometric scanning of haemolymph polypeptides of control (a: positive control, b: negative control) and larvae treated with different sublethal concentrations of RH-5992 (c: 1/20, d: 1/10, e: 1/5, of LD\(_{50}\)) on day 4 of fifth instar silkworm, \textit{Bombyx mori}.
of *B. mori* at the late larval and early pupal stages\(^{18}\). In the female pupae, 30 kDa proteins are also detectable in the mature oocytes together with vitellin, a female specific yolk glycolipoprotein\(^{19}\). *Aedes* is the only species known in which ecdysone appeared to stimulate vitellogenin synthesis\(^{20,22}\). It is noteworthy that ecdysone stimulated both the female specific and general protein synthesis in fatbody culture of *Bombbyx*, but egg-specific proteins were not synthesised. Ecdysone exerts widespread metabolic control of fat body function, as it is known to stimulate bulk synthesis of RNA and proteins\(^{23,24}\). The decrease in 30kDa protein, might be related to inhibition of protein synthesis at high concentration of RH-5992. It is shown that there is no major changes in other haemolymph proteins in the RH-5992 treated larvae. The present results suggest RH-5992 is specific to 30kDa protein and further investigations are required to elucidate the effect of RH-5992 on the 30kDa protein gene expression and synthesis.

The substituted dibenzoyl hydrazine RH-5992 acts similarly to that of ecdysteroid in its direct action on gene expression early in the molting process and its suppressive actions at the end of molt\(^{1}\). The morphogenetic effect is ecdysteroid specific and can be evoked by RH-5849\(^{25}\) and also by RH-5992\(^{26}\), at much lower concentrations than 20-hydroxyecdysone and RH-5849. The biological activity is RH-5992>>20-hydroxyecdysone>RH-5849\(^{27}\). In addition to its hormonal effects, RH-5992 may cause some neurotoxic side effects\(^1\). RH compounds also inhibit chitin synthesis\(^{28,29}\). *B. mori* seems to be more sensitive to the ingested ecdysteroids than other insects\(^{32}\) and thus provides a suitable model for exploring different effects of ecdysone and 20-hydroxyecdysone. Hence, the use of lepidopteran specific bioinsecticide RH-5992 mainly near silkworm rearing centres should be done very cautiously. Present study would probably initiate more understanding of the control of synthesis of 30 kDa protein specifically by RH-5992.

Fig. 8—Densitometric scanning of haemolymph polypeptides of control (a: positive control, b: negative control) and larvae treated with different sublethal concentrations of RH-5992 (c: 1/20, d:1/10, e: 1/5, of LD\(_{50}\)) on day 5 of fifth instar silkworm, *Bombbyx mori*.

to unravel the mysteries of gene expression in fat body cell and application of this information in developing a strategy for insect control by interfering with the process of molting and metamorphosis. In addition to its potential as a powerful, Lepidoptera-specific, pest control agent, its stability relative to that of the ecdysteroids should make it an important tool in the elucidation of the mode of ecdysteroid action in the initiation and coordination of the molting process.

Acknowledgement

The authors are grateful to Dr. Tarlochan S. Dhadialla, Rohm and Haas Company, U.S.A for the generous gift to technical grade RH-5992. Financial assistance from International Foundation for Science, Sweden to M.K (B/2520) is acknowledged.

References

1 K.P Kashi, J Seric Sci, Japan, 41 (1971) 301
5 Couillard F, in Recent advances in insect endocrine research, edited by D. Muraleedharan and Mariamna Jacob (Association for Advancement of Entomology, India) 1995, 141.
16 Kodrik D & Schnal F, Int J Morphol Embryol, 23 (194) 311.