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Non-steroidal ecdysteroid agonist, methoxyfenozide (RH-2485) arrests hatching and yolk protein degradation in silk moth *Bombyx mori* L

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Abstract
Toxicological data on silkworm *Bombyx mori* are quite comparable to those of other epidopteron pest insects, therefore it is considered as a suitable model for exploring effects of new synthetic formulations. In this study, egg cards of silk moth *B.mori* were chosen to evaluate the lethal and sublethal toxicity effects of RH-2485 on yolk protein degradation. Protein profile of eggs were carried out on 7% native PAGE and further separated on 7% SDS PAGE. Freshly laid eggs of silk moth *B.mori* were treated with three different concentrations *viz* 0.1%, 1.0 % and 10.0 ppm of methoxyfenozide (RH -2485). Protein profile of freshly laid eggs of day 3, day 5 and day 7 were studied on 7% Native PAGE and 7%SDS-PAGE.

Keywords: RH-2485, *Bombyx mori*

Introduction

New approaches to the development of insect control agents have led to the discovery of natural and synthetic compounds capable of interfering with the process of development and reproduction of target insects. One of the goal of the insecticide industry is to develop chemicals that are not only effective but also pest selective. Ecdysteroids are the key regulators in insect development. They play a crucial role in coordinating molting, metamorphosis and reproduction in insects. Therefore they are important in each and every cycle of insect life cycle and in the regulation of many developmental, biochemical and physiological process, the search for new insect pest control agents mimicking or interfering ecdysteroid action in an alternative.

Rohm and Haas Company (1984) [48] discovered a group of synthetic non-steroidal ecdysteroid agonists mimicking the action of 20-hydroxyecdeysone (20E). These dibenzoylhydrazine derivative, RH-5849 and tebufenozide are the first synthetic endysteroid agonists. They competitively bind with endysteroid receptors and showing hyperecdysonic properties (Wing *et al*., 1988) [60]. The lepidopteran larvae treated with these mimics exhibited symptoms of premature lethal molting (Carlson et as., 2001; Smagghe and Degheele 2003) [5, 54]. Therefore, these agents were toxic and represent a new class of insect growth regulators acting selectively against lepidopteran pests.

In the past decades tebufenozide under the brand names Mimic Confirm is used as an insect growth regulator to control caterpillar pests in vegetables, fruits, ornamentals anf forest, with no adverse effects on various predators and beneficial insects like honey bees (Carlson *et al*.,2001) [5].

Recently, two new structural analogs, methoxyfenozide (RH-2485 marketed as INTERPID) and halofenozide (RH-0345 marketed as MACH 2) are also widely used in the control of caterpillars and grubs. The non- steroidal agonists include early and early late gene expression in lepidopteran target tissues in a fassion similar to that of naturally occurring molting hormone 20E, but the response induced by these mimics become subsequently arrested and the expression of gene that required a decline in hormone titre is suppressed. During the early stages of molting process, the effects of dibenzoylhydrazine derivatives and 20E and the mimics start at a stage in the molting cascade where the falling phase of 20E, RH-5992 induces the expression of Manduca sexta hormone receptor 3, suppresses the 14kDa larval cuticular protein and prevent the expression of decarboxylase enzyme necessary for cuticular melanization and sclerotization (Truman and Riddiford, 1990) [59]. Since the agonist persist for long time and cause the suppression of genes which require ecdysteroid withdrawl, the precocious molting is induced, but incomplete due to lack of chitin synthesis and eclosion hormone release.

Ecdysteroid mimicking action of dibenzoylhydrazine derivatives are also shown in the reproduction of insects. These agents were also found to have antifertility and antifecundity effects (Knight 2000; Knight 2001) [28, 29]. Though antifertility and ovicidal has been observed in the eggs subjects to the application of bisacylhydrazine derivatives the exact mechanism involved in these effects are not fully elucidated.

Ninety percent of the egg of insects by weight is comprised of yolk and it is comprised of lipid and protein. 60-90% of yolk proteins are derived from special proteins called vitellogenins. In most oviparous insects vitellogenin (Vg) is synthesized in the fat body and accumulated in yolk granules in the oocytes. Once inside the oocyte, it undergoes further refinement in structure and is called as vitellin (Englemann, 1979). In *Bombyx mori* two specific proteins have been demonstrated one in the haemolymph, termed as female specific protein and the other exclusively in the egg, called as egg-specific proteins. The female specific protein accumulates in the oocyte from the haemolymph and the egg - specific protein is sythesised solely by the oocyte itself (Ono *et al.,* 1975) [41]. In *B.mori* during embryonic development the major yolk proteins namely vitellin, egg specific protein and 30 kDa proteins stored in the yolk granules undergo programmed degradation by proteolytic enzyme into a set of peptides which are then accessed by the embryo during development (Liu *et al.,* 1996) [36]. The exact mechanism regulating yolk protein degradation is not fully known. In *B.mori* the embryonic development takes place in 2 stages. 1) From fertilization to the formation of embryo (Fertilization, cleavage, blastulation, yolk cleavage, germ band formation and elongation and 2) the development of embryo into a larva (appearance of abdominal appendaages and tubercles, tracheal colouration, body pigmentation). Thus organogenesis, cuticulogenesis are important in embryonic development. The embryonic development is followed by hatching. Eggs including *B.mori* contain various ecdysteroids and their amount fluctuates during embryonic development and their peaks coincident with the production of cuticle (Fournier *et al.,* 1987) [13]. The interaction between the ecdysteroid receptor, BmEcR/BmUSP heterodimer with eggs of ecdysteroids of *B.mori* showed that ecdysteroids could control embryonic development through their receptors (Makka *et al*., 2002) [38]. In addition the enzyme ecdysteroid phosphate phosphatase which catalyse the dephosphorylation of ecdysteroid phosphates which catalyse the dephosphorylation of ecdtsteroid phosphates which catalyse the dephosphorylation of ecdysteroid phosphates increases during embryonic development.

If ecdysteroids play a role in embryogenesis, treatment of eggs with ecdysteroids agonist is anticipated to interfere with the process of development. It is not known whether ecdysteroids play a role in the sequential degradation of yolk proteins or in the synthesis of proteins necessary for the formation of embryonic structure and subsequent hatching.

Therefore the present study was initiated with a keen interest to find the effect of methoxyfenozide (RH-2485) on the degradation of yolk proteins during embryonic development. Eggs of *B.mori* is chosen since the yolk proteins and their programmed degradation during embryogenesis are well studied and therefore emerging as an important model for the study of embryogenesis at the molecular level.

Materials and Methods

The model organism selected for this study is the mulberry silk moth, *Bombyx mori* belonging to the order Lepidoptera, Class Insecta and family Bombicidae. The race chosen was a hybrid of Lx NB_4D_2 (L-Local multivoltine variety, NB_4D_2-New bivoltine with dumbbell shaped cocoon, which feeds, mainly on mulberry leaves. It is a holometabolous insect, whose life cycle has four distinct stages namely the egg, larva, pupa and adult. The silk moths were obtained from Government sericulture Grainage Centre, Tiruchirapalli, for the present study.

The study was designed to find the effect of RH -2485 on the egg hatchability and the protein profile of eggs during embryonic development in silk moth *Bombyx mori* L. The experiment consisted of 80 egg cards (ie each egg card contained eggs laid by a single adult female). A group of 20 egg cards served as control and other 3 group of 20 eachas experimental. The experimental groups were sprayed with 0.1, 1.0 and 10, 0 ppm of RH-2485 in acetone. Care was taken to ensure that each egg was fully drenched in the test solution for 5 seconds. Then the different groups of eggs were incubated separately and the observations were made on the changes in colour, duration of egg development and hatchability. The eggs were also subjected to the study on the changes in protein profile by scraping the eggs that belong to a single female from the egg cards after nine days (the day on which the control eggs hatched out). The protein extract, were subjected to electrophoretic separation both in native and SDS-PAGE and immunodiffusion.

Preparation of methoxyfenozide

5mg of methoxyfenozide (RH-2485) was dissolved in 1ml acetone and considered as stock. From the stock solutions 0.1ppm, 1.0ppm and 10.0 ppm was prepared (Yamada R., 2005) [61].

Incubation of eggs

Since conditions of incubation affect the development, hatchability and viability of embryos, enough care was taken to incubate the eggs at a temperature of 25 ± 1^0C and a relative humidity of 75 ± 5%. Incubation was done inside a mud pot kept buried in a basket of sand after being sprinkled with water and the mouth of the pot was covered with porous cloth to allow aeration.

Egg development and hatchability

The incubated control and treated eggs were observed for development on the 9th day of incubation, the larvae hatched out from the eggs of the control.

Estimation of protein

Protein content of the sample was estimated by adopting the method of Lowry *et al.,* 1951. Bovine serum albumin was used as standard.

Non–dissociating polyacrylamide gelelectrophoresis (Native PAGE)

The nondissociating system of electrophoresis and elution of protein fractions were done by adopting the method of Homes and Rickwood (1981) [18]. The eluted fractions were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis according to the method of Laemmli *et al*., 1970 [33].

Immunodiffusion

Qualitative immunodiffusion was done following the method of Ouchterlony 1949 [42].

Results

The study aimed at finding the effects of methoxyfenozide (RH-2485) on yolk protein utilization during embrygenesis and subsequent hatching in the model organism *Bombyx mori.*

Effect of RH-2485 on egg hatchability

The freshly laid eggs of *B.mori* were yellow in colour, the colour changed to gray on day 5 and the black on 8th day of embryonic development. The control eggs hatched only 91.00 ±0.19% of eggs treated with 0.1ppm hatched out. The egg hatch was only 70.25±0.96% in 1.0ppm treated group and 31.09 ± 0.33% in 10.0ppm. The treated unhatched eggs were observed for three more days, but these eggs failed to hatch.

Effect of RH-2485 on protein profile during embryogenesis

The metabolic fate of proteins were studied by observing the changes in native and subunit fractions during embryonic development in *B.mori* by subjecting eggs to topical application of RH-2485 in acetone. The proteins extracted from freshly laid eggs showed six major proteins of high intensity in Coomassie blue (R-250) stained gel resolved in the region of molecular weight ranging from 700 to 25 kDa in 7% native PAGE. These proteins had an apparent molecular weight of 690, 640, 280, 180, 140 and 100 kDa (Fig.1, lane 1). The Yolk proteins of yolk proteins of day 0 eggs separated in 7% native gel were eluted and subsequently electrophoresed on 7% SDS-PAGE. The protein band 1 (690 kDa eluted from native gel got resolved into four fractions at 180, 80, 44 and 30 (31 and 29) kDa regions (Fig.2, lane 1). The second native protein with a molecular weight of 640 kDa got separated into two fractions at 180 and 44 kDa regions (lane 2). The third protein (280 kDa) in the native gel showed two fractions at 74 and 70 kDa regions (lane 3). The fourth native protein (180 kDa) showed three polypeptides at 74, 70 and 30 kDa regions (lane 4). The fifth protein (140 kDa) in native gel got eluted and showed only a single polypeptide at 70 kD region (lane 5). The last protein (100 kDa) in native gel showed two fractions at 74 and 30 kDa regions (lane 6).

The proteins extracted from the eggs on day 3 of embryonic development also got resolved into six Coomassie blue stained fractions in 7% native PAGE (Fig 1, Lane 2). These fractions had similar molecular weights (690, 640, 280, 180, 140 and 100 kDa) as that of day 0 egg. Native protein profile of the eggs treated with 0.1, 1.0 and 10.0 ppm of RH-2485 (Fig.1, lane 3, 4 and 5) were similar to control eggs of day 3. Since there was no difference in protein profile of the eggs of day 0 and day 3 control, it is inferred that the yolk proteins are not utilized during first three days of embryonic development.

Native protein profile of control eggs of day 5 showed seven fractions (Fig. 1, lane 6) six fractions were similar to day 3 but there was the appearance of a protein at 160 kDa region. All the three treated groups of the same age group also showed similar proteins (Fig.1, lane 7, 8 and 9). Native protein profile of eggs on day 7 of embryonic development showed three fractions at 690, 200 and 90 kDa regions (Fig 3, lane 1). The eggs treated with 0.1ppm showed four fractions at 690, 640, 200 and 90 kDa regions (lane 2). In contrast, the eggs treated with 1.0ppm showed three major fractions at 690, 640 and 120 kDa regions (lane 3). The eggs treated with 10.0ppm showed four fractions at 690, 640, 140 and 100 kDa region (lane 4).

Towards the completion of embryonic development on day 9, though proteins extracted were positive to quantitative estimation, the extract did not show any positive staining of egg protein fraction observed previously to Coomassie blue (Fig. 3, lane 5). But the eggs treated with 0.1ppm showed 2 fractions at 640 and 120 kDa region (Fig 3. lane 6). The eggs of 1.0 ppm treated group showed three proteins at 640, 140 and 100 kDa regions (lane 7). The eggs of 10.0 ppm treated groups showed four fractions at 690, 640, 140 and 100 kDa regions (lane 8).

Native proteins separated on 7% native PAGE from 10.0 ppm treated eggs on day 9 of embryonic development (day of hatching in the control eggs) were eluted and separated in 7% SDS-PAGE and presented in Fig 4. Protein 1 (690 kDa) showed five fractions at 180, 70, 58, 44 and 30 (31, 29, 28) kDa regions (lane 2). The protein 2 (640 kDa) showed two fractions at 180 and 44 kDa regions (lane 3). The third protein (140 kDa) showed only fraction of 80 kDa region (lane 4). The eluted fourth protein (100 kDa) did not show any band in 7% SDS-PAGE. This could be due to insufficiency or loss. The protein profile of day 0 control egg was given in lane 1 for comparision.

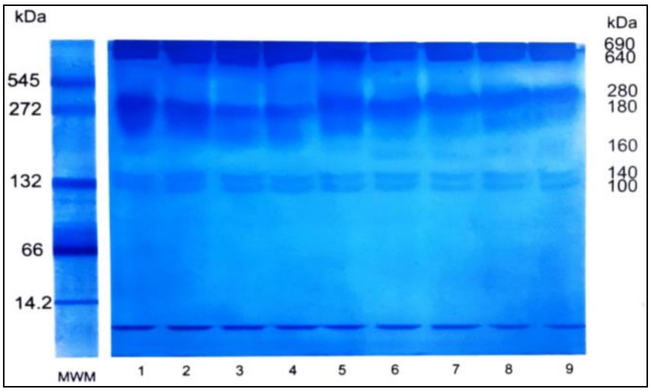

Fig 1: Effect of RH-2485 on the protein profile of the eggs of silk moth *B.mori*. Each lane was loaded with 100 µg of protein in 7% Native -PAGE (mini slab gel) and stained with Coomassie brilliant blue. Lane 1-Protein profile of freshly laid control eggs, Lane-2 control eggs of day 3, Lane 3,4 and 5 RH-2485 treated (0.1ppm, 1.0ppm and 10.0 ppm) eggs of similar age, Lane 6- day 5 control eggs, Lane 7- treated (0.1ppm), Lane 8- treated (1.0ppm), Lane 9- treated (10.0 ppm) eggs of day 5.MWM-molecular weight marker proteins (Urease hexamer-545 kDa, Urease trimer-272 kDa, bovine serum albumin dimer -132 kDa, bovine serum albumin monomer-66 kDa, lactalbumin-14.2 kDa).

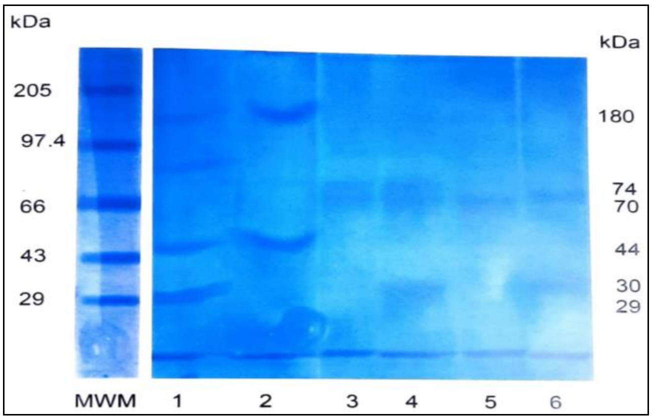

Fig 2: Subunit of native proteins eluted from freshly laid control eggs of silk moth *B.mori* in 7% SDS-PAGE. Each lane was loaded with protein eluted from individual native protein and stained with Coomassie blue. Lane 1-Subunits of native protein 1 of freshly laid control eggs, Lane 2-Subunits of native protein 2 of similar age, Lane 3-subunit profile of native native protein 3, Lane 4-Subunits of native protein 4, Lane 5-subunits of native protein 5. MWM-Molecular weight marker proteins (Myosin-205 kDa, Phosphorylase b-97.4 kDa, Bovine serum albumin-66 kDa, Ovalbumin-43 kDa, Carbonic anhydrase-29 kDa).

The protein extracted from *B.mori* eggs treated with different concentration of RH- 2485 revealed changes in the number and intensities of polypeptides in 7% SDS-PAGE.

The protein profile of the egg homogenate showed seven Coomassie blue stained polypeptides resolved in the region of molecular weight ranging from 205-20 kDa. The molecular weights of these protein were determined by refering to the standard molecular weight marker proteins. The protein profile of fresly laid eggs is given in lane 1 of Fig 5. The polypeptides with molecular weights of 205, 180, 74, 70, 58, 44 and 30 kDa showed very high intensities. The protein profile of the eggs subjected to topical application of different concentration (0.1, 1.0 and 10.0 ppm) of RH- 2485 are presented in lane 2, 3 and 4 of Fig 5. These treated eggs of day 3 did not differ from control eggs. The protein profile of the eggs after 5 days of treatment showed the appearance of a polypeptide at 54 kDa region in the control as well as different treatment groups (Fig. 5 lane 6, 7, 8 and 9).

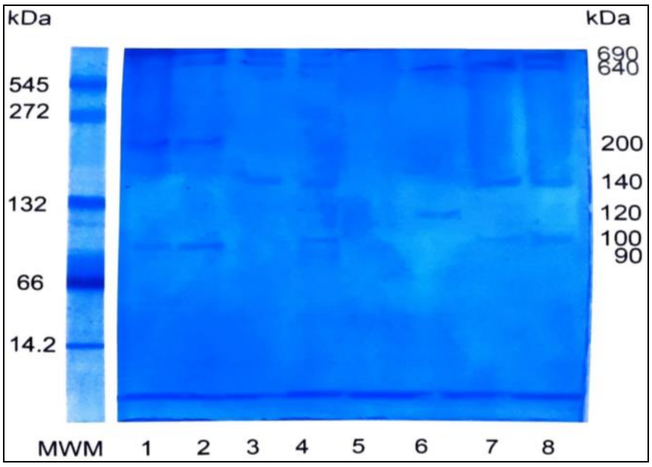

Fig 3: Protein profile of the eggs of B. mori subjected to topical application of RH-2485 in 7% Native-PAGE. Lane 1-Control eggs of day 7, Lane 2, 3 and 4-protein profile of day 7 eggs treated with 0.1, 1.0 and 10.0 ppm. Lane 5- protein profile of day 9 control eggs, Lane 6-0.1 ppm treated, Lane 7- 1.0 ppm treated, Lane 8 - 10.0 ppm treated, MWM-molecular weight marker proteins as in Fig 1.

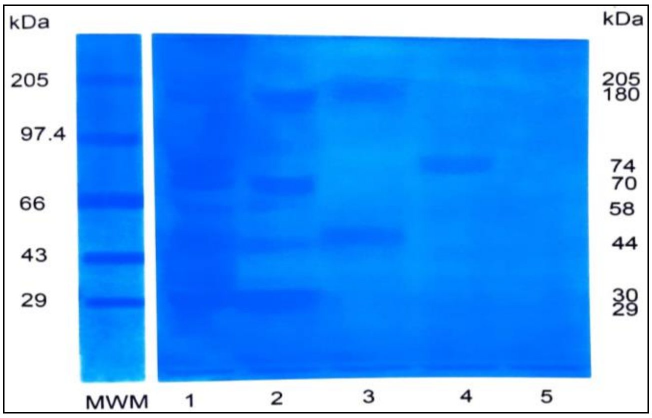

Fig 4: Effect of RH-2485 (10.0 ppm) on the protein profile of the eggs on day 9 of development in silk moth *B.mori* in 7% SDS-PAGE. Each lane was loaded with proteins eluted from 7% Native-PAGE (mini slab gel) and stained with Coomassie blue. Lane 1- Protein profile of day 0 egg, Lane 2-subunits of native potein 1, Lane 3-subunits of native protein 2, Lane 4-subunit of Native protein of 3, MWM –molecular weight marker as in Fig 2.

As embryonic development reached day 7, out of seven major polypeptides observed in day 0 control eggs all six polypeptides. (205, 180, 74, 70, 58 and 44 kDa) disappeared completely leaving onlythe polypeptide at 30 kDa region (Fig6, lane 1). The eggs treated with 0.1ppm showed polypeptides at 180, 44 and 30 kDa regions. In contrast to this, the eggs treated with 1.0 and 10.0 ppm showed a large number of faintly staned fractions in addition to intensely stained polypeptide at 205, 180, 54, 44 and 30 kDa (lane 3). In 10.0 ppm treated eggs only five major fractions were observed.

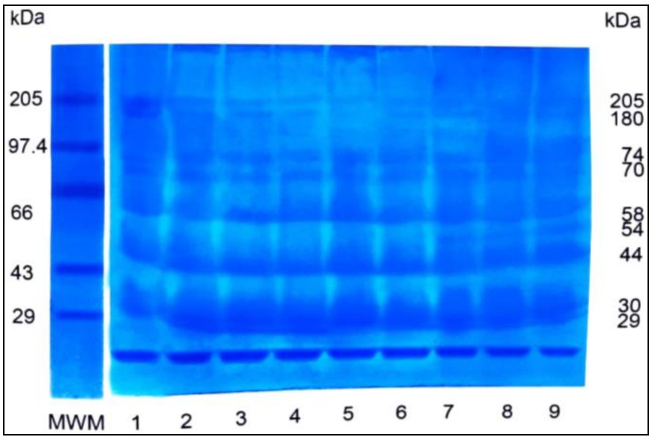

Fig 5: Effect of RH-2485 on the protein profile of the eggs of silk moth *B.mori*. Each lane was loaded with 100 µg of protein in 7% SDS-PAGE (mini slab gel) and stained with Coomassie blue. Lane 1- control eggs of day 3, Lane 2-control eggs of day 3, Lane 3,4 and 5-RH-2485 treated (0.1ppm, 1.0 ppm and 10.0 ppm) eggs of similar age, Lane 6-day 5 control eggs, Lane 7-treated (0.1 ppm) Lane 8-treated (1.0 ppm) Lane 9-treated (10.0 ppm) eggs of day 5. MWM-molecular weight marker proteins as in Fig 2.

Fig 6 gives the protein profile of eggs on the 9th day the day of hatching of control eggs of treatment. The hatched larvae and the remains of the control eggs did not show any polypeptides which were observed previously (lane 5). Similar was the observation in the eggs treated with 0.1ppm (lane 6). But the eggs treated with 1.0ppm (lane 7) showed polypeptides at 58, 44 and 30 kDa regions and 10.0 ppm treated group had polypeptides at 205, 180, 80, 58, 44 and 30 kDa regions (lane 8). Further, the polypeptides at 180, 44 and 30 kDa regions were of very high intensity.

Immunodiffusion

Female specific protein is the precursor of yolk protein. Since egg protein antiserum of painted grasshopper of *Poecilocera picta* was found to cross react with *B.mori* egg protein, a basic immunologic assay namely immunodiffesion was carried out to confirm the extend of degradation of yolk protein during embryonic development in this study.

When immunodiffusion assay was carried out with the egg homogenate of day 0 egg against antiegg protein antibody, a precipitin line was formed (Fig. 7a) the egg homogenate from day 3 control and RH-2485 (0.1, 1.0 and 10.0 ppm) treated eggs also formed precipitin lines (Fig. 7, b, c, d and e). These precipitin lines were of same intensity. On day 9 of embryonic development when the control eggs hatched out, the egg homogenate (both freshly hatched larvae and the remains in the egg) of control group and 0.1 ppm treated group did not show any precipitin line in the immunodiffusion assay (Fig. 8, a, b). Whereas thin precipitin lines were formed between the egg- protein antibody and egg homogenate of 1.0 and 10.0 ppm treated eggs (Fig. 8, c, d).

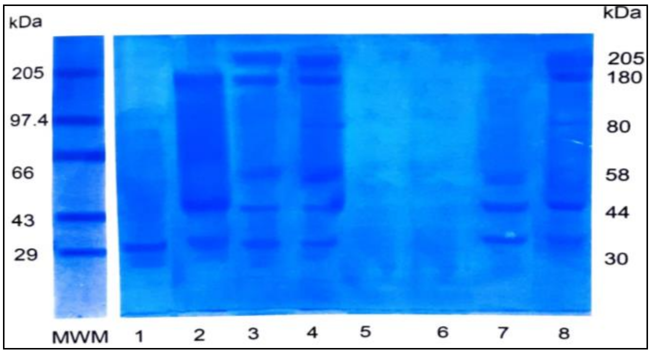

Fig 6: Protein profile of the eggs of *B.mori* subjected to topical application of RH-2485. Lane 1- control eggs of day 7, Lane 2,3 and 4-Protein profile of day 7 eggs with 0.1, 1.0 and 10.0 ppm. Lane 5-control egg of day 9, Lane 6-0.1 ppm treated group of similar age, Lane 8-1.0 ppm treated eggs, Lane 9- 10.0 ppm treated group. Each lane was loaded with 100 µg of protein in 7% SDS-PAGE and stained with Coomassie blue. MWM- molecular weight marker proteins as in Fig. 2.

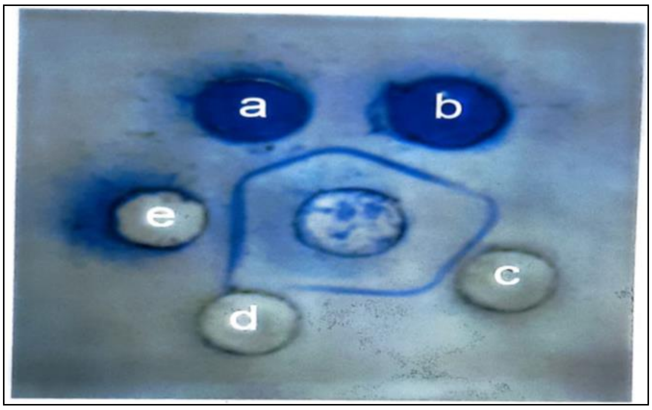

Fig 7: Immuno diffusion assay of antiserum of yolk protein during embryonic development. Yolk protein of day 0 control egg (a), protein extract from day 3 control egg (b) and RH-2485 (0.1 ppm (c), 1.0 ppm (d), and 10.0 ppm (e) treated eggs of day 3 embryonic development in *B.mori*.

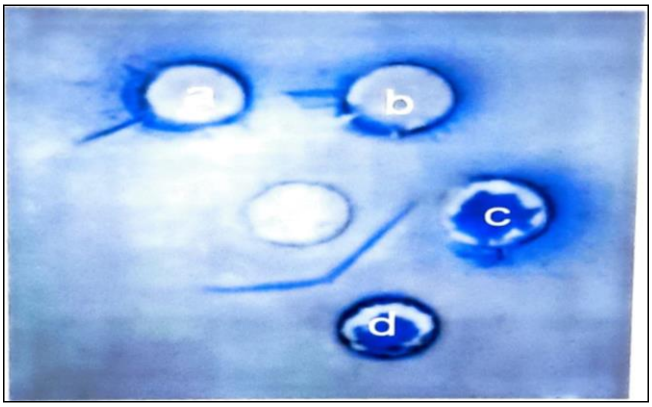

Fig 8: Immunodiffusion assay of antiserum of yolk protein against egg protein extract of *B.mori* on day 9 of embryonic development. Protein extract of freshly hatched larva and the egg remains (a) protein extract freshly hatched larva and the egg remains of 0.1 ppm treated group (b), protein extract from 1.0 ppm treated group (c) protein extract from 10.0 ppm treated group (d).

The occurrence of protein at 640 kDa region in the native PAGE, polypeptides at 180, 44 and 30 kDa region in SDS-PAGE and occurrence of precipitin line in immunodiffusion assay of the eggs indicate that the major yolk protein vitellin was not utilized fully in the eggs treated with 10.0 ppm.

Discussion

Methoxyfenozide (RH-2485) an insect growth regulator category of insecticide is used efficiently in lepidopteran pest control and the studies on antifertility and antifecundity in lepidopterans are in progress. In this study an attempt was made to find the influence of RH- 2485 on programmed degradation of yolk protein during embryogenesis and egg hatch in the model insect silk moth *B.mori*.

The topical application of RH-2485 at higher doses was highly effective in preventing egg hatchability. In support of this finding, reduction in egg hatchability was reported in tufted apple moth *Platynota idaeusalis* (Biddinger and Hall, 1999) [1] codling moth *C.pomonella* (Pons *et al.*, 1999; Knight, 2000) [44, 29], S.littrolis (Budia *et al.*, 1999) [3] and *E.viteana* (Rufus *et al.*, 2005) [5] exposed to tebufenozide or methoxyfenozide.

The yolk protein profile showed 6 fractions (690, 640, 280, 180, 140 and 100 kDa) in 7% native PAGE and 7 fractions (205, 180, 74, 70, 58, 44 and 30 kDa) in 7% SDS-PAGE in the freshly laid eggs of *B.mori*. Among these seven fraction in SDS-PAGE five polypeptides with molecular weight of 180, 74, 70, 44 and 30 kDa were separated in the eggs of *B.mori* by different authors (Ono *et al*., 1975; Yamashita, 1986; Zhu *et al.*, 1986; Indrasith *et al.*, 1998 and Rajathi, 2002) [41, 64, 64, 45]. The polypeptides at 180 and 44 kDa were the heavy and light subunit of vitellin (Yamashita, 1986; Rajathi, 2006) [64]. Similarly, polypeptides at 74 and 70 kDa region were the subunits of egg specific protein (Indrasith *et al.*, 1998). These fractions did not show any change upto three days of embryonic development. A native protein of 160 kDa appeared on day 5 of embryogenesis and disappeared on subsequent days, similarly polypeptides less than 30 kDa appeared in SDS-PAGE on day 7 and disappeared later.

Appearance of 54 kDa polypeptide and its disappearance during later days of embryogenesis observed in this study could be supported from the studies of Indrasith *et al.*, 2003 [24] who have reported the appearance of 96, 55, 46 and 36 kDa polypeptides and the subsequent disappearance before hatching in *B.mori*.

The results of this study indicate that there is sequential proteolysis of yolk proteins, in this process the egg- specific proteins are degraded completely before the degradation of vitellin and 30 kDa protein. A unique trypsin like protease was responsible for this degradation (Indrasith *et al.*, 1988), since developmental changes in protease activity are parallel to the decrease in egg specific protein but not with vitellin and 30 kDa protein (Zhu *et al*., 1986) [64].

Studies also demonstrated proteolytic processing of vitellin into polypeptides, after 3-4 days of post ovulation which are then accessed by embryo. Enzyme cysteine protease is involved in this degradation (Liu *et al.*, 1996) [36]. Therefore, it is evident that programmed degradation of egg specific proteins and vitellin starts after 3 days and the former is utilized fully during mid embrygenesis and the later towards the completion of embryonic development.

Topical application of different concentration 0.1, 1.0 and 10.0 ppm of RH-2485 had induced changes in the yolk protein degradation. The eggs treated with 0.1 ppm did not show marked difference in yolk protein degradation. Since the polypeptide at 74 and 70 kDa regions disappeared fully in the eggs treated with 1.0 ppm, the processing of egg-specific protein is not affected at these 2 concentrations.

Presence of 180, 44 and 30 kDa polypeptide in 7% SDS-

PAGE and the occurrence of precipitin line in immunodiffusion assay of the egg treated with 10.0 ppm clearly states impairment of vitellin processing during embryogenesis in response to RH-2485 treatment.

At this juncture, it is important to analyse the reasons for restricted vitellin processing. It has been well established that eggs of insects including *B.mori* contain various ecdysteroids and the amount of these ecdtsteroids fluctuates during embryonic development (Ohinishi, 1987) [40]. Ecdysone and 20-hydroxyecdysone occur in the egg almost exclusively in conjucated form (Mizuno *et al*., 1981). Ecdysteroids are reported to play a role in cellular differentiation in embryonic stages and cuticulogenesis. In *Blaberus cranifer* three ecdysteroids peaks were observed during embryonic development, the first peak coinincided with the end of metamaerisation the second peak with the first cuticle and the third with transition between the first and the second (Bulliare *et al*., 1979). Recently, Yamada *et al*., 2005 [61] have reported the occurrence of physiologically inactive phosphoric esters of ecdtsteroids in the newly laid eggs of *B.mori*. An enzyme ecdysteroid phosphate phosphatase (EPPase) catalyses the dephosphorylation of ecdysteroid phosphates into free ecdysteroids, during early embryonic development. Further ecdysteroid-phophates are localized in yolk granules being bound to vitellin. But EPPase is not able to hydrolyse vitellin bound ecdysteroid phosphate. The mechanism of dissociation investigated by them show that the acidification of yolk granules cause this dissociation.

The results of this study along with other available evidences support the fact that the occurrence of ecogenous ecdysteroid agonist might have restricted the need for the release of maternal ecdysteroids from vitellin and the processing of native vitellin into their subunits which could be accessed by the embryos. Since the embryos do not have an access to vitellin their development and subsequent hatching are affected.

Since 30 kDa protein also occur in high intensities in the unhatched eggs of RH-2485 treated group the processing of 30 kDa protein is also inferred by the treatment of 30 kP protease A is responsible for 30 kDa protein degradation in silkworm (Maki and Yamashita, 2005). 30 kP preotease A was reported to rise at the last phase of embrygenesis and reached a maximum level at larval hatching. As 30 30 kDa protein occurred at high intensities in 10.0 ppm treated group the enzyme (30 Kp protease) might have not been synthesized.

An enzymes cysteine protease with molecular mass of 47 kDa which catalyses the degradation of different protein substrates such as haemoglobin, vitellogenin, vitellin and lipophorin was shown to be involved in protein degradation during embryonic development in silkworm (Kageyama & Takahashi, 1990) [27]. It is not known whether the treatment has affected the synthesis or release of this enzyme or both. If cysteine protease activity is assayed in response to RH-2485 it could be possible to give a concrete evidence.

The occurrence of 180, 44 and 30 kDa polypetides in the unhatched eggs from 10.0 ppm treated group indicates that programmed degradation of major yolk protein vitellin is affected by RH-2485. But it is not known whether its effect is direct or indirect. The evidence of this study and other reports that the effect could be indirect since mRNA responsible for the production of various enzyme were produced from the maternal source and packaged in the eggs. RH-2485 might specifically interfere with the post transcriptional or translational processes involved in the formation of enzymes.

The results of this study revealed the occurrence of programmed degradation of yolk proteins during embryonic development and the yolk proteins were utilized completely in control eggs. But the eggs subjected to topical application of 10.0 ppm RH-2485 showed yolk protein degradation during early stages of embryonic development. But the major egg protein vitellin was not degraded fully during later stages of embryonic development. Therefore, RH-2485 at higher doses could restrict programmed degradation of yolk protein during embryogenesis.

Conclusion

Topical application of 0.1, 1.0 and 10.0 ppm of methoxyfenozide (RH-2485) in acetone to freshly laid eggs of silk moth *B. mori* had reduced egg hatchability among different treatment groups. Egg homogenate of freshly laid eggs showed six proteins at 690, 640, 280, 180, 140 and 100 kDa regions in 7% native -PAGE and 7 polypeptides at 205, 180, 74, 70, 58, 44 and 30 kDa in 7% SDS-PAGE stained with Coomassie blue (R.250). On elution, native protein 1 (690 kDa) of day 0 eggs showed four polypeptides at 180, 80, 44 and 30 kDa regions in 7 % SDS-PAGE. Protein 2 (640 kDa) got separated into two subunits at 180 and 44 kDa regions. The third native protein (280 kDa) showed two subunits at 74 and 70 kDa regions. The fourth native protein (180 kDa) showed three polypeptides at 74, 70 and 30 kDa regions. The fifth native protein (140 kDa) showed only a single polypeptide at 70 kDa region. The last protein in native gel (100 kDa) showed two subunits at 74 and 30 kDa regions. There was no change in protein profile of eggs in control as well as treated group on day 3 of embryonic development both in native and SDS-PAGE.

As the development reached day 5, new protein appeared at 160 kDa region in native-PAGE and 54 kDa region in SDS-PAGE in all the groups. On day 7 of embryonic development, three proteins at 690, 200 and 90 kDa regions were observed in native- PAGE of control eggs, but only 30 kDa region was observed in SDS-PAGE. On the same day 0.1ppm treated eggs showed four native proteins at 690, 640, 200 and 90 kDa regions, whereas 1.0ppm RH-2485 treated eggs three major proteins at 690, 640 and 120 kDa region. 10.0 ppm treated group had major proteins at 690, 640, 140 and 100 kDa regions. On the same day 0.1 ppm treated eggs showed 180, 44 and 30 kDa polypeptides in SDS-PAGE. 1.0 and 10.0 ppm had polypeptides at 205, 180, 80, 54, 44 and 30 kDa regions. On the day of hatching, all native proteins and their subunits disappeared both native and SDS-PAGE in control egg group. On the same day, the eggs are treated with 0.1 ppm showed only two proteins at 640 and 120 kDa regions in native PAGE, whereas 1.0 ppm treated eggs showed three proteins at 640, 140 and 100 kDa regions. The unhatched eggs of 10.0 ppm treated group showed three native proteins at 690, 640 and 280 kDa regions. Native protein 1 (690 kDa) on elution gave five subunits at 205, 180, 80, 44 and 30 kDa. The second native protein (640 kDa) had only two subunits at 180 and 44 kDa regions. The third protein at 280 kDa showed only one subunit (80 kDa). The unhatched eggs of 10.0 ppm treated eggs showed polypeptide at 205, 180, 80, 58, 44 and 30 kDa region.

In immuno diffusion assay, well marked precipitin lines were formed between egg homogenate and anti egg protein antiserumon day 0 and day 3 of embryonic development. On the day of hatching, there was no precipitin line formed between egg homogenate and antibody of egg protein in the control and 0.1 ppm treated group as well. But thin precipitin lines were observed both 1.0 and 10.0 ppm treated unhatched eggs. To sum up, the eggs subjected to topical application of methoxyfenozide (RH-2485) was found to arrest yolk protein degradation and hatching during embryogenesis in the model insect *B.mori*.

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