Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

J E Z S Journal of Entomology and Zoology Studies

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2015; 3(5): 285-290

Received: 01-08-2015
Accepted: 04-09-2015

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Biochemical analysis of silk proteins of the mango leaf webber, *Orthaga exvinacea* Hampson (Lepidoptera: Pyralidae)

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Abstract
The mango leaf webber, *Orthaga exvinacea* makes silken webs and galleries during its larval stage. Silk is produced from the silk gland of this insect. Quantitative and qualitative analysis of proteins from the silk glands of last three larval stages and pre-pupae were conducted. Significant differences were observed among different stages. SDS-PAGE of proteins from anterior, middle and posterior regions of silk glands of final instar larvae revealed that the posterior region had several proteins than the other two regions of silk glands. Anterior region had no visible protein bands. The proteins of molecular weights 269 kDa, 251 kDa, 175 kDa, 125 kDa and 66 kDa were common to posterior region of silk glands, web and cocoon of the mango leaf webber. The regenerated protein obtained from the web had a molecular weight of 125 kDa, which may be a fibroin like web and cocoon except 125 kDa molecular weight protein may be sericins, which are the members of a glue protein family. The regenerated liquid silk had two UV absorption peaks at 214 nm and 272 nm. The anti-UV properties of silk protein of *Orthaga exvinacea* can be exploited in cosmetic industry.

Keywords: silk glands, silk proteins, *Orthaga exvinacea*, SDS-PAGE, sericin, fibroin

Introduction
Silks are protein polymers produced by various species of insects and spiders. It is used for different purposes which include construction of protective shelter, structural support for developing eggs and egg sacs, reproduction, foraging and dispersal [1-3]. In the insect order Lepidoptera, Bombycidae and Saturniidae are the two important families utilized for commercial silk production. These two families are characterized by low silk production in early larval stage and enormous silk production in the last instars. During the production of cocoon, around 20% of the body mass is converted to silk.

The tasar silkworm, *Antheraea mylitta* has the highest silk producing capacity among all silk spinning insects [4]. Silk glands in insect larvae are ectodermal in origin, which is anatomically and physiologically divided into three distinct regions, viz., anterior, middle and posterior regions [5]. The anterior, middle and posterior region of silk glands of *B. mori* larvae consist of 200 cells, 255 cells and 520 cells respectively [6]. The morphogenesis [7] of silk glands are completed within eight days after egg laying. Silk is a natural fiber, up to 95% of which is composed of fibroin and sericin and the remaining 5% constituted by other proteins, waxes, fats, salts and ash [8]. Fibroin is the major structural protein formed by two different polypeptide chains, i.e., heavy (H) and light (L) chains of molecular weights 350 kDa and 25 kDa respectively. These two chains are linked together by di-sulfide bonds [3, 9]. A glycoprotein, P25, has also been associated with H-L complex by non-covalent interactions [10-13]. In *B. mori*, fibroin was identified as the product of the posterior region of the silk gland, whereas sericin is produced in the middle region that serves as silk reservoir [14]. In the posterior region of silk gland the concentration of fibroin protein is around 12-15% by weight, while fibroin and sericin is 30% by weight in the middle region of silk gland [15]. Sericin accounts for 20-30% by weight of *B. mori* cocoon fibers [16, 17]. Sericins include sericin P (150 kDa), sericin M (400 kDa) and sericin A (250 kDa) identified in the distal, central and anterior of the middle regions of silk gland respectively [18]. In the lumen of gland silk proteins accumulate as a concentrated gel. During spinning, the liquid silk is subjected to stress and elongation, thus forming silk fibers. The unique properties of silk are mainly due to long storage of silk in the form of gel followed by its rapid conversion to silk filament [19].

Tasar, muga, eri, fagaria and shashe silks are produced by the non-mulberry silkworms *A. mylitta, A. assama, Philosamia ricini, Attacus atlas* and *Gonometa postica* respectively.

In tasar silk (*A. mylitta*), fibroin have a molecular weight of 395 kDa under native condition. But in reduced condition, fibroin is a homodimer, each having a molecular weight of 197 kDa [20-22], while the molecular weight of sericin ranges in between 70-200 kDa [23, 24]. Fibroin protein in muga silk (*A. assama*), consists of two units having molecular weights of about 220 kDa and 20 kDa respectively [25, 26], whereas the sericin protein is about 66 kDa. *P. ricini* possess sericin protein, whose molecular weight is same as that of *A. assama* but fibroin consists of two units, having molecular weights of 97 kDa and 45 kDa [21, 22, 25].

In tasar silkworm *A. mylitta*, the content of protein in the silk glands vary with ecoraces. The Daba ecoraces of tasar silk worms showed high amount of protein than the Sukinda and Sarihan ecoraces. In addition to it, the content of the silk gland also varied with sexes. The female larvae of tasar silkworm have more of proteins compared to male larvae [27].

The present study is an attempt to determine the molecular weights of proteins in the silk glands, web, cocoon and degummed web, the UV absorption properties of the regenerated fibroin like liquid silk from web and also to quantify proteins present in the different developmental stages of silk glands of the mango leaf webber, *O. exvinacea*.

Materials and Methods

Experimental animal

Orthaga exvinacea, the mango leaf webber was used in this experiment. The larvae of *O. exvinacea,* were collected from different parts of Kozhikode and Malappuram districts of Kerala, India from infested mango trees. They were fed on mango leaves and reared in wooden cages with glass sides, wire gauze top and wooden bottom (30×30×30 cm) in the laboratory at a room temperature of 27±2 ºC and 60%-70% relative humidity.

Isolation of silk glands

Insect larvae were cut open longitudinally along the mid-dorsal line and the digestive system was removed. Paired silk glands were collected from V, VI and VII instar larvae and pre-pupal stage, washed in ice cold insect ringer and kept in separate Eppendorf tubes containing 200 µl of 0.01% SDS and stored at -20 ºC.

Extraction and quantification of proteins from silk glands

Frozen silk glands collected from the various stages were thawed and homogenized in 200 µl of 0.01% SDS solution by ultra-sonication. The homogenates were centrifuged at 12000 rpm at 4 ºC for 10 min. The supernatant was collected for quantification of proteins. Quantifications were done using a modified method of Lowry *et al*. [28] using BSA as the standard. The data obtained were subjected to statistical analysis.

Electrophoretic analysis of proteins

SDS PAGE-the samples of silk gland homogenates prepared as mentioned above were used for PAGE analysis. The supernatents were collected and stored at -20ºC. Web and cocoons were collected from culture cages and cleaned off from excretory pellets and other debris. Cleaned samples of 100 mg of each were dissolved in 1.0 ml of sample loading buffer (Tris-HCl, Glycerol, bromophenol blue, β-mercaptoethanol and SDS) by boiling for 30 min. All samples containing about 150 µg of proteins were electrophoresed in 10% polyacrylamide gel of thickness 1.5 mm according to the method described by Laemmeli *et al*. [29]. All the extracted proteins from the silk glands of different stages were separately loaded on to the gel. The running of gel was performed at 200 V for 45 min. The gels were stained with Coomassie brilliant blue G250. Destaining of gels was done in a mixture of methanol (5%) and glacial acetic acid (7.5%).

Purification of fibroin like protein from the web of *O.exvinacea* were done according to the method described by Sajitha and Gokuldas [30]. After dialysis, the regenerated protein solution was used for further studies. The quantification of protein solution was done by Bradford [31]. The protein sample purified by dialysis was also subjected to SDS-PAGE analysis.

UV spectrophotometric analysis

The regenerated protein solution prepared as described above was scanned in JASCO double beam UV–visible spectrophotometer, Model: V-630, with a fixed band width (2 nm) and 1 cm quartz cell. The solution was taken at a concentration of 10 mg/ml.

Results

Quantitative changes of protein in the silk glands in various development stages of *O. exvinacea*

Total protein content in the silk glands of V instar larvae was found to be 12.39±0.14 µg/mg of tissue (Table 1). But the content of proteins becomes almost half in the VI instar. A significant decrease in the amount of protein in the silk glands has been observed (P<0.0001) (Table 1). The protein content was 6.67±0.22 µg/mg (Table 1). As the larvae moulted into VII instar, there was a slight increase in protein which was around 7.17±0.21 µg/mg. The increase was not statistically significant (P=0.1421). However, a remarkable increase was seen in the level of protein at the pre-pupal stage (P<0.0001). The protein content in the pre-pupal stage had shown a sharp increase upto 22.36±0.23 µg/mg.

Table 1: The amount of protein present in the silk glands of various developmental stages of *O. exvinacea*

S. No.	Different stages	Amount of proteins (µg/mg of tissue)
1	V	12.33 ± 0.142
2	VI	6.67 ±0.227
3	VII	7.17 ± 0.210
4	Pre-pupa	22.36 ± 0.230

Values are expressed as means ±SEMs, n=7.
Paired 't'test of 1 & 2, P<0.0001
Paired 't'test of 2 & 3, P=0.1421
Paired 't'test of 3 & 4, P<0.0001
Paired 't'test of 4 & 1, P<0.0001

Silk protein maps of silk proteins of *Orthaga exvinacea*

The molecular weight of proteins in the bands observed in polyacrylamide gel were determined by plotting the relative mobility of protein bands with respect to antilog of standard marker run in the same gel and confirmed by Quantity One program (Lane 5; Fig. 1). The result showed that sample loaded in Lane 1, which was from V instar has numerous bands compared to proteins from VI and VII instars and pre-pupal stage. The molecular weight of proteins in the bands

ranged from 125 kDa-30 kDa. Altogether 15 bands were seen on this lane. Some protein bands showed higher intensities compared to others. The molecular weights of intense bands were of 84 kDa, 54 kDa, 48 kDa, 46 kDa, 39 kDa, 37 kDa, 32 kDa and 30 kDa respectively. In VI instar (Lane 2; Fig. 1) 13 protein bands were seen. But in the case of VII instar, (Lane 3; Fig. 1) there were only 10 protein bands, the intensities of 4 protein bands were found to be relatively high, which showed molecular weights of 80 kDa, 77 kDa, 54 kDa and 48 kDa respectively. Even though the amount of protein in pre-pupal stage was significantly high, the numbers of protein bands were only few (Lane 4; Fig. 1). However, the intensities of the following proteins of molecular weight 77 kDa, 46 kDa, 32 kDa and 38 kDa were found high.

In another experiment, protein profile of the different regions of the silk glands of the VII instar larvae was analyzed on PAGE. Proteins extracted from the anterior, middle and posterior region of the silk glands were loaded on to gel. Extracts from anterior region (Lane 1, Fig. 2) had no visible bands but that from middle region (Lane 2 and 4, Fig. 2) showed 9 visible bands having molecular weights ranging from 104 kDa to 45 kDa. Posterior regions (Lane 3, Fig. 2) of silk gland had 20 visible bands ranging from 269 kDa to 45 kDa. From an analysis of protein profile of web and cocoon, it was observed that the separated protein bands had molecular weights 269 kDa, 251 kDa, 175 kDa 125 kDa and 66 kDa (Lane 1 and 3, Fig. 3).

Properties of purified web protein

Around 30% of weight loss has been observed after degumming of the web. Before degumming and purification, the web contained 5 distinct protein bands (Lane 1, Fig. 3). The purified regenerated silk protein showed a single distinct band of 125 kDa (Lane 1 and 2, Fig. 4). All the proteins except 125 kDa were lost during the purification process. The UV absorption spectra of regenerated liquid silk showed absorption peaks at 214 nm and 272 nm (Fig. 5).

4. Discussion

Secretion of silk by modified salivary glands is a common phenomenon in Lepidoptera. In pyralids and some other groups of lepidopterans, silk is used to make webs that protect the larvae that live in resilient silky tubes. Construction of cocoon in which the larvae pupate is a general character of lepidopterans especially in moths. The present work is an investigation on the silk proteins of silk glands and the web silk of *O. exvinacea,* a pyralid moth. The pattern and amount of protein from the silk glands of different developmental stages and separate analysis of different region of silk glands of seventh instar larvae of *Orthaga* showed major differences (Figs. 1 and 2).

The pattern of total protein content of the silk gland and its distribution in various developmental stages of *O. exvinacea* differed from that of *B. mori*. *Orthaga exvinacea* which produces silk throughout its larval stages for nesting, also make use of silken tubes to escape from predators and parasitoids. During the V instar, larvae voraciously feed on mango leaves and spin silken threads to make galleries. Due to the utilization of large amount of proteins for spinning towards the end of VI instar the amount of protein in salivary gland decreases considerably, to almost half of the quantity in the V instar. This lower plateau is maintained during VII instar also because feeding and web formation is relatively feeble in these stages. In contrast to *Orthaga*, the silk gland of *B. mori* [32] showed maximum level of protein at the V instar and minimum at III instar. The high level of protein in the silk glands of V instar larvae account for the increased level of the silk protein synthesis required for spinning cocoon. There is a steady of increase in the amount of soluble proteins with the growth of the silk glands during the first phase of V instar larvae of *B.mori* [33]. During second phase, the protein shows a plateau. The amount of soluble protein in the silk gland decreased rapidly after spinning the cocoon.

The VII instar, which is the last larval stage of *Orthaga*, make cocoon with the help of silken threads, excreta and veins of mango leaves. The cocoon formed is very small and the amount of protein required for spinning cocoon is relatively low. Hence the use of silken threads is less pronounced in the cocoon than in the larval stage. After the formation of cocoon, larvae enter the pre-pupal stage. The amount of protein in the silk gland observed at this stage was very high, which may be due to the production of enzymes involved in autolysis and phagocytosis of silk glands. A similar phenomenon has been reported in *Galleria mellonella* [34], where the silk glands undergo autolysis and phagocytosis in the pupal stage. The enzymes like acid and alkaline DNAases and phosphatases, which are responsible for autolysis and phagocytosis showed high activity in the pupal stage of *G. mellonella*. A complete absence of silk gland in the adult stage of *B. mori* had been reported earlier [35]. After spinning the cocoon, the silk gland cells of *B. mori* undergoes lysis and gradually it disappears. The apoptotic features of silk glands of *B. mori* [36] appeared at the pre-pupal stage or early pupal stage. The situation appeared to be similar in *O. exvinacea* also as there were no silk glands in the pre-pupal stage of this insect.

The electrophoretic patterns of silk proteins of different stages of *O. exvinacea* obtained from PAGE also support the above observations. The number of protein bands present in V instar (Lane 1, Fig. 1) is much more than the bands present in the other instars. This change in the protein composition in the silk glands of different stages of *O. exvinacea* indicates that the cellular metabolism vary with development in compliance with the physiological functions during different stages. The large number of protein bands of V instar silk glands presumably indicates and correspond to the high activity of larvae and its silk production during V instar. There are some proteins common to middle and posterior region of silk glands suggesting the possibility of the proteins being synthesized in the posterior region and getting transported through the middle region of silk gland for the secretion of silk. But storage of proteins in the middle region of the silk glands prior to the secretion of silk has been reported in *B. mori* [14]. The posterior region of silk glands of final instar larvae of *Orthaga* showed several protein bands as has been reported in the case of *B. mori*. In sweet potato hornworm, *Agrius convolvuli*, the lumen protein [37] recovered from middle and posterior region of labial gland showed no significant difference in its composition. Unlike the sweet potato horn worm, a remarkable difference in the pattern of protein between the middle and posterior regions of silk glands has been observed in *B. mori* [38]. The difference in the pattern of protein between middle and posterior region that has been noticed in *O. exvinacea*

(Lane 2 and 3, Fig. 2) thus resembles the difference observed in *B. mori*. The difference between the protein content of the different region of the silk glands in these insects reflect the requirement of the various proteins during development and related physiological activities.

A protein of molecular weight of 66 kDa has been detected in the posterior and middle regions of silk glands of final instar larvae, web and cocoon of *O. exvinacea* (Lane 2 and 3, Fig. 3; Lane 1 and 3, Fig. 3). All the protein bands detected in web and cocoon were found to be present in the posterior region of silk glands of the final instar larvae of *O. exvinacea.* This is suggestive of the origin of web and cocoon proteins in the posterior region of silk gland. In non-mulberry silkworms like *P. ricini* and *A. assama* [25, 26] the protein of molecular weight 66 KDa has been reported earlier and it was identified as sericin. The protein of similar molecular weight noticed in *Orthaga*, may belong to sericin family. In *O. exvinacea*, the protein from the web of molecular weight 125 kDa remains unchanged before and after regeneration (Lane 3 Fig. 3; Lane 1 and 2 Fig. 4). But in *B. mori* [39] the regenerated heavy chain fibroin appeared as diffused bands on SDS-PAGE. The regenerated protein from the web of *O. exvinacea* showed two UV absorption peaks (Fig. 4). The peak at 272 may correspond to aromatic amino acids like tyrosine present in the protein. Anti-UV properties of the regenerated liquid silk have been reported earlier in *B. mori* [40].

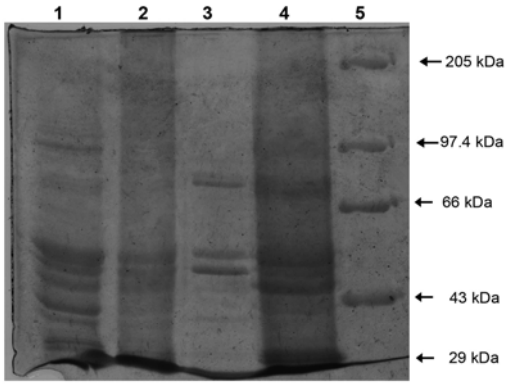

Fig. 1. SDS-PAGE analysis of proteins from silk glands of *O. exvinacea* stained with Coomassie Brilliant Blue G-250
Lane 1 Fifth instar, Lane 2 Sixth instar, Lane 3 Seventh instar, Lane 4 Pre-pupal stage and Lane 5 Standard protein marker

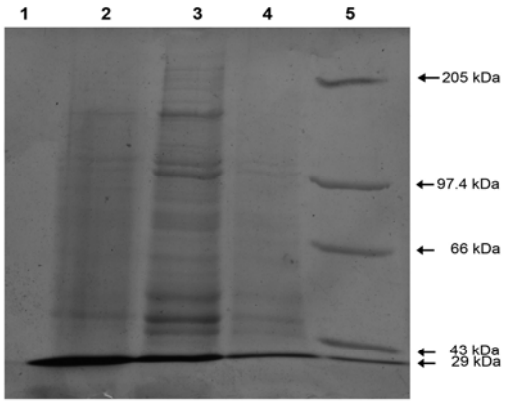

Fig. 2. SDS-PAGE analysis of proteins from different regions of silk glands of seventh instar larvae of *O. exvinacea* stained with Coomassie Brilliant Blue G-250
Lane 1 Anterior, Lane 2 & 4 Middle, Lane 3 Posterior region and Lane 5 Standard protein marker

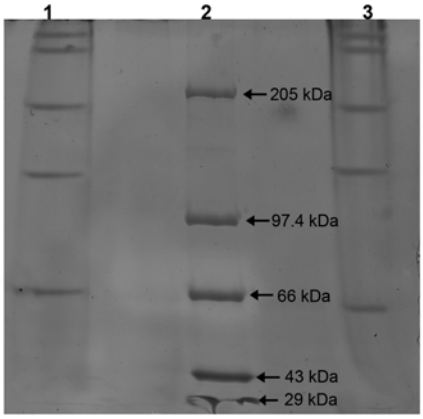

Fig. 3. SDS-PAGE analysis of proteins from web and cocoon of *O. exvinacea* stained with Coomassie Brilliant Blue G-250
Lane 1 Web, Lane 2 Standard protein marker and Lane 3 Cocoon

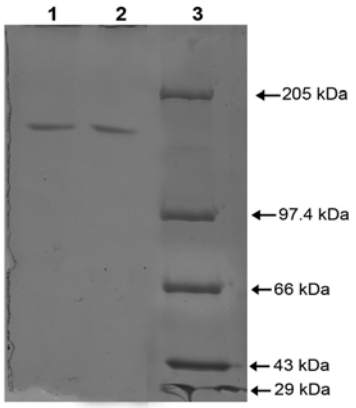

Fig. 4. SDS-PAGE analysis of regenerated fibroin like protein from the web of *O. exvinacea* stained with Coomassie Brilliant Blue G-250
Lane 1 & 2 regenerated (dialyzed protein sample) and Lane 3 Standard protein marker

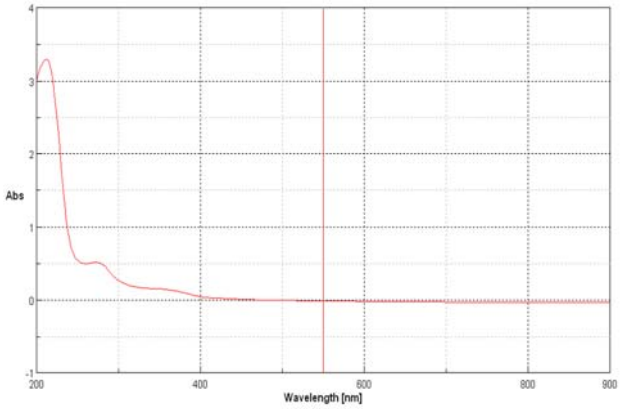

Fig. 5. UV absorption spectrum of regenerated silk solution from the web of *O. exvinacea*

Conclusion

The results revealed that the production and pattern of proteins in the modified labial glands *O. exvinacea* varied according to the use of silk at various stages of development. The most striking feature observed in this study was the production of silk in the larval stage of this insect, especially in V instar. The situation was quite different from *B. mori*, which produce silken cocoon only at the end of the larval stage. The

regenerated protein from the web of *Orthaga* showed UV absorption properties. This anti-UV property of silk of *O. exvinacea* can be exploited in the cosmetic industries as an alternative to mulberry silk protein and other harmful chemicals.

Acknowledgements
The authors acknowledge financial support by the University of Calicut and the equipment fund provided by KCSTE Thiruvananthapuram, Kerala.

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