

# ELECTROPHORETIC SEPARATION AND COMPARATIVE ANALYSIS OF SILK GLAND PROTEINS FROM BOMBYX AND PHILOSAMIA

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Received for publication: June 09, 2014; Revised: June 11, 2014; Accepted: July 16, 2014

**Abstract:** A comparative analysis of protein extracted from different regions of silk glands in the *Bombyx mori* L. and *Philosamia ricini* Hutt was performed employing single-dimensional-electrophoresis technique. Notably, a protein extracted directly from the lumen of the middle silk gland yielded two discrete protein bands with molecular mass of 325 and 26 kDa representing fibroin heavy (H) and low (L) chains than whole silk gland of *B. mori*. Contrastingly, such differentiation in protein separation could not be achieved due to undifferentiated regions of silk gland in *P. ricini*. Interestingly, copious amount of proteins with different molecular weight were noticed in the whole silk gland of *B. mori* and *P. ricini*, whose functional properties in the silk gland remain enigmatic. Thus, middle and whole silk glands of *B. mori* shall be an ideal source for the extraction of fibroin and sericin respectively as potent bio-materials for biotechnological and biomedical applications.

Key Words: Bombyx mori, Electrophoresis, Fibroin, Sericin, Silk Gland

#### **INTRODUCTION**

Among insects, Bombyx mori (Lepidoptera) and Drosophila melanogaster (Diptera) has widely been used in a choice of experimental studies including basic, molecular and biotechnological aspects. However, unlike any other insects, the mulberry silkworm has been domesticated over the centuries owing its economic value and this has been an object of research since medieval times [1]. In recent years, it is recognized as a molecular model organism for various proteomic and genomic investigations among lepidopterans [2] and also for the synthesis of recombinant proteins [3] considering silk gland as a potential source unlike microorganisms. All the more, development of germ line transformation technique in B. mori [4] explicit the worth of this insect as an investigational organism to uncover the developmental mechanism and regulation of tissue specific expression of silk protein genes in the silk gland.

There are many insects that produce fibers, but the term 'SILK' generally refers to fibers produced by the domestic mulberry silkworms (*B. mori*) whereas Wild silk or Vanya silk is from non-mulberry silkworms (*Philosamia* and *Antherea*). The chief compositions of silk fiber are fibroin and sericin wherein fibroin composed of two proteins; light- (25 kDa) and heavychains (325 kDa), [5] and sericin covers fibrous fibroin and function as glue in all types of silkworm cocoons. While fibroin forms the core of 72-80% and sericin is about 28-20% of silk both of them contain same 18 amino acid residues with assorted amounts [6]. But, the major difference is crystalline structure, which makes the fibroin insoluble in water while sericin readily soluble in hot alkaline soap solution due to its loose structural property and rich hydrophilic amino acid [7]. Among many proteins, fibroin and sericin known to synthesize in the posterior and middle regions of the silk gland respectively, are stored in the middle silk gland until cessation of synthesis and then ooze out through an exclusive orifice "spinneret" in the process of spinning.

Fibroin has a wide range of biomedical applications-silk screws to repair bone fractures, silk protein scaffolds for tissue engineering and bone repair, wound dressing, material cell culture and wound healing drug systems [8]. Sericin on the other hand has attracted researchers in diverse fields - fiber processing to increase launder ability, protect cell death in insect cell culture, etc. Therefore, concerted efforts are in force to extract and analyze fibroin and sericin separately from the cocoon upon polymerization into intact silk fiber. However, there were many constraints in separation of sericin and fibroin from the silk fiber and also dissolving these proteins for which significant progress has not been made in biochemical and biomedical research. In view of this, the present study was not only undertaken to develop appropriate procedures for separation and extraction of fibroin and sericin but also comparative analysis of associated proteins disjointedly in mulberry and eri silkworms.

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# MATERIALS AND METHODS

#### **Experimental insects**

The fifth instar larvae of mulberry (Bombyx mori) and non-mulberry (Philosamia ricini) were utilized for comparative proteomic analysis in different parts of silk glands.

# Extraction of silk gland proteins from Bombyx

Healthy fifth instar day-6 larvae were dissected for whole silk gland in the pre-cooled 0.6 M saline solution. The middle and posterior silk glands as shown in figure-1 were excised separately for extraction of protein. The gel like liquid silk was collected removing the surface layer of the middle silk gland and the homogenate was prepared in Tris buffer (pH 6.8). The homogenate was centrifuged at 4000 RPM for 10 minutes at  $4^{\circ}$ C and the supernatant thus obtained from whole, middle and posterior silk glands were used for electrophoresis.



**Figure 1:** The schematic représentation of the silk glands in *Bombyx mori*. S-Spinneret, F- Filippi's gland, A- Anterior silk gland, M- Middle silk gland, P- Posterior silk gland.

## Extraction of silk gland protein from Eri silkworms

Eri silkworm larvae (5<sup>th</sup> instar – day 6) were dissected in the pre-cooled o.6 M saline solution for silk glands. Since there is no clear differentiation as anterior, middle and posterior regions as in *B. mori*, the whole silk gland was homogenized in Tris buffer (pH 6.8) and silk protein was extracted as described above.

# Sodium dodecyl sulphate - Polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoretic separation of proteins extracted from the silk glands was performed following the procedure established by Vasudha *et al.* [9]. Precisely, the running gel (12%) was prepared with a mixture of 4 ml of solution 'a' (14.6 g Acrylamide + 0.4 Bis-acrylamide + 50 ml double distilled water) with 2.5 ml of 'b' (18.15 g Tris + 100 ml double distilled water, pH 8.8) and 3.38 ml distilled water. The mixture was degassed and 0.1 ml of solution 'c' (10 g Sodium dodecyl sulphate + 50 ml distilled water), 20 µl of TEMED and 6.0 mg of ammonium per sulphate were added. The components mixed carefully was poured between the assembled glass plates and allowed to polymerize at room temperature. Consequently, stacking gel (5%) was prepared mixing solutions 'a' (0.83 ml) and 'd' (1.25 ml - 3 g Tris + 50 ml double distilled water, pH 6.8) with distilled water (3.0 ml). The degaussed mixture was added to 0.05 ml of solution 'c', 10  $\mu$ l TEMED and 2 mg ammonium per sulphate and poured above the polymerized running gel. The polymerization of the gel was achieved at room temperature.

Samples were prepared dissolving protein in solution 'd' (0.1ml) containing SDS (4%),  $\beta$ -mercaptoethanol (10%) and glycerol (20%). To this, bromophenol blue was added and heated in boiling water bath for 5 mins. ~20  $\mu$ l of cool samples were loaded into the wells immersed in solution 'e' (0.3 g Tris + 1.44 g glycine + 0.1 g SDS + 100 ml distilled water). Electrophoresis was performed at constant voltage (100V) until the tracking dye reaches the lower end of the gel. Protein molecular markers were run along with samples. Thereafter, the gel was stained with Coomassie brilliant blue and de-stained with destaining solution for clarity of the protein bands [9].

# Densitometric analysis

Gels were analyzed comparing the banding patterns and their molecular mass against protein molecular markers run parallel along with samples using gel image analysis software-ALFA installed in gel documentation unit.

## **RESULTS AND DISCUSSION**

Considering the biomedical importance of fibroin and sericin, which is biologically blended into a fiber, concerted efforts have been made for extraction of these two naturally occurring biopolymer possessing biocompatibility and biodegradability following various methods from cocoons [10] than directly from the silk glands of silkworm larvae. But, information on proteins present in the silk gland apart from fibroin and sericin, which might have been associated with silk proteins synthesis remain unknown.

# Structure of B. mori and P. ricini silk glands

The structure of the glands explicit differentiated and undifferentiated regions in *B. mori* (Fig. 1) and *P. ricini* (Fig. 2) respectively. In *B. mori*, prominent middle and long-winded posterior part while obvious, the same could not discriminate in *P. ricini* with ease, although both belong to order lepidoptera. However, the structural differences of silk glands between these silkworms indicate genusspecific variation, but the biological significance remains enigmatic.



**Figure 2:** The schematic representation of the silk glands in the eri silkworm, *Philosamia ricini*.

S-Spinneret, F- Filippi's gland, A- Anterior silk gland, M- Middle silk gland, P- Posterior silk gland.

#### Proteome of silk gland

Electrophoretic separation of proteins from different parts of the silk glands exhibit a separate entity of sericin, fibroin and allied proteins in *B. mori* (Fig. 3) compared to *P. ricini* (Fig. 4). Notably, the silk glands being a special organ in the larvae known to involve in the synthesis of fibroin and sericin, structural properties and biological functions of other proteins observed in the whole silk glands of *B. mori* and *P. ricini* has not reported till date.

#### Proteomic analysis of whole silk gland

Interestingly, sericin and fibroin though major proteins of interest, the whole silk gland, which can be differentiated as anterior, middle and posterior in *B. mori* but not so in *P. ricini*, is loaded with abundant proteins with molecular mass ranging from 10 to 350 kDa as obvious in figures 3 (lane-2) and 4 (lane-2) respectively. To support, the densitometric analysis of 1-DE gel revealed discreetly resolved ~21 peaks in *B. mori* while *P. ricini* exhibit ~32 peaks that correspond to different protein bands (Table 1, Fig. 5). Whereas, Kundapur *et al.* [11] reported 54 protein spots that resolved by 2-DE indicating higher resolution of proteins in *B. mori* but not *P. ricini*. In addition, various methods followed earlier revealed a different protein profile with three components 1.0, 1.5 and 1.8 S [12] and 14 bands [13] in the whole silk gland and fibroin alone from the posterior part of the middle gland [14]. Besides, the Rf values were ranging from 0.0021 – 0.9599 in *B. mori* and 0.0063 - 0.9497 in *P. ricini* with banding area from 576 - 35845 and 141 - 4252 respectively (Table 1, Figs. 3-5). The more interesting inference is that as the silk gland known to synthesis two proteins (fibroin and sericin) the other proteins noticed in both the gels might be cellular proteins but their importance in the silk gland is unknown.



**Figure 3:** Total protein profile of *Bombyx mori* silk gland. Lane 1-Molecular marker; 2 - Whole silk gland; 3- Middle silk gland; 4 - Posterior silk gland. Arrow indicates a differential banding pattern.

**Table 1:** Densitometric analysis of differential silk gland protein banding pattern in Bombyx mori and Philosamia ricini.

	Region	No. of Peaks	Distance	Width	Height	Area	%	Rf
Bombyx mori	whole silk gland	21	6 - 2803	3 - 170	143 -231	576 -35845	0.4-26.0	0.0021-0.9599
	middle silk gland	2	107 - 565	5 - 40	107 -146	616 -5580	5.2-47.4	0.0365-0.5345
Philosamia ricini	whole silk gland	32	20 - 3024	3 - 169	12 - 70	141 - 4252	0.3-10.1	0.0063-0.9497



**Figure 4:** Total protein profile of silk gland in Philosamia ricini.

Lane 1-Molecular marker; 2 - Whole silk gland; 3- Middle silk gland; 4-Posterior silk gland. Arrow indicates a differential banding pattern.

Among different proteins, the intensity of a 25 kDa protein band while quite prominent in B. mori (Fig. 3, lane-2), it was faint in P. ricini (Fig. 4, lane-2). Sequentially, two protein bands with molecular mass 29 and 33 kDa distinct in the whole silk gland gel profile were unnoticed in the middle part, but found as light bands in the posterior part of B. mori and also discernible in P. ricini. Yet again, 40 kDa protein band found prominently in the whole silk gland, whilst absent in the middle and appeared as a faint band in the posterior part of the silk gland in B. mori. Further, two bands of 50 and 54 kDa while obvious with same intensity both in whole and posterior, but absent in the middle parts of the silk gland of B. mori. Unequivocally, of the two, a 54 kDa protein band was unseen in the posterior part of the Eri silk gland, although, both of them with same intensity obvious in whole and middle part of the silk gland. Notably, two proteins with molecular mass 150 and 190 kDa were found unique, few other proteins differ with the intensity of the band in the whole silk gland 1-DE gel (Fig. 3). While such elite features were undetectable in the Eri silk gland (Fig. 4), some of these could be sericin proteins [15] distributed between 97 to 14 kDa in the SDS-PAGE gel, which undetermined due to its complex nature.

Thus, the total protein profile reported in the present study for the first time for the whole silk glands of two genus found reasonably different to denote species-specific variation in silk and cellular proteins. However, since characterization of cellular proteins has not been made offers further investigation employing advanced proteomic techniques to assign the biological function.

#### Proteomic analysis of middle silk gland

Comparatively, we have extracted protein directly from the lumen of the middle silk gland by removing its membrane for the first time. The silk-gelmatrix dissolved in protein extraction buffer, as in another case (whole and posterior silk gland), was separated into two discrete protein bands with molecular mass 325 and 26 kDa that corresponds unequivocally two protein bands in the whole silk gland protein profile (Fig. 3). It was further substantiated by densitometric analysis with two prominent peaks (Fig. 5, Table 1). These two proteins (325 and 26 kDa) bands are ascribed as fibroin heavy and low chain units or fibroin and sericin devoid of cellular proteins in between (Fig. 3, lane-3) which offer mass spectrometry based approach for precise identification of a protein [2]. Unlike B. mori, no fibroin and sericin could be separated from cellular proteins in the silk glands of P. ricini due to undifferentiating regions and intact glandular wall (Fig. 4, lane-3).



**Figure 5:** Densitometric profile of proteins derived from different parts of *Bombyx mori* and *Philosamia ricini* silk glands.

A – Bombyx mori whole silk gland; B - Philosamia ricini whole silk gland; C - middle silk gland of B. mori.

## Proteomic analysis of posterior silk gland

From the posterior silk gland its cell wall could not be removed, thus protein extracted was resolved into many protein bands. Of which, 2 discrete protein bands (325 and 26 kDa) closely matched with protein bands of middle silk gland and another two bands which were not found in middle silk gland but obvious in the whole silk gland with same amount (Fig. 3, lane 4). Notably, a 25 kDa protein band appeared prominently in correlation with its mass with the protein band of whole and middle silk gland. Hence, this 25 kDa protein band shall be fibroin L-chain as posterior silk gland involved in synthesis of only fibroin but not sericin; whereas large number proteins observed in the SDS-PAGE gel could be cellular proteins, which might support the glandular cells for fibroin synthesis. Comparatively, none of the proteins found specific unless their quantity differs in the posterior silk gland of P. ricini (Fig. 4).

Collectively, we have uncovered the structural and proteome differences between B. mori and P. ricini for the first time in the present investigation. The silk gland may not have only two - fibroin and sericin proteins but has large amount of proteins linked with synthesis of mammoth quantity of silk proteins. Due to this, identification of specific sericin protein in the present study remain obscure that represents a family of protein with wide-ranging molecular mass distributed all along the gel unlike middle silk gland protein. Interestingly, the middle silk gland devoid of glandular membrane exhibit presence of two discrete protein bands in B. mori, which are ascribed as fibroin H- and L-chain since  $\beta$ -mercaptoethanol used in the present study could cleave disulfide bonds between these two chains. Thus, the method standardized here is differ from other protocols and simple for discrete separation of fibroin and sericin, which can be isolated through gel extraction for 'high value fibroin protein' from B. mori than Philosamia silk glands for biotechnological and biomedical applications.

## **ACKNOWLEDGMENTS**

HBM thanks to the University Grants Commission for funding under MRP, Punyavathi thanks to DST for INSPIRE fellowship and MAB thanks to University of Mysore for granting NON-PHD fellowship.

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Source of support: University of Mysore NON-PHD fellowship, DST-INSPIRE fellowship and UGC-MRP Conflict of interest: None Declared